Physiol Genomics 45: 89–97, 2013.
First published December 4, 2012; doi:10.1152/physiolgenomics.00070.2012.

Functional genomics of the rat neuromedin U receptor 1 reveals a naturally occurring deleterious allele

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Submitted 24 May 2012; accepted in final form 4 December 2012

Panetta R, Meury L, Cao CQ, Puma C, Mennicken F, Cassar PA, Laird J, Groblewski T. Functional genomics of the rat neuromedin U receptor 1 reveals a naturally occurring deleterious allele. *Physiol Genomics* 45: 89–97, 2013. First published December 4, 2012; doi:10.1152/physiolgenomics.00070.2012.—Neuromedin U (NMU) plays an important role in a number of physiological processes, but the relative contribution of its two known receptors, NMUR1 and NMUR2, is still poorly understood. Here we report the existence of a SNP $T^{1022} \rightarrow A$ ($Val^{341} \rightarrow Glu$) in the third exon of the rat *Nmur1* gene that leads to an inactive receptor. This SNP is present within the coding region of the highly conserved NPXXY motif found within all class A type G protein-coupled receptors and translates to an NMUR1 receptor that is not expressed on the cell surface. Genetic analysis of the *Nmur1* gene in a population of Sprague-Dawley rats revealed that this strain is highly heterogeneous for the inactivating polymorphism. The loss of functional NMUR1 receptors in Sprague-Dawley rats homozygous for the inactive allele was confirmed by radioligand binding studies on native tissue expressing NMUR1. The physiological relevance of this functional genomics finding was examined in two nociceptive response models. The pronociceptive effects of NMU were abolished in rats lacking functional NMUR1 receptors. The existence of naturally occurring NMUR1-deficient rats provides a novel and powerful tool to investigate the physiological role of NMU and its receptors. Furthermore, it highlights the importance of verifying the NMUR1 single nucleotide polymorphism status for rats used in physiological, pharmacological or toxicological studies conducted with NMUR1 modulators.

neuromedin U; rat gene polymorphism; nociception; GPCR structure-function

NEUROMEDIN U (NMU) is a bioactive peptide first isolated from porcine spinal cord (28) in 1985. Although different species express different molecular forms of NMU (1), all NMU peptides share a common COOH-terminal segment of eight residues essential for their biological activity (14). NMU administration in vivo has numerous effects including smooth muscle contraction (24, 27, 46), blood pressure changes (5, 27), modulation of neurohypophysial hormone levels (10, 25, 35, 48), feeding (18, 20, 21, 33), gastric secretion (30), and nociception (4, 32, 50) (for complete review see Refs. 1, 29). NMU also exhibits antiproliferative effects on an esophageal squamous carcinoma cell line (49).

Through extensive ligand identification campaigns, two orphan G protein-coupled receptors (GPCRs), FM-3 (or GPR66) and FM-4 (or TGR-1), were identified as neuromedin U receptor-1 (NMUR1) and -2 (NMUR2), respectively (9, 15, 17, 18, 20, 37, 41, 43). NMUR1 mRNA is predominantly found in peripheral tissues (9, 15, 19, 50) including lung, GI tract, pancreatic islets, testis, adipose, immune tissues, and dorsal root ganglia, whereas NMUR2 mRNA appears to be mainly expressed in the central nervous system (CNS) (1, 10, 13, 18, 37, 43). The human genes for *Nmur1* and *Nmur2* have been mapped to SHGC-33253 (chromosome 2q34-q37) and SHGC-8848 (chromosome 5q31.1-q31.3), respectively (37). The rat genes have been mapped to chromosome 9q35 and 10q22 for *Nmur1* and *Nmur2,* respectively, as reported by the National Center for Biotechnology Information Rat Genome Resources website (http://www.ncbi.nlm.nih.gov/genome/guide/rat/). Both receptors are members of the class A or rhodopsin-like GPCR subfamily harboring the highly conserved D/ERY and NPXXY sequences located at the end of the third and seventh transmembrane domains. The NPXXY sequence is essential for the structural and functional integrity of Rhodopsin-like GPCRs (8). Evidence for dual coupling to $G\alpha_{q/11}$ and $G\alpha_i$ proteins has been reported for both receptors (2). Activation of NMUR1 and NMUR2 stimulates inositol-triphosphate and ERK phosphorylation pathways in a pertussis toxin-insensitive manner and inhibits adenylyl cyclase via a pertussis toxin-sensitive mechanism. NMU binds with equal subnanomolar affinities to either receptor subtype (2, 18, 38); however, the relative role of each receptor in the biological effects of NMU is poorly understood. Advances in understanding the biological role of each NMUR subtype have been limited to the use of knockout mice and comparative expression studies (for review Ref. 29).

A structurally related peptide to NMU, known as neuromedin S (NMS), has recently been identified and proposed as a second ligand for these receptors (31). NMS has been reported to bind with greater affinity to NMUR2, compared with NMU (31). To date, NMU and NMS appear to functionally have overall similar physiological actions (29, 52).

Our interest in NMUR1 as a potential drug target prompted us to establish cellular recombinant expression systems to develop binding and functional assays. In the course of these studies, we discovered a naturally occurring rat single nucleotide polymorphism (SNP) within the coding region of the highly conserved NPXXY motif that translates to a nonfunctional NMUR1 receptor that is not expressed on the cell surface. Analysis of the *Nmur1* gene in a commercially available population of Sprague-Dawley rats revealed that this strain is highly heterogeneous for the inactivating polymorphism. Rats homozygous for the *Nmur1* variant allele were healthy and viable but devoid of membrane-expressed NMUR1

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as shown by the loss of specific NMU binding in the lung. Given the established pronociceptive effects of NMU, we confirmed the loss of function in vivo by examining the physiological consequences of expression of the variant allele using behavioral and electrophysiological nociceptive models. Results obtained in both models revealed that NMU could no longer exert its pronociceptive effects in rats lacking the functional NMUR1 receptor.

