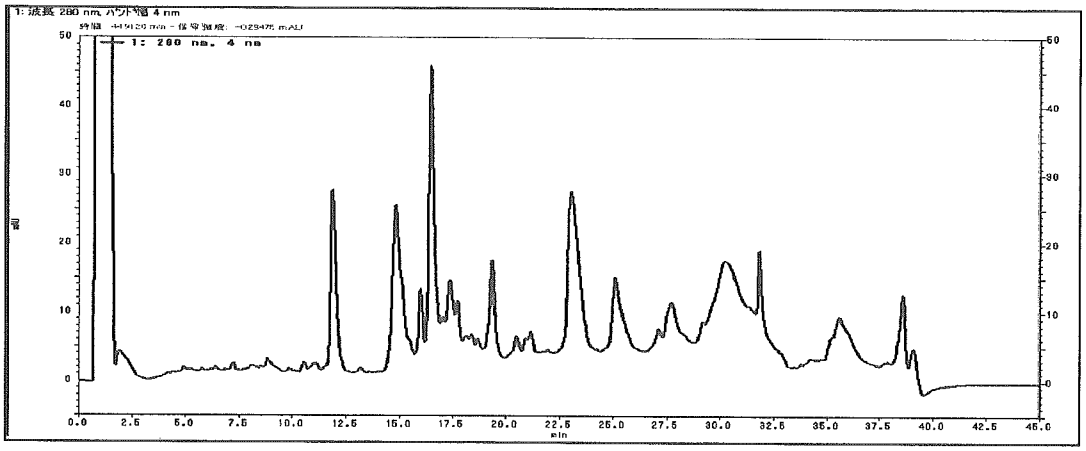
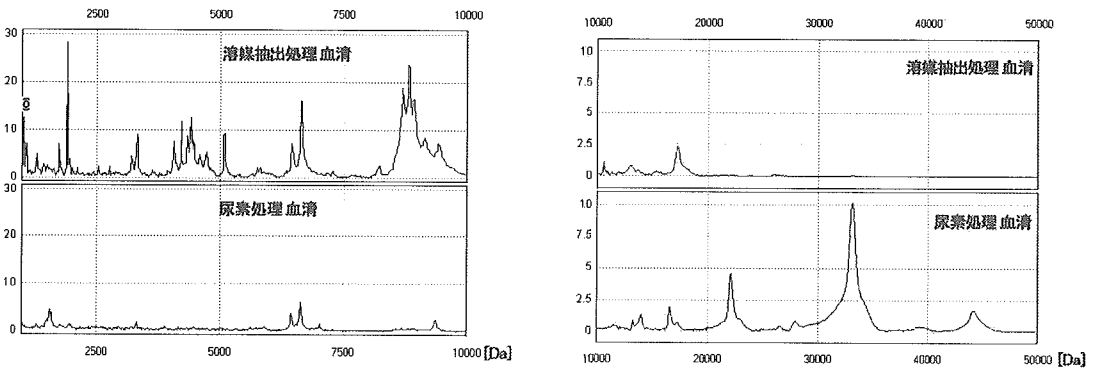


図 15. アジレント抗体カラム処理血清の C18 逆相分画クロマトによる分離

3）低分子量分画法（溶媒抽出法）の検討：

標準血清（CT02S）あるいは購入血漿(Rockland 社) 200μl に 0.1%TFA/アセトニトリル 400 μl を添加して高分子量たんぱく質を沈殿させた。遠心上清を SpeedVac により乾固し、0.1%TFA/30%アセトニトリル 50 μl で再溶解した。これを試料として SELDI-TOF 測定（Ciphergen 社）を行った。一方、溶媒抽出法の効果を確認するために、Ciphergen 社推奨プロトコール通りに尿素処理した血清あるいは血漿も SELDI-TOF 測定を行い、溶媒抽出処理と比較した。

溶媒抽出処理による分画効果を Ciphergen 社推奨のプロトコールである尿素処理と比較した。カチオン交換チップを使用した場合、溶媒抽出処理により高分子領域（10kDa～50kDa）の測定では大部分のピークが消失したが、低分子領域（1kDa～10kDa）の測定ではピーク数およびピーク強度が増大した（図 16）。これは溶媒抽出処理により 20kDa 以上のタンパク質が除去され 10kDa 以下のペプチドが濃縮されたこと、および 20kDa 以上のタンパク質が除去されたことにより 10kDa 以下のペプチドに対するイオン化効率が高まったためと考えられた。以上から低分子量画分に焦点を絞って解析する上で、溶媒抽出処理の効果が高いことが明らかとなった。



低分子領域（1～10kDa）　　高分子領域（10～50kDa）

図 16. 溶媒抽出法および尿素処理法による血清の SELDI-TOF 法による解析

次に溶媒抽出処理を施した血清と血漿を比較したところ、ピーク数は血清の方が多く観察された。血液を血清に処理する段階で凝固系が働き、プロセッシングを受けて生じたフラグメントが主に観察されていると考えられた。凝固系が活性化されているような疾病においては特に低分子量域を調べることにより変動たんぱく質が発見できることが期待される。

以上より、溶媒抽出によりペプチド画分が濃縮され感度よく SELDI-TOF 解析が可能であることが明らかとなった。今後は、実際に疾患サンプルを測定して比較することを計画している。

4）アフィニティー分画法（ヘパリンカラム）の検討：

購入血清（Lot. CT02S）あるいは購入血漿（Lot. CT03P）500 μl を 3 倍希釈後にヘパリンカラム（Amersham HiTrap Heparin HP 1mL）に注入し 10mM Tris, 200mM NaCl, pH7.5 で十分に wash した後、 10mM Tris, 1.0M NaCl, 5M 尿素, pH7.5 により溶出させた。溶出液は、10mM Tris, 5M 尿素, pH8.5 で 10 倍希釈して脱塩濃縮、続いて 10mM Tris, 2M 尿素, pH8.5 で 10 倍希釈して脱塩濃縮を行った。濃縮液は定法に従って cICAT 化を行った。現在、血漿と血清を比較サンプルとして、同定タンパク質や再現性について検討中である。

E．考察：

本年度は研究協力医療機関より送付された各疾患患者の血清高発現たんぱく質（100-150 種類）の cICAT 法による発現解析を最優先に行った。この場合、基準となる標準血清として購入健常外国人血清(プール血清)を用いたが、日本人健常人血清と比較した結果、血清の調製の際に遊離する血小板由来のたんぱく質を除くと、殆どのたんぱく質（全体の 94%）がほぼ同等であることが分かった。また、すでに個体間で変異が大きいことが知られている数種のたんぱく質は本解析でも同様の変異が観察された。このことから、本標準血清を用いて日本人患者血清を比較することは基本的に満足するものであると考えた。今後、疾患により、日本人患者の Disease control serum や Age-match control serum との比較検討が必要になる場合があると思われるが、その場合でも本標準血清をリンクすることにより相対的な比較検討が可能になるものと考えられる。

現在、PF で測定している血清高発現たんぱく質(100-150 種類)は血清たんぱく質全体の 99.9%を占める。その中には、補体系たんぱく質、線溶・凝固系たんぱく質、血清糖たんぱく質系、リポたんぱく質系など機能が解明されている主要成分の他に、いわゆる関連たんぱく質、あるいは類似たんぱく質など遺伝子の配列情報のみからその機能が類推されているたんぱく質が多数存在している。従って、研究協力医療機関から送付される各疾患の血清を網羅的に解析することにより、この未だ機能が充分に解明されてないたんぱく質群が想定外の疾患との関連性がみられ、新規な機能を発見する糸口を提供する可能性は充分にあると思われる。特に、血清あるいは血漿が病気の発症原因に深く関与する可能性のある循環器疾患やネフローゼ等の腎疾患等では、この可能性は少なくないと

考えられる。実際、ネフローゼ患者血清の場合、cICAT の誤差の閾値を越えて変動しているたんぱく質が 8.4%～11.4%も存在する。その中には、患者の治療前・中・後の比較により、経時的変化を示すものが観察されている。この変化が、疾患特有なものなのか、非特異的なものなのか、薬剤投与のためなのか等の詳細な検討は、今後、協力研究機関と相談の上、症例数を増やし解析することおよび他の腎疾患（Focal and segmental glomerulsclerosis (FSFG), IgA nephropathy）等を比較解析することによって明らかになるものと考えられる。

