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Species-dependent smooth muscle contraction to Neuromedin U and determination of the receptor subtypes mediating contraction using NMU1 receptor knockout mice

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1 The peptide ligand neuromedin U (NMU) has been implicated in an array of biological activities, including contraction of uterine, intestinal and urinary bladder smooth muscle. However, many of these responses appear to be species-specific. This study was undertaken to fully elucidate the range of smooth muscle-stimulating effects of NMU in rats, mice and guinea-pigs, and to examine the extent of the species differences. In addition, the NMU1 receptor knockout mouse was used to determine which receptor subtype mediates the contractile responses generated by NMU in the mouse.

2 A range of isolated organ *in vitro* bioassays were carried out, which were chosen to re-confirm previous literature reports (uterine and stomach fundus contraction) and also to explore potentially novel smooth muscle responses to NMU. This investigation uncovered a number of previously unidentified NMU-mediated responses: contraction of rat lower esophageal sphinster (LES), rat ileum, mouse gallbladder, enhancement of electrically evoked contractions in rat and mouse vas deferens, and a considerable degree of cross-species differences.

3 Studies using the NMU1 receptor knockout mice revealed that in the mouse fundus and gallbladder assays the NMU contractile response was mediated entirely through the NMU1 receptor subtype, whereas, in assays of mouse uterus and vas deferens, the response to NMU was unchanged in the NMU1 receptor knockout mouse, suggesting that the NMU response may be mediated through the NMU2 receptor subtype. NMU receptor subtype-selective antagonists are required to further elucidate the role of the individual receptor subtypes.
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Abbreviations: DMSO, dimethyl sulfoxide; GI, gastrointestinal; GPCR, guanine nucleotide protein-coupled receptor; LES, lower esophageal sphincter; 5-mef, 5-methylfurmethide; NMU, neuromedin U; NMU-LIR, NMU-like immunoreactivity

Introduction

The peptide ligand neuromedin U (NMU) was first isolated from porcine spinal cord in 1985 (Minamino *et al*., 1985) and named neuromedin 'U' on account of its ability to contract rat uterine smooth muscle *in vitro*. After 15 years the orphan G-protein-coupled receptors FM-3 and FM-4 (now known as the NMU1 and NMU2 receptors) were identified as the cognate receptors for NMU (Fujii *et al*., 2000; Hosoya *et al*., 2000; Howard *et al*., 2000; Raddatz *et al*., 2000; Shan *et al*., 2000; Szekeres *et al*., 2000). Both receptors are seven-transmembrane, guanine nucleotide protein-coupled receptors (GPCRs) that activate phospholipase C and mobilize intracellular calcium *via* the G_q class of G protein (Raddatz *et al*., 2000; Shan *et al*., 2000; Szekeres *et al*., 2000) and, additionally, have been demonstrated to inhibit adenylyl cyclase *via* the G_i class of G protein (Brighton *et al*., 2004b).

The NMU peptide and its receptors have been implicated in an array of biological activities, ranging from contraction of isolated smooth muscle (such as rat uterus and fundus (Minamino *et al*., 1985; Benito-Orfila *et al*., 1991)), regulation of intestinal ion transport (Brown & Quito, 1988), alteration in heart rate, blood pressure and blood flow (Sumi *et al*., 1987; Gardiner *et al*., 1990a, b; Chu *et al*., 2002), to modification of feeding behavior (Howard *et al*., 2000; Nakazato *et al*., 2000), release of stress-mediating hormones (Hanada *et al*., 2001; Wren *et al*., 2002) and enhancement of nociception (Cao *et al*., 2003; Yu *et al*., 2003). The smooth muscle stimulating effects of NMU reported to date include contraction of chicken crop, duodenum, ileum and rectum (Sakura *et al*., 1991), canine urinary bladder, stomach, colon and ileum (Westfall *et al*., 2002), and human urinary bladder and ileum (Maggi *et al*., 1990), as well as the rat uterus and fundus mentioned above. Many of these responses appear to be species-specific, as no response to NMU was observed in guinea-pig ileum, porcine jejunum, rat ileum and colon, frog stomach muscle or small intestine, canine uterus and rat, mouse, guinea-pig, rabbit or ferret urinary bladder (Minamino *et al*., 1985; Brown & Quito,

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1988; Bockman *et al*., 1989; Benito-Orfila *et al*., 1991; Westfall *et al*., 2002). This study was undertaken to fully elucidate the (non-vascular) smooth muscle stimulating effects of NMU in rats, mice and guinea-pigs, and to examine the extent of the species differences. To this end, we carried out a comprehensive survey of the responses of smooth muscle preparations from common laboratory animals to NMU peptides. In addition to re-confirming some of the previous literature reports (contraction of uterus and fundus tissue), we now report a variety of novel smooth muscle responses to NMU, including contraction of rat lower esophageal sphincter (LES), rat ileum, mouse gallbladder and enhancement of electrically evoked contraction of rat and mouse vas deferens. To determine the receptor subtype mediating these effects, we conducted additional assays using tissue from NMU1 receptor knockout mice. These studies revealed that in the mouse fundus and gallbladder assays the NMU response is mediated entirely through the NMU1 receptor subtype, whereas in assays of mouse uterus and vas deferens the response to NMU was unchanged in the NMU1 receptor knockout mouse, suggesting that the NMU response may be mediated through the NMU2 receptor subtype.

An apparent variation in tissue responses across species was confirmed in this study and this highlights the need for cross-species NMU receptor subtype-selective antagonists as pharmacological tools for characterization of this receptor system.

Methods

Isolated organ in vitro *bioassays*

For all assays, rats (Sprague–Dawley: 250–300 g), mice (CD1: 20–25 g; NMU1R−/−: 20–50 g) and guinea-pigs (Hartley: 300–350 g) of both sexes were used where possible. The same basic protocol was employed for each smooth muscle preparation from all species, unless otherwise stated. All procedures were performed according to the internationally accepted guidelines for the care and use of laboratory animals in research and were approved by the local IACUC.

All tissues, except the uterus, were mounted in 20-ml organ baths containing Krebs–Henseleit solution (composition (mM): NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, Na_2HPO_4 1.0, $NaHCO_3$ 25 and D-glucose 10) thermostatically controlled at 32°C (37°C for vas deferens assay) and gassed with 95% O_2, 5% CO_2. The uterine smooth muscle was mounted in 20-ml organ baths containing Tyrode's solution (composition, mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, Na_2HPO_4 0.36, $NaHCO_3$ 12 and D-glucose 5.6) thermostatically controlled at 28°C and also gassed with 95% O_2, 5% CO_2. After an equilibration period (60–90 min), the tissues were incubated with rat NMU-23, mouse NMU-23 or guinea-pig NMU-9 using a cumulative dosing regime, and only one concentration–effect curve was obtained per preparation. Where no response to NMU was obtained, tissue viability was assessed by addition of a near-maximal concentration of the muscarinic agonist, 5-methylfurmithide (5-mef; 10 μM) to the bath. Responses in the fundus and gastric corpus smooth muscle assays were measured using isotonic transducers. All other responses were measured using isometric force displacement transducers; both transducer types being coupled through an amplifier system (Harvard Apparatus, Holliston, MA, U.S.A.) to a flatbed chart recorder (Kipp & Zonen, Delft, The Netherlands).

Uterus assay preparation

Both uterine horns were removed, cleared of any adhering tissue and divided into longitudinal strips measuring 2 × 5 mm. Tissues were mounted between a fixed tissue holder and a stainless steel hook attached to the isometric transducer and placed under 2 g resting tension. After a 60–90-min equilibration period, tissues were exposed to the appropriate NMU peptide.