MATERIALS AND METHODS

Cloning and sequencing of rat NMUR1 variants. NMUR1 coding region was amplified by PCR from rat lung cDNA (Clontech) as follows: 3 min at 95°C, 30 cycles of 95°C for 30s, 60°C for 45 s, and 72°C for 1 min in the presence of 0.2 mM dNTPs, 50 pmol of forward (5′-ATGCTCTCCCCAAATGCTTCAAC-3′) and reverse (5′-TTATTCAGGAGGGTCTGTCTCTTG-3′) primers and 1 U of TAQ DNA polymerase (GE Healthcare, Amersham). The 1.2 kb PCR fragment obtained was cloned into the pGEM-T vector (Promega) and sequenced using ABI BigDye technology (Applied Biosystems, Life Technologies).

Generation of expression constructs. The identified rat NMUR1 PCR fragment was subcloned in the expression vector pcDNA3.0 (Invitrogen) by endonuclease restriction digestion and subsequently used as a template to generate expression constructs exhibiting the four possible point mutation combinations. Transformer site-directed mutagenesis (Clontech) was used following the manufacturer's protocol and the appropriate mix of the following primers: 5′-GCTAACCCGGTGCTCTACAACCTC-3′ (A^{1022}→T); 5′-GAGGGAGAGGATTTTGCTCCAAGAGG-3′ (A^{750}→G).

Heterologous expression of rat NMUR1 constructs. All rat NMUR1 receptor variants were transiently expressed in HEK-293s cells using Lipofectamine 2000 transfection (Invitrogen) following manufacture's protocol. Cells were tested in various assays 48 h posttransfection.

Calcium mobilization. Activation of the receptor by NMU was measured using a calcium mobilization assay on a FLIPR platform as previously described (23). Transfected cells were loaded with 2 μM FLUO-3 AM (TEF Labs, Austin, TX) for 1 h and washed extensively with HBSS buffer containing 20 mM HEPES pH 7.4/0.1% BSA. Fluorescence was measured upon addition of NMU-8 (Bachem Bioscience) at various concentrations. EC_{50} values were calculated with PRISM (GraphPad, La Jolla, CA) software using the nonlinear regression analysis method.

Immunocytochemistry and confocal microscopy. The rat NMUR1 variants Val^{341} and Glu^{341} cDNAs were subcloned into the NH_2-terminal FLAG expression vector, pCMV-Tag2A (Stratagene, San Diego, CA), which were used for transient transfection into HEK-293s cells and subsequent plating onto poly-D-lysine-coated glass coverslips. To exclusively visualize cell surface expression of the FLAG constructs, cells were initially incubated at 4°C with 20 μg/ml murine anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), subsequently fixed with cold 4% paraformaldehyde (PFA), exposed to 1% BSA/5% goat serum blocking solution at room temperature, and lastly treated with goat anti-mouse Alexa 488-conjugated secondary antibody (Life Technologies, Molecular Probes) at 1:400 dilution. To visualize intracellular expression of the tagged receptors, cells were initially fixed with 4% PFA, permeabilized for 10 min with 0.3% Triton X-100, treated with 5 μg/ml of murine anti-FLAG M2 monoclonal antibody solution at room temperature, and lastly exposed to both the secondary antibody, Alexa Fluor 488 F(ab)′ goat anti-mouse antibody (1:400 dilution) and the nuclear stain DRAQ5 (Alexis Biochemicals). Coverslips were mounted with Fluorsave (Calbiochem) and observed under a Leica DM IRBE laser scanning confocal microscope (Leica). Images were captured at ×40 magnification in oil immersion and displayed on screen by the Leica TCS NT software.

Genotyping. Genotyping was carried on genomic DNA extracted from blood of 244 Sprague-Dawley rats using QIAamp DNA Blood kit (Qiagen). A FRET-based real-time PCR protocol on LightCycler (Roche Diagnostics) previously described (38) was used to detect the two possible nucleotides at position 1022. Briefly, a 353 bp fragment encompassing position 1022 was amplified using the sequences 5′-GCTGGGTTCCATTCCATGCTGACC-3′ and 5′-TTATTCAGGAGGGTCTGTCTCTTG-3′ as forward and reverse primers, respectively. The PCR reaction includes 2 detection probes: 5′-RED640-GCCGCTGAGCCGAGGTACAAG-p-3′ and 5′-TGAGGTTGTAGAGCTCCGGGTT-Fluorescin-3′ (Proligo, Paris, France) that emit fluorescence when hybridizing at the polymorphism site on the sense strand. The reaction is carried out in glass capillaries and includes 100 ng of DNA, 0.5 μM of primers, 0.2 μM of probes, 1 mM $MgCl_2$, and 2 μl of 10× LC FastStart DNA Master Hybridization probe mix (Roche Diagnostics) in a final volume of 20 μl. The protocol was as follows: enzyme activation at 96°C for 10 min, amplification at 96°C for 0 s (20°C/s), 58°C for 15 s (20°C/s), and 72°C for 16 s (20°C/s) for 35 cycles. We then ran a renaturation curve as follows: 96°C for 0 s (20°C/s), 80°C for 30 s (20°C/s), and a slow ramping to 52°C at 0.05°C/s. Melting temperatures of the probe were 62 and 66°C when hybridizing to the allelic forms T^{1022} and A^{1022}, respectively, as calculated by plotting the derivative of the renaturation curve against the temperature. Validation of this assay was confirmed by sequencing the PCR products.

In situ hybridization. Rats expressing the NMUR1 wild-type variant were euthanized by decapitation, and the lung tissue was rapidly dissected and snap-frozen for sectioning. Fresh frozen lung tissue was transversely cryosectioned at 14 μm and thaw-mounted onto Superfrost Plus slides (VWR, Montreal, Quebec, Canada). Slides were stored at −80°C until processed for in situ hybridization (ISH). A full-length riboprobe for rat NMUR1 (1239 pb; amplified from rat lung polyA+ RNA) and a partial riboprobe for rat NMUR2 (743 pb; 5′ coding region amplified from rat brainstem/spinal cord cDNA library) were transcribed in vitro using appropriate RNA polymerases (Promega), in the presence of [^{35}S]UTP and [^{35}S]CTP (~800 Ci/mmol; Amersham, Oakville, Ontario, Canada). ISH was carried out as previously described (26), and hybridized sections were exposed to Kodak Biomax MR film for 2 wk.