一方、病変部位が血液以外である場合、たとえば、各種臓器がん、パーキンソン病（黒質、線条体）、アルツハイマー病（海馬、皮質神経）等では、その病変部位のたんぱく質の発現差異が上述の高発現血清たんぱく質に反映する可能性は比較的低いと考える方が妥当である。これらの変異を捉えるためには、より病変部位に近い体液、例えば脳神経疾患の場合では脳脊髄液での解析が必要であり、また血清中で捉えるためには、より低発現の血清たんぱく質を解析する必要がある。　実際、現在までに国立精神神経センターから送付されたパーキンソン病の血清の解析結果から、高発現血清たんぱく質では特に差異のあるものは見出せず、また逆に、変異ユビキチン化酵素など注目たんぱく質は本高発現血清たんぱく質群には含まれていなかった。今後は、ペプチドマッピング技術を有効に利用し、より低発現たんぱく質（例えば 200-300 種類まで）も同定・定量可能にすることを目標にしたい。

一方、さらに低濃度の低発現たんぱく質解析に関しては、たんぱく質レベルでの分画（網羅的、群特異的）を導入することが必要であると思われる。　現在、限外ろ過膜、逆相カラム、ヘパリンアフィニティカラム、溶媒抽出等の手法を用いて予備検討中であるが、今後は具体的な戦略と戦術を選択して要望に応えることが重要である。

さて、創薬ターゲット探索の観点からすると、いわゆる組織・細胞のたんぱく質発現解析が非常に重要である。実際、例えば、ある種のがん疾患で非常に特異的な細胞たんぱく質ががん細胞の表面に局在した場合には、ヒト型モノクロナル抗体医薬のターゲットの一つになる可能性がある。本プロジェクトの残存期間を考えると、cICAT 法による組織・細胞たんぱく質発現解析系を至急に構築する必要がある。

そこで、LMD 法を使用するワークフローを設定し、実際のヒト組織解析の準備段階として、ヒト培養胃がん細胞株(KATO-III, MKN-45, MKN-74 等)を用いて基礎的検討をおこなった。まず、可溶化に関してであるが、実際の難溶の組織にも対応できるように、出来る限り多くのたんぱく質を可溶化する方法を目指した。すなわち、通常(8M)よりも濃い(9.8M)の尿素含有の 4%CHAPS で細胞を可溶化(全体の 80～90%程度)し、それでも不溶な画分は 0.1%SDS で熱処理を行い可溶化(全体の 10～20%程度)し、両画分を合わせたものを可溶画分とした。これにより殆ど全て（100%近く）の細胞たんぱく質が可溶化できるものと考えた。さて、我々は、本可溶画分のアセトン沈殿処理等を行い、完全に尿素を除去したたんぱく質を用いて各

種実験（ショットガン、cICAT 法）を行っている。実際に、ショットガン法で解析すると、いずれの細胞の場合でも多数のたんぱく質が同定され（図 11）、またイソ尿素による副反応で生じるカルバミル化ペプチドは全ペプチドの 1%以下であった。同様に、cICAT 法で解析しても、多数のペプチド・たんぱく質の同定および比較定量が可能であり、またその中でカルバミル化した cICAT ペプチドは見出されなかった。従って、9.8M 尿素を使用することにより、30-40%のペプチドがカルバミル化するとするクレームは当たらない。　おそらく、尿素が残存し、トリプシン消化中にカルバミル化したものと思われる。以上のことから、本可溶化法は基本的には一応満足するものであると考える。　但し、今後、難溶な組織を解析する場合には、尿素濃度などの一部条件を修正する可能性はあり得る。

さて、前述のごとく、実際のヒト組織のルーチン解析には LMD 法により採取した病変および正常部位（各 100 μg protein）を用いて cICAT 解析を行う予定であるが、実際にはどの程度の細胞たんぱく質が同定され比較定量可能なのであろうか？そのための基礎実験を、性質の異なる胃がん細胞株すなわち、スキルス型印環型の KATO-III と低分化型 MKN-74（各 100μg protein）を cICAT 法を用いて検討した。その結果、図 13 に示すように、SCX カラムの 10 分画の段階で約 290 種類が同定され、そのうち約 260 種が比較定量可能であった。この結果は、Chen Li らによって報告(9)されたヒト肝臓がん部位と正常部位で cICAT 法により得られた結果（261 種類が同定・定量可能）とほぼ同等であることを示す。また、図 13 に示すように、同定たんぱく質のカテゴリー分類を調べたところ、主要な細胞骨格系、核酸結合系、酸化還元系たんぱく質の他に、細胞膜に微量に存在すると思われる受容体、イオンチャネル系、トランスポーターも多数含まれていた。一方、Chen Li らの報告では膜受容体などの微量成分の同定はされていなかった(9)。

以上のことより、PF で検討した cICAT 法による細胞たんぱく質の同定比較定量ワークフローは基本的には一応満足するものであると考えた。但し、現段階では、SCX カラムの分画数は 10 分画しか行っていない。　今後は、SCX カラムのスケールダウン、cICAT 反応・TFA 切断条件、cICAT ペプチドの溶出条件の最適化を検討し、最終的には血清の場合と同様に 25 分画を用いて解析したい（なお、現時点でも、SCX カラムの 1 分画でも 130 種類のたんぱく質の同定・定量が可能な最適化が進んでいる）。　今後は、基本方針に従い、購入ヒト組織を用いて検討した上で、次いで実際の臨床検体の解析を開始する予定である。なお、対象疾患としては、日本人に多い胃がん（特に予後が悪いスキルス型）から開始し、次いで他のがん、他疾患の病変組織を検討したい。

統合データベースに関しては、本年度は、QSTAR XL の測定で観察される飽和ピークを排除し、適性ピークを自動的に選別する機能を導入することにより、cICAT 比較定量の精度を大幅に向上させた。また、生データの参照機能を導入し、真偽があいまいなデータに関しては、研究者が個別に検証・確認ができる支援機能を充実させ、

さらに、PF 独自スコアリングおよび自動フィルターリング機能を導入することにより、個人差のない共通の基準に沿って検証された高信頼データの登録を可能にした。以上のことにより、信頼性の高い疾患関連たんぱく質発現データベースを構築することが可能であると考える。現在、各種疾患検体のたんぱく質発現データが順次統合データベースに蓄積され、それを利用して疾患特異的なたんぱく質を見出す研究が進行している。　今後、データベース/バイオインフォマティクス部門としては、①個体ごとに集積されたたんぱく質発現データを疾患群単位で集約して、有意に疾患特異的な変動を示すたんぱく質の抽出や検定を支援する半自動化システムの構築、②臨床情報等を活用した大規模なクラスター解析システムの構築、③特許出願したペプチドマップ技術を活用するための技術開発、④疾患関連たんぱく質ネットワークデータベースの導入、⑤疾患関連・創薬ターゲット候補たんぱく質の上記ネットワーク上へのマッピングなどを検討する予定である。すなわち、プロテオームファクトリーで生み出される多種類かつ大量なたんぱく質データから、良質なマーカー候補、創薬ターゲット候補を効率よく発見して検証するシステムの構築を目指していく。

F．結論：

疾患関連血清たんぱく質解析フロー（cICAT 法）に基づき、各研究協力機関から提供されたヒト血清試料（糖尿病、がん、認知症、腎疾患及び免疫・アレルギー疾患等）の同定・比較解析研究を実施し、そのうち、腎疾患ネフローゼ患者とパーキンソン病患者血清の解析結果を関係研究協力機関に開示し、病態・治療に伴う変化の解析および追加研究等の対応について検討した。　ヒト胃がん細胞株等を用いて、細胞可溶化法、cICAT 法、発現たんぱく質の同定・比較定量解析の検討を行い、cICAT 法によるヒト組織(細胞)たんぱく質解析ワークフロー法を構築の基礎データとした。統合データベースに関しては、統合データ解析システム(HiSpec)に独自スコアリング機能を導入することにより、たんぱく質同定の効率化とたんぱく質同定・比較定量結果の高信頼化を図った。さらに、同定・定量結果の網羅性を高めるペプチドマップ法を検討し、その解析法を特許出願した。以上の実績をもとに、今後、各研究協力機関のヒト試料（血清、組織）のたんぱく質発現解析を鋭意実行する。