Fundus assay preparation

The stomach fundus was removed, cleared of any adhering tissue and divided into circular strips measuring 2 × 7 mm. Tissues were mounted in organ baths and placed under 0.3 g isotonic tension. After a 90-min equilibration period, rat and guinea-pig tissues were directly stimulated with either rat NMU-23 or guinea-pig NMU-9. Mouse fundic smooth muscle failed to contract to NMU unless the tissue was initially exposed to a concentration of KCl sufficient to produce a threshold contractile response, achieved by incremental addition of KCl to each tissue over the concentration range 6–10 mM. Once the KCl contraction had stabilized, cumulative concentration–response curves to mouse NMU-23 were obtained.

Gastric smooth muscle assay preparation

In the rat and guinea-pig assays, circular strips measuring 2 × 7 mm were prepared from the stomach corpus, with the mucosal layer removed. In the mouse assay, it proved very difficult to remove the mucosa without damaging the muscle layer; therefore intact strips were used. Tissues were placed under 0.3 g isotonic tension. After a 90-min equilibration period, rat tissues were directly stimulated with rat NMU-23. For guinea-pig and mouse gastric smooth muscle, tissues were both exposed directly to NMU (as for the rat tissue) or initially exposed to a concentration of KCl sufficient to produce a threshold contractile response (mouse: 6–10 mM; guinea-pig: 10 mM).

Gallbladder assay preparation

Circular muscle strips (1 × 4 mm) were prepared from the guinea-pig gallbladder. In the organ bath, the tissues were placed under an initial resting tension of 1 g. The mouse gallbladder was mounted whole, in the longitudinal orientation and placed under 0.5 g resting tension. All tissues were allowed to equilibrate for 60 min, during which time the organ bath fluid was replaced with pre–warmed Krebs–Henseleit solution at 30-min intervals, after which cumulative concentration–response curves to NMU peptides were obtained.

LES assay preparation

The ring of sphincter muscle was cleared of surrounding esophageal and stomach tissue, mounted between two parallel, stainless steel wires and placed under 1 g resting tension. Tissues were allowed to equilibrate for 60 min, during

which time the organ bath fluid was replaced with pre-warmed Krebs–Henseleit solution at 30-min intervals. Cumulative concentration–response curves to NMU peptides were obtained.

Ileum, duodenum, colon and urinary bladder assay preparations

Both longitudinal and circular muscle strips were prepared from the duodenum, ileum and colon (2×5 mm). Longitudinal strips (2×5 mm) were prepared from the bladder. In the organ bath, all tissues were placed under an initial resting tension of 1 g and allowed to equilibrate for 60 min, during which time the organ bath fluid was replaced with pre-warmed Krebs-Henseleit solution at 30-min intervals. Cumulative concentration–response curves to NMU were obtained.

Vas deferens assay preparation

Portions of prostatic vas deferens (8 mm) were mounted whole and placed under 1 g resting tension for the rat and guinea-pig and 0.5 g tension for the mouse. Tissues were allowed to equilibrate for 60 min, during which time the organ bath fluid was replaced with pre-warmed Krebs–Henseleit solution at 30-min intervals. Tissues were electrically stimulated using Grass stimulators (0.2 Hz, 0.5 ms, 80–100 V) and then exposed to NMU peptides.

Trachea assay preparation

Rings of trachea (3 mm) were mounted between two parallel, stainless steel wires and placed under 1 g resting tension. Tissues were allowed to equilibrate for 60 min, during which time the organ bath fluid was replaced with pre-warmed Krebs–Henseleit solution at 30-min intervals. Tissues were either exposed to NMU directly to evaluate the contractile effects of the peptide, or the rings were precontracted (40 mM KCl or 10 μM 5-mef) and any relaxation responses to NMU were measured.

Esophagus assay preparation

Portions of intact esophagus (5 mm) were mounted whole and placed under 1 g resting tension. Tissues were allowed to equilibrate for 60 min, during which time the organ bath fluid was replaced with pre-warmed Krebs–Henseleit solution at 30-min intervals. Cumulative concentration–response curves to NMU were obtained.

Generation of NMU1 receptor gene knockout mice (NMU1R−/−)

NMU1 knockout mice were generated by Lexicon Genetics (Woodlands, TX, U.S.A.). In brief, the NMU1 receptor knockout mice were generated by insertion of a neomycin-resistant gene cassette into the NMU1 gene to replace nucleotides 4117–4868. Chimeric mice were generated from embryos injected with embryonic stem cells containing the modified gene. Germline mice were obtained by breeding chimeric male mice with C57BL/6 females. Mice heterozygous for the disrupted NMU1 receptor gene were identified by PCR (wild-type primers: forward 5′-CCGGTGCCCGACTCAGC TATATGTT-3′ and reverse 5′-GTCCCTGCTAAGTCTGGC CCATAGG-3′ giving a 559-bp product; knockout primers: forward 5′-GATTCGCAGCGCATCGCCTTCTATC-3′ and reverse 5′-GTCCCTGCTAAGTCTGGCCCATAGG-3′, giving a 435-bp product). Wild-type and NMU1 receptor−/− mice were obtained from cross–breeding of heterozygous mice.

Quantitative RT–PCR

Samples of uterus and fundus were collected from wild-type, heterozygous and knockout mice ($n = 3$ for each). The Qiagen RNeasy Mini Kit was used to prepare total RNA (DNaseI treatment performed). RNA quantity was assessed using *uv*-spectrophotometry and RNA quality confirmed using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, U.S.A.). Two-tube quantitative real-time RT–PCR was utilized throughout the study. RT reactions utilized the TaqMan Reverse Transcription kit (Applied Biosystems). Control reactions not containing reverse transcriptase were also performed to ensure that contaminating DNA did not influence PCR. Quantitative real-time PCR assays were performed using the iCycler iQ detection system (Bio-Rad, Hercules, CA, U.S.A.). PCR was performed using 300 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 50 μl. Reactions were incubated as directed for the SYBR Green PCR Master Mix. Levels of mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, as preliminary studies indicated that this mRNA species did not change with any of the treatments (data not shown). Primer sequences were as follows: mouse NMU1 receptor, forward 5′-CGGAGACAGGTGACCAAGAT-3′, reverse 5′-GTCCAT ACACCAGGCTCCAC-3′; mouse NMU2 receptor, forward 5′-CCCTCATCAGCGTCCTCTAC-3′, reverse 5′-GCAAAC ACGAGGACCAAGAC-3′; mouse GAPDH primers, forward 5′-GAGGACCAGGTTGTCTCCTG-3′, reverse 5′-ATGTAG GCCATGAGGTCCAC-3′.

Materials

Rat NMU-23 was obtained from Bachem (Torrance, CA, U.S.A.). Guinea-pig NMU-9 was obtained from Phoenix Pharmaceutical Inc. (Belmont, CA, U.S.A.). Mouse NMU-23 was synthesized by Synpep Corp (Dublin, CA, U.S.A.). 5-mef was obtained from RBI (U.K.). All other buffers and reagents used in the *in vitro* studies were from Sigma-Aldrich (St Louis, MO, U.S.A.). The TaqMan Reverse Transcription kit and SYBR Green PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, U.S.A.). The RNeasy Mini Kit was obtained from Qiagen (Valencia, CA, U.S.A.).

