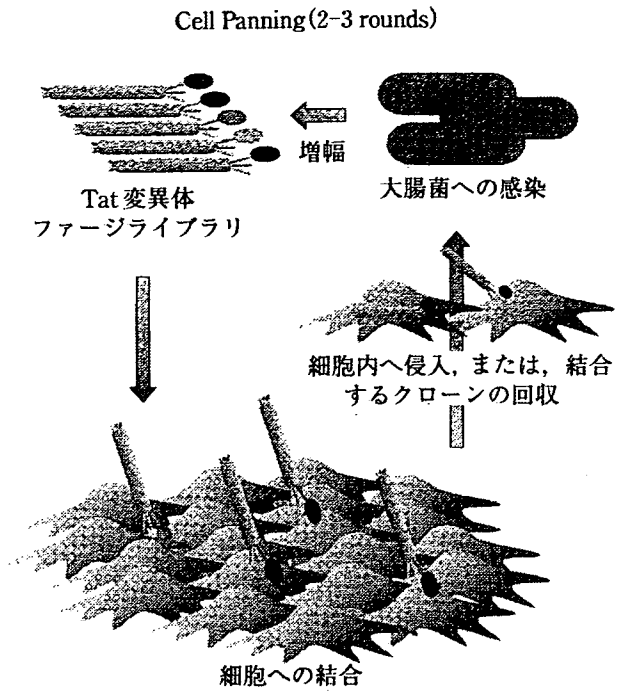

図4 Schematic diagram of the cell panning

まず18アミノ酸のランダムペプチドを提示したファージライブラリ(18a.a.-ファージライブラリ)を作製し，特定細胞に結合，あるいは細胞内へ侵入する活性を有するペプチドのスクリーニングを試みた．

b. 細胞パンニングによるスクリーニング(図4)

細胞内移行活性に優れたPTDを創製するために，18個のアミノ酸をランダムに並べたランダム18a.a.ペプチドライブラリを用い，モデル標的細胞(A431細胞)に対するパンニングを行った．パンニングを繰り返すにしたがって，A431細胞に結合するペプチド提示ファージが濃縮されていることを確認した．濃縮されたライブラリの中から更に細胞内移行活性を有するクローンだけを選び出し，その遺伝子配列を解析した結果，これまでに報告されているPTD活性を有するペプチドとは全く異なるペプチドが提示されている数種類のクローンの存在を明らかにした．PTDは一般にアルギニンなどの塩基性アミノ酸に富んだペプチド[11,12]であるが，今回濃縮されたペプチドのなかにはプロリンを多く含むペプチドが複数クローン存在していた．現在，その各クローンのペプチドの細胞内移行活性を評価しているが，少なくともTATペプチドよりも優れた細胞内移行活性を有するペプチドを得ることに成功している．

今回得られた新規PTDの細胞内移行機序や細胞特異性などを，既存のPTDと比較検討することで，細胞質内微粒子送達や，薬物送達の最適化にかなうPTDの分子設計指針において重要な知見が得られるものと考えており，この点については，別な機会に報告させて頂きたい．

おわりに

以上，本稿では細胞内DDSの確立を目指した細胞質内ナノ粒子デリバリー法の開発について紹介した．FLやPTDを用いたナノ粒子の細胞質内デリバリー法は，今後続々と開発されてくることが予想される様々な機能性ナノマテリアルに適用可能な汎用性の高い方法論である．そして細胞質内ナノ粒子デリバリーに基づく細胞内DDSは，次世代の細胞内ナノ治療を展開していくうえで多大な貢献をもたらすものと期待される．

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第27回　血小板機能シリーズ No.10

MC-FAN　血液さらさら度への血小板の寄与

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本邦における死亡原因のトップは依然として悪性腫瘍であるが，第二，第三の死亡原因は心筋梗塞や脳梗塞などの血栓性疾患であり，現在，これらの疾患の背景となる生活習慣病の増加が深刻な問題となっている．生活習慣病は，運動不足や過剰な栄養摂取，精神的・肉体的ストレスなどの不健康な生活習慣により誘発される疾患(高血圧や動脈硬化，肥満，糖尿病など)であり，こうした病態では恐らく血液は「どろどろ」状態となり，微小循環系の障害，そして組織の機能障害がスパイラル的に進行し，血栓症や臓器障害を来すと考えられている．しかし，実際に血液「どろどろ」状態がどのようにして生じるのか，そのメカニズムは明らかではない．一般に，健康の維持には血液「どろどろ」状態を改善して血液「さらさら」状態にすることが重要であると信じられている．現在，血液の「どろどろ」状態や「さらさら」状態を解析できる分析機器としては，血液流動性測定装置 MC-FAN (microchannel array flow analyzer)が汎用されている[1]．本稿では，血液の「どろどろ・さらさら」状態と血小板との関わりを論じ，筆者らが得た測定結果の意義を考察してみたい．

血小板の機能と病態

血小板は，生理的な止血と病的な血栓形成のいずれにも関与する細胞成分である．直径 2～4 μm の核のない円盤型の血球で，骨髄巨核球から産生される．血小板の細胞表面にはインテグリンに代表される各種の糖蛋白質 glycoprotein (GP)が発現しており，細胞外基質との接着や血小板同士の凝集に関与している．

血管が損傷され内皮下組織が露呈される状況下では，血小板は様々な因子によって活性化され，細胞膜 GP を発現し，あるいは GP が活性化される．同時に，血小板の α 顆粒と濃染顆粒 dense body からは von Willebrand 因子(VWF)や第 V 因子，フィブリノゲンなどの凝固因子とともに，ADP やトロンボキサン A_2(TXA_2)などの血小板凝集促進物質が放出され，より多くの血小板が活性化される．そして，活性化血小板膜 GPIbα は VWF を仲介して内皮下コラーゲンに結合し，また血小板 GPVI は直接コラーゲンに結合して傷害部位に血小板が集積する．また，GPIIb/IIIa はフィブリノゲンを介して連結し，血小板凝集が惹起される．さらに，血小板膜上に発現した P-selectin は活性化白血球や白血球由来マイクロパーティクル(微小顆粒)に発現する P-selectin glycoprotein I ligand (PSGL)-1 に結合し，止血血栓の形成や病的血栓の形成に関与し，さらには動脈硬化にも関わる可能性も示唆されている[2]．さらに，活性化血小板膜上にはホスファチジルセリンのような陰性荷電リン脂質が露呈し，このリン

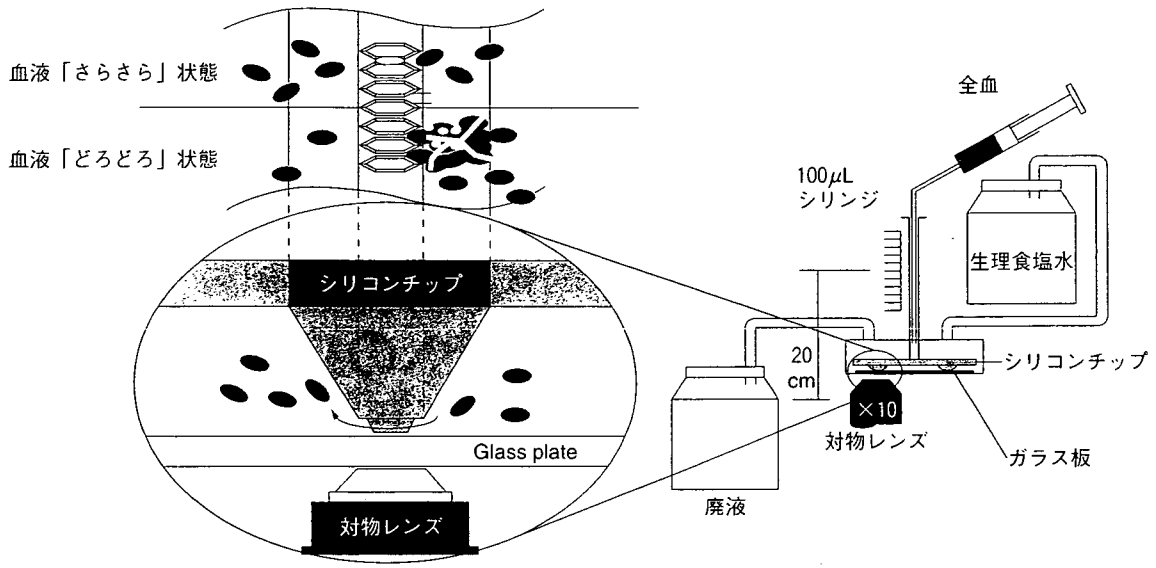

図1　MC-FAN の概略

採血した全血をシリンジからシリコンチップへと添加し，血液の流れ（血液「さらさら」度）をモニターで観察しながら，定量的に測定できる．血液「さらさら」状態では，シリコンチップに刻まれた微小流路内を流れていくが，血液「どろどろ」状態では微小流路に血栓が詰まることで，血液が流れない様子が見られる．

脂質に血漿中や顆粒から分泌された凝固因子が結合し，血液凝固反応が効率的に進展する．こうして，血小板は血小板血栓の形成だけでなく，凝固血栓の形成にも重要な役割を果している．

血液の粘度について

血液粘度の上昇は心血管系疾患の危険因子であり，血液粘度を測定する医学的重要性は増している．血液粘度の上昇によって起こる循環障害を過粘性症候群（hyperviscosity syndrome）と呼び，様々な病態の基盤になることが多い[3]．

血液粘度は，ずり速度に依存した非ニュートン流体であると考えられており，この性質は，血液構成成分の流体力学的特性だけでなく，血球成分および血漿成分の間の生化学的ならびに摩擦的相互作用に基づいている．血流速度の高い，高ずり速度領域では，流動抵抗を減らすため赤血球は楕円板に変形し，血液の流れる方向に対して平行に配位する．他方，低ずり速度領域では，赤血球と血漿成分との相互作用が増して赤血球を含む凝固塊が形成される．これにより血液の流れは乱れ，流動性抵抗は大きくなるため血液粘度は増加する．血小板は細胞成分の中で最も小さく，数も赤血球に比べて少ないので血液の流動性に，そのままの状態ではほとんど影響しない．しかし，傷害された血管壁に接触することにより血小板が活性化され凝集すると，血液の流れの妨げになる．とりわけ，血小板凝集塊の下流域には，ずり速度のきわめて小さい血液の溜まりが形成され，赤血球集合の形成が促進されると考えられ，こうした状態では微小血管は閉塞し血流は低下する．