G.　健康危険情報：

特になし。創薬プロテオームファクトリー施設では万全なバイオハザード対策設備を施工している。

H.　研究発表：

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I.　知的財産権の出願・登録状況：

１．特許出願：２件

１）同位体標識法、出願日：平成１７年７月１４日　　出願番号：特願2005-205749

２）質量分析システムおよび質量分析法、
出願日：平成１７年１２月２１日
出願番号：特願 2005-368472

２．実用新案登録：
なし。
３．その他

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III 研究成果の刊行に関する一覧表

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Neuromedin S Is a Novel Anorexigenic Hormone

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A novel 36-amino acid neuropeptide, neuromedin S (NMS), has recently been identified in rat brain and has been shown to be an endogenous ligand for two orphan G protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1. These receptors have been identified as neuromedin U (NMU) receptor type 1 and type 2, respectively. In this study, the physiological role of the novel peptide, NMS, on feeding regulation was investigated. Intracerebroventricular (icv) injection of NMS decreased 12-h food intake during the dark period in rats. This anorexigenic effect was more potent and persistent than that observed with the same dose of NMU. Neuropeptide Y, ghrelin, and agouti-related protein-induced food intake was counteracted by co-administration of NMS. Icv administration of NMS increased proopiomelanocortin mRNA expression in the arcuate nucleus (Arc) and CRH mRNA in the paraventricular nucleus (PVN). Pretreatment with SHU9119 (antagonist for α-MSH) and α-helical corticotropin-releasing factor-(9–41) (antagonist for CRH) attenuated NMS-induced suppression of 24-h food intake. After icv injection of NMS, Fos-immunoreactive cells were detected in both the PVN and Arc. When neuronal multiple unit activity was recorded in the PVN before and after icv injection of NMS, a significant increase in firing rate was observed 5 min after administration, and this increase continued for 100 min. These results suggest that the novel peptide, NMS, may be a potent anorexigenic hormone in the hypothalamus, and that expression of proopiomelanocortin mRNA in the Arc and CRH mRNA in the PVN may be involved in NMS action on feeding. (*Endocrinology* 146: 4217–4223, 2005)

NEUROMEDIN U (NMU), originally isolated from porcine spinal cord, is a brain-gut peptide that has potent contractile activity on uterine smooth muscle (1). In previous studies, two orphan G protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1, were identified as NMU receptor type 1 (NMU1R) and type 2 (NMU2R), respectively (2–5). Recently, a novel 36-amino acid neuropeptide was identified in rat brain as another endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique (6). This neuropeptide was designated neuromedin S (NMS) because it is specifically expressed in the suprachiasmatic nucleus (SCN). Although the NMS shares a C-terminal core structure (seven-amino acid residues) with NMU and activates both recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, NMS is not a splice variant of NMU because both NMS and NMU genes were mapped to discrete chromosomes. In addition, although NMU mRNA was detected in peripheral and central organs (7), the distribution of NMS was limited to the testis, spleen and SCN (6). NMS was recently suggested to be involved in circadian oscillation systems because intracerebroventricular (icv) administration of NMS induces phase-dependent phase shifts in the circadian rhythm of locomotor activity in rats kept under constant darkness (6).

NMU1R is located in a wide range of peripheral tissues such as intestine, testis, pancreas, uterus, lung, and kidney. On the other hand, expression of NMU2R is limited to areas of the brain such as the paraventricular nucleus (PVN), along the wall of the third ventricle in the hypothalamus and the CA1 region of the hippocampus (2, 5, 8, 9). Immunohistochemical and *in situ* analysis has revealed NMU-immunoreactive neurons or NMU mRNA expression in the ventromedial hypothalamic region including the arcuate nucleus (Arc), pituitary, caudal brainstem region including the nucleus of the solitary tract, area postrema, dorsal motor nucleus of the vagus nerve and inferior olive, and spinal cord (2, 10, 11). NMU-immunoreactive fibers project prominently into the PVN, ventromedial nucleus, dorsomedial nucleus, and Arc. It has been well documented that the PVN and Arc of the hypothalamus play pivotal roles in the regulation of feeding behavior through a complex neuronal network composed of several orexigenic neuropeptides such as neuropeptide Y (NPY), agouti-related protein (AGRP) and ghrelin, and anorexigenic neuropeptides such as α-MSH, cocaine- and amphetamine-regulated transcript, CRH, and leptin (12, 13). Icv administration of NMU suppresses both dark-phase food intake and fasting-induced feeding, suggesting that NMU acts as anorexigenic hormone (2, 3). Conversely, disruption of the *NMU* gene in mice [NMU knockout (KO) mice] resulted in severe obesity (14). Although ob/ob mice (mutant leptin-deficient mice) are known to be obese through a decrease in proopiomelanocortin (POMC) mRNA and an in-

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Abbreviations: AGRP, Agouti-related protein; Arc, arcuate nucleus; α-hCRF, α-helical corticotropin-releasing factor-(9–41); icv, intracerebroventricular; KO, knockout; MUA, multiple-unit activity; NMS, neuromedin S; NMU, neuromedin U; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus.

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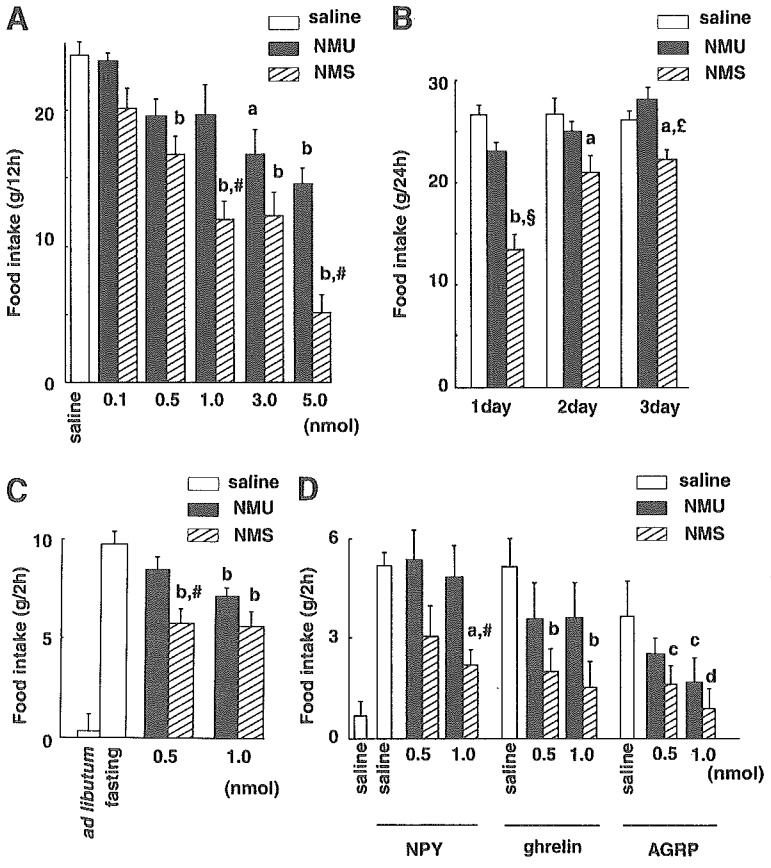

FIG. 1. Comparison of food intake after intracerebroventricular administration of NMS and NMU to rats (n = 8–10 in each group). Each *bar* and *vertical line* represents the mean ± SEM. A, Dark-phase feeding. Food intake of free-feeding rats was examined during a period of 12 h from 1900 h to 0700 h. Each reagent was injected at 1845 h (a, $P < 0.05$; b, $P < 0.01$ *vs.* saline; #, $P < 0.01$ *vs.* NMU at the same dose as NMS). B, The inhibitory effects on food intake after injection. Food intake was examined over a period of 24 h from 1900 h to 1900 h for 3 d after injection. Each reagent (1 nmol) was injected at 1845 h. (a, $P < 0.05$; b, $P < 0.01$ *vs.* saline; £, $P < 0.05$; §, $P < 0.01$ *vs.* NMU at the same dose as NMS). C, Two-hour food intake in rats that had fasted for 8 h and then received each reagent at 0900 h (b, $P < 0.01$ *vs.* saline in fasting rats; #, $P < 0.01$ *vs.* NMU at same dose as NMS in fasting rats). D, Effect of coadministration of NPY (0.5 nmol), ghrelin (0.5 nmol), or AGRP (1 nmol) with NMU or NMS on 2-h food intake in free-feeding rats. Each reagent was injected at 0845 h (a, $P < 0.05$ *vs.* NPY + saline; #, $P < 0.05$ *vs.* NPY + 1.0 nmol NMU; b, $P < 0.01$ *vs.* ghrelin + saline; c, $P < 0.05$; d, $P < 0.01$ *vs.* AGRP + saline).

crease of NPY and AGRP mRNA in the Arc (15–17), obesity in NMU KO mice results specifically from a decrease of CRH mRNA in the PVN. Therefore, NMU and leptin share the mechanism of feeding suppression (14).