In vitro *data analysis*

Where possible, individual concentration–effect (E/[A]) curve data were fitted to the following Hill equation (1), to provide estimates of midpoint location ($[A]_{50}$), maximal asymptote (α) and Hill slope (n_H) parameters, where [A] is the agonist concentration and E is the measured effect:

$$E = \frac{\alpha[A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}} \quad (1)$$

For display purposes the mean fitted parameter estimates were used to generate a single $E/[A]$ curve shown superimposed on the mean experimental data. All data were analyzed using the software package GraphPad Prism® (version 3.01). Potency estimates for agonists are expressed as the negative logarithm of the midpoint locations ($p[A]_{50} \pm$ s.e.m.).

Results

Isolated organ in vitro *bioassays*

A total of 16 different smooth muscle preparations from three different species were investigated using *in vitro* bioassay techniques to evaluate the contractile effects of NMU. For all assays where both male and female animals were used, there was no apparent gender difference in the response to stimulation with NMU. The uterine tissue preparation consistently contracted to NMU in all studies, whereas for the other NMU-responsive smooth muscles there was significant species variation in response (see Table 1 for summary). All smooth muscle preparations produced a contractile response upon addition of 10 μM 5-mef.

Contraction of uterine tissue in response to NMU

Rat and mouse uterine muscle strips responded in a concentration-dependent manner to rat and mouse NMU-23, respectively (rat uterus: $p[A]_{50} = 7.99 \pm 0.11$, $n = 5$; mouse uterus: $p[A]_{50} = 7.83 \pm 0.08$, $n = 7$; Figure 1a, b and e, Table 1). Guinea-pig uterus contracted upon administration of a single high concentration (1 μM) of guinea-pig NMU-9 (no response to rat NMU-23), but no contraction was seen when NMU-9 was added cumulatively from 1 nM to 1 μM. A significant level of basal spontaneous contractile activity was apparent in all uterine tissues (see Figure 1e). For the measurement of responses, the baseline was taken as the bottom of the spontaneous contractions (which tended to be more consistent than the peaks) and, thereafter, any increase in tone produced by addition of NMU was measured as the difference between this baseline and the bottom of the spontaneous contractions remaining once the response had attained a plateau.

Contraction of fundic tissue in response to NMU

Circular strips of fundus smooth muscle from rat and mouse responded in a concentration-dependent manner to rat and mouse NMU-23, respectively (rat: $p[A]_{50} = 8.15 \pm 0.14$, $n = 5$; mouse: $p[A]_{50} = 8.15 \pm 0.13$, $n = 4$; Figure 1c, d and f, Table 1). No response to guinea-pig NMU-9 or rat NMU-23 was obtained in the guinea-pig fundus preparation.

Contraction of gastric smooth muscle tissue in response to NMU

A concentration-dependent contraction to rat NMU-23 was observed in rat circular gastric smooth muscle strips where the mucosal layer had been removed ($p[A]_{50} = 8.39 \pm 0.17$, $n = 16$; Figure 2a and b, Table 1). Guinea-pig circular gastric smooth muscle strips (with or without KCl pre-contraction) failed to respond to either guinea-pig NMU-9 or rat NMU-23. Similarly, mouse gastric smooth muscle strips did not respond to stimulation with mouse NMU-23 (with or without KCl pre-contraction). In contrast, all of these preparations contracted in response to 10 μM 5-mef.

Contraction of gallbladder in response to NMU

Mouse gallbladder, mounted whole and in the longitudinal orientation, gave a small contraction to mouse NMU-23 ($p[A]_{50} = 8.05 \pm 0.27$, $n = 5$; Figure 2c and d, Table 1). A high failure rate was observed in this assay (60% of tissues failed to respond to NMU), due to a combination of the very small size of the tissue and the small maximal contraction that NMU can produce in the gallbladder (which is at most $\sim$15% of the 5-mef response). Thus, the signal-to-noise ratio in this tissue was often particularly small and, consequently, the isometric transducers may not have been sensitive enough to pick up the NMU-mediated response over background noise. Guinea-pig gallbladder did not contract upon stimulation with either guinea-pig NMU-9 or rat NMU-23, though it did contract upon stimulation with both 5-mef and cholecystokinin octapeptide (data not shown). This assay was not conducted in the rat due to the absence of a gallbladder in this species.

Contraction of LES in response to NMU

Rat LES responded in a concentration-dependent manner to rat NMU-23 ($p[A]_{50} = 7.59 \pm 0.35$, $n = 6$; Figure 3a and b, Table 1). The response of the mouse LES was less reliable, with only 5 out of 23 tissues giving a small contraction to mouse NMU-23, though the response, when present, did appear to be more potent ($p[A]_{50} \sim 9$; data not shown). Guinea-pig LES did not respond to administration of guinea-pig NMU-9 or rat

Table 1 Summary of NMU-mediated smooth muscle contractile responses

	Rat		*Mouse*	
Tissue	$p[A]_{50}$	n_H	$p[A]_{50}$	n_H
Uterus	7.99 ± 0.11	1.12 ± 0.24	7.83 ± 0.08	1.51 ± 0.39
Stomach – fundus	8.15 ± 0.14	1.11 ± 0.73	8.15 ± 0.13	1.01 ± 0.31
Stomach – corpus	8.39 ± 0.17	1.05 ± 0.36	NR	
LES	7.59 ± 0.35	1.07 ± 0.77	ND	
Ileum – longitudinal	6.40 ± 0.18	1.04 ± 0.38	NR	
Gallbladder			8.05 ± 0.27	0.93 ± 0.40
Vas deferens	ND		7.97 ± 0.32	0.96 ± 0.53

NR = no response, ND = dose–response curve not defined, but response observed.
The mid-point location ($p[A]_{50} \pm$ s.e.m.) and slope ($n_H \pm$ s.e.m.) of the curves are given where possible.