Radioligand binding. Tissue membrane preparations and radioligand binding were performed as previously described (12, 23). Three individual rats homozygous for either the NMUR1 active or inactive allele were used for membrane preparation. Membranes were isolated separately from each rat lung and tested for binding individually. We used 30 μg of lung membrane protein per well in a 96-well format plate and incubated it with increasing amount of rat ([^{125}I]Tyr)-NMU-23 (2,200 Ci/mmol, radiolabeled in-house) with or without 10 μM of unlabeled NMU-23. K_d and Bmax values were calculated with PRISM (GraphPad) software by the nonlinear regression analysis method.

In vivo experiments. Male adult Sprague-Dawley rats [250–280 g from Charles River SD colonies (Crl: CD), Canada] were used in this study. All procedures and experimental manipulations were conducted according to protocols that have been approved by the AstraZeneca ethical committee, whose members include a certified veterinary physician and senior bench scientists working with animals. The animals were kept and experiments were performed at the AstraZeneca site in Montréal, which has accreditation from the Canadian Council on Animal Care, the Association for the Assessment and Accreditation of Laboratory Animal Care and approval by the AstraZeneca Global Veterinary Council for Study Conduct. All in vivo experiments were performed by experimenters blind to the genotype of the test subjects.

Rat nociceptive behavior. NMU-23 (Bachem Bioscience) was dissolved in pH 6.5 sterile saline and 3.8 nmol or 10 μg/10 μl was delivered by intradermal injection into the paw. The drug solution for delivery was prepared daily before the experiment and conserved on ice. Rats were acclimatized to the test room for at least 1 h prior to testing. Nociception was assessed using the plantar test. The nociceptive effect of NMU was determined according to the latency (in seconds) of hind paw withdrawal to a noxious radiant heat stimulus (mean of 2 trials). The cut-off value of plantar test was set to 25 s to prevent tissue injury. The plantar test was performed at before (baseline) and 30 min after the intradermal administration. Data expressed as means ± SE were analyzed by either a *t*-test or ANOVA followed by the post hoc Bonferroni *t*-test multiple-comparisons test. Differences between means were considered statistically significant if $P < 0.05$ (***$P < 0.001$).

In vivo electrophysiology. Extracellular recordings of flexor α-motoneuron activity were made from a filament of the nerve to the posterior biceps femoris-semitendinosus muscles in decerebrate-spinal rats as previously described (50). The excitability of the flexor reflex was determined by measuring efferent activities evoked by touch, pinch, and heat (52°C water) stimuli applied to the hind paw. NMU-23, prepared in sterile saline at pH 7.4, was administered by slow infusion intrathecally (5 nmol/10 μl) followed by a saline flush (50). The nociceptive responses were analyzed as the total number of action potentials counted, minus the extrapolated spontaneous activity over the same period. Statistical analysis was performed as previously described (50).

RESULTS

Polymorphisms of the rat Nmur1 receptor gene. Two different primary sequences for the rat NMUR1 receptor, previously known as FM-3 or GPR66, are registered in GenBank under the accession numbers AF242873 (18) and AB038649 (9). Alignment of the two primary sequences revealed three significant differences. The AF242873 sequence lacks the first 10 residues at the NH_2 terminus of the AB038649 sequence, does not contain the expected initiation codon, and is likely an incomplete open reading frame. The AB038649 sequence, containing 412 residues, likely corresponds to the full-length NMUR1 receptor protein. This latter sequence contains methionine and glutamic acid residues at positions 250 and 341, respectively, whereas isoleucine and valine residues are found at the equivalent positions in AF242873 sequence (Fig. 1). PCR amplification and sequencing of rat NMUR1 complete open reading frame cDNA from commercial sources confirmed the existence of these two polymorphic sites (G^{750}→A and T^{1022}→A) in the rat *Nmur1* gene coding region (data not shown). Searching the human and mouse public SNP databases revealed that these polymorphic variants are rat specific with no equivalent found in the human and mouse coding sequences.

Characterization of rat NMUR1 Ile^{250}→Met and Val^{341}→Glu variants. Our interest in NMUR1 as a potential drug target prompted us to establish cellular recombinant expression systems to develop binding and functional assays. Our first attempts to express the rat NMUR1 receptor in transfected cell lines were conducted with the AB038649 sequence, but no functional expression was obtained as judged by the lack of intracellular calcium responses to NMU application. We predicted that the ablated function was associated with one, or both, of the variant residues at position 250 and 341 (Fig. 1). We therefore decided to explore the functional consequences of these two naturally occurring amino acid substitutions in the rat NMUR1 receptor. Proteins with the four possible permutations at position 250 and 341 were transiently expressed in HEK-293s cells, and mobilization of intracellular calcium in response to NMU-8 challenge was used to assess their function. As shown in Fig. 2*A*, the presence of methionine or isoleucine residue at position 250 does not affect the functionality of the receptor, whereas the presence of glutamic acid residue at position 341 led to a nonfunctional receptor unable to respond despite the high concentration of agonist applied. Concentration-response analysis of the Val^{341} or Glu^{341} variants (Fig. 2*B*) confirmed these initial observations. NMU-8 displays subnanomolar potency at the Val^{341} construct, whereas the Glu^{341} construct does not respond to NMU-8 at concentrations up to 10 μM.