The fact that receptors for NMU have a high affinity for NMS suggests that NMS may also act on feeding. The *NMS* gene was mapped to chromosome 2q11.2 in humans, and this locus is consistent with one potential location of the quantitative trait loci implicated in obesity (18). These data also lead to speculation that NMS may play an important role in central regulation of feeding.

To examine whether NMS is involved in feeding regulation, the effects of central administration of NMS and NMU on food intake were investigated in rats, and the cellular mechanisms involved were analyzed.

Materials and Methods

Animals

Male Wistar rats (Charles River Japan, Inc., Yokohama, Japan), weighing 300–350 g, were housed in individual Plexiglas cages in an animal room maintained under a constant light-dark cycle (light on from 0700–1900 h) and temperature (22 ± 1 C) for at least 1 wk. Food and water were provided *ad libitum* except during the fasting experiments. All procedures were done in accordance with the Japanese Physiological Society's guidelines for animal care.

Feeding experiments

Cannulation for icv injection was performed described previously (19). After surgery, all rats were housed individually in Plexiglas cages. During a 6-d postoperative recovery, the rats became accustomed to the handling procedure. In the first experiment, various doses of rat NMS and NMU were dissolved in saline, and 10 μl of solution was injected through a 27-gauge injection cannula connected to a 50-μl Hamilton syringe into each free-moving rat at 1845 h; 12-h food intake was then examined. We also examined the diurnal effect of NMS on food intake by icv injection of NMS at 0900 h. Rat NMS and NMU were synthesized by an Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA). In the second experiment, rats were fasted for 8 h from 0100 h at night, and then centrally injected with NMS or NMU (0.5 or 1 nmol) at 0845 h. In the third experiment, single NPY (0.5 nmol), ghrelin (0.5 nmol) or AGRP (1 nmol), and mixed NPY, ghrelin or AGRP + NMS (0.5 or 1 nmol) or NMU (0.5 or 1 nmol) (each peptide was mixed in 10 μl of saline solution) was administered to free-feeding rats at 0845 h and 2-h food intake was measured. NPY, ghrelin and AGRP were purchased from the Peptide Institute, Inc. (Osaka, Japan). In the fourth experiment, 1 nmol NMS was injected 1 h after pretreatment with 1, 5, or 10 μg α-helical corticotropin-releasing factor-(9–41) (α-hCRF) (Sigma, St. Louis, MO) or 0.1, 0.5, or 1 nmol SHU9119 (Bachem, Budendorfm, Switzerland) at 0745 h to 8-h fasted rats or intact rats, and 2-h and 24-h food intake was examined, respectively.

c-Fos immunohistochemistry

Ninety minutes before perfusion, rats were injected with NMS, NMU (1 nmol per rat) or saline (n = 3 per group) in the lateral ventricle to study the immunostaining of c-Fos-expressing neurons. After the rats had been perfused with fixative [4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)], the brain was removed immediately, fixed in fixative and embedded in O.C.T. compound (Tissue-Tek, Tokyo, Japan) at −20 C. Frozen serial brain sections (40 μm thick) were incubated for 1 d with goat anti-c-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; final dilution 1:1500) and visualized by the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) using 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.005% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6).

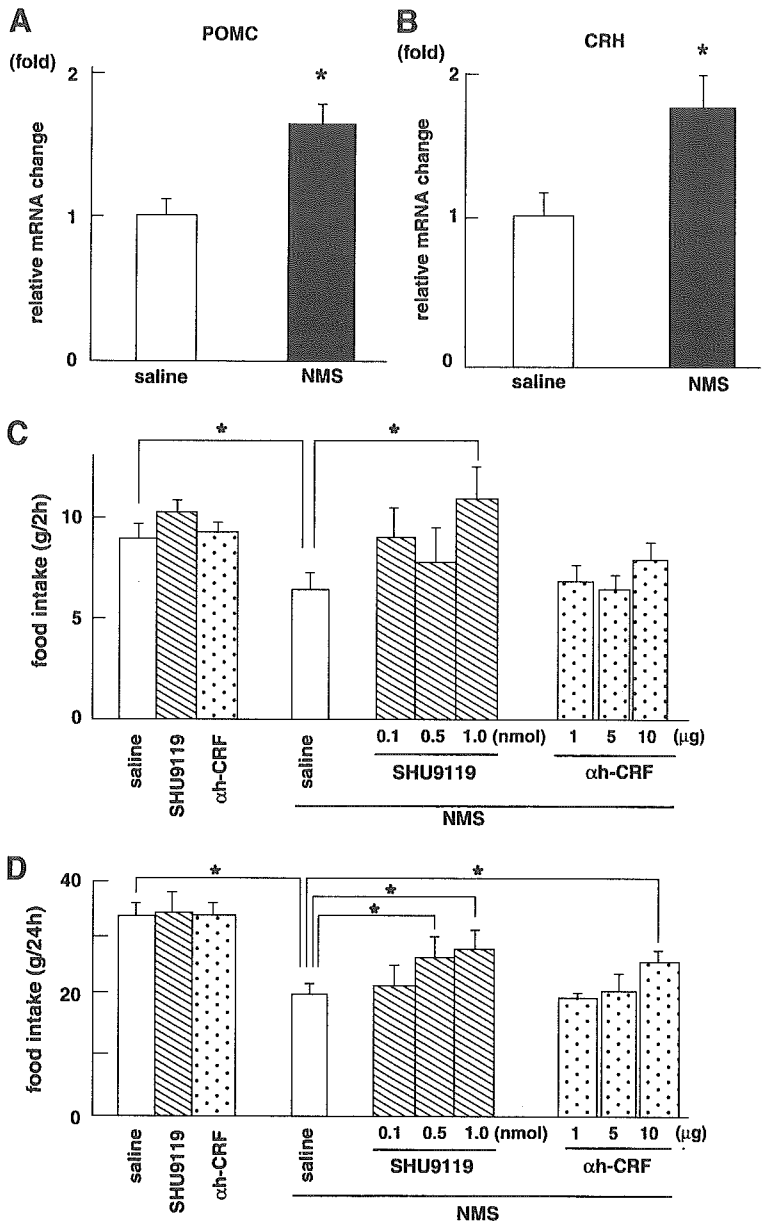

FIG. 2. Involvement of α-MSH and CRH in NMS-elicited feeding behavior. Quantitative RT-PCR on NMS administered rats (n = 8 per group). Level of POMC mRNA (A) and CRH mRNA (B). C, Effect of pretreatments with α-MSH antagonist (SHU9119) or CRH antagonist [α-helical CRF-(9–41)] on food intake reduction by NMS in 8-h fasted rats. Each antagonist was injected at 0745 h, and then NMS was injected at 0845 h. Food intake was examined during a 2-h period from 0900–1100 h. *Asterisks* indicate the significant difference ($P < 0.05$). D, Effect of pretreatments with α-MSH antagonist (SHU9119) or CRH antagonist [α-helical CRF-(9–41)] on food intake reduction by NMS in intact rats. Each antagonist was injected at 0745 h, and then NMS was injected at 0845 h, then, 24-h food intake was examined. *Asterisks* indicate the significant difference ($P < 0.05$).

Quantitative RT-PCR

To quantify POMC and CRH mRNA in the Arc and PVN after icv injection of NMS, 1 nmol NMS was injected into rats at 1845 h, 4 h before collection of Arc and PVN tissue for mRNA extraction. After the brain tissues had been frozen, the Arc and PVN were dissected out. Total RNA was extracted from the Arc and PVN using an RNeasy Mini kit (QIAGEN, Hilden, Germany) and then synthesized into first-strand cDNA. Quantitative RT-PCR was conducted with a LightCycler system (Roche, Basel, Switzerland) using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche). The primer set used for rat POMC was 5′-GACCTCACCACGGAAAGCAACCTG-3′ and 5′-ACTTCCGGGGATTTTCAGTCAAGGG-3′, and for rat CRH was 5′-ATCTCACCTTCCACCTTCTG-3′ and 5′-GTGTGCTAAATGCAGAATCG-3′. Known amounts of rat POMC and CRH cDNA were used to obtain a standard curve. Rat glyceraldehyde-3-phosphate dehydrogenase mRNA was also measured as an internal control. The primer set used for rat glyceraldehyde-3-phosphate dehydrogenase was 5′-CGGCAAGTTCAACGGCACA-3′ and 5′-AGACGCCAGTAGACTCCACGACA-3′.