他方，血漿の粘度は，主に血漿中の高分子物質の種類と濃度に依存しており，血漿蛋白質，とりわけフィブリノゲンのような繊維状分子は，流れの中での配向や温度の影響，血漿蛋白質の性質や状態によって変化する．したがって，フィブリノゲン濃度の増加は，血漿粘度を増加させる要因であり，心筋梗塞，脳虚血，脳梗塞，高血圧症などの心血管系疾患の危険因子になると考えられている[4]．また，血漿中の脂質濃度も血栓症，狭心症，心筋梗塞など循環系の病気と密接に関連することから，

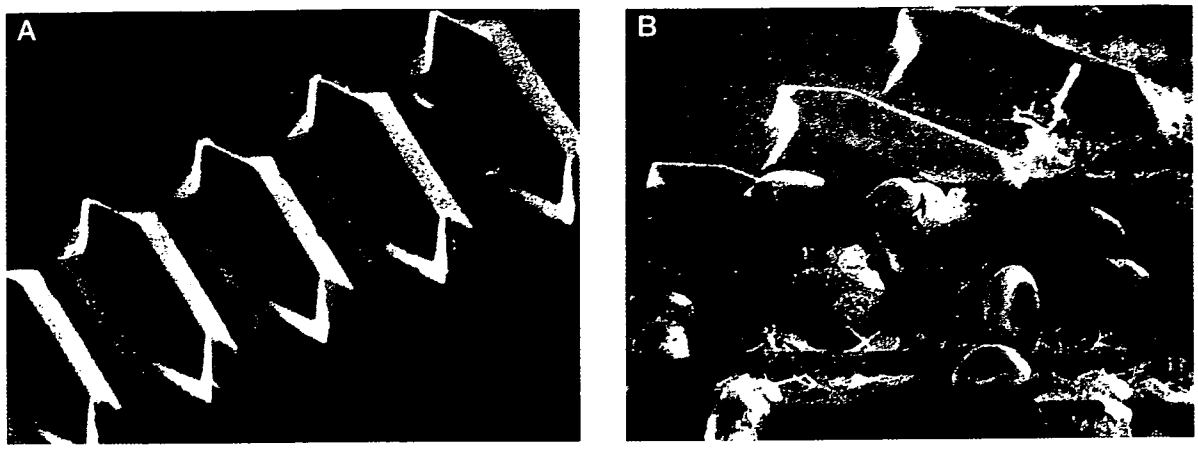

図2　シリコンチップの電子顕微鏡写真

A：血液添加前のシリコンチップ
B：血液にADPを添加し、MC-FANにて血流測定を行った後のシリコンチップ．血小板凝集塊とフィブリンネットが形成され，その内部に赤血球が巻き込まれている．

血漿に含まれる各種の蛋白質，脂質などの成分の変化は，血液粘度に影響を及ぼし，血栓性疾患の直接的あるいは間接的な要因になると考えられる．

MC-FANの特性

これまで，血小板の機能を解析するには，主に多血小板血漿(platelet rich plasma：PRP)を用いて，血小板の凝集状態を可視光の透過度の変化として測定する血小板凝集計が用いられてきた．また，血小板浮遊液の微小流路の通過時間を計測する方法もあるが，いずれの方法も他の血球成分が含まれない条件下で測定されるので，血管内でみられるような多様な血球成分の共存下での血小板の凝集状態を解析するものではない．

血液流動性測定装置MC-FANは，半導体微細加工技術を用いて，シリコン単結晶基板に毛細血管(平均径6 μm)とほぼ同じ幅(7 μm)と深さ(4.5 μm)(等価径6 μm)の微小流路が多数(約8,700個)並列している溝の中を血液(全血)が通過する状態をカメラで撮影し，血液(主に赤血球)の通過状態から血液の流動性(粘度)を測定する装置である(図1)．流路の表面はシリコン加工されており，固相面と血球の相互作用は最小限にとどめてある．実際にMC-FANで固相面と血小板の相互作用はほとんど観察されないが，血小板の凝集塊を解析することはできる．しかし，MC-FANの特徴は，血小板の凝集状態を他の血球の存在下に観察できる点にあり，単に血小板の凝集状態を解析するというよりは，赤血球を中心とする全血の凝血状態を観察できることである．MC-FANによる血液「どろどろ」状態では，多数の並列した微小流路に血小板の凝集塊や，白血球の活性化によって生じた赤血球を巻き込んだ血液凝固塊が流路を塞ぎ，血流が妨げられる状況が映像として見られる(図2)．こうした画像が，血管内での血液の流れとしてイメージ化され，血液「さらさら」や「どろどろ」として表現される．実際には，MC-FANでは，血液そのものの流動性を観察するというよりは，「微小流路を閉塞する血液凝固塊の形成過程(あるいは凝固塊のできやすさ)を定性・定量的に観察する」と表現するほうが真実により近いといえよう．

血液さらさら度と血小板の関係

それでは，MC-FANでみられる血液「どろどろ」の生理的意義はどのようなものだろうか？　先述したように，MC-FANにおける血液流動性には，微小流路を通過する血球成分，特に存在比の多い赤血球の変形能が大きな影響を及ぼすと考えられる．しかし，一般の健常者には，抗体などの赤血球凝集素の影響を除けば，赤血球の変形能の低下を招くような後天的あるいは先天性要因が

あるとは考えられない．したがって，血小板凝集塊や血液凝固塊によって流路が閉塞されて生じる血流低下を観察する本装置においては，赤血球よりもむしろ血小板や白血球がどれだけ活性化されたかを測定しているものと考えられる[5]．

多くの生活習慣病では，健常状態と比較して，血小板の機能的変化が認められている[6]．実際，糖尿病患者における血小板の機能変化と心血管障害の発症との関連性を示唆する報告がなされており，糖尿病に罹患することで血小板がコラーゲンに対して高感受性になるとの報告がある[7]．また，高脂血症においても糖尿病と同様に，血小板は高感受性になっていることが知られている．Tailorらは，血漿コレステロール値の上昇が，血小板および血管内皮細胞の機能的変化を惹起し，特に血小板上に発現するP-セレクチンにより，血小板と血管内皮の接着が促進されることで，向血栓性へと変化すると報告している[8][9]．したがって，MC-FANの測定により観察される流路の閉塞は，例えば，糖尿病性網膜症の末梢血管の状態を一部反映しているかもしれない．すなわち，目の網膜には眼球や周囲組織に栄養や酸素を供給するための細小血管が張りめぐらされているが，このような血管が糖尿病性機能障害を受けると血小板凝集塊やフィブリンからなる微小血栓が，微小血管を梗塞して糖尿病性網膜症が発症すると考えられている．MC-FANによって観察される微小流路の閉塞と血流低下はこのような過程を*in vitro*で再現しているものと考えられる．実際に血管内で起こる血液凝固過程は非常に複雑であり，MC-FANでの解析画像が微小血管内の病態変化そのものを忠実に再現しているとは考えられない．しかし，MC-FANで見られる血液「どろどろ」状態は，糖尿病や高脂血症などの生活習慣病患者に見られるアゴニスト高感受性血小板の活性化状態を反映している可能性があると考えられる．

MC-FANの医薬品・機能性食品開発への応用

血小板を人為的に活性化し，MC-FANを用いて血流が低下した状態を定量化できれば，血流を改善させる薬物の開発に応用できる可能性がある．血液の凝固過程は，前記したように，異物により活性化された白血球あるいは血小板が，血漿成分と相互反応しながら進行するものであり，こうして生じた血栓は，微小循環障害を来す．われわれは，このような状態を想定し，バクテリア由来のリポ多糖(LPS，エンドトキシン)等で全血を刺激し，白血球を活性化させたときの血液流動性をMC-FANを用いて測定した．その結果，LPSは濃度依存性に血流低下を惹起し，この血流低下は，凝固阻害剤や血小板凝集阻害剤の濃度依存性に改善されることを示した[10]．また，ADP等の血小板凝集惹起物の添加によりMC-FAN観察で血流が低下し，この血液「どろどろ」状態を血小板凝集阻害剤は濃度依存性に有意に抑制した(投稿中)．こうした結果から，MC-FANは，高感度に白血球や血小板の活性化に基づく微小血栓の形成を血流低下として定量化できる点で有用であり，また，凝固阻害剤や血小板凝集阻害剤などの治療薬のスクリーニングにも有用であることが示唆された．われわれは，現在，生活習慣病における血流障害を改善する機能性食品の開発にもMC-FANが有用であることを認めている．

展　望

本稿では，MC-FANによる血流測定に及ぼす血小板や白血球の影響について，われわれの私見を中心に概説した．MC-FANを用いた解析による血液「さらさら」状態は，血液中の血小板や白血球が活性化されていないことを示唆するものであろうが，患者の健康状態を診断できるか否か，その科学的根拠はあまりない．高脂血症や高血圧，糖尿病などの生活習慣病では血小板機能は影響を受けており，これが血液「さらさら」度に影響を及ぼすことは想像できるが，生活習慣病では血管内皮の機能障害も，血栓形成における重要な要因になっている．すなわち，被験者の血管内皮に問題があれば，仮にMC-FAN測定により血液「さらさら」であっても，健康とは言えない．さらに，われわれの結果でも，生活習慣病と無縁な若年健常者の血液でも，採血状態や採血後の血液の保管状態によって，血液「どろどろ」状態になることがあり，MC-FAN測定では，採血法とその後の血液管理には細心の注意を払う必要がある．また，MC-FAN測定による血液「さらさら」と「どろどろ」が真に

医学的に意味のあるものか否かを明確化するためには，種々の疾患患者の血液流動性を測定するとともに，MC-FAN 測定の結果が，生活習慣病の発症や増悪化に関連するかどうかプロスペクティブな調査検討も必要である．

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Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord

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Abstract

Expressions of the growth hormone secretagogue receptor (GHS-R) mRNA and its protein were confirmed in rat fetal spinal cord tissues by RT-PCR and immunohistochemistry. *In vitro*, over 3 nM ghrelin and des-acyl ghrelin induced significant proliferation of primary cultured cells from the fetal spinal cord. The proliferating cells were then double-stained using antibodies against the neuronal precursor marker, nestin, and the cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU), and the nestin-positive cells were also found to be co-stained with antibody against GHS-R. Furthermore, binding studies using [^{125}I]des-acyl ghrelin indicated the presence of a specific binding site for des-acyl ghrelin, and confirmed that the binding was displaced with unlabeled des-acyl ghrelin or ghrelin. These results indicate that ghrelin and des-acyl ghrelin induce proliferation of neuronal precursor cells that is both dependent and independent of GHS-R, suggesting that both ghrelin and des-acyl ghrelin are involved in neurogenesis of the fetal spinal cord.