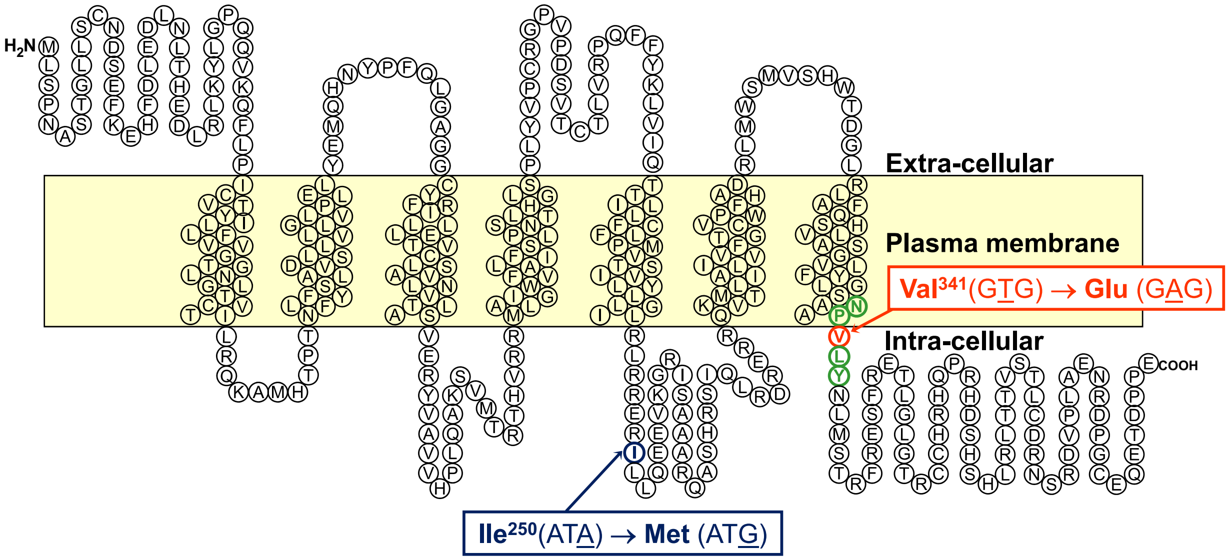

Fig. 1. Schematic representation of the rat neuromedin U receptor-1 (NMUR1) primary sequence. The diagram highlights the position of the 2 naturally occurring single nucleotide polymorphisms (SNPs, underlined in codon sequence shown in parentheses) leading to amino acid changes found in the rat NMUR1 receptor. The residue at position to 250 is located within the 3rd intracellular loop, and the residue at position 341 is part of the highly conserved NPXXY motif in class A or rhodopsin-like G protein-coupled receptor.

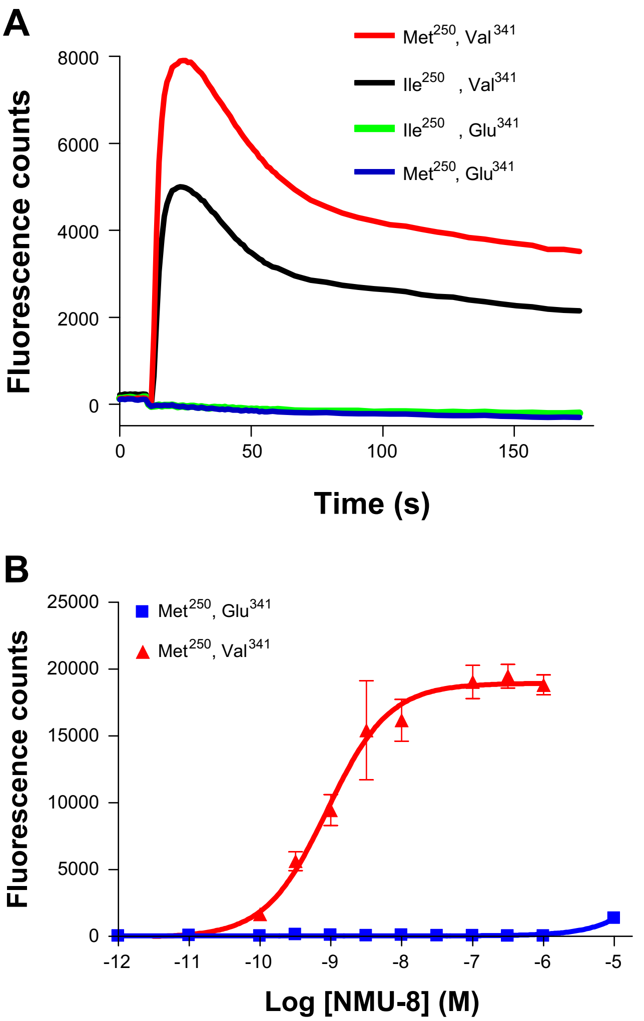

Fig. 2. Calcium mobilization induced by neuromedin U (NMU) on cells transiently expressing the various rat NMUR1 polymorphic variants. HEK-293s cells stably expressing the chimeric G protein Gαqi5 were transiently transfected by various plasmids coding for the rat NMUR1 polymorphic variants and challenged with neuromedin U (NMU-8). *A:* Cells expressing the 4 possible amino acid combinations at position 250 and 341 were challenged with 1 μM NMU-8. Receptors with a glutamic acid at position 341 showed no response to the NMU-8 stimulation regardless of the amino acid found at position 250. *B*: illustration of a full concentration response evoked by NMU-8 stimulation of the Met^{250}, Glu^{341} (blue square) and Met^{250}, Val^{341} (red triangle) variants. Whereas NMU-8 displayed a high potency ($EC_{50} = 0.9 \pm 0.5$ nM) at the Val^{341}-rat NMUR1 receptor, no agonist effects were evident on the Glu^{341}-rat NMUR1 variant.

We next explored whether the nonfunctional variant was able to bind NMU, despite the lack of functional effect. Radioligand binding studies were performed on membranes from cells transfected with either the Val^{341} or Glu^{341} variants. Whereas the Val^{341} receptor displayed the expected binding profile, no specific binding could be detected when the plasmid harboring the Glu^{341} variant was expressed in the cells (data not shown). This suggested that the nonfunctional receptor may not be expressed on the cell membrane; we thus assessed the cell surface expression of the two position 341 receptor variants. Since no receptor-specific antibody was available, we introduced the 12 amino acid Flag epitope at the NH$_2$-terminal receptor extremity and studied cellular localization by immunocytochemistry. Confocal microscopy of HEK-293s cells transiently transfected with the two NH$_2$-flag rat NMUR1 constructs (Fig. 3, *A* and *B*) revealed that the Glu^{341} variant does not reach the plasma membrane, whereas the functional Val^{341} variant does. Permeabilized cells (Fig. 3, *C* and *D*) showed intracellular staining for both variants, demonstrating that the absence of surface expression of the Glu^{341} mutant is not due to a failure to synthesize the protein but is likely a receptor trafficking defect.