Multiple unit activity (MUA) recording

Rats were fitted with chronically implanted electrode arrays as described previously (20). Briefly, the electrode assembly consisted of four 75-mm Teflon-insulated platinum (90%)-iridium (10%) wires (A-M Systems, Inc., Sequim, WA) encased in a stainless steel guide tube (650 mm diameter; Inter Medical, Fukuoka, Japan). The stainless steel tube served as a ground. The impedance of each platinum-iridium electrode measured at 1 kHz was 50–100 kΩ. According to the stereotaxic atlas of the rat brain (Paxinos and Watson, Ref. 27) described by Albe-Fessard *et al.*, the electrodes were implanted unilaterally into the left side of the PVN and fixed to the skull with anchor screws and dental cement. At the same time, an icv cannula was implanted slantingly into the right lateral cerebral ventricle. After a recovery period of 5 d, MUA was recorded as follows: signals were passed through a buffer amplifier, amplified by a biophysical amplifier (MEG-2100; Nihon Kohden, Tokyo, Japan) with low and high cutoff frequencies of 500 Hz and 10 kHz, respectively, and displayed on an oscilloscope (DS-8812; Iwatsu, Tokyo, Japan). Neural spikes were discriminated by their amplitude, and the number of spikes was counted with a pulse counter (ET-612J; Nihon Kohden) and inte-

grated for 1 sec. Outputs were recorded as a histogram on a thermal recorder (WR8500; Graphtec, Tokyo, Japan) and with a powerLab (AD Instrument, Castle Hill, Australia), respectively. On the day of the experiment, the MUA electrode was attached to the buffer amplifier under isoflurane inhalation anesthesia (Univentor 400; Univentor, Zejtun, Malta). Rats were maintained under anesthesia with 1.5% isoflurane (Abbott Laboratories, Abbott Park, IL). At 15 min after the beginning of stable MUA volley, rats received icv administration of 1 nmol NMS, NMU, or saline. At 120 min after administration, electrical stimulation was applied for 1 sec through the MUA electrode with pulses (1 mA) from an electric stimulator (RGF-4A; Radionics, Burlington, MA) to check the site of the electrode.

Statistical analysis

The data (mean ± SEM) were analyzed statistically by ANOVA with the *post hoc* Fisher's test. $P < 0.05$ was considered statistically significant.

Results

Intracerebroventricular injection of NMS reduced 12-h food intake during the dark period in a dose-dependent manner (Fig. 1A). This effect of NMS was more potent than that of NMU because a smaller dose of NMS was effective at suppressing feeding (Fig. 1A). We also measured 12-h water intake after NMS or saline injection before the onset of dark period. A quantity of 1 nmol of NMS, but not 0.5 nmol, significantly decreased water intake during dark phase [NMS 1 nmol, 34.75 ± 3.26 ml ($P < 0.05$ *vs.* saline); 0.5 nmol, 44.74 ± 4.89 ml; saline, 47.17 ± 4.54 ml]. Although feeding suppression by 1 nmol NMU recovered completely within 2 d, suppression by the same dose of NMS continued at least for 3 d starting from 1845 h (Fig. 1B). Icv injection of 1 nmol NMS and NMU into 8-h fasted rats also resulted in a decrease in food intake for 2 h. On the other hand, at a dose of 0.5 nmol, only NMS injection suppressed food intake (Fig. 1C).

Although icv injection of NPY, ghrelin, and AGRP significantly increased food intake, this peptide-induced food intake was reduced by coadministration of NMS or NMU (Fig. 1D). In these cases, the suppressive effect with NMS was more potent than that with NMU. We also examined the diurnal effect of NMS on food intake by icv injection of NMS at 0900 h. There was no significant difference in food intake during the 12-h light period on the first, second, and third day between the NMS- and saline-treated groups (first 12-h light period 1.9 ± 0.62 *vs.* 2.4 ± 0.64 g; second 12-h light period 2.4 ± 0.52 *vs.* 2.5 ± 0.44 g; third 12-h light period 2.5 ± 0.72 *vs.* 2.4 ± 0.48 g; NMS *vs.* saline). However, NMS suppressed significantly 12 h dark food intake for 3 d starting from 0900 h.

To understand the cellular mechanisms involved in NMS-induced suppression of feeding, POMC and CRH mRNA expression and the expression of c-Fos protein were investigated. Icv administration of NMS augmented the levels of Arc POMC and PVN CRH mRNA (Fig. 2, A and B). The involvement of POMC and CRH in NMS-induced suppression of feeding was therefore investigated using an antagonist for these peptides. Pretreatment with both SHU9119 (an antagonist for α-MSH) and α-hCRF (an antagonist for CRH) attenuated NMS-induced suppression of food intake in a dose-dependent manner in fasted rats. Whereas only SHU9119 significantly blocked the effect of NMS on 2-h food intake (Fig. 2C), both α-hCRF and SHU9119 blocked the effect of NMS on 24-h food intake (Fig. 2D). The central distributions of c-Fos immunoreactive cell were similar in NMS- and NMU-injected rats. The hypothalamic PVN (Fig. 3, A and D), Arc (Fig. 3, B and E), supraoptic nucleus (Fig. 3, C and F) and SCN (data not shown) expressed the c-Fos protein strongly. In saline-treated rats, no c-Fos immunoreactivity was observed in any of these regions (data not shown).

Neuronal electrical activity in the PVN was then measured before and after icv administration of 1 nmol NMS and NMU using a MUA recording system. This method has practical advantages, in that continuous and real-time analysis of hypothalamic neural activity can performed *in vivo*. In the frequency-time histograms, MUA could be influenced within 5 min by NMS and NMU (Fig. 4, A and B). The most active MUA induced by NMS was observed between 20 min and 100 min and decreased gradually thereafter. Although NMU also increased MUA immediately after injection, the effect was weaker than that of NMS. We analyzed the total spike count at 30-min intervals for 120 min (Fig. 4C). Although a significant increase in the spike count was observed only between 30 and 60 min after icv injection of NMU, the increase continued for at least 120 min in NMS-treated rats. As shown in Fig. 4D, the recording sites of these MUA volleys were located adjacent to the PVN.

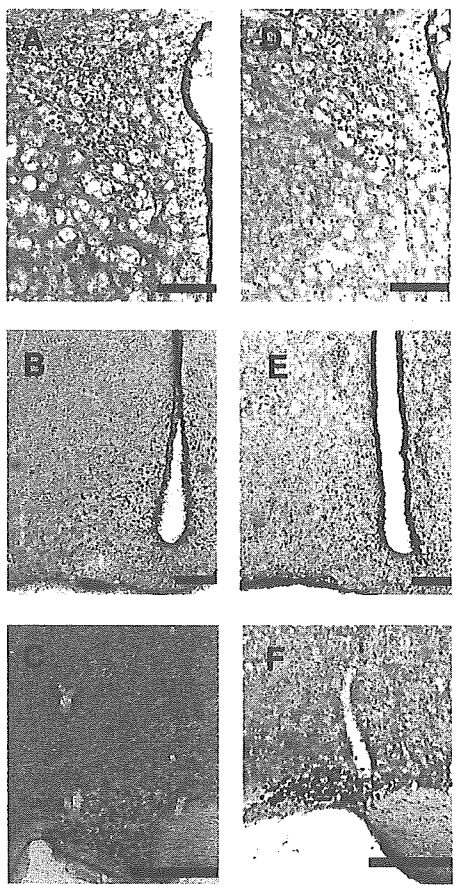

FIG. 3. Representative photomicrographs of c-Fos-immunoreactive cell nuclei in selected brain regions after administration of 1 nmol NMS (A–C) or NMU (D–F). The brain regions shown include the PVN (A and D), Arc (B and E), and supraoptic nucleus (C and F). *Scale bar*, 200 μm (for all panels).