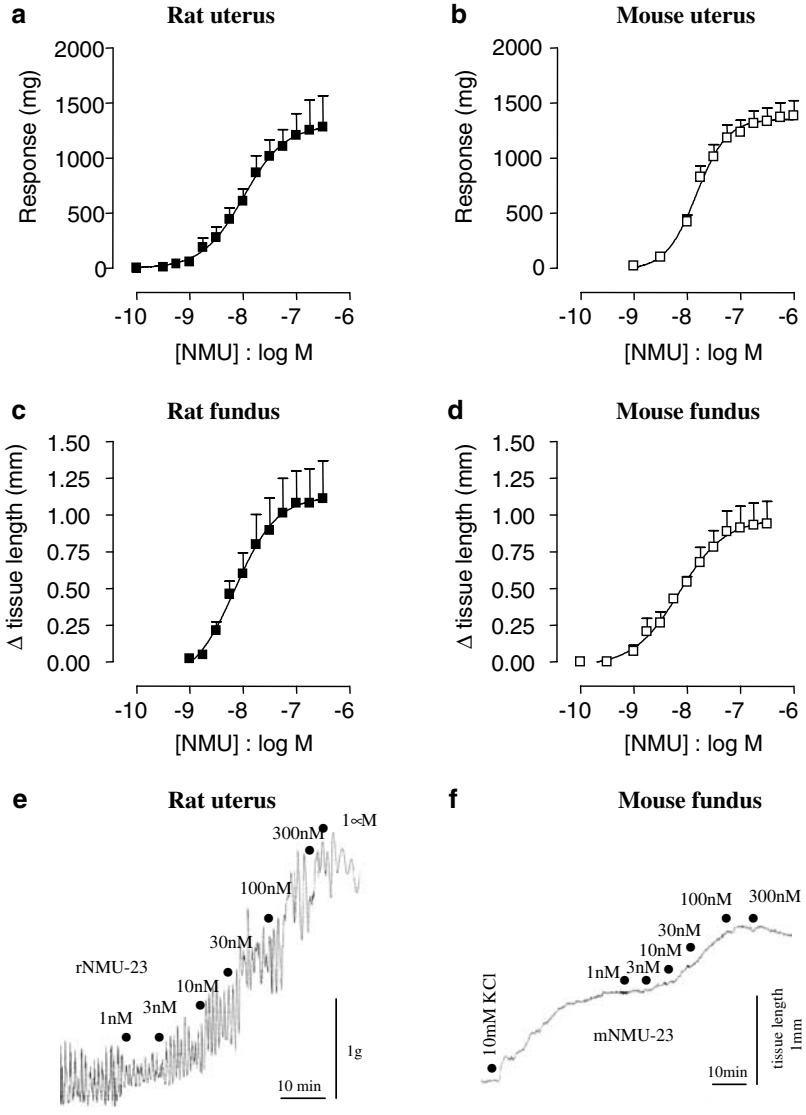

Figure 1 (a) Concentration–effect (E/[A]) curve for rat NMU-23 obtained in the rat uterus preparation, $n=5$. (b) E/[A] curve for mouse NMU-23 obtained in the mouse uterus preparation, $n=7$. Uterine responses are expressed in terms of isometric tension (mg). (c) E/[A] curve for rat NMU-23 obtained in the rat fundus preparation, $n=5$. (d) E/[A] curve for mouse NMU-23 obtained in the mouse fundus preparation, $n=4$. Responses in the fundus are measured isotonically and thus expressed as the change in the length of the tissue (mm). (e) Experimental trace showing response of the rat uterus to rNMU-23. (f) Experimental trace showing response of the mouse fundus to mNMU-23.

NMU-23, though again it did respond to stimulation with 5-mef.

Contraction of ileum, duodenum, colon and urinary bladder in response to NMU

No response to NMU was observed in circular or longitudinal strips from the colon and duodenum or the urinary bladder tissue of all three species investigated, although all these tissues did respond to 10 μM 5-mef. However, a concentration-dependent contraction to rat NMU-23 was observed in rat ileal longitudinal muscle strips ($p[A]_{50}=6.40\pm0.18$, $n=4$; Figure 3c and d, Table 1). No NMU-23-mediated contraction was observed in rat circular ileal strips. Additionally, no NMU-mediated contraction was observed in guinea-pig or mouse circular or longitudinal ileal strips. Similar to the situation in the uterine tissue, there was a high level of basal spontaneous contractile activity in the rat ileal longitudinal strips (see Figure 3d). For analysis, responses were measured from the bottom of the spontaneous contractions each time the response attained a plateau.

Contraction of vas deferens in response to NMU

Rat, mouse and guinea-pig vasa deferentia were electrically stimulated at 0.2 Hz, 0.5 ms and 80–100 V. These stimulation parameters produced large electrically evoked contractions in the rat tissue. Addition of a high concentration of rat NMU-23 (1 μM) produced approximately a 30% increase in the size of the evoked contractions (Figure 4c), though it proved difficult to distinguish responses to low NMU-23 concentrations and

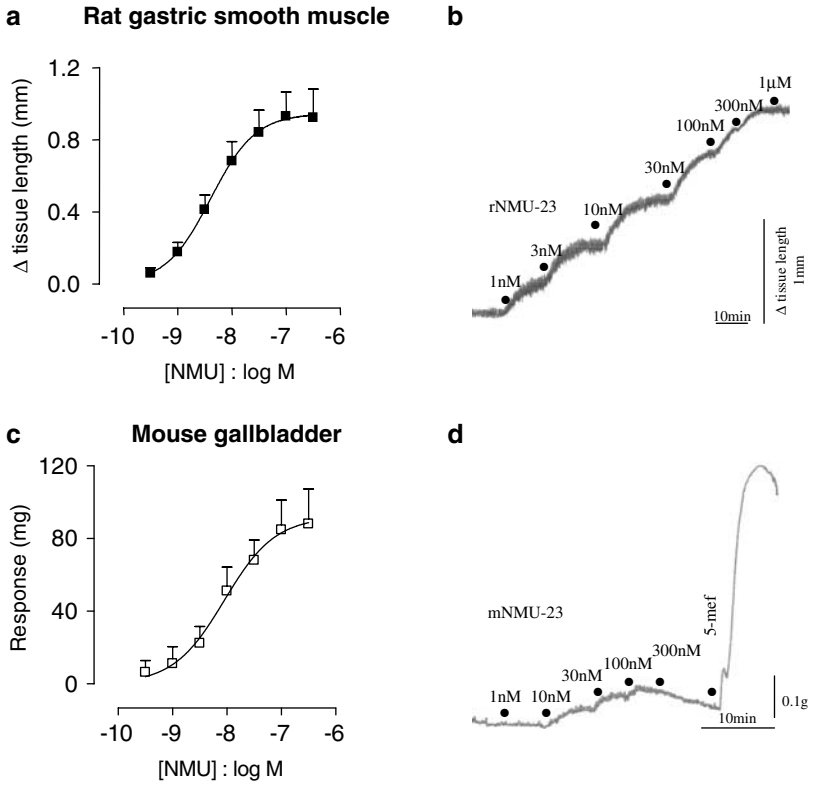

Figure 2 (a) E/[A] curve for rNMU-23 obtained in the rat gastric smooth muscle preparation, $n=16$. Gastric smooth muscle responses are measured isotonically and expressed as the change in the length of the tissue (mm). (b) Experimental trace showing response of rat gastric smooth muscle to rNMU-23. (c) E/[A] curve for mNMU-23 obtained in the mouse gallbladder preparation, expressed in terms of isometric tension (mg), $n=5$. (d) Experimental trace showing response of mouse gallbladder to mNMU-23.

generate a complete concentration–response curve. In the mouse tissue, occasionally a very small electrically evoked contraction was apparent, but more frequently the tissue was quiescent despite the stimulation. Notwithstanding the response to stimulation, nanomolar concentrations of mouse NMU-23 caused either the appearance of and/or a significant increase in the size of existing electrically evoked contractions ($p[A]_{50}=7.97\pm0.32$, $n=6$; Figure 4a and b, Table 1). Similar to mouse tissue, guinea-pig vas deferens failed to demonstrate electrically evoked contractions when initially stimulated; however, neither guinea-pig NMU-9 nor rat NMU-23 produced any further contraction. In all species, no response to NMU was observed when the tissues were not electrically stimulated.

Contraction/relaxation of trachea in response to NMU

In rat, mouse and guinea-pig, we examined both contraction of tracheal rings and relaxation of rings pre-contracted with either KCl (40 mM) or 5-mef (10 μM) and in all cases no response to NMU peptides was observed.

Contraction of esophagus in response to NMU

In rat, mouse and guinea-pig, we examined the effect of NMU peptides on intact segments of esophagus. No contraction was generated upon administration of NMU in any species.