Frequency of Val^{341}→Glu substitution for NMUR1 in Sprague-Dawley rats. Extensive bioinformatics analysis of the published rat genomic DNA sequence (RGSC v3.4 and Celera assembly) suggests a unique copy of *Nmur1* gene in the rat genome located on chromosome 9 (9q35) and a pseudogene with 90% homology on chromosome 1. The amino acid change at position 341 is caused by the SNP T^{1022}→A converting the valine codon GTG into the glutamic acid codon GAG (Fig. 1). A genotyping assay based on melting point assessment of specific fluorescent oligonucleotide probes (38) was developed to screen rat genomic DNA samples for the inactivating SNP T^{1022}→A. This method allows for the simultaneous analysis of T^{1022}→A^{1022} nucleotide changes for both alleles and was used to genotype a population of 244 Sprague-Dawley rats. We found that 47% of animals were homozygous for the active allele T^{1022} (Val^{341}), 40% were heterozygous and expressed both variants (Val^{341} and Glu^{341}), and 13% of the population was homozygous for the inactive allele A^{1022} (Glu^{341}), identifying the frequency of the minor allele as 0.33 in this population. The distribution is not significantly outside of the Hardy-Weinberg equilibrium ($\chi^2 = 2.27$, where threshold for $P < 0.05 = 3.84$), suggesting that there is no overt selection on the mutation.

This genotyping analysis demonstrated that it is possible to find, in a given rat Sprague-Dawley colony, animals carrying two inactive alleles for the *Nmur1* gene. These animals, theoretically devoid of any functional NMUR1 receptor, can be considered as natural NMUR1-deficient rats. From this point, we refer to the active allele as the rat *Nmur1* T^{1022} allele (Val^{341}) and to the inactive allele as rat *Nmur1* A^{1022} allele (Glu^{341}).

Characterization of NMUR1 receptor variants in rat tissue. To characterize the native membrane expression of the rat NMUR1 variants by radioligand binding, we chose lung as a tissue since it was initially reported to express NMUR1 mRNA at higher levels compared with NMUR2 in rat lung in a quantitative RT-PCR approach (9, 10, 17). We confirmed the expression of NMUR1 and NMUR2 in fresh-frozen lung sections from rats homozygous for the *NMUR1* T^{1022} active allele by ISH using specific radiolabeled riboprobes. As shown (Fig. 4, *A* and *B*), NMUR1 mRNA is strongly expressed throughout the slice of lung tissue examined, while the same was not observed for NMUR2 in an adjacent lung section since we were unable to identify specific labeling for NMUR2 as we have for NMUR1. Absence of ISH labeling for NMUR2 was confirmed by cellular analysis of the tissue sections after dipping in an autoradiographic emulsion (data not shown). With quantitative RT-PCR, it was reported that rat lung has low-level NMUR2 mRNA expression (10, 17). It is possible that the specific signal for NMUR2 may be too low to detect by our ISH approach. The ISH data reveal that NMUR1 mRNA is expressed at much higher levels in rat lung compared with NMUR2, allowing us to use lung as a control tissue to assess

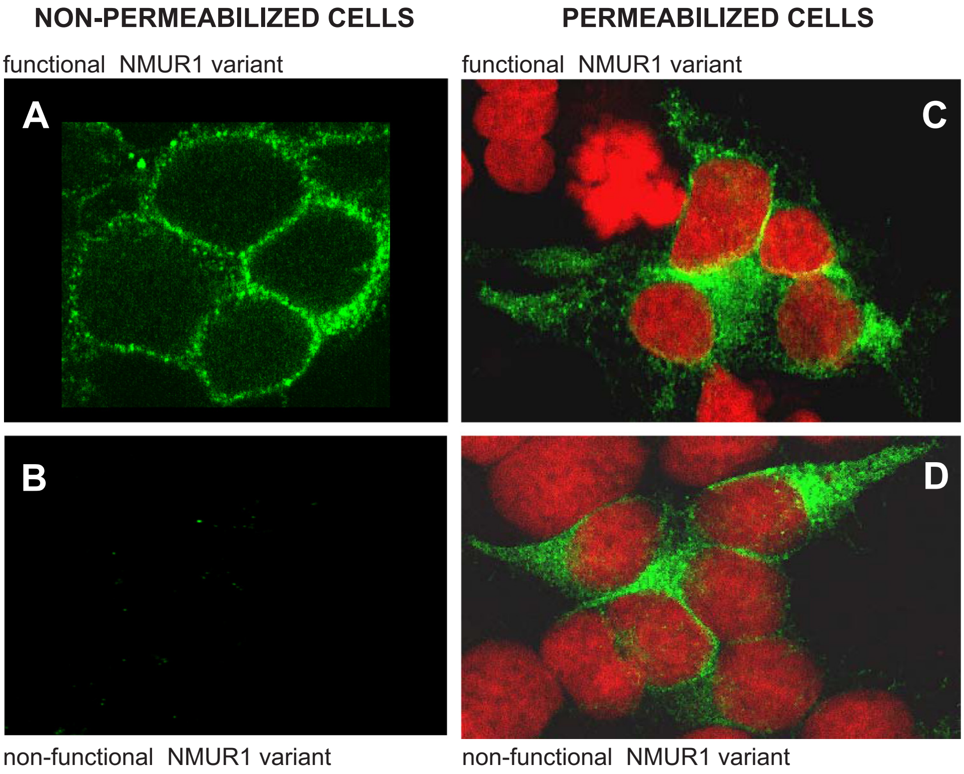

Fig. 3. Cell surface and intracellular expression of Glu^{341} and Val^{341} rat NMUR1 receptor variants in transfected cells using immunocytochemistry and confocal microscopy. *A* and *B*: cell surface expression of the Val^{341} and the Glu^{341} N-FLAG rNMUR1 variants, transiently expressed in HEK-293s cells, was assessed by immunocytochemistry of non-permeabilized cells. Whereas the Val^{341} (*A*) variant shows the expected plasma membrane expression, no cell surface expression could be detected for the Glu^{341} variant (*B*). *C and D*: under permeabilized conditions in the presence of the nuclear staining dye DRAQ5, intracellular expression can be detected for both variants, Val^{341} in *C* and Glu^{341} in *D*, indicating that the 2 proteins are synthesized normally. Confocal microscopic images shown are representative of 50–100 cells per experiment (n = 3) viewed at ×40 magnification in oil immersion using a Leica DM IRBE laser scanning confocal microscope.

the membrane expression of the NMUR1 variants by radioligand binding without having significant inference from NMUR2. Membrane extracts from rat uterus are known to display specific and high maximal binding capacity for NMU (34), but the reported high expression of NMUR2 mRNA compared with NMUR1 (9, 17) in rat uterus did not make it an ideal native tissue for this study.