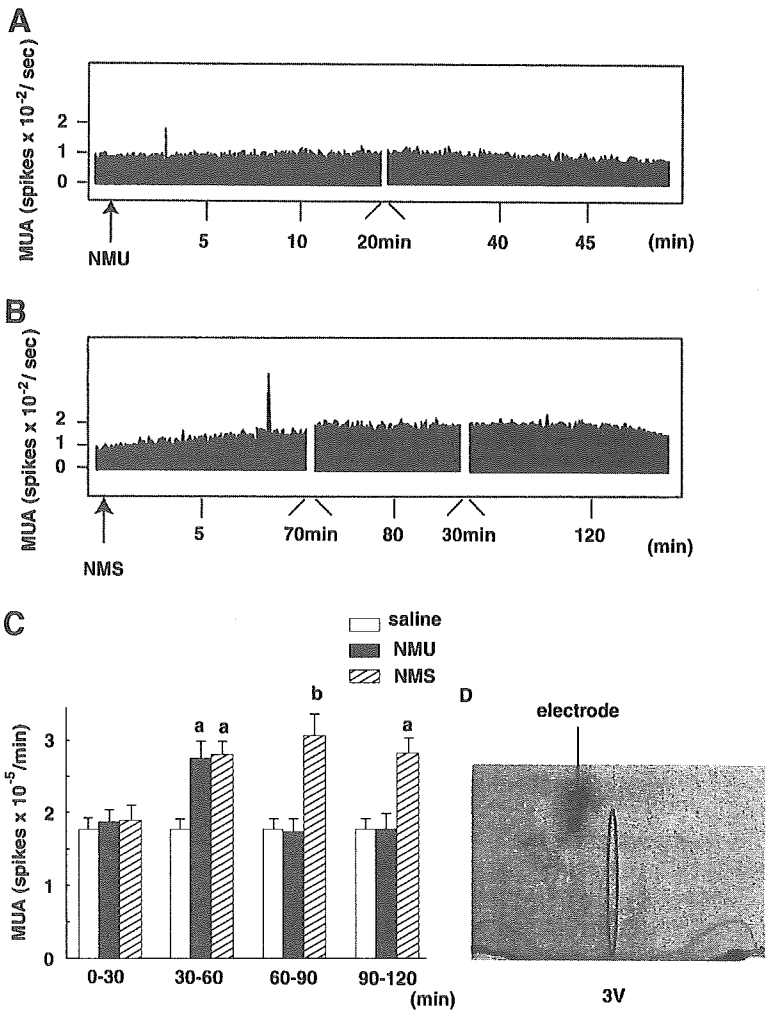

FIG. 4. Effect of icv injection of NMS or NMU on MUA in the PVN. A and B, Frequency-time histograms of firing rate of the PVN after administration of NMS or NMU in representative rats. NMS (A) or NMU (B) was injected 15 min (*arrows*) after stable MUA volley. Abscissa: time (min), ordinate: spike/sec. MUA profiles in one representative rat are shown. C, Summary of the effects of icv injection of NMS or NMU on MUA volley frequency at 30-min intervals for 2-h. Each *column* and *vertical bar* represents the mean ± SEM (n = 3–4) (a, $P < 0.05$ *vs.* saline; b, $P < 0.01$ *vs.* saline). D, Location of the MUA electrode tip. Recording sites of MUA volleys were located adjacent to the PVN.

Discussion

In the present study, the novel peptide, NMS, was demonstrated to be a potent anorexigenic hormone in rats. Central administration of NMS reduced the daily dark period food intake and 8-h fasting-induced food intake. This suppression of feeding is unlikely to be due to any side effects of NMS because NMS-injected rats did not show any abnormal behavior (such as glooming behavior, searching behavior, attaching behavior, and barrel rolling). Although NMU has been well documented to reduce food intake in rats (2, 3), the relative potency of NMS on suppression of food intake was stronger than that of NMU because a smaller dose of NMS significantly suppressed food intake. Considering that NMS contains the core active C terminus of NMU and binds to the same receptors (NMU1R and NMU2R) as NMU (6), NMS-induced suppression of food intake can be assumed. In a previous study, the distribution of NMS mRNA was investigated in various rat tissues by quantitative RT-PCR (6). NMS mRNA was expressed mainly in the hypothalamus, spleen, and testis. In the hypothalamus, however, NMS mRNA was expressed predominantly in the SCN, with only very slight expression in other brain regions including the PVN and Arc. *In situ* hybridization histochemistry also showed that NMS mRNA expression was restricted to the SCN. No hybridization signal was observed in any other brain region. The fact that the relative potency of NMS in suppressing food intake was stronger than that of NMU despite the lower expression of NMS mRNA than NMU mRNA in the PVN and Arc suggests that the feeding regulation effect may differ between NMS and NMU. Especially, in the case of NMS, its action on the PVN and Arc through the NMS projection from the SCN may be important.

When the NMS was injected at 0900 h, there was no significant difference in food intake during the 12-h light period, suggesting the diurnal variation in the anorexigenic effect of NMS. Although the interpretation of these data is difficult because of the very low feeding activity in the beginning of the light period, this diurnal difference may be due to diurnal variation of NMU receptors in SCN (21) or diurnal variation of NMS secretion (6) in autocrine regulation.

It is not known why NMS-induced suppression of food intake is more potent and continues for a longer time than with NMU. There was no difference in the distribution of c-Fos expression between NMS- and NMU-injected rats. However, neural MUA records showed a clear difference between the rats. There was a greater increase in firing rate of PVN neurons in NMS-treated rats than in NMU-treated rats, and this increased effect continued for a long period of

time after NMS injection. This potent and long-term increase of firing rate by NMS may cause the powerful and long-term suppression of food intake. Alternatively, the possibility that NMS may act on another unknown receptor cannot be excluded.

NPY, ghrelin, and AGRP-induced food intake was counteracted by coadministration of NMS, suggesting that the NPY, ghrelin, and AGRP are independently antagonistic with NMS for feeding regulation.

Hanada *et al.* (14) reported that icv injection of NMU in rats did not affect POMC mRNA expression in the Arc but augmented CRH mRNA expression in the PVN. In addition, CRH KO mice did not show any reduction in food intake after NMU injection (22). Therefore, it has been speculated that an increase in CRH, but not α-MSH, is the primary cause of NMU-induced suppression of food intake. In the present study, NMS increased both POMC and CRH mRNA expression. These results indicate that the cellular mechanism of suppression of food intake by NMS may be different from that by NMU, and both CRH and α-MSH may be involved in NMS-induced suppression of food intake. This hypothesis is supported by the following results: pretreatment with antagonists for α-MSH and CRH blocked NMS-induced suppression of food intake.

It is questionable why receptors for NMS and NMU are the same; nevertheless, the downstream mechanism of feeding regulation by NMS and NMU is different. Recent studies demonstrate that NMU, NMS, NMU1R, and NMU2R mRNA each have an intrinsic rhythmic expression in the SCN with a different circadian pattern (6, 21). Because the SCN sends neural projections into the PVN and Arc (23, 24), these different rhythmic expressions may relate to the different effects of NMS and NMU. Of course, as mentioned above, NMS may act on a receptor other than NMU1R and NMU2R. Either way, it is unknown why NMS, but not NMU, stimulates the POMC system in the Arc, but a different downstream mechanism may explain the difference in effectiveness and duration of action between NMU and NMS.

Wren *et al.* (25) reported that leptin was able to stimulate NMU release in hypothalamic explants *in vitro*. In contrast, Hanada *et al.* (14). showed that the anorexigenic effect of NMU is independent of leptin in NMU KO mice because NMU and leptin reduced food intake in ob/ob mice and NMU KO mice, respectively. Wren *et al.* measured NMU content using an antibody raised in a rabbit immunized with synthetic NMU-8. Because NMU-8 is the core active C terminus of NMS and NMU, the antibody must recognize both NMS and NMU. We had also raised antiserum against synthetic NMU-8 and established a RIA for NMU (26). Rat NMS and NMU were equally recognized with the serum on a molar basis (data not shown) and could not separate NMS and NMU in this RIA system. Therefore, NMU release stimulated by leptin in hypothalamic explants presented by Wren *et al.* might be NMS. If this is the case, NMS is the downstream signal pathway for leptin. NMS is a novel anorexigenic hormone, and further investigation of the function of NMS will help in our understanding of weight control mechanisms and should facilitate the study of eating disorders.