Characterization of mouse NMU-mediated contractile responses using NMU1 receptor KO mice

To further investigate the NMU-mediated contractile responses observed in mouse tissue, these *in vitro* assays were repeated using tissue obtained from NMU1 receptor knockout ($-/-$), heterozygous ($+/-$) and wild-type mice ($+/+$, littermate controls). In the mouse uterus and vas deferens assay, no significant difference was observed between the NMU concentration–effect curves obtained for NMU-23 in tissue from knockout, wild-type or heterozygous NMU1 receptor mice (mean $p[A]_{50}\pm$s.e.m.: uterus; $+/+7.80\pm0.17$, $n=8$; $+/-7.74\pm0.12$, $n=9$; $-/-7.82\pm0.08$, $n=9$; vas deferens: $+/+8.18\pm0.35$, $n=6$; $+/-7.72\pm0.13$, $n=8$; $-/-7.98\pm0.35$, $n=5$; Figure 5a and d). Conversely, in the mouse fundus and gallbladder assays, no response to NMU was observed in tissue obtained from the knockout mice (Figure 5b and c). For the fundus assay, the NMU curve generated in the heterozygote tissue was located approximately midway between the knockout and wild-type assays (mean $p[A]_{50}\pm$ s.e.m: $+/+7.58\pm0.22$, $n=8$; $+/-7.04\pm0.15$, $n=5$; $-/-$ no response, $n=8$). In the gallbladder assay, the response in the wild-type and heterozygote tissue was variable between preparations. Thus, for the wild-type gallbladders six out of 10 tissues responded and in the knockout gallbladders six out of 12 tissues responded to NMU administration. All tissues contracted in response 5-mef. This variation was not gender dependent. There was no significant difference between the midpoint locations estimated for the responding tissues from

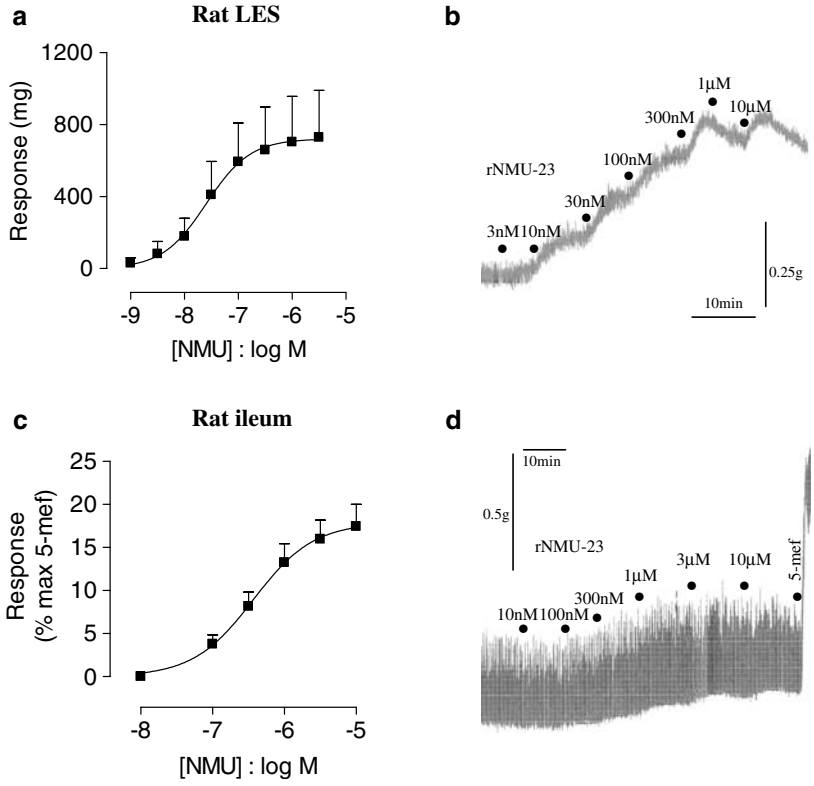

Figure 3 (a) *E*/[A] curve for rNMU-23 obtained in the rat LES preparation, expressed in terms of isometric tension (mg), $n=6$. (b) Experimental trace showing response of rat LES to rNMU-23. (c) *E*/[A] curve for rNMU-23 obtained in the rat ileum longitudinal smooth muscle preparation, expressed as a percentage of the maximal 5-mef response, $n=4$. (d) Experimental trace showing response of rat ileum to rNMU-23.

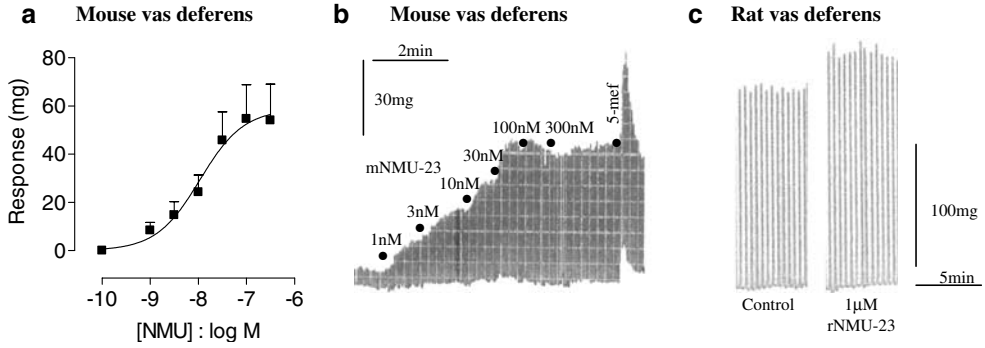

Figure 4 (a) *E*/[A] curve for mNMU-23 obtained in the electrically stimulated mouse vas deferens preparation, expressed in terms of isometric tension (mg), $n=6$. (b) Experimental trace showing the effect of mNMU-23 on electrically evoked contractions of the mouse vas deferens. (c) Experimental trace showing the effect of rNMU-23 on electrically evoked contractions of the rat vas deferens. Stimulation parameters were 0.2 Hz, 0.5 ms, 80–100 V.

wild-type or heterozygote mice (mean $p[A]_{50} \pm$ s.e.m.: $+/+$ 7.70 ± 0.15, $n=6$; $+/-7.66 \pm 0.37$, $n=6$; $-/-$ no response, $n=9$).

Quantitative RT–PCR

Levels of NMU1 and NMU2 receptor mRNA were assessed in uterus and fundus samples from the wild-type, heterozygous and knockout mice using quantitative RT–PCR. NMU1 receptor mRNA expression was detected at low levels in samples of both uterus and fundus from wild-type mice. Data were normalized to GAPDH mRNA levels, which did not vary across the treatment groups (data not shown), and were expressed relative to wild-type levels. Expression was reduced in the heterozygote samples and below the limit of detection in the NMU1 receptor knockout samples (Figure 6). NMU2 receptor mRNA expression was detected in samples of wild-type uterus, though there was no significant difference in the expression level of this mRNA species in wild-type, heterozygote or NMU1 receptor knockout uterus samples (data not shown). No NMU2 receptor mRNA expression was detected in wild-type, heterozygote or NMU1 receptor knockout fundus samples.

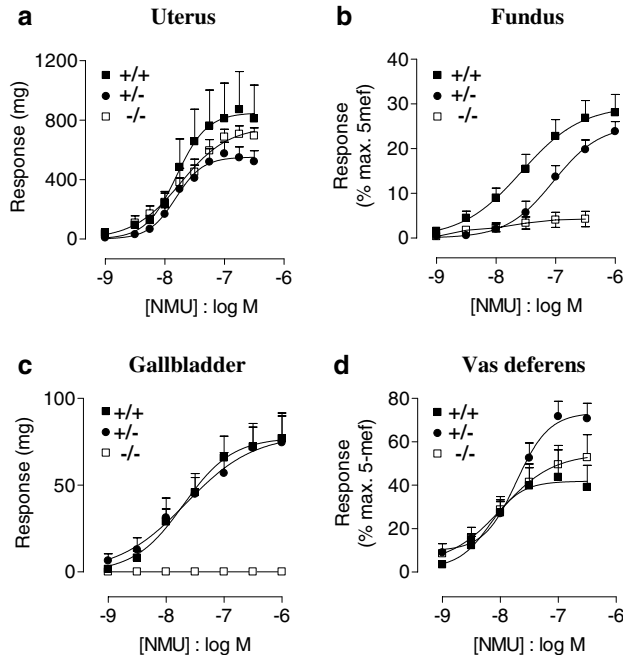

Figure 5 (a) *E*/[A] curves for mouse NMU-23 obtained in uterus preparations from wild-type mice (+/+, $n=8$), NMUR1−/− mice ($n=9$) and heterozygous mice (+/−, $n=9$). (b) *E*/[A] curves for mouse NMU-23 obtained in fundus preparations from wild-type mice ($n=8$), NMUR1−/− mice ($n=8$) and heterozygous mice ($n=5$). (c) *E*/[A] curves for mouse NMU-23 obtained in gallbladder preparations from wild-type mice ($n=6$), NMUR1−/− mice ($n=9$) and heterozygous mice ($n=6$). (d) *E*/[A] curves for mouse NMU-23 obtained in vas deferens preparations from wild-type mice ($n=6$), NMUR1−/− mice ($n=5$) and heterozygous mice ($n=8$). Uterine and gallbladder responses are expressed in terms of isometric tension (mg). Responses in the fundus and vas deferens are expressed as a percentage of the maximal 5-mef response in that tissue.