The presence of functional NMUR1 receptor was examined by measuring NMU binding in lung membrane preparations from the homozygous NMUR1 variant rats. Lung membranes prepared from rats homozygous for the *NMUR1* T^{1022} active allele displayed a specific and saturable [^{125}I]NMU-23 binding (Bmax = 70 fmol/mg, K_d = 0.83 ± 0.08 nM), whereas no specific nor saturable binding could be observed on lung membranes prepared from rats homozygous for the *NMUR1* A^{1022} inactive allele (Fig. 4*C*).

Contribution of NMUR1 to the pronociceptive effects of NMU. When injected into the skin NMU induces spontaneous pain-like behaviors and hypersensitivity to external painful stimuli in rats (50). These effects are presumed to be mediated by NMUR1 receptors, since NMUR2 expression is largely restricted to the CNS. We explored the impact of the variant alleles on the expected effects of peripheral NMU injection in rats homozygous for each of the variants. There were no differences in the responses of either group of rats to a painful heat stimulus applied to the paw (baseline bar graph, Fig. 5*A*). After an intradermal administration of NMU into the paw, the rats homozygous for the active *Nmur1* allele T^{1022} exhibited significant heat hypersensitivity (Fig. 5*A*). In contrast, the pronociceptive effect of intradermal NMU was absent in homozygous rats for the inactive *Nmur1* allele; paw withdrawal latency remained unaffected by the injection of NMU and was comparable to the baseline (Fig. 5*A*).

Administration of NMU to the spinal cord in the rat also has pronociceptive effects, manifested both behaviorally in awake animals and as increased spinal reflex excitability as measured in in vivo electrophysiological preparations (50). The NMU receptor underlying this pronociceptive effect is unclear, since NMUR2 is expressed by intrinsic neurones in the spinal cord, whereas NMUR1 is expressed by peripheral afferent neurons that innervate the spinal cord (50). The pronociceptive effects of NMU at the spinal cord level were investigated with in vivo electrophysiology recordings using the rat flexor-reflex model in rats homozygous for each of the variant alleles. Extracellular recordings of flexor α-motoneuron activity were made in decerebrate-spinal rats. We determined the excitability of the flexor reflex by measuring the number of action potentials evoked by touch, pinch, and heat stimuli applied to the hind paw. As expected, intrathecal administration of NMU markedly increased the excitability of nociceptive flexor reflex in rats homozygous for the active *Nmur1* gene allele T^{1022}. This was evidenced by a significant increase in heat (Fig. 5*B*), touch (Fig. 5*C*), and pinch (Fig. 5*D*) responses of the flexor reflex. However, intrathecal injection of NMU in rats homozygous for the inactive *Nmur1* gene allele A^{1022} had no effect on the excitability of nociceptive flexor reflex for any of the stimuli (Fig. 5, *B–D*).

DISCUSSION

The rat has been used primarily as a physiological model, but with the advent of the Rat Genome Project, it is emerging as a genetic model. The physiological relevance of the rat genomic information made available is being explored by either genome-wide association studies or functional genomics. These two approaches can probe the impact of naturally

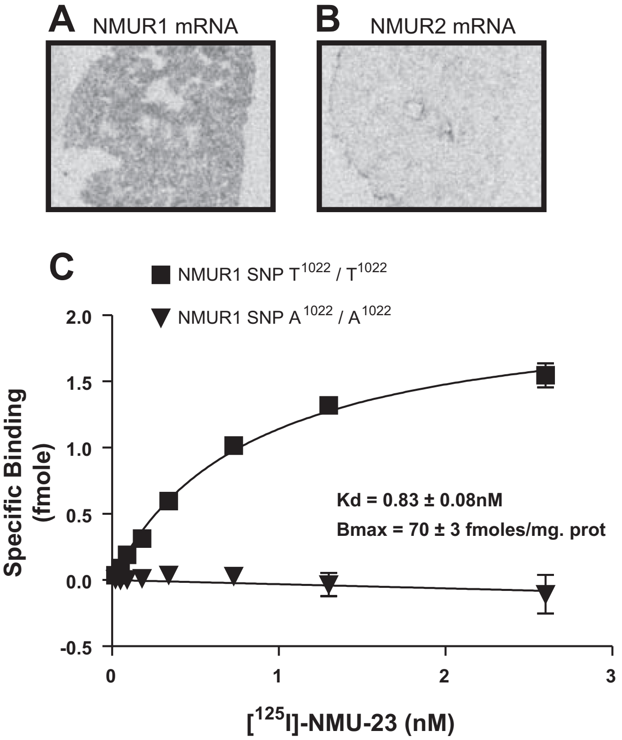

Fig. 4. NMUR1 expression in rat lung using in situ hybridization (ISH) and radioligand binding. *A*: positive ISH film autoradiography labeling of NMUR1 mRNA on lung tissue from rats homozygous for the *NMUR1* active allele T^{1022}. *B*: no specific ISH labeling for rat NMUR2 mRNA in an adjacent lung section. *C*: saturation binding analysis conducted with $[^{125}I]$NMU23 on rat lung membranes revealed an absence of saturable binding sites on rats homozygous for the *NMUR1* A^{1022} inactive allele (▼). $[^{125}I]$NMU23 binding on lung membranes from rat homozygous for the T^{1022} allele (■) was saturable (Bmax = 70 ± 3 fmol/mg protein) with a K_d value of 0.83 ± 0.08 nM. Each symbol represents a mean value with an SE based on individual data obtained using lung membrane from $n = 3$ rats homozygous for the NMUR1 active or inactive allele.

occurring SNPs in this important model organism. Indeed over the last decade, a large number of SNP genotypes have been discovered not only for human and mouse but also for rat (for reviews see Refs. 3, 7, 39, 47).