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Effect of neuromedin S on feeding regulation in the Japanese quail

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Abstract

Neuromedin S (NMS) was recently isolated from the brains of humans, mice and rats as an endogenous ligand for the orphan G protein-coupled receptors FM-3 and FM-4, which have been identified as neuromedin U (NMU) receptors 1 and 2, respectively. To investigate the role of NMS in avian species, we elucidated the effect of intracerebroventricular (i.c.v.) administration of rat NMS on food intake, body weight, body temperature and gross locomotor activity in adult Japanese quails. NMS significantly decreased food intake (and consequently body weight) in a time-dependent manner during 12-h light period, but increased both body temperature and gross locomotor activity. On the other hand, i.c.v. injection of rat NMU showed the reverse effects of NMS in Japanese quail. These results suggest that NMS may play an important role in regulating food intake and sympathetic nerve activity in the Japanese quail.

Keywords: Food intake; Body temperature; Neuromedin S; Japanese quails

Recently, a novel 36-amino acid residue neuropeptide was isolated from rat brain and was identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique [9]. FM-3/GPR66 and FM-4/TGR-1 have been already identified as neuromedin U (NMU) receptor type-1 (NMUR1) and NMU receptor type-2 (NMUR2), respectively [3]. The novel peptide was designated neuromedin S (NMS), because it is specifically expressed in the suprachiasmatic nucleus (SCN) [9]. Although NMS shares a C-terminal core structure (seven amino acid residues) with NMU, and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, it is not a splice variant of NMU because the genes for NMS and NMU have been mapped to discrete chromosomes [9].

The physiological functions of NMU have recently been clarified. Its most marked effect is on feeding regulation [3,7,10]. Intracerebroventricular (i.c.v.) administration of NMU decreases both the daily food intake during dark period and fasting-induced food intake in rats [5,12]. Conversely, injection of anti-NMU IgG increases dark-phase feeding compared with preimmune serum IgG [7]. Recently, our group has also demonstrated that NMU-knockout mice become obese [2]. These results indicate that NMU is a potent endogenous anorexigenic peptide in rats. In addition to feeding regulation, NMU increases gross locomotor activity, body temperature and heat production in rats, suggesting that it is a catabolic signaling molecule [10]. We previously reported that synthetic Japanese quail NMU decreased food intake and increased both body temperature and gross locomotor activity in Japanese quails [11], thus implying that avian NMU also plays important physiological roles.

As mentioned above, NMS shows homology of the C-terminal core structure with NMU and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells. It may therefore also play important roles in feeding regulation, locomotor activity and body temperature. With this possibility in mind, we compared the effects of NMS with those of NMU in avian species. In this study, we chose to use adult Japanese quails instead of chickens because the growth curve in chickens is steep and so their body weight and food intake vary widely on a daily basis, whereas in the adult Japanese quail, the growth curve is relatively constant and therefore body weight and food intake are not subject to such great variability. In addition, it is possible to chronically implant an i.c.v. cannula into adult Japanese quails.

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Adult male Japanese quails (*Coturnix coturnix japonica*), weighing 110–120 g, were reared in individual net cages (*W*: 14 × *L*: 26 × *H*: 17 cm) in a room with a 12-h light (300 lx)/12-h dark (dim light, 25 lx) period (lights on at 07:00 h), at a temperature of 28 ± 1 °C. The birds were given free access to food and water. Rat NMS or rat NMU (Peptide Institute, Osaka, Japan) was dissolved in 0.9% saline and several doses were administered i.c.v. to each of six free-feeding male birds in each experimental group. Each experiment was set for measurement only one parameter to avoid the effect of one parameter on the other. All the experiments were performed twice in order to confirm the results obtained in each experiment. We performed 1-week interval between the first and second time experiment to avoid the residual effects of repeated injection. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

For implantation of the i.c.v. cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 μl/g body weight) and placed in a stereotaxic frame. A stainless steel guide cannula (outer diameter: 550 μm; length: 14 mm) was stereotaxically implanted into the third cerebral ventricle using a modification of a previously reported method [1]. The coordinates were 5 mm anterior to the interaural axis and 6.5 mm below the dura at the midline. One stainless steel anchoring screw was fixed to the skull, and the guide cannula was secured in place with acrylic dental cement. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments. The i.c.v. injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds. At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 μl), followed by sacrifice and brain sectioning (20 μm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

Before the feeding experiment, the birds were weighed and assigned to an experimental group based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible. To examine the orexigenic or anorexic effect of NMS, rat NMS (0.1, 0.5 or 1.0 nmol/10 μl saline) or saline (control) was administered i.c.v. at 07:00 h. Food consumption was determined in the free-fed birds at 2, 4 and 12 h after administration by measuring the disappearance of food from a pre-weighed feeder placed in each individual cage. Care was taken to collect and weigh any spillage, thus making the determination of food intake as accurate as possible.

The quails' body temperature was measured at 0 min (before injection), then at 5, 10, 20, 40, 60 and 120 min after i.c.v. injection of rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol/10 μl saline) or saline vehicle ($n = 6$ in each group) at 10:00 h using a previously reported method [1]. Briefly, temperature was measured electronically with a small sensor (measurable range: 25–50 °C; measurement error: 0.05 °C) connected to a line (outer diameter: 0.7 mm; length: 45 cm). The sensor tip was inserted into the cloaca, and part of the line was fixed to the body of the bird.

Locomotor activity was measured in each bird under light/dark conditions for 1 week, and thereafter under constant dim light at an intensity of about 30 lx. Locomotion was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer [8]. The infrared sensors were placed above the cages and measured all locomotor activity (e.g. eating, perch-hopping and flying). Each cage with its infrared sensor was placed in an isolated chamber with a controlled light/dark cycle. Data were collected at 15-min intervals and analyzed using CompactACT AMS software (Muromachi Co.). Rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol/10 μl saline) or saline vehicle was administered i.c.v. at 10:00 h ($n = 8$ per group). After the injections, the birds were immediately returned to their individual cages. Locomotor activity counts were made every 15 min and summed for the 2-h period following administration.

All results are expressed as mean ± S.E.M. The data were analyzed using analysis of variance and the post hoc Fisher's test.

I.c.v. administration of NMS 0.5 and 1 nmol significantly ($P < 0.05$) decreased food intake in a time-dependent manner compared with saline alone (Fig. 1A). This anorexigenic action of NMS was apparent by 2 h and continued for 12 h after i.c.v. administration. The effect was no longer observable on the following day (data not shown). Concomitantly, a significant ($P < 0.05$) decrease in body weight was observed at 2, 4 and 12 h after i.c.v. injection of NMS (Fig. 1B). The decrease in body weight was more pronounced than the decrease in food intake, and became quite considerable by 12 h after the injection. The effect of a smaller dose of NMS (0.01 nmol i.c.v.) was examined, but this dose effected no significant change in food intake ($n = 6$; data not shown).

I.c.v. injection of NMS also significantly ($P < 0.05$) increased body temperature and locomotor activity (Fig. 1C and D). An increment of about 2 °C was observed in body temperature 40–60 min after i.c.v. injection of 1.0 nmol NMS. Although 0.1 nmol NMS also caused an increase in body temperature, the change was not significantly different from that seen with saline alone. Locomotor activity was increased 1.5-fold during the 2-h period following i.c.v. injection of 1 nmol NMS.

When the effects of i.c.v. injection of the same doses of rat NMU and rat NMS on food intake, body temperature and locomotor activity were compared in Japanese quails, opposite effects were observed. Fig. 2 shows that rat NMU produced an increase in food intake but decreases in body temperature and locomotor activity.

The present study demonstrates that rat NMS suppresses food intake but promotes locomotor activity and increases body temperature in avian species. The suppression of feeding is unlikely to be due to any side effect of NMS, since the quails in the treated group did not show any abnormal behavior. The noticeable decrease in body weight after i.c.v. injection of NMS may be due to both a decrease in food intake and an increase in energy expenditure. These results therefore suggest that central NMS may play important roles in the regulation of feeding and the sympathetic nervous system in avian species.