Keywords: Ghrelin; Des-acyl ghrelin; GHS-R; Neurogenesis; Spinal cord; Fetal development

Ghrelin, a peptide hormone secreted from the stomach, has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), through which ghrelin stimulates GH release in the pituitary [1]. Two types of GHS-R, type1a and 1b (GHS-R1a and 1b, respectively), have so far been found, and only the former is able to activate signal transduction of the receptor downstream linking to phospholipase C, resulting in an increase of intracellular calcium [2]. Ghrelin consists of 28 amino acids and is characterized by esterified modification with octanoic acid on serine 3, which is essential for activation of GHSR-1a, although the modification mechanism remains unknown. On the other hand, the level of des-acyl ghrelin, which is inactive on GHS-R1a because of a lack of octanoic acid, is 4 times as high as that of ghrelin in the blood [3].

Many studies have reported that ghrelin has multiple effects other than GH secretion, including regulation of food intake [4] and energy metabolism [5], and gastrointestinal coordination [6,7], as well as facilitation of cell survival, and/or inhibition of apoptosis [8–15]. Although these multiple functions of ghrelin would account for the very wide distribution of GHS-R1a, it is debatable whether GHS-R1a contributes to all of the actions of ghrelin, i.e. that ghrelin may act as a ligand for other types of receptors [16]. So far, however, this possibility remains uninvestigated, and no such alternative receptor has been identified.

We have previously demonstrated that rat fetal growth was increased by treatment of the mother with exogenous ghrelin, and that the effect of ghrelin on fetal growth is diminished by immunization against ghrelin *in vivo* [17]. In addition, we have found that amniotic fluid contains a

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large quantity of des-acyl ghrelin, and that proliferation of cells from rat fetal skin is also stimulated by treatment with both ghrelin and des-acyl ghrelin *in vitro*. On the basis of these findings, we speculate that both ghrelin and des-acyl ghrelin play a crucial role in fetal growth, both peptides acting to facilitate fetal growth not only by enhancement of maternal appetite but also via direct stimulation of fetal cell proliferation by transfer of maternal ghrelin to the fetus.

In the present study, we demonstrated that both ghrelin and des-acyl ghrelin facilitate neural cell proliferation in cultured cells from the fetal spinal cord, which express both the GHS-R gene and its protein, and identified these proliferating cells as neuronal precursor cells. Furthermore, in binding studies using [^{125}I]des-acyl ghrelin, we clarified that des-acyl ghrelin has at least one binding site in the membrane fraction from fetal spinal cord. These results suggest that ghrelin and des-acyl ghrelin can facilitate neurogenesis in the rat fetal spinal cord through both the GHS-R and also an unidentified GHS-R-independent alternative pathway.

Materials and methods

Primary culture of embryonic spinal cord cells. Embryonic spinal cords were obtained from a pregnant rat at day 17. The uterus usually contained 10–14 embryos, 10 of which were utilized for primary culture. The whole spinal cords were mechanically and enzymatically dissociated in papain solution, and the digestion was stopped by addition of culture medium. Cells were passed though a strainer, then centrifuged at 1000 rpm at 4 °C for 10 min and resuspended in DMEM supplemented with $NaHCO_3$, antibiotics (penicillin, streptomycin; Sigma, MO), and 5% fetal calf serum, followed by plating onto laminin-coated 96-well plates at 10^5 cells per well.

Ghrelin and des-acyl ghrelin treatment and cell proliferation assay. Cell proliferation was measured by Cell Proliferation ELISA with BrdU (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions with some optimization for the present cell conditions as follows. Briefly, after incubation for four days, the cells were treated with ghrelin or des-acyl ghrelin at a final concentration of 0.003–300 nM for 12 h. Subsequently, BrdU was added to the cells to label newly synthesized DNA, followed by further incubation for 6 h. After incubation, the cells were fixed and denatured, and incubated with anti-BrdU antibody for 90 min. Each well was washed out and reacted with substrate solution until color development. The absorbance of the reaction was measured by an immunoreader. Data were expressed as means ± SEM. The significance of differences between the control and treated cells was analyzed by Student's *t* test. Differences at $P < 0.05$ were considered statistically significant.

Immunohistochemistry. Frozen sections of the embryonic spinal cord 14 μm thick were prepared from embryos at embryonic day (ED) 17 and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. After washing with 0.1 M phosphate buffer, the preparations were incubated with 2% normal goat serum in PBS for 30 min at room temperature, washed with PBS three times, and incubated overnight at 4 °C with each of the following primary antibodies: Polyclonal rabbit anti-microtubule-associated protein 2 (Map2; 1:1000, Chemicon International, Inc., CA), anti-neurofilament H (NF-H; 1:1000, Chemicon International), and anti-GHS-R and monoclonal mouse anti-nestin (1:10,000, Chemicon International). Subsequently, all the sections were washed in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG, except the sections that were incubated with the anti-nestin antibody, for which FITC-conjugated goat anti-mouse IgG (1:200, Chemicon International) was used as the secondary antibody. After washing out the residual antibodies and embedding of the sections, they were observed using a light microscope.

Cultured spinal cord cells, which had incorporated BrdU after incubation for 4 days during the ELISA preparation procedure, were fixed with methanol and glacial acetic acid at −20 °C for 20 min. After DNA denaturation with 2 M HCl and blocking with 2% normal goat serum in PBS for 30 min at room temperature, the fixed cells were incubated overnight at 4 °C with either anti-Map2 or anti-nestin as the primary antibody. Afterwards, the cells were incubated at RT for 1 h with the same secondary antibodies as those used for staining the frozen sections. After the washing step, the cells were further incubated with rat anti-BrdU monoclonal antibody (1:1000, Abcam, Cambridge, UK) as a primary antibody for double staining, followed by incubation with Cy™3-conjugated donkey anti-rat IgG polyclonal antibody (1:1000, Jackson Immuno Research Laboratories, Inc., PA) as the secondary antibody. For double staining of the GHS-R for either Map2 or nestin, cells fixed with 4% paraformaldehyde in 0.1 M phosphate buffer were first incubated with either mouse anti-Map2 or anti-nestin primary antibody, and then with rabbit anti-GHS-R antibody.

RT-PCR. Total RNA was isolated from the spinal cord of embryos at ED 13, 15, 17, 19, and postnatal day (PD) 0 using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD). Single-strand DNA was generated from 1 μg of total RNA with the use of Superscript 3 preamplification reagent (Life Technologies, Inc., Bethesda, MD) according to the manufacturer's instructions. PCR was carried out using a BD advantage™ 2 PCR Enzyme System (BD Science, CA). The PCR primers specific for GHS-R1a were 5′-GATACCTCTTTTCCAAGTCCTTCGAGCC-3′ for sense and 5′-TTGAACACTGCCACCCGGTACTTCT-3′ for antisense (nucleotides 842–869 and 1001–1025; Accession No. AB001982, GenBank), and those specific for GAPDH were 5′-CGGCAAGTTCA ACGGCACA-3′ for sense and 5′-AGACGCCAGTAGACTCCACGA CA-3′ for antisense (nucleotides 1002–1020 and 1125–1147; Accession No. AF106860, GenBank).

Des-acyl ghrelin binding assay. Binding of des-acyl ghrelin to tissue membranes of fetal spinal cord was studied using [^{125}I]des-acyl ghrelin as a radioligand. Membrane fractions (30,000g pellet) were isolated from fetal spinal cord tissue as described previously [18–20]. Membranes with a protein content of 10 μg, as determined by the Lowry method, were incubated at 4 °C for 1 h with increasing concentrations (0.13–16.64 nM) of [^{125}I]des-acyl ghrelin in a final volume of 0.5 ml assay buffer (50 mM Tris–HCl, 2.5 mM EGTA, 0.1% BSA, and protease inhibitor cocktail (Sigma, MO), pH 7.4). Parallel incubations in the presence of 1.0 μM unlabeled des-acyl ghrelin were used to determine nonspecific binding, which was subtracted from total binding to yield specific binding values. For competition assay, tissue membranes were incubated with 0.1 nM labeled des-acyl ghrelin and either unlabeled des-acyl ghrelin or ghrelin at 4 °C for 1 h. After incubation, the reaction solution was filtered through Whatman GF/B filters, which were then rinsed three times with assay buffer. The radioactivity of the membranes on the filter was measured with a gamma counter. Saturation isotherms were transformed using the method of Scatchard and the maximal number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated using the GraphPAD Prism 4 program (GraphPAD Software, CA).

Results

GHS-R mRNA and protein expression in fetal spinal cord

Using RT-PCR, we examined GHS-R mRNA expression in spinal cords obtained from rat fetuses at ED 13, 15, 17, 19, and PD 0 (Fig. 1a). Abundant levels of GHS-R mRNA were expressed in the spinal cord in fetuses at all ages examined, as we have reported previously [17]. We then investigated GHS-R expression at the protein level by immunohistochemistry. At the same time, we detected neuronal cells by using antibodies against the neuron-specific markers Map2 and NF-H, as well as nestin for

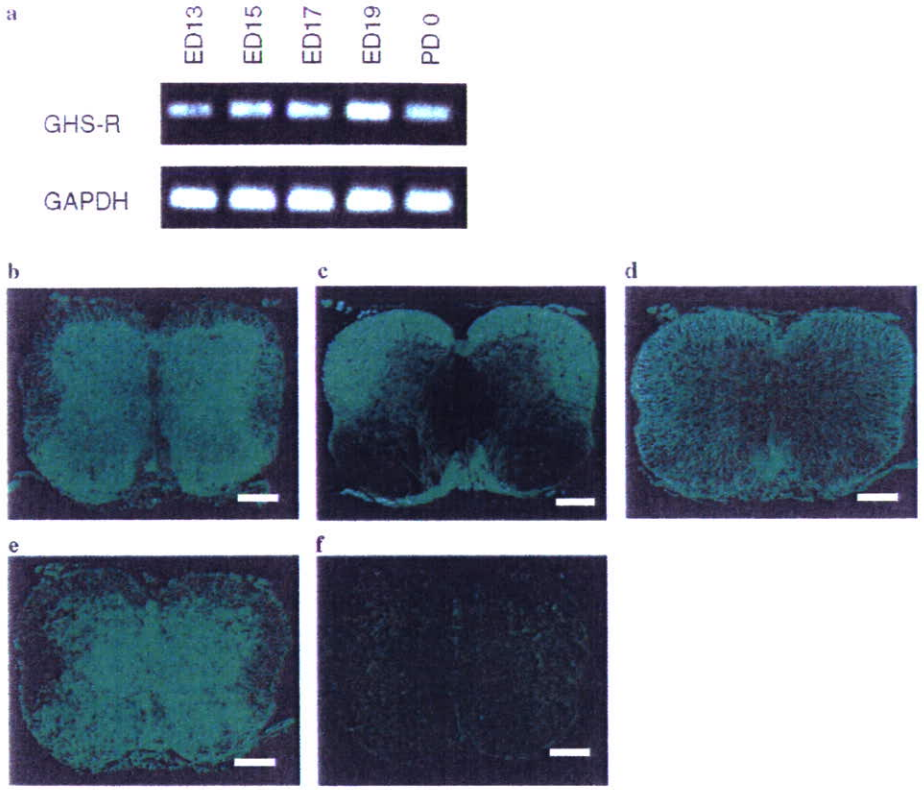

Fig. 1. (a) Detection of GHS-R mRNA in the rat fetal spinal cord by RT-PCR. The PCR product amplified with primers specific for GHS-R1a was detected from embryonic day (ED) 13 to postnatal day (PD) 0. GAPDH mRNA was also detected as an internal control. (b–f) Immunofluorescence staining with antibodies against neuron marker and GHS-R proteins in the rat fetal spinal cord. Map2-positive (b) and Neurofilament H-positive cells (c) were localized in the gray and white matters, respectively. Immunoreactivity of the neuronal precursor cell marker, nestin, was found throughout the spinal cord but with strongest staining in the white matter (d) GHS-R immunoreactivity was localized in the gray matter (e) and was not observed in sections that had been exposed to the preadsorbing antibody (f). Bars, 200 μm.