Outbred rodent strains used as laboratory models, such as Sprague-Dawley, are maintained under conditions where many phenotypes are not under active selection. Hence, it is possible for mutations that are nonlethal, but otherwise expected to be rare, to accumulate and become fixed at high levels in particular strains or colonies. This presumably occurs by genetic drift acting on a relatively high level of inbreeding. These mutations offer the possibility to study functional genetic change, akin to a human monogenic trait. Here we have applied functional genomics to study the impact of the SNP found in the coding sequence of the *Nmur1* gene in Sprague-Dawley rats.

The existence of two rat-specific SNPs in the coding sequence of the *Nmur1* gene prompted us to explore their impact on receptor function, pharmacology, and in vivo physiology since the NMU system has been shown to play an important role in numerous biological areas. We present evidence that the SNP G^{750}→A (substitution of methionine residue at position 250 by an alanine) does not produce any apparent changes in the functionality of the NMUR1 receptor, whereas the other SNP T^{1022}→A (replacement of valine residue at position 341 by a glutamic acid residue) generates a nonfunctional receptor. The substitution of the Val^{341} residue by a charged amino acid, within the highly conserved NPXXY motif of class A GPCR, prevents the proper receptor trafficking to the plasma membrane and the response to agonist stimulation in vitro. Conservation of the NPXXY motif is one of the key characteristics of the class A or rhodopsin-like GPCR subfamily (11), and hydrophobic residues (Val, Leu, Iso, Ala, or Phe) are commonly found at the position adjacent to the conserved proline residue. Since charged or polar residues are not found at this position within the NPXXY motif for any mammalian class A rhodopsin-like GPCR (see Ref. 16 for online sequence alignment), the Val^{341}→Glu mutation can be expected to have a major impact on the protein structure. The very high degree of motif conservation across the GPCR rhodopsin-like subfamily suggests a key structural and functional role (42). This role was indeed verified for rhodopsin where it was shown to be essential for the stabilization of the ground and activated states of the protein (8, 36).

The in vitro demonstration of the combined loss of protein function and proper plasma membrane expression of the rat NMUR1 Glu^{341} variant led us to identify rats homozygous for the *Nmur1* inactivating allele A^{1022}, as naturally occurring NMUR1-loss of function rats. The membranes prepared from the lung of these rats no longer bind radiolabeled NMU, whereas those from the animals carrying the two active alleles T^{1022} displayed high affinity for $[^{125}I]$NMU-23.

Several functional polymorphisms in the rat genome have already been reported. A total of 16 functional polymorphisms linked to three coat color and 13 disease-associated genes in inbred rat strains have been characterized altogether with their allele frequencies in commercially available outbred stocks (22). Other examples of naturally occurring inactivating SNPs or gene insertion/deletion associated with marked phenotypic change have also been discovered in rat. For instance, the insipidus diabetes found in Long-Evans rats (Brattleboro rats) has been linked to a G nucleotide deletion in the vasopressin precursor gene, giving rise to an inactive hormone (40). A mutation in the rat protein kinase Cγ gene generating a premature stop codon was found to be the cause of the altered behavior and brain pathology resembling human Parkinsonian syndromes observed in Albino Swiss/AGU rats (6). Naturally occurring CCK-1R gene-deficient rats have also been discovered in the Otsuka Long-Evans Tokushima Fatty rat strain (44). Deletion of a part of the promoter region and the first two exons of the rat CCK-1 receptor gene is associated with adult-onset diabetes and obesity observed in this rat strain.

To explore the potential phenotypic changes arising for this identified inactivating SNP on *Nmur1*, we chose to examine the impact that allele A^{1022} may have on the NMU pronociceptive response. The physiological significance of the *Nmur1* inactivating allele A^{1022} was investigated in electrophysiological and behavioral nociceptive response models, exploring both spinal and peripheral mechanisms. On the basis of the known rat tissue expression profile of NMUR1 and NMUR2 receptors (50), it appears that NMU can act on these receptors either in the peripheral or CNS to produce its pronociceptive effects. In the absence of selective pharmacological tools, knockout studies in mice have been used to show that NMUR2 plays a predominant role in