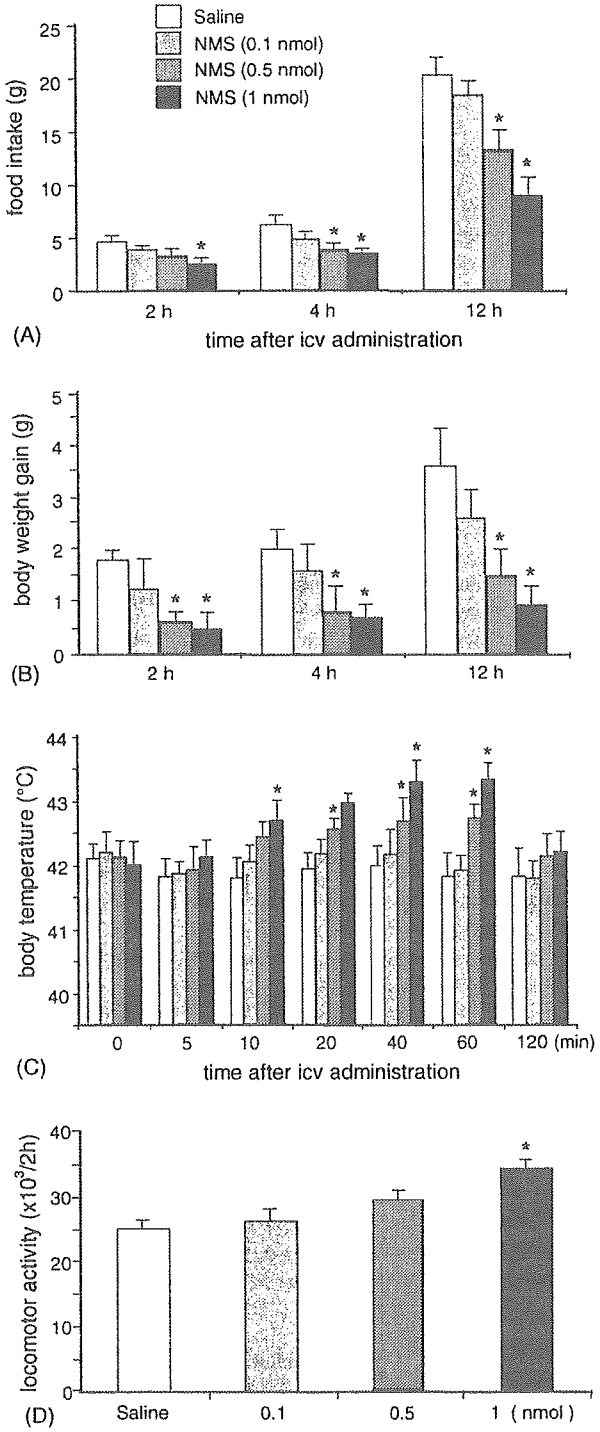

Fig. 1. Effect of intracerebroventricular (i.c.v.) administration of rat NMS on food intake (A), body weight change (B), body temperature (C), and gross locomotor activity (D) in the Japanese quail. Saline (vehicle control) or NMS (0.1, 0.5 or 1.0 nmol) was injected i.c.v. at 07:00 h for food intake assessments or 10:00 h for body temperature and gross locomotor activity assessments. Each bar and vertical line represents the mean ± S.E.M. ($n = 12$ for food intake assessments; $n = 6$ for body temperature assessments and $n = 8$ for gross locomotor activity assessments). *Significantly different from the saline-treated group; $P < 0.05$.

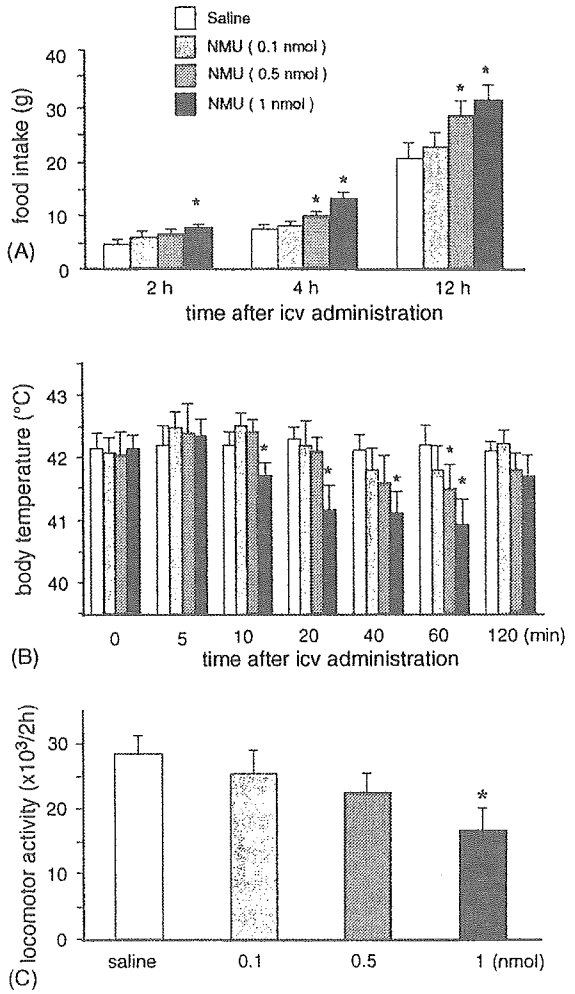

Fig. 2. Effect of i.c.v. administration of rat NMU on food intake (A), body temperature (B) and gross locomotor activity (C) in the Japanese quail. Saline (vehicle control) or NMU (0.1, 0.5 or 1.0 nmol) was injected i.c.v. at 07:00 h for food intake assessments or 10:00 h for body temperature and gross locomotor activity assessments. Each bar and vertical line represents the mean ± S.E.M. ($n = 12$ for food intake assessments; $n = 6$ for body temperature assessments and $n = 8$ for gross locomotor activity assessments). *Significantly different from the saline treated group; $P < 0.05$.

In a previous study, the distribution of NMS mRNA in various rat tissues was investigated using a quantitative reverse-transcriptase polymerase chain reaction technique [9]. NMS mRNA was expressed mainly in the hypothalamus, spleen and testis. Within the hypothalamus, NMS mRNA was expressed predominantly in the SCN; there was only very slight expression in other brain regions, such as the paraventricular nucleus (PVN) and arcuate nucleus (Arc) [9]. In situ hybridization histochemistry also showed that NMS mRNA expression was restricted to the SCN. No hybridization signal was observed in any other brain region [9]. In the case of NMS, therefore, its action on the PVN and Arc through the NMS projection from the SCN may be important.

Recently, we observed that i.c.v. NMS also suppressed food intake in rats [4]. In that case, cFos expression was detected in preopiomelanocortin (POMC)-neuron in the arcuate nucleus and corticotropin-releasing hormone (CRH)-secreting cells in

the paraventricular nucleus. This suggests that neuron containing POMC (a precursor of α-melanocyte-stimulating hormone; α-MSH) and CRH may be the targets for suppression of food intake by NMS, because CRH and α-MSH are known to be anorexigenic hormones in chicken [6,13]. However, further study is required to elucidate the mechanism of action of NMS in avian species.

Because NMS contains the active core C-terminus of NMU and binds to the same receptors (NMU1R and NMU2R), rat NMS and rat NMU would be expected to have very similar actions on food intake, locomotor activity and body temperature in Japanese quails. However, opposite effects were observed. Previously, we reported that Japanese quail NMU, but not rat NMU, suppressed food intake in Japanese quails, and that pretreatment with rat NMU inhibited the Japanese quail NMU-induced suppression of food intake [11]. Rat NMU therefore appears to have an antagonistic action on Japanese quail NMU, possibly through competition for NMU receptors. If this is so, why did rat NMS not show similar antagonism? The reason for the discrepancy is unclear from the present study; however, the following considerations may provide possible explanations. First, the structure of avian NMS may be close to that of rat NMS. If this is so, rat NMS may not act antagonistically at NMU receptors, and may be able to have same physiological function as avian NMS. Although we tried cloning Japanese quail NMS using essentially the same method as that used for cloning Japanese quail NMU [11], we were unsuccessful and could not therefore perform direct experiments with Japanese quail NMS. Second, there may be a specific receptor for NMS other than the NMU1R and NMU2R, and NMS may act on feeding and locomotion through it.

In conclusion, NMS, a novel peptide, appears to play important roles in the regulation of feeding, locomotor activity and body temperature in avian species. As this is the first paper to describe the actions of NMS in avian species, further research will be required to elucidate the exact mechanisms of action of NMS and any further physiological functions that it may have.

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