neuroprogenitor cells. Cells immunoreactive for Map2 were located in the core of the spinal cord, the so-called gray matter (Fig. 1b), while NF-H immunoreactivity was confirmed in the white matter (Fig. 1c). Nestin-positive cells were located in all regions, but the most intense staining was observed in the white matter (Fig. 1d). GHS-R-positive staining was located in the gray matter of the spinal cord (Fig. 1e), and the staining was abolished by pre-absorption of the antibody (Fig. 1f).

Proliferation of spinal cord cells upon treatment with ghrelin and des-acyl ghrelin

Primary culture of spinal cord cells from rat fetus at ED17 was performed. The cells were cultured with BrdU for 6 h after initial incubation for 4 days, and then treated with ghrelin and des-acyl ghrelin for a further 12 h. BrdU is incorporated into DNA when cells synthesize DNA during the S phase of the cell cycle and can be immunodetected using anti-BrdU antibody. BrdU-positive cells were detected under all conditions, irrespective of treatment, although BrdU positivity was more abundant in cells that had been cultured with ghrelin and des-acyl ghrelin than in non-treated cells. To quantify the increase in the number of cells positive for BrdU, we measured cell proliferation by BrdU ELISA. Treatment with both ghrelin (Fig. 2a) and des-acyl ghrelin (Fig. 2b) at over 3 nM significantly increased the incorporation of BrdU.

Identification of the proliferative cell type and cell type expressing GHS-R

Immunofluorescence double staining of cultured cells treated with ghrelin that had incorporated BrdU into their DNA was performed to identify proliferating cells among cultured rat fetal spinal cord cells. Cells with Map2 positivity showed a typical neuron-like shape with extended dendrites and did not show BrdU positivity in their nuclei (Fig. 3a). Cells with nestin positivity were pleomorphic and showed BrdU positivity in their nuclei, i.e., neuronal precursor cells (Fig. 3b). In addition, BrdU positivity was also found in cells that were unstained by antibodies against both Map2 and nestin (data not shown). GHS-R-expressing cells were then examined by immunofluorescence double staining, as was the case for cultured cells without BrdU treatment. GHS-R immunoreactivity was observed in the nestin-positive cells (Fig. 3c).

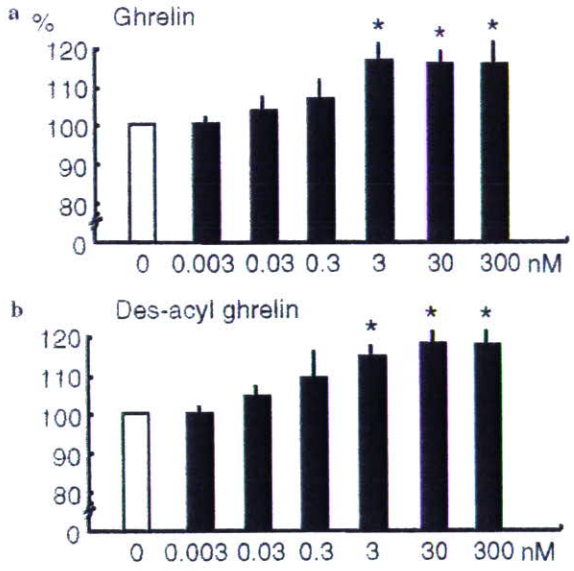

Fig. 2. Cell proliferation effects of ghrelin and des-acyl ghrelin on cultured fetal spinal cells. Proliferative cells were quantified by BrdU ELISA. Significant proliferation was indicated in cells treated with over 3 nM ghrelin (a) and des-acyl ghrelin (b). Values are presented by means + SEM ($^*P < 0.05$).

Binding assay

To identify the presence of the binding site of des-acyl ghrelin, [^{125}I]des-acyl ghrelin binding to membranes from fetal spinal cord was assayed. Specific, high affinity and saturable binding of labeled ghrelin were observed ($K_d = 3.467$, $B_{max} = 1.061$ fmol/mg protein) (Fig. 4a). The binding of labeled des-acyl ghrelin was displaced by unlabeled des-acyl ghrelin and ghrelin (Fig. 4b). The IC_{50} values for des-acyl ghrelin and ghrelin were 23.52 and 41.60 nM, respectively.

Discussion

Our previous study showed that ghrelin, as well as des-acyl ghrelin, play important roles in fetal growth, and that GHS-R mRNA is abundantly expressed in the spinal cord of rat fetus compared with other tissues [17]. Therefore, we reasoned that these ligands and their receptor might exert important actions during neurogenesis of the embryonic spinal cord. In the present study, in fact, ghrelin and des-acyl ghrelin both facilitated the proliferation of cells from fetal spinal cord. In addition, GHS-R mRNA and

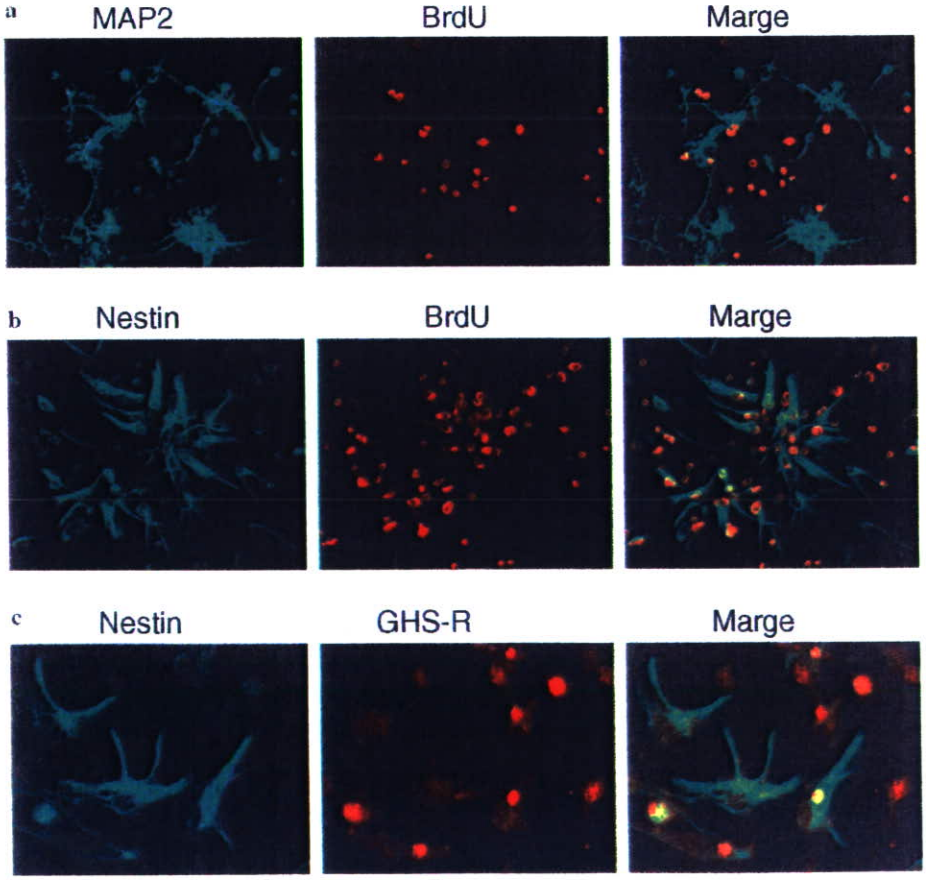

Fig. 3. Identification of proliferative cells in the rat fetal spinal cord. Double immunofluorescence staining demonstrated immunoreactivity for both Map2 and BrdU in distinct cells (a), and co-localization of nestin and BrdU in the same cells (b) Co-localization of nestin and GHS-R was also observed in neuron precursor cells (c).

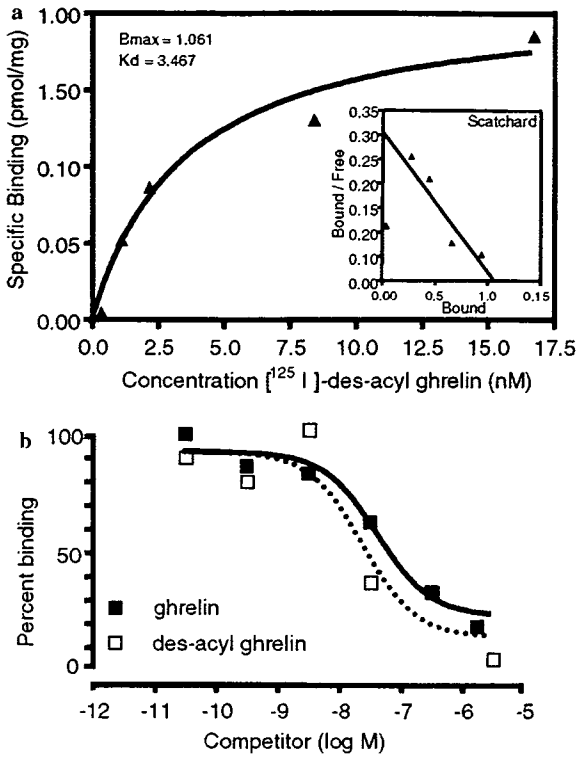

Fig. 4. (a) Representative saturation curve and the Scatchard plot of [^{125}I]des-acyl ghrelin binding to membranes from the fetal spinal cord. Binding assay was performed by incubating a fixed amount of membranes with increasing concentrations (0.13–16.64 nM) of the radioligand. Specific binding values were obtained by subtracting non-specific binding from total binding. (b) Displacement curve of [^{125}I]des-acyl ghrelin binding in the presence of unlabeled des-acyl ghrelin or ghrelin. Binding assay was performed by incubation of fixed amounts of membranes and labeled ligand with increasing concentrations of either of the unlabeled ligands.