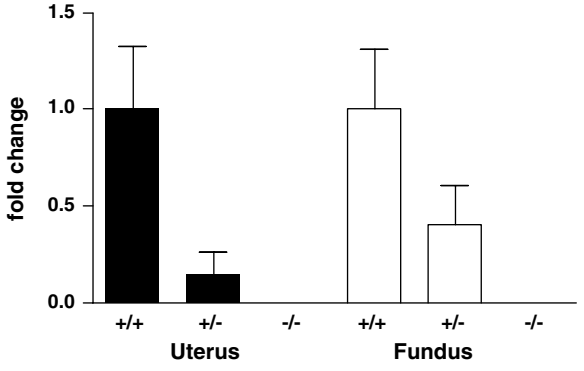

Figure 6 Uterine and fundus smooth muscle NMU1 receptor mRNA expression data from wild-type, heterozygote and NMU1R knockout samples, assessed using RT–PCR. Data were normalized to GAPDH mRNA expression levels. Error bars indicate s.e.m. with $n=3$.

Discussion

NMU is a peptide hormone that was originally isolated from porcine spinal cord and was termed 'U' because of its contractile action on isolated uterine smooth muscle (Minamino *et al*., 1985). Subsequently, NMU has been shown to mediate contractile effects on smooth muscle preparations of stomach, ileum, colon and urinary bladder, although many of these effects appear to be species-specific (Maggi *et al*., 1990; Benito-Orfila *et al*., 1991; Westfall *et al*., 2002). Here we describe a systematic investigation of the contractile effects of NMU in 16 different smooth muscle preparations from rat, mouse and guinea-pig.

Consistent with the original naming of the peptide (Minamino *et al*., 1985), NMU elicited a contractile response in the uterus of all species investigated. The uterus was the only smooth muscle preparation in the guinea-pig that responded to NMU administration, although the response in this tissue was difficult to define. It may be that this NMU receptor undergoes rapid internalization upon administration of NMU, which would make a cumulative dosing regime difficult to conduct. Alternatively, the receptor expression may be so low that the midpoint location of NMU in this tissue is less than 1 μM. The guinea-pig NMU peptide is 9 a.a. compared to the 23-a.a. rat mature sequence (Conlon *et al*., 1988; Murphy *et al*., 1990). However, there is a high degree of amino-acid conservation across species in the C-terminal domain of the NMU peptide and, as such, the C-terminal heptapeptides of guinea-pig NMU-9 and rat NMU-23 are identical (for a review, see Brighton *et al*., 2004a). Guinea-pig NMU-9 is capable of causing contraction in both rat uterus and fundus assays (albeit with lower potency than NMU-23; data not shown), though it seems that the reverse is not true, as NMU-23 was unable to stimulate the guinea-pig uterine receptors here. It would be interesting to evaluate the affinity of the NMU peptides at homogenous populations of NMU1 and NMU2 receptors; however, there are no reports of the cloning of the guinea-pig NMU receptors to date.

This study also revealed a number of novel smooth muscle preparations that responded to NMU stimulation. The rat and mouse LES, the longitudinal muscle of the rat ileum, the mouse gallbladder and the mouse and rat electrically stimulated vas deferens all responded to NMU administration and, to our knowledge, have not been previously reported in the literature. The potency of NMU in these tissues was remarkably consistent, with the exception of the rat ileum, where NMU had an approximately ~13-fold lower potency (Table 1). In a previous study by Benito-Orfila *et al*. (1991), no contraction to NMU was observed in longitudinal muscle strips from the small or large intestine of the rat. It may be that the decreased potency shown here and the failure of the previous study to demonstrate contraction result from low NMU receptor expression in this tissue or a reduced receptor–effector coupling efficiency. In addition, there may be differences in the relative expression levels of NMU1 and NMU2 receptors between this tissue and the other smooth muscles investigated. Unfortunately, in the absence of receptor-selective pharmacological ligands, the exact contribution of each receptor subtype to the observed contractions in these assays is difficult to delineate.

Knockout mice provide a useful tool for studying the involvement of a given receptor in the response elicited by an agonist ligand. In this study, the uterine, stomach fundus, gallbladder and vas deferens *in vitro* assays were repeated in the NMU1 receptor knockout tissues, in order to determine which receptor subtype was implicated in the contractile responses observed to NMU in the mouse. These studies revealed that in the mouse fundus and gallbladder assays, the NMU response is mediated entirely through the NMU1

receptor subtype. In the fundus assay, the NMU curve generated in the heterozygote tissue was less potent than in the wild-type assay, behavior consistent with reduced receptor density. However, in the gallbladder assay, the response was more variable, with only 50% of the heterozygote tissues and 60% of the wild-type tissues producing a response. Interestingly, in this assay, the responses obtained in the functioning heterozygotes and wild-type gallbladders were not significantly different. Therefore, in this tissue it would appear that only one copy of the gene is required to produce a full effect. It would be interesting to investigate the levels of NMU receptor protein expression in these tissues; however, currently there are no available pharmacological or biochemical tools available for this study.

In the mouse uterus and vas deferens assays, the response to NMU was unchanged in the NMU1 receptor knockout mouse or in the heterozygote mouse. This suggests that the NMU response is mediated entirely through the NMU2 receptor subtype. However, it does not rule out the possibility of both receptor subtypes being involved in the response of the wild-type tissue, since in the absence of NMU1 receptors the NMU2 receptors may simply still be able to attain the maximal response in that tissue. An NMU2 receptor-selective antagonist would be required to confirm this possibility. Either way, it is evident that the NMU2 receptors play a significant role in the NMU-mediated contraction of the uterus and vas deferens.

Due to the lack of suitable antibodies for the detection of the receptor proteins and potential species differences, a definitive pattern of NMU receptor expression has not yet been determined. That said, a general picture has emerged. In the periphery, significant amounts of NMU-like immunoreactivity (NMU-LIR) have been identified in the gastrointestinal (GI) tract of the rat (Domin *et al*., 1987; Ballesta *et al*., 1988) and guinea-pig (Augood *et al*., 1988; Furness *et al*., 1989) and the genitourinary tract of the rat (including the uterus and vas deferens; Domin *et al*., 1987). While studies clearly show the NMU2 receptor to be predominantly expressed in the CNS (Howard *et al*., 2000; Raddatz *et al*., 2000; Shan *et al*., 2000; Guan *et al*., 2001; Funes *et al*., 2002), peripheral detection has been noted in the GI tract and testes (Shan *et al*., 2000) and based on the tissue distribution of mRNA, the greatest level of NMU2 receptor expression in the rat is found in the uterus (Hosoya *et al*., 2000). Quantitative RT–PCR studies have detected the NMU1 receptor in human CNS (Raddatz *et al*., 2000; Szekeres *et al*., 2000), though Northern blotting and RT–PCR techniques have shown the predominant expression to be peripheral, with highest levels in the small intestine and lung, but also present in other areas of the GI and genitourinary tracts (Fujii *et al*., 2000; Hedrick *et al*., 2000; Howard *et al*., 2000; Raddatz *et al*., 2000; Szekeres *et al*., 2000; Westfall *et al*., 2002).