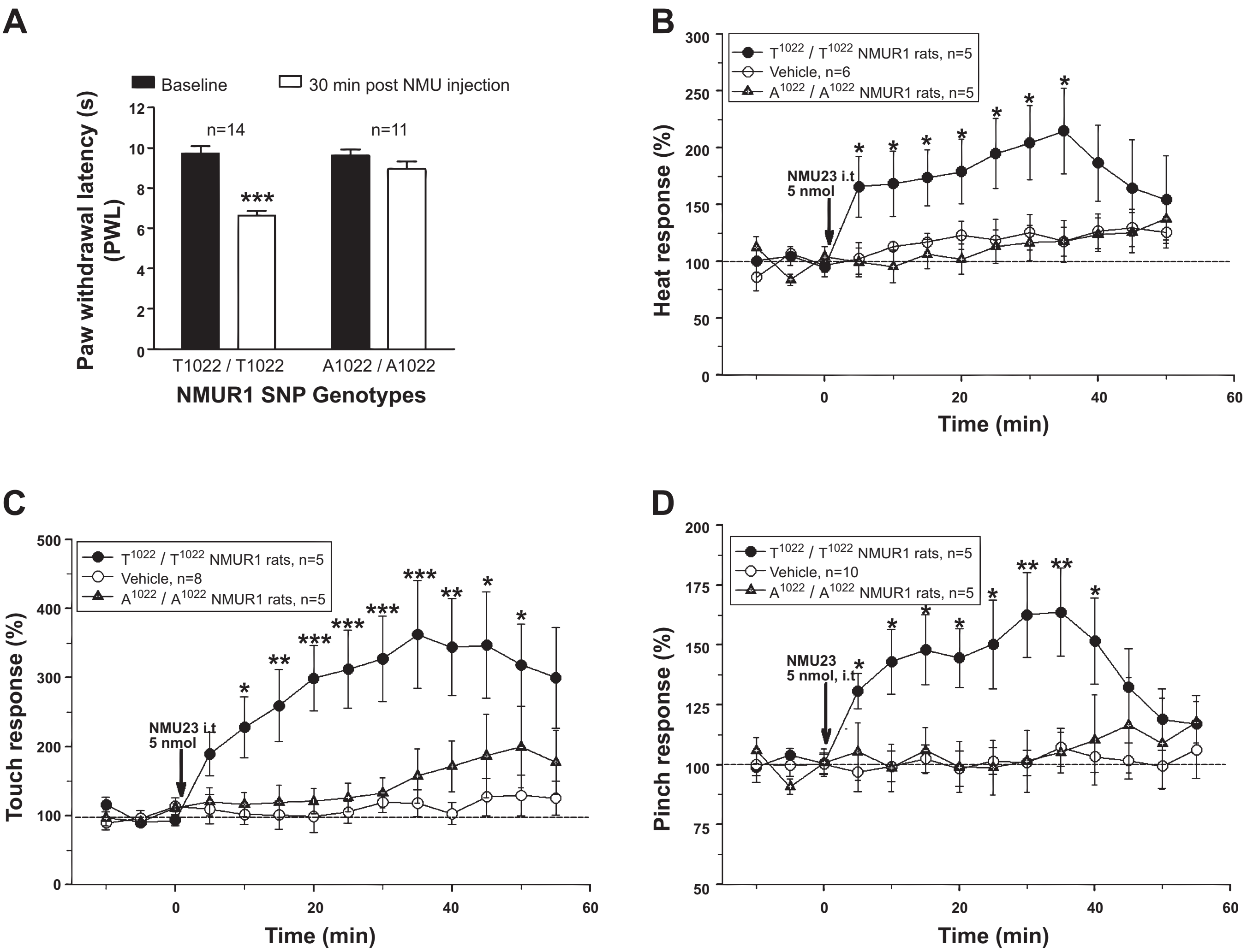

Fig. 5. Comparative effects of intradermal and intrathecal injections of NMU on paw withdrawal latency and spinal excitability in rats homozygous for the 2 *NMUR1* alleles. *A*: intradermal injection of NMU-23 (3.8 nmol, 10 μg/10 μl) produced a significant decrease of the paw withdrawal latency in rats homozygous for the *NMUR1* active allele T^{1022} but not in rats homozygous for the *NMUR1* inactive allele A^{1022} (data expressed as means ± SE; ***$P < 0.001$). Time-course potentiation for heat (*B*), touch (*C*), and pinch (*D*) responses of flexor α-motoneurons following intrathecal injection of NMU-23 (5 nmol) is observed in rats homozygous for the active NMUR1 T^{1022} allele (●) but not in rats homozygous for the inactive NMUR1 A^{1022} allele (△). Vehicle (○) was used as a control. Excitability is expressed as percentage change from baseline taken as an average of the 3 responses immediately prior to NMU application. Each response is the total number of action potentials evoked by 8 light touches applied to the plantar surface of the foot, each lasting for 2 s. NMU-23 was administered (i.t.) at the time indicated by the arrow. Data are illustrated as means ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ compared with vehicle control.

mediating nociception (45, 51) via central mechanisms. It has been shown that NMUR2 mRNA is found at highest levels in CNS regions (10), while NMUR1 mRNA is predominantly found in the periphery including dorsal root ganglion (DRG) (50). Localization of NMUR1 mRNA in DRG implies that NMUR1 protein is expressed in peripheral nociceptive fibers innervating the spinal cord. We took advantage of the naturally occurring NMUR1-loss of function rats to test for the first time the contribution of NMUR1 to nociception triggered by NMU. Our data show that the hypersensitivity to heat normally evoked by the peripheral injection of NMU is no longer observed in rats homozygous for the *Nmur1* inactivating allele. Similarly, following administration of NMU directly to the spinal cord, the expected increase in spinal excitability was absent in rats homozygous for the NMUR1-loss of function allele.

The outcome of the knockout mouse studies (45, 51) and the present results using NMUR1-loss of function rats underline a complex role for NMU in nociception and for potential differences across species. Taken together, our data suggest that NMU could exert its pronociceptive effects at both peripheral and spinal levels via the NMUR1 subtype and at supraspinal levels via the NMUR2 subtype. No studies have been published yet that examine the potential pronociceptive effect of the peptide NMS. To better understand the role of NMU, NMS, and their receptors in nociception, more extensive studies are required.

The existence of naturally occurring NMUR1-loss of function rats represents a powerful tool to investigate other biological and physiological processes where NMU plays a role. For instance, given the high expression of NMUR1 (compared to NMUR2) in the lung and pancreatic islets (19), the NMUR1-

loss of function rats could also be used to explore the potential role of NMUR1 in respiratory function and insulin secretion.

Given the importance of the rat as a model organism and the growing number of SNPs that are being identified in the rat genome from various strains (39), more studies need to be done to understand their functional significance on gene translation, mRNA stability, and protein function. Besides Sprague-Dawley rats, we have also found the *Nmur1* inactive allele in other rat strains which include Lewis, Wistar, Long-Evans, and Fischer. Thus, the impact of the naturally occurring SNPs leading to NMUR1 loss of function in rat has to be taken into consideration in future rat pharmacological and toxicological studies involving compounds targeting the NMUR1 receptor. The results of these studies cannot be correctly interpreted without proper verification of the NMUR1 SNP status for each of the tested rats.

ACKNOWLEDGMENTS

We thank Stéphane Faubert for veterinary support and Drs. Sultan Ahmad and Dajan O'Donnell for critical review of the manuscript. We are grateful to Dr. Hugh Salter for expert input on the manuscript and for conducting the Hardy-Weinberg analysis.

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DISCLOSURES

All authors were employed by AstraZeneca when research work was conducted.

AUTHOR CONTRIBUTIONS

Author contributions: R.P., L.M., C.Q.C., C.P., F.M., P.A.C., and T.G. performed experiments; R.P., L.M., F.M., J.L., and T.G. interpreted results of experiments; R.P., L.M., C.Q.C., C.P., F.M., P.A.C., and T.G. prepared figures; R.P., L.M., C.Q.C., C.P., F.M., P.A.C., J.L., and T.G. drafted manuscript; R.P. and T.G. edited and revised manuscript; L.M., C.Q.C., C.P., F.M., P.A.C., J.L., and T.G. analyzed data; T.G. conception and design of research; T.G. approved final version of manuscript.

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