GHS-R protein were detected in spinal cord tissue, and neuronal precursor cells in primary culture possessed GHS-R immunoreactivity, indicating that ghrelin stimulated the proliferation of neuronal precursor cells through GHS-R. Although the receptor recognizing des-acyl ghrelin has not yet been characterized, the present binding study indicated that there was at least one binding site specific for des-acyl ghrelin in membranes from fetal spinal cord tissue. Several recent studies have reported that not only ghrelin but also des-acyl ghrelin exert a biological effect even in tissues or cells that do not express GHS-R, suggesting that these reactions would not require octanoic acid modification and could be achieved without GHS-R [11,17,21,22]. Interestingly, many of the effects induced by both peptides at the cellular level are associated with cell fate, such as cell survival and/or apoptosis as well as cell proliferation, although activation or inhibition of the cell survival and proliferation pathways appear to be independent of cell type [11]. Thus, it is assumed that the effect of ghrelin and des-acyl ghrelin on spinal cord cells observed in the present study could be induced through both the GHS-R and another unknown pathway.

Recently, Zhang et al. demonstrated that ghrelin acted directly on dorsal motor nucleus of the vagus neurons to stimulate neurogenesis, and concluded that neuronal proliferation would result from an increase of calcium concentration associated with cellular depolarization through activation of GHSR-1a by ghrelin [15]. In the present study, however, diltiazem, a blocker of L-type voltage-dependent calcium channels, did not inhibit proliferation of spinal cord neuronal cells, inconsistent with dorsal motor nucleus of the vagus neurons, suggesting that the proliferation effect was likely mediated via a pathway other than the calcium increase caused by depolarization of L-type calcium channels (data not shown). Some studies of the molecular mechanism involved in the induction of cell proliferation and adhesion by ghrelin have suggested cascades of intracellular events, such as the MAPK and/or PI3 K/Akt pathways. In hepatoma cells expressing GHS-R, ghrelin has been shown to activate the IRS-1-GRB2-MAPK pathway, downstream from the insulin receptor, but to inhibit Akt activity [23]. Also in cardiomyocytes and endothelial cells, ghrelin induces phosphorylation of tyrosine, and both ghrelin and des-acyl ghrelin activate the MAPK and Akt pathways [9]. In addition, activation of the MAPK pathway by ghrelin has also been reported in a rat pituitary somatotroph cell line [13] and human adrenal zona glomerulosa cells [12]. Nanzer has explained the possible pathways leading to MAPK activation, resulting from stimulation of phospholipase C and PKC, or transactivation of tyrosine kinase receptors via the beta and gamma subunits of the G protein. Similarly, it is assumed that the cell proliferation effect of ghrelin and des-acyl ghrelin in the rat fetal spinal cord might involve activation of MAPK and/or PI3K/AKt.

In this study, not only neuronal precursor cells but also neurons seemed to possess GHS-R protein, because the localization of Map-2-positive cells was consistent with that of GHS-R-positive cells in sections of spinal cord tissue. These results suggested that ghrelin would play an unidentified role via GHS-R in neurons of the rat fetus, for instance during formation of the neuronal network. Although there is no evidence of any abnormality in GHS-R-knockout mouse fetus [24], this function as well as the cell proliferative effect may be concealed by compensating actions of growth factors such as nerve growth factor.

In summary, we have demonstrated that both the GHS-R gene and protein are expressed in the rat fetal spinal cord from ED 13 to PD 0. In primary cultures of fetal spinal cord cells, ghrelin and des-acyl ghrelin induced cell proliferation effects, whereby neuronal precursor cells possessing GHS-R protein were increased. Moreover, a binding study using labeled des-acyl ghrelin showed that specific binding to des-acyl ghrelin could be displaced by unlabeled ghrelin and des-acyl ghrelin in membranes from fetal spinal cord. Taken together, our findings suggest that in the rat fetal spinal cord, ghrelin and des-acyl ghrelin are involved in neurogenesis via both GHS-R and an unidentified receptor

for des-acyl ghrelin. Further examinations to identify this unknown receptor for des-acyl ghrelin are warranted.

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Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus

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Summary

Ghrelin, a gastrointestinal peptide, stimulates feeding when administered peripherally. Blockade of the vagal afferent pathway abolishes ghrelin-induced feeding, indicating that the vagal afferent pathway may be a route conveying orexigenic ghrelin signals to the brain. Here, we demonstrate that peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) at least in part via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus, thereby stimulating feeding at least partially through α-$_1$ and β-$_2$ noradrenergic receptors. In addition, bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine β hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. These findings provide new evidence that the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin.

Introduction

Ghrelin, a newly discovered member of the family of gut-brain peptides, functions in feeding control and growth hormone (GH) secretion by binding to the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999; Nakazato et al., 2001; Tschöp et al., 2000; Wren et al., 2000). This peptide, which is produced primarily by endocrine cells of the stomach, is released into the circulation (Date et al., 2000; Dornonville de la Cour et al., 2001). Ghrelin is also produced by neurons of the hypothalamus, where it serves as part of the neural networks (Cowley et al., 2003). GHS-R is extensively distributed throughout the brain, including the hypothalamus and brainstem where are essential for energy homeostasis. Given the GHS-R expression pattern, ghrelin, when given centrally, peripherally, or both, may increase food intake directly via effects on neurons present in the hypothalamus or brainstem. We recently demonstrated, however, that blockade of the gastric vagal afferent pathway abolished peripheral ghrelin-induced feeding (Date et al., 2002). A similar study demonstrated that intraperitoneal injection of ghrelin into vagotomized mice did not stimulate food intake (Asakawa et al., 2001). These findings suggest that the gastric vagal afferent pathway as well as the humoral pathway may have some significant part in conveying ghrelin-mediated orexigenic signals to the brain.

Several gastrointestinal hormones, including ghrelin, cholecystokinin (CCK), peptide YY, and glucagon-like peptide 1, transmit signals of starvation and satiety to the brain at least in part via the vagal afferent system (Date et al., 2002; Smith et al., 1981; Koda et al., 2005; Abbott et al., 2005). Feeding-related information, travels directly to the nucleus tractus solitarius (NTS), where it can be converted to additional signals that transmit a feeling of hunger or fullness to the hypothalamus. In the present study, we focused on the importance of the neural pathways from the NTS to the hypothalamus in transmitting peripheral ghrelin signals.

To investigate the neural pathways involved in the transmission of ghrelin orexigenic signals from the NTS to the hypothalamus, we examined the effects of bilateral midbrain transections on ghrelin-induced feeding. The NTS contains the A2 noradrenergic cell group, which projects to regions of the hypothalamus that include the arcuate nucleus (ARC) (Sawchenko and Swanson, 1981). Therefore, we examined the role of the central noradrenaline (NA) system in peripheral ghrelin feeding stimulation. Using real-time PCRs, we quantified the expression of dopamine β hydroxylase (DBH), an enzyme necessary to convert dopamine into NA, within the NTS. We also measured overflow NA within or near the ARC after intravenous administration of ghrelin using in vivo microdialysis. We studied the effects of adrenergic antagonists and the elimination of NA innervation within the ARC on ghrelin-induced food intake. Using immunohistochemical techniques, we demonstrated that the NPY neurons activated following intravenous administration of ghrelin are innervated by DBH-containing fibers.

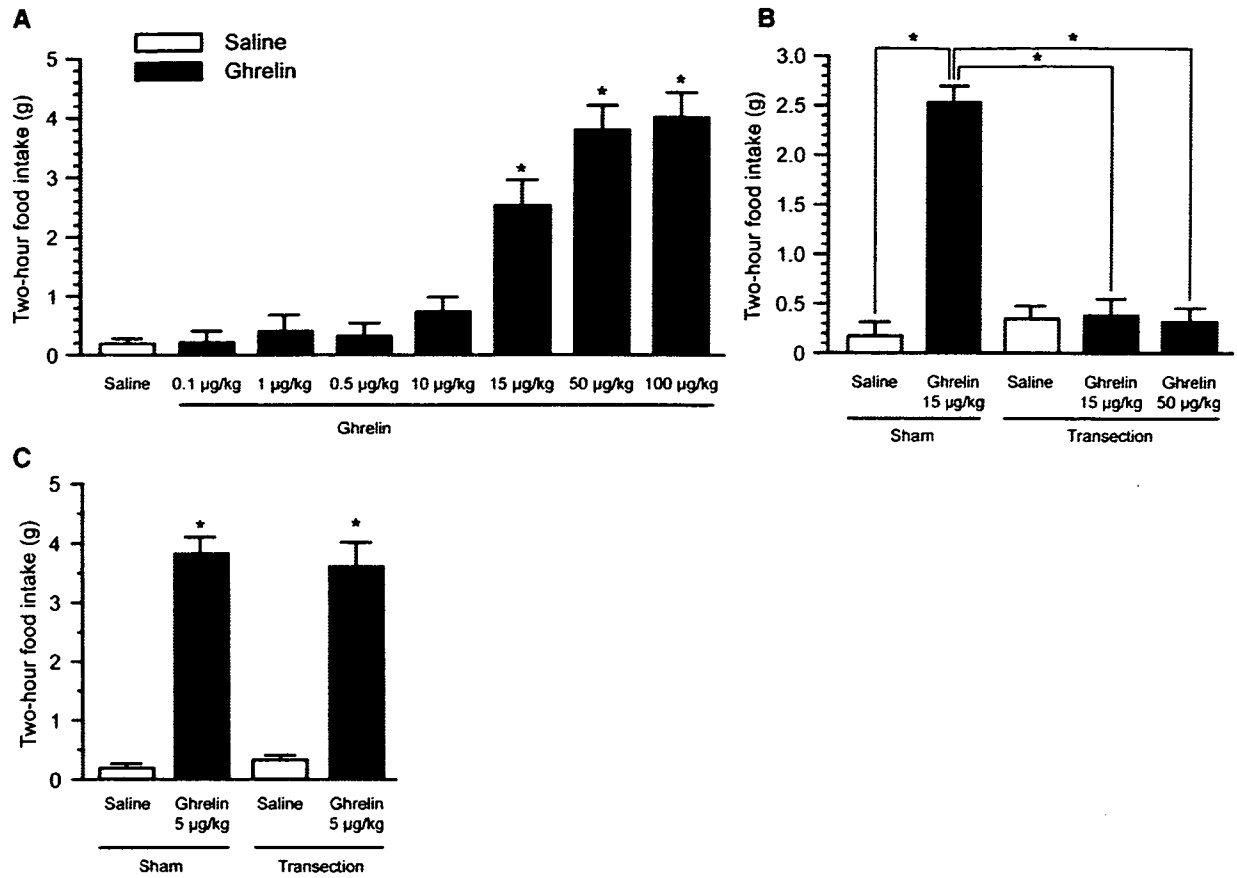

Figure 1. Effect of bilateral midbrain transections on ghrelin-induced feeding behavior

A) Two hour food intake (mean ± SEM) of sham-treated rats after a single intravenous administration of ghrelin (0.1–100 μg/kg). *$p < 0.0001$ versus saline.
B) Food intake of rats with bilateral midbrain transections after a single intravenous administration of ghrelin (15 and 50 μg/kg). *$p < 0.0001$.
C) Food intake of rats with bilateral midbrain transections after single intracerebroventricular administration of ghrelin (5 μg/kg). *$p < 0.0001$.
Error bars represent the SEM.