In this study, we chose to examine NMU1 and NMU2 receptor mRNA expression in uterus and fundus samples from wild-type, heterozygous and NMU1 receptor knockout mice, using quantitative RT–PCR. This choice of tissues was representative of the two distinct pharmacological responses observed in the knockout study; namely, the fundus demonstrating an ablated response to NMU in the knockout mouse and the uterine response to NMU being resistant to NMU1 receptor knockout. It was determined that in the fundus tissue from wild-type mice, NMU1 receptor mRNA, but not NMU2 receptor mRNA, was detectable. The NMU1 receptor mRNA expression level was reduced in the heterozygote mouse (though the response did not reach significance) and below the limit of detection in the knockout mouse, which is consistent with the *in vitro* results, and indicates that the NMU response in this tissue is NMU1 receptor-mediated. NMU2 receptor mRNA expression remained undetectable in the heterozygote and knockout fundus samples. In the wild-type uterus tissue, both NMU1 and NMU2 receptor mRNA expressions were detected. NMU2 receptor mRNA expression was unchanged across the heterozygote and knockout samples, suggesting that there is no compensatory increase in the levels of NMU2 receptor mRNA expression in the NMU1 receptor knockout mouse. However, as was observed in the fundus tissue, NMU1 receptor mRNA levels were reduced in the heterozygote uterine samples and below the limit of detection in the uteri from knockout mice. Despite the presence of NMU1 receptors in the uterus, our *in vitro* studies in the knockout mice demonstrate that the NMU2 receptors alone are sufficient to maintain the full NMU-mediated contractile response.

It is clear from the many knockout studies in the literature that once a target protein has been ablated, many complex compensatory changes can develop as a result; for example, dopamine D1 receptors and NMDA receptors are up-regulated in the D4 knockout model (Gan *et al*., 2004) and β-1-adrenoceptors are up-regulated in the β-3-adrenoceptor knockout mouse (Hutchinson *et al*., 2001). Using RT–PCR, we demonstrated that there did not appear to be any compensatory increases in the NMU2 receptor at the level of the message. However, it is possible that changes in the translation of the NMU2 mRNA to protein or, indeed, alterations in the coupling of the NMU receptor proteins occur as a means of physiological adaptation to gene deletion. Further studies will be required to shed light on these possibilities.

The results obtained in this study, showing NMU-mediated contraction of uterus, stomach (fundus and corpus smooth muscle), LES, ileum, gallbladder and vas deferens, are all consistent with the projected distribution of NMU and its receptors in the GI and genitourinary tracts. The use of NMU1−/− mice allowed us to determine that NMU1 receptors mediated the contractile response to NMU in the mouse fundus and gallbladder assays, and that the response in the uterus and vas deferens was dominated by NMU2 receptors. While it may be likely, based on the receptor distribution described above, that the other peripheral responses to NMU determined in this study are mediated by the NMU1 receptor simply by virtue of the peripheral location of the tissues involved, it should be noted that we could not confirm this in the NMU−/− mice (due to the apparent species differences) and current lack of receptor-selective agents. Therefore, it is still premature to make the assumption that all responses to NMU in the periphery will be mediated by NMU1 receptors.

Although this study emphasizes the differences in the response to NMU across species, it is slowly becoming apparent that there is some overlap between the responses observed in these rodent species and in humans. It has been demonstrated that the longitudinal muscle of the human ileum (Maggi *et al*., 1990), circular muscle of the human ascending colon and longitudinal muscle of the human gallbladder (personal communication; Dr Neil Jones, Dr Simon Hollingsworth)

contract upon stimulation with NMU-8 and NMU-25, respectively, suggesting that, when carefully chosen, some animal models may provide a suitable and useful replacement to human tissue when screening compounds targeted at the NMU receptors. Overall, we have identified a number of novel smooth muscle preparations that respond to NMU and, using the NMU1 receptor knockout mice, implicated the NMU1 receptor subtype in contraction of the stomach fundus and gallbladder and the NMU2 receptor subtype in the contraction of uterus and vas deferens. To further determine the receptor subtype contributing to these novel responses, it will be necessary to characterize these assays using receptor-selective pharmacological tools, which to date have not been reported in the literature.

References

AUGOOD, S.J., KEAST, J.R. & EMSON, P.C. (1988). Distribution and characterization of neuromedin U-like immunoreactivity in rat brain and intestine and in guinea pig intestine. *Regul. Pept.,* **20,** 281–292.

BALLESTA, J., CARLEI, F., BISHOP, A.E., STEEL, J.H., GIBSON, S.J., FAHEY, M., HENNESSEY, R., DOMIN, J., BLOOM, S.R. & POLAK, J.M. (1988). Occurrence and developmental pattern of neuromedin U-immunoreactive nerves in the gastrointestinal tract and brain of the rat. *Neuroscience,* **25,** 797–816.

BENITO-ORFILA, M.A., DOMIN, J., NANDHA, K.A. & BLOOM, S.R. (1991). The motor effect of neuromedin U on rat stomach *in vitro*. *Eur. J. Pharmacol.,* **193,** 329–333.

BOCKMAN, C.S., ABEL, P.W., HICKS, J.W. & CONLON, J.M. (1989). Evidence that neuromedin U may regulate gut motility in reptiles but not in mammals. *Eur. J. Pharmacol.,* **171,** 255–257.

BRIGHTON, P.J., SZEKERES, P.G. & WILLARS, G.B. (2004a). Neuromedin U and its receptors: structure, function, and physiological roles. *Pharmacol. Rev.,* **56,** 231–248.

BRIGHTON, P.J., SZEKERES, P.G., WISE, A. & WILLARS, G.B. (2004b). Signaling and ligand binding by recombinant neuromedin U receptors: evidence for dual coupling to Galphaq/11 and Galphai and an irreversible ligand–receptor interaction. *Mol. Pharm.,* **66,** 1544–1556.

BROWN, D.R. & QUITO, F.L. (1988). Neuromedin U octapeptide alters ion transport in porcine jejunum. *Eur. J. Pharmacol.,* **155,** 159–162.

CAO, C.Q., YU, X.H., DRAY, A., FILOSA, A. & PERKINS, M.N. (2003). A pro-nociceptive role of neuromedin U in adult mice. *Pain,* **104,** 609–616.

CHU, C., JIN, Q., KUNITAKE, T., KATO, K., NABEKURA, T., NAKAZATO, M., KANGAWA, K. & KANNAN, H. (2002). Cardiovascular actions of central neuromedin U in conscious rats. *Regul. Pept.,* **105,** 29–34.

CONLON, J.M., DOMIN, J., THIM, L., DIMARZO, V., MORRIS, H.R. & BLOOM, S.R. (1988). Primary structure of neuromedin U from the rat. *J. Neurochem.,* **51,** 988–991.

DOMIN, J., GHATEI, M.A., CHOHAN, P. & BLOOM, S.R. (1987). Neuromedin U – a study of its distribution in the rat. *Peptides,* **8,** 779–784.

FUJII, R., HOSOYA, M., FUKUSUMI, S., KAWAMATA, Y., HABATA, Y., HINUMA, S., ONDA, H., NISHIMURA, O. & FUJINO, M. (2000). Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J. Biol. Chem.,* **275,** 21068–21074.