Results and Discussion

Midbrain transections and peripheral ghrelin-induced feeding

To investigate if intravenous administration of ghrelin stimulates feeding via the ascending efferent fibers of the NTS, we examined ghrelin-induced food intake in rats with bilateral midbrain transections (Crawley et al. 1984). Before this experiment, we confirmed that there were no significant differences in body weight or food intake between control and actual transected groups up to eight days after the surgery (see Supplemental Results and Figure S1 in the Supplemental Data available with this article online). There were also no significant differences in the feeding response after fasting for 12 hr, energy expenditure, locomotor activity, body fat, or food preference between the two groups seven days after surgery (Supplemental Results and Figure S2). Therefore, we performed feeding experiments using rats seven days after the surgery. The lowest effective dose of intravenously (i.v.) administered ghrelin for rats subjected to sham surgery (sham-treated rats) was 15 μg/kg; this value was used as the standard dose in the subsequent experiments (Figure 1A). Intravenous administration of ghrelin (≥ 15 μg/kg) significantly increased food intake (10:00–12:00 hr) in sham-treated rats, whereas ghrelin-induced feeding was absent in midbrain transected rats (Figure 1B) (n = 10 per group). Because bilateral midbrain transections may nonspecifically suppress feeding in response to ghrelin, we tested the orexigenic effect of centrally administered ghrelin in the midbrain transected rats. Intracerebroventricular administration of ghrelin similarly increased food intake in the transected and control groups (Figure 1C) (n = 7 per group). This finding demonstrates that bilateral midbrain transections specifically blocked peripherally administered ghrelin-induced feeding, but did not affect centrally administered ghrelin-induced feeding. Centrally and peripherally administered ghrelin may therefore stimulate feeding by distinct mechanisms. Midbrain transections severing the ascending efferent fibers of the NTS block feeding reduction of CCK that transmits satiety signals to the brain via the afferent limb of the vagus nerve (Crawley et al., 1984). In contrast, Grill and Smith showed that CCK-induced feeding reduction is still observed in chronic decerebrate rats (Grill and Smith, 1988). We described some differences in the surgery between midbrain transection and chronic decerebration in Supplementary Methods (Grill and Norgren, 1978) (Supplemental Experimental Procedures).

We have already shown the possibility that peripheral ghrelin signals for starvation are transmitted to the neuropeptide Y

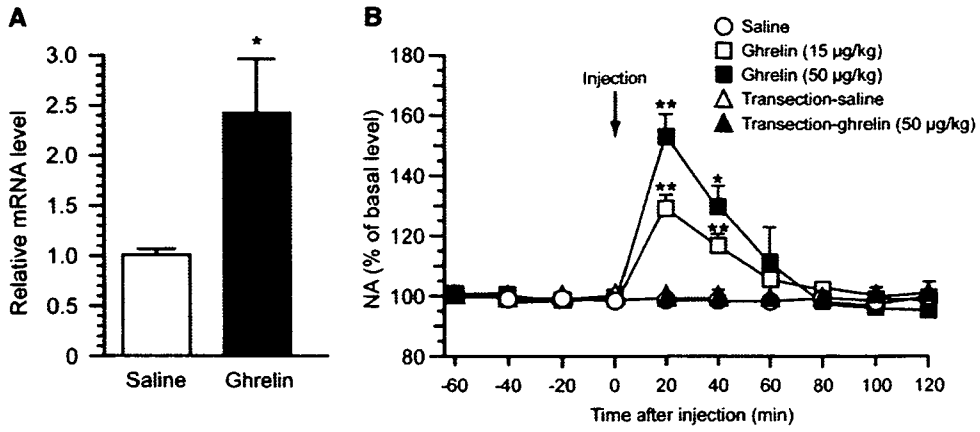

Figure 2. Ghrelin stimulates feeding via the NA system

A) DBH mRNA levels in rats receiving either ghrelin (15 μg/kg, i.v.) or saline. *$p < 0.03$ versus saline.

B) Effect of intravenous ghrelin on NA levels within the ARC in sham-treated and midbrain-transected rats. NA levels are represented as percentages of the mean concentration of NA in four consecutive dialysate samples taken before ghrelin injection. *$p < 0.01$, **$p < 0.0001$ versus sham saline.

Error bars represent the SEM.

neurons of the ARC at least partially via the vagal afferent pathway (Date et al., 2002). The possibility remains, however, that i.v. administered ghrelin may bind directly to receptors present on neurons in the ARC, as the ARC, situated at the base of the hypothalamus, is incompletely isolated from the general circulation by the blood-brain barrier (Banks and Kastin, 1985; Merchenthaler, 1991). The present study shows that ghrelin-induced feeding was abrogated in transected rats. This result indicates that neural pathways ascending from the NTS may play an important role in the transmission of ghrelin orexigenic signals to the hypothalamus. Conveyed to the NTS, these signals could be relayed to the hypothalamus through other transmitters produced by neurons located in the NTS.

NA system and peripheral ghrelin-induced feeding

Although afferent projections from the NTS to the hypothalamus are not exclusively noradrenergic, the noradrenergic pathway is the major constituent. We here showed that DBH mRNA levels increased significantly in the NTS after ghrelin (15 μg/kg) administration (Figure 2A). Considering that the NTS is the termination area of the vagal afferent fibers that receive vicerosensory information from the gastrointestinal tract, it seems reasonable to expect that peripheral ghrelin induces Fos expression in the NTS. We were not, however, able to detect any increase in the number of Fos-expressing neurons in the NTS (Date et al., 2005). This finding is consistent with previous data from other groups (Wang et al., 2002; Rüter et al., 2003). These results may depend on the fact that peripherally administered ghrelin decreases the firing rate of gastric vagal afferent fibers by binding to its receptor present in the vagal afferent terminals (Asakawa et al., 2001; Date et al., 2002). Thus, inhibitory signals caused by peripherally administered ghrelin may affect DBH expression in the NTS.

Peripherally administered ghrelin and synthetic GHSs primarily activate neurons located in the ARC (Hewson and Dickson, 2000). Most peripheral ghrelin-induced Fos-positive neurons in the ARC express NPY (Wang et al., 2002; Date et al., 2002). NPY and agouti-related protein (AgRP), which are colocalized in neurons of the ARC, have been implicated in the stimulation of feeding behavior. Pharmacological examinations indicated that centrally administered NPY Y-1 receptor antagonists block the orexigenic effect of ghrelin injected peripherally (Asakawa et al., 2001). Furthermore, Chen et al. demonstrated that peripherally administered ghrelin does not induce food intake in $NPY^{-/-}$, $AgRP^{-/-}$ double-knockout mice (Chen et al., 2004). These findings suggest that the ARC plays a crucial role in regulating peripheral ghrelin signals. In order to examine whether peripherally administered ghrelin affects the release of NA in the ARC, which is not only a noradrenergic terminal area but also a target site of peripheral ghrelin signals, we monitored overflow NA within or near the ARC using a microdialysis system. Overflow NA is thought to include both newly released NA and NA that was not subject to reuptake. Examination of overflow NA within or near the ARC after intravenous administration of 15 and 30 μg/kg ghrelin to sham-treated rats ($n = 7$ per group) revealed significantly increased NA concentrations within and near the ARC, reaching 129.7 ± 4.7% and 152.8 ± 7.5% of the control levels, respectively (Figure 2B). Ghrelin administration, however, did not induce NA release in transected rats. Hindbrain noradrenergic neurons innervating the hypothalamus are implicated in mediation of the feeding response to glucose deprivation (Ritter et al., 2001), suggesting that the NA system in the brain contributes significantly to feeding regulation and/or energy homeostasis. The present study demonstrates that ghrelin, an orexigenic signal produced in the periphery, increases DBH mRNA levels in the NTS and increases NA levels within the ARC. These results suggest that noradrenergic inputs, projecting from the hindbrain to the ARC, are critical for the feeding behavior induced by peripheral ghrelin. This study, however, has yet to elucidate whether peripheral ghrelin signals transmitted via the vagal afferent pathway affect the NA system in the ARC or whether ghrelin bound to the receptor present in the area postrema or NTS stimulates it. To clarify this issue, further examinations to evaluate NA overflow in the ARC of vagotomized animals are needed.

CCK, an anorectic peptide produced by the gastrointestinal tract, increases the firing rate of the vagal afferent fibers, and thereby transmits satiety information to the NTS. Recently, Sutton et al. showed that the CCK-induced reduction in feeding is modulated by a melanocortinergic pathway through extracellular signal-regulated kinase signaling in the NTS (Sutton

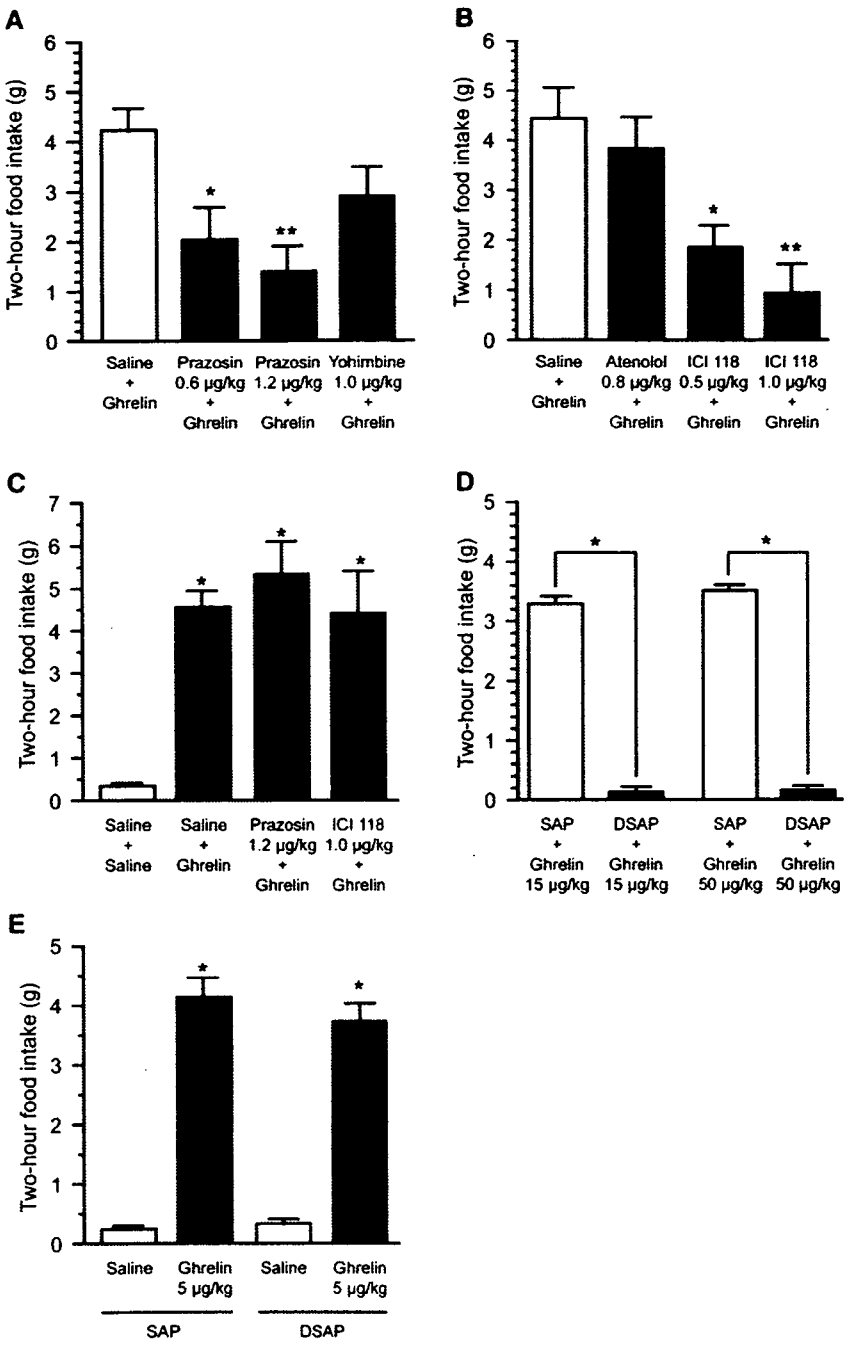

Figure 3. The effects of either pretreatment with adrenoceptor antagonists or disruption of DBH-containing neurons on ghrelin-induced feeding

A) Effect of i.c.v.-administered α_1 or α_2 antagonists on feeding induced by ghrelin (15 µg/kg). $^*p < 0.05$, $^{**}p < 0.005$ versus rats given saline plus ghrelin.

B) Effect of i.c.v.-administered β_1 or β_2 antagonists on feeding induced by ghrelin (15 µg/kg). $^*p < 0.005$, $^{**}p < 0.001$ versus rats given saline plus ghrelin.

C) Food intake of rats treated with an α_1 or a β_2 antagonist after a single intracerebroventricular administration of ghrelin (5 µg/kg). $^*p < 0.0005$ versus saline.

D) Effect of DSAP treatment on ghrelin-induced feeding. $^*p < 0.0001$.

E) Food intake of DSAP-treated rats after a single intracerebroventricular administration of ghrelin (5 µg/kg). $^*p < 0.0001$.

Error bars represent the SEM.

et al., 2005). There is also a report that fourth ventricular administration of the MC4-melanocortin receptor antagonist SHU9119 completely blocked the peripherally administered CCK-induced decrease in feeding (Fan et al., 2004). These findings indicated that the NTS is crucial for the integration of peripheral ascending signals with descending signals from the hypothalamus that relate to feeding. The precise molecular mechanisms that underlie the effect of peripheral ghrelin signals on NTS function remain to be elucidated. To fully understand the noradrenergic pathway from the NTS to the hypothalamus, further investigations into the identities of the intracellular signaling systems in the NTS that are mobilized by peripheral ghrelin, and the signals from the forebrain that modulate peripheral ghrelin signaling in the NTS are required.

NA can utilize at least four distinct receptor subtypes: α_1, α_2, β_1, and β_2 (O'Dowd et al., 1989). We examined which of these receptors was involved in ghrelin-induced feeding by treatment with an antagonist for each adrenoceptor. Ghrelin-induced feeding was attenuated in rats pretreated with either the specific α_1 antagonist prazosin or the specific β_2 antagonist ICI 118, but not the α_2 antagonist yohimbine or the β_1 antagonist atenolol (Figures 3A and 3B) (n = 7 per group). After injection of these adrenergic antagonists intracerebroventricularly (i.c.v.), rats were observed for behavioral signs of nausea (elongation of the body, gaping, raising the tail, and lowering the belly to the floor), ataxia, sedation, and anxiety (locomotion within the cage and avoidance of the front of the cage). The rats did not exhibit any of these signs during the testing period. We also tested

the orexigenic effect of centrally administered ghrelin in rats treated with prazosin or ICI 118, as these antagonists may nonspecifically suppress feeding in response to ghrelin. Centrally administered ghrelin increased feeding similarly in the prazosin- and ICI 118-treated groups and the control group (Figure 3C). This result suggests that although NA antagonists specifically suppressed feeding induced by peripherally administered ghrelin, centrally administered ghrelin induces feeding by a mechanism that is independent of the noradrenergic system. Considering that NA excites approximately 50% of the neurons in the ARC, probably due to a direct postsynaptic response through α_1- or β-adrenoceptors (Kang et al., 2000), peripherally administered ghrelin may activate NPY/AgRP neurons in the ARC through the NA system. A recent study also suggested the possibility that the GABAergic system is involved in ghrelin-induced feeding. Cowley et al. showed that ghrelin induced depolarization of ARC NPY neurons and hyperpolarization of ARC pro-opiomelanocortin (POMC) neurons using hypothalamic slices (Cowley et al., 2003). Given that NPY/AgRP neurons expressing are GABAergic, central ghrelin may induce the release of GABA from NPY axonal terminals and thereby modulate the activity of postsynaptic POMC neurons.

NA exerts a variety of responses that depend on the type of neurons and the expression of different adrenoceptor subtypes (Nicoll et al., 1990). Infusion of exogenous NA can cause either increases or decreases in food intake (references in Wellman, 2000), which may depend on the site of application or changes in the numbers of adrenoceptors according to the circadian cycle. For example, NA injection into the hypothalamic paraventricular nucleus (PVN) increases feeding through PVN α_2-adrenoceptors, whereas it decreases feeding through PVN α_1-adrennoceptors (Goldman et al., 1985; Wellman et al., 1993). The circadian pattern in the number of α_2-adrenoceptors within the PVN exhibits a sharp increase in α_2-adrenoceptors at the onset of the dark phase, a time when feeding is greatly enhanced. Taken together, it may be difficult to determine whether microinjection of an NA agonist or antagonist into the hypothalamic nuclei results in a physiologically significant effect. Therefore, in the present study, we focused on the role of endogenous NA induced by peripherally administered ghrelin in the control of food intake. We demonstrated that α_1- and β_2-receptor antagonists attenuated feeding induced by ghrelin. This result indicates that α_1- and/or β_2-adrenoceptors in the ARC play an important role in peripheral ghrelin-induced feeding.

To eliminate NA innervation of the ARC, we used DSAP, a monoclonal antibody specific for DBH, the enzyme that converts dopamine into NA, conjugated to saporin (SAP) (Fraley and Ritter, 2003). DSAP, an immunotoxin that allows an antibody against the NA synthetic enzyme DBH to selectively deliver the saporin toxin, can successfully destroy hindbrain neurons that contain DBH (Rinaman, 2003). Bilateral DSAP injections into the ARC induced an approximately 70% reduction in DBH-positive neurons in the NTS in comparison to the number of DBH neurons present in rats treated with an SAP control solution (data not shown). DSAP injections also completely disrupted peripherally administered ghrelin-induced feeding (Figure 3D) (n = 7 per group). We also tested the orexigenic effect of centrally administered ghrelin in the DSAP-treated rats. Centrally administered ghrelin increased feeding similarly in the DSAP-treated group and the control group (Figure 3E) (n = 7 per group). This finding suggests that the noradrenergic system in the ARC is not involved in centrally administered ghrelin-induced feeding.

There are several catecholaminergic neuronal cell groups in the hindbrain. DBH-positive neurons projecting to the hypothalamus are found within the A2 cell group located in the caudal medial and commissural NTS and the A1/C1 cell group located in the ventrolateral medulla (VML). Most NA neurons within the A2 group directly project to the hypothalamus, central nucleus of the amygdala, and bed nucleus of the stria terminalis, whereas the A2 NA neurons also project to these forebrain areas in part via the A1/C1 group. As viscerosensory signals from the gastrointestinal tract are carried to the caudal medial and commissural NTS via the vagal afferent pathway, NA neurons in A2 may be an integral component of the brainstem circuits that mediate ghrelin-induced feeding. Given the projection from the A2 group to the A1/C1 group, these integrative circuits would include a role for NA neurons in the VML. Our findings suggest that NA neurons in the hindbrain are necessary to convey ghrelin-related orexigenic signals to the hypothalamus.

Innervation of NPY neurons by DBH-containing fibers

To examine the effect of peripheral ghrelin signals ascending from the NTS on neurons in the ARC, we investigated DBH innervation and ghrelin-induced Fos expression using unilateral midbrain-transected rats as described previously (Ericsson et al., 1994; Sawchenko, 1988). We compared DBH innervation and Fos expression in the ARC ipsilateral and contralateral to the lesion. Midbrain transections significantly decreased the DBH-imunoreactive innervation ipsilateral to the lesion (Figures 4A and 4B). This finding is consistent with the fact that the ascending catecholamine input to the hypothalamus is largely unilateral. In lesioned rats, peripherally administered ghrelin resulted in a significant increase in Fos expression in the ARC that was contralateral to the lesion (ipsilateral side, 24.3 ± 1.8 neurons; contralateral side, 50.6 ± 1.9 neurons; $p < 0.001$) (Figures 4C and 4D). When saline was injected i.v. to lesioned rats, Fos expression did not differ significantly on the two sides of the brain (ipsilateral side, 11.6 ± 1.3 neurons; contralateral side, 11.9 ± 1.0 neurons; $p > 0.1$) (data not shown). These results suggest that the midbrain transections that were effective in reducing DBH-positive innervation blocked the response of neurons in the ARC to peripherally administered ghrelin.