FUNES, S., HEDRICK, J.A., YANG, S., LIXIN, S., BAYNE, M., MONSMA, F.J. & GUSTAFSON, E.L. (2002). Cloning and characterization of murine neuromedin U receptors. *Peptides,* **23,** 1607–1615.

FURNESS, J.B., POMPOLO, S., MURPHY, R. & GIRAUD, A. (1989). Projections of neurons with neuromedin U-like immunoreactivity in the small intestine of the guinea-pig. *Cell Tissue Res.,* **257,** 415–422.

GAN, L., FALZONE, T.L., ZHANG, K., RUBINSTEIN, M., BALDESSARINI, R.J. & TARAZI, F.I. (2004). Enhanced expression of dopamine D(1) and glutamate NMDA receptors in dopamine D(4) receptor knockout mice. *J. Mol. Neurosci.,* **22,** 167–178.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990a). Differential effects of neuropeptides on celiac and superior mesenteric blood flows in conscious rats. *Regul. Pept.,* **29,** 215–227.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., DOMIN, J. & BLOOM, S.R. (1990b). Regional hemodynamic effects of neuromedin U in conscious rats. *Am. J. Physiol.,* **258,** R32–R38.

GUAN, X.M., YU, H., JIANG, Q., VAN DER PLOEG, L.H. & LIU, J. (2001). Distribution of neuromedin U receptor subtype 2 mRNA in the rat brain. *Gene Expr. Patterns,* **1,** 1–4.

HANADA, R., NAKAZATO, M., MURAKAMI, N., SAKIHARA, S., YOSHIMATSU, H., TOSHINAI, K., HANADA, T., SUDA, T., KANGAWA, K., MATSUKURA, S. & SAKATA, T. (2001). A role for neuromedin U in stress response. *Biochem. Biophys. Res. Commun.,* **289,** 225–228.

HEDRICK, J.A., MORSE, K., SHAN, L., QIAO, X., PANG, L., WANG, S., LAZ, T., GUSTAFSON, E.L., BAYNE, M. & MONSMA JR, F.J. (2000). Identification of a human gastrointestinal tract and immune system receptor for the peptide neuromedin U. *Mol. Pharmacol.,* **58,** 870–875.

HOSOYA, M., MORIYA, T., KAWAMATA, Y., OHKUBO, S., FUJII, R., MATSUI, H., SHINTANI, Y., KUKUSUMI, S., HABATA, Y., HINUMA, S., ONDA, H., NISHIMURA, O. & FUJINO, M. (2000). Identification and functional characterization of a novel subtype of neuromedin U receptor. *J. Biol. Chem.,* **275,** 29528–29532.

HOWARD, A.D., WANG, R., PONG, S-S., MELLIN, T.N., STRACK, A., GUAN, X-M., ZENG, Z., WILLIAMS, D.L., FEIGHNER, S.D., NUNES, C.N., MURPHY, B., STAIR, J.N., YU, H. & JIANG, Q. (2000). Identification of receptors for neuromedin U and its role in feeding. *Nature,* **406,** 70–74.

HUTCHINSON, D.S., EVANS, B.A. & SUMMERS, R.J. (2001). beta(1)-Adrenoceptors compensate for beta(3)-adrenoceptors in ileum from beta(3)-adrenoceptor knockout mice. *Br. J. Pharmacol.,* **132,** 433–442.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S., TURINI, D., BARBANTI, G., ROVERO, P. & MELI, A. (1990). Motor response of the human isolated small intestine and urinary bladder to porcine neuromedin U-8. *Br. J. Pharmacol.,* **99,** 186–188.

MINAMINO, N., KANGAWA, K. & MATSUO, H. (1985). Neuromedin U-8 and U-25: novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.,* **130,** 1078–1085.

MURPHY, R., TURNER, C.A., FURNESS, J.B., PARKER, L. & GIRAUD, A. (1990). Isolation and microsequence analysis of a novel form of neuromedin U from guinea pig small intestine. *Peptides,* **11,** 613–617.

NAKAZATO, M., HANADA, R., MURAKAMI, N., DATE, Y., MONDAL, M.S., KOJIMA, M., YOSHIMATSU, H., KANGAWA, K. & MATSUKURA, S. (2000). Central effects of neuromedin U in the regulation of energy homeostasis. *Biochem. Biophys. Res. Commun.,* **277,** 191–194.

RADDATZ, R., WILSON, A.E., ARTYMYSHYN, R., BONINI, J.A., BOROWSKY, B., BOTEJU, L.W., ZHOU, S., KOURANOVA, E.V., NAGORNY, R., GUEVARRA, M.S., DAI, M., LERMAN, G.S., VAYSSE, P.J., BRANCHEK, T.A., GERALD, C., FORRAY, C. & ADHAM, N. (2000). Identification and characterization of two neuromedin U receptors differentially expressed in peripheral tissues and the central nervous system. *J. Biol. Chem.,* **275,** 32452–32459.

SAKURA, N., OHTA, S., UCHIDA, Y., KUROSAWA, K., OKIMURA, K. & HASHIMOTO, T. (1991). Structure–activity relationships of rat neuromedin U for smooth muscle contraction. *Chem. Pharm. Bull. (Tokyo),* **39,** 2016–2020.

SHAN, L., QIAO, X., CRONA, J.H., BEHAN, J., WANG, S., LAZ, T., BAYNE, M., GUSTAFSON, E.L., MONOSMA, F.J.J. & HEDRICK, J.A. (2000). Identification of a novel neuromedin U receptor subtype expressed in the central nervous system. *J. Biol. Chem.,* **275,** 39482–39486.

SUMI, S., INOUE, K., KOGIRE, M., DOI, R., TAKAORI, K., SUZUKI, T., YAJIMA, H. & TOBE, T. (1987). Effect of synthetic neuromedin U-8 and U-25, novel peptides identified in porcine spinal cord, on splanchnic circulation in dogs. *Life Sci.,* **41,** 1585–1590.

SZEKERES, P.G., MUIR, A.I., SPINAGE, L.D., MILLER, J.E., BUTLER, S.I., SMITH, A., RENNIE, G.I., MURDOCK, P.R., FITZGERALD, L.R., WU, H.-L., MCMILLAN, L.J., GUERRERA, S., VAWTER, L., ELSHOURBAGY, N.A., MOONEY, J.L., BERGSMA, D.J., WILSON, S. & CHAMBERS, J.K. (2000). Neuromedin U is a potent agonist at the orphan G protein-coupled receptor FM3. *J. Biol. Chem.,* **275,** 20247–20250.

WESTFALL, T.D., MCCAFFERTY, G.P., PULLEN, M., GRUVER, S., SULPIZIO, A.C., AIYAR, V.N., JYOTI, D., CONTINO, L.C., MANNAN, I.J. & HIEBLE, J.P. (2002). Characterization of neuromedin U effects in canine smooth muscle. *J. Pharmacol. Exp. Ther.,* **301,** 987–992.

WREN, A.M., SMALL, C.J., ABBOTT, C.R., JETHWA, P.H., KENNEDY, A.R., MURPHY, K.G., STANLEY, S.A., ZOLLNER, A.N., GHATEI, M.A. & BLOOM, S.R. (2002). Hypothalamic actions of neuromedin U. *Endocrinology,* **143,** 4227–4234.

YU, X.H., CAO, C.Q., MENNICKEN, F., PUMA, C., DRAY, A., O'DONNELL, D., AHMAD, S. & PERKINS, M. (2003). Pronociceptive effects of neuromedin u in rat. *Neuroscience,* **120,** 467–474.

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