Electron microscope immunohistochemistry demonstrated that NPY-immunoreactive perikaryon and dendritic process often received synapses from DBH-containing axon terminals (Figures 4E–4G). Approximately 40%–50% of hypothalamic NPY neuron innervation arises from catecholaminergic neurons in the hindbrain (Everitt and Hokfelt, 1989). NPY, a potent orexigenic peptide, is thought to be the final mediator of ghrelin feeding signals. To examine the anatomical linkage of NPY neurons, which are activated by ghrelin, with DBH-immunoreactive fibers, we performed immunohistochemistry. Intravenous ghrelin injection significantly increased Fos expression in 53% of the NPY neurons in the ARC (Figure 4H), in accordance with previous studies (Date et al., 2002). Triple labeling immunofluorescence demonstrated that 54% of these NPY neurons in the ARC induced to express Fos by ghrelin treatment were innervated by DBH-immunoreactive fibers (Figure 4I). These results suggest that ghrelin signals activate NPY neurons via the noradrenergic pathway ascending from the NTS to the ARC, resulting in increased feeding.

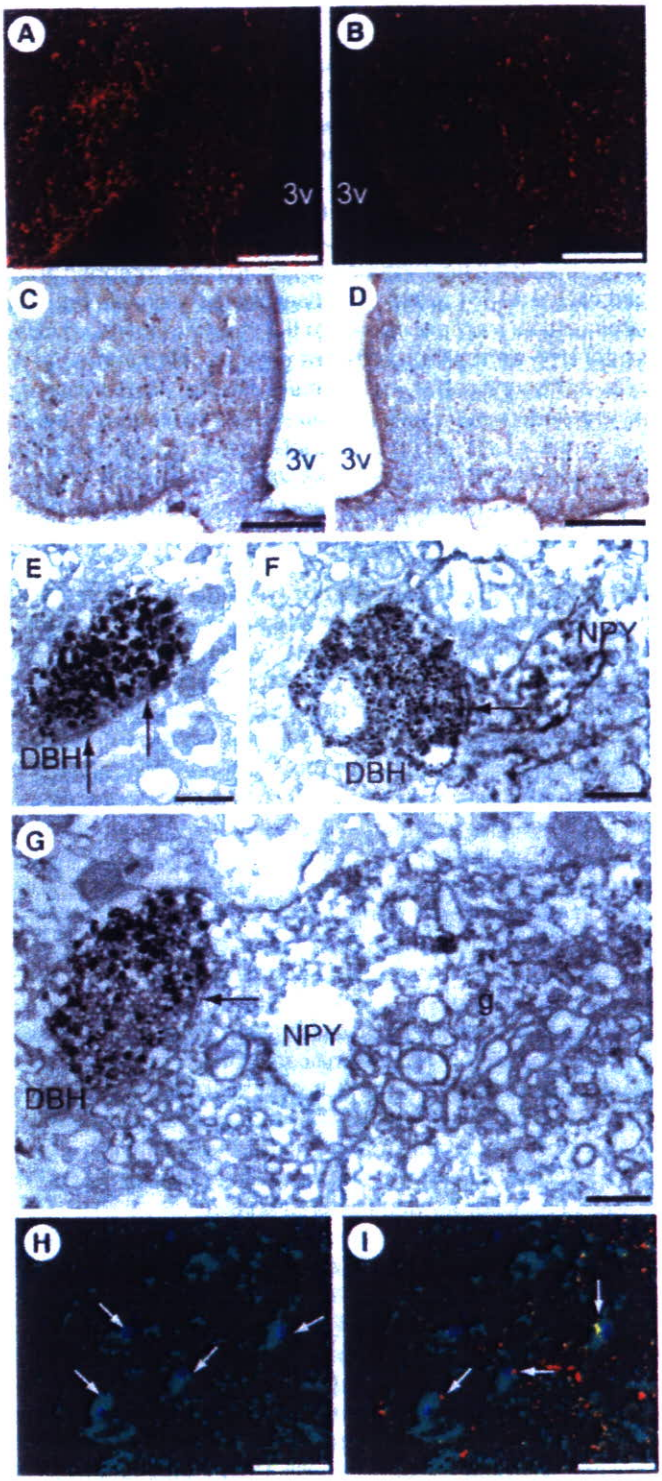

Figure 4. The effect of unilateral midbrain transections on ghrelin-induced Fos expression and activation of NPY neurons by ghrelin via the catecholaminergic pathways

A) DBH-immunoreactive fibers project to the ARC contralateral to the lesion.

B) DBH-immunoreactive innervation ipsilateral to the lesion decreases as compared to that on the contralateral side.

C) Peripherally administered ghrelin (15 μg/kg) induces Fos protein expression contralateral to the lesion.

D) Ghrelin-induced Fos expression ipsilateral to the lesion decreases as compared to that on the contralateral side.

E) DBH-immunoreactive axon terminal making synapses with immunonegative dendritic process (arrow, synapse).

F) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive dendritic process (arrow, synapse).

G) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive perikaryon (arrow, synapse).

H) Intravenous administration of ghrelin (15 μg/kg) upregulates Fos expression in NPY neurons of the ARC (arrows) (blue, Fos; green, NPY).

I) Fifty-four percent of ghrelin-activated NPY neurons receive projections from DBH-immunoreactive fibers (arrows) (blue, Fos; green, NPY; red, DBH). g, Golgi apparatus; 3v, third ventricle. The scale bar represents, respectively, 100 μm (**A and B**), 200 μm (**C and D**), 400 nm (**E–G**), and 50 μm (**H and I**).

The present study focused on the hypothesis that the neural pathway from the brainstem to the ARC plays a crucial role in transmitting peripheral ghrelin signals and peripheral ghrelin regulates feeding at least partially via NA-mediated neuronal transmission. Although the central circuits for feeding may have been altered in response to bilateral midbrain transections or DSAP treatment, the results shown here are consistent with the hypothesis. The hypothesis, if correct, is a counterpoint to the most widely accepted model for neuroendocrine energy balance regulation. We have shown previously that peripheral ghrelin-induced feeding is absent in either vagotomized or capsaicin-treated rats. We showed here that ghrelin-induced feeding is also canceled in midbrain transected rats. Thus, it may seem that peripheral ghrelin signals for starvation are transmitted to the hypothalamus only via the vagal afferent pathway and neural pathways from the NTS. However, we have to consider the possibility that vagotomy and/or midbrain transections affect several peripheral substances as well as central circuits relative to feeding. In addition, the present study has yet to address the direct relationship between peripheral ghrelin signals via the vagal afferent pathway and the NA system in the ARC. Taken together, it may be difficult to assert that peripheral ghrelin signals are transmitted only via the neural pathways. We, therefore, think that the humoral pathway and the neural pathway are important routes to convey peripheral energy balance information to the brain. Very recently, we found that peripherally administered leptin decreased 2 hr and 4 hr food intake in vagotomized, midbrain transected, and sham-operated rats, and the leptin-induced reduction in feeding was less pronounced in vagotomized and transected rats than in the sham-operated rats (unpublished data). These findings suggest that the vagal afferent pathway and/or the ascending efferent pathway from the brainstem to the hypothalamus are necessary elements for the effectual action of leptin on feeding and energy homeostasis. Feeding is regulated by a complicated interaction of many orexigenic and anorectic signals; sophisticated interactions between humoral pathways and neural pathways may be necessary to maintain energy homeostasis. We have shown that the central noradrenergic system is a candidate to mediate peripheral ghrelin signals. Although the pathways linking peripheral ghrelin to NA transmission are likely to be more complicated given the remarkable number of signals that provide input to the NTS and ARC, we believe that this study provides an important clue to understanding the feedback loops linking the brain and peripheral tissues in the control of feeding and energy homeostasis.

Experimental procedures

Experimental animals

We maintained male Wistar rats (Charles River Japan, Inc.), weighing 255.9 ± 2.0 g, under controlled temperature and light conditions (0800–2000 hr light)

For feeding and microdialysis experiments, an intravenous cannula was implanted into the right jugular vein of each rat under anesthesia. We performed unilateral or bilateral midbrain transections 5 days after implantation, as described details in the Supplemental Experimental Procedures. To confirm that the transection surgeries were successful, the brains were immunostained using an anti-DBH antiserum diluted 1:1000 (Chemicon International, Inc.) by the avidin-biotin complex method (Date et al., 1999) after the feeding tests were completed (Figure S3A). To facilitate the penetration of a microdialysis probe, a guide cannula (500 μm outside diameter; AG-12, Eicom) was stereotaxically implanted 1.0 mm above the ARC (0.2 mm lateral to the midline, 2.4 mm caudal to the bregma, and 9.0 mm ventral to the dura), fixed to the skull with acrylic dental cement, and sealed with a dummy cannula (350 μm external diameter; AD-12, Eicom). To inject adrenergic receptor antagonists into the rats, we implanted intracerebroventricular cannulae into the lateral cerebral ventricle. To block noradrenergic innervation of the ARC, we microinjected either a SAP-conjugated DBH-specific mouse monoclonal antibodies (DSAP; Advanced Targeting Systems; 42 ng/0.2 μl in phosphate buffer [pH 7.4], n = 6) or SAP-conjugated normal mouse IgG (SAP control solution) (Advanced Targeting Systems; 8.82 ng/0.2 μl, n = 6) bilaterally into the ARC (Ritter et al., 2001). Only animals exhibiting progressive weight gain after these surgeries were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Food intake

First, rat ghrelin (Peptide Institute, Inc.) at 0.1–100 μg/kg (100 μl), or saline alone (100 μl) was administered i.v. at 1000 hr to ad libitum-fed rats that had undergone a sham operation (n = 7 per group). Second, rat ghrelin (15 or 50 μg/kg [100 μl]) was administered i.v. to rats that had undergone bilateral midbrain transection. Third, rat ghrelin (15 or 50 μg/kg [100 μl]) was administered i.v. to rats that had been treated with either DSAP or SAP control solution. Fourth, ghrelin (5 μg/kg [10 μl]) was injected i.c.v. at 1000 hr into rats that had undergone bilateral midbrain transections or sham operations, or into rats that had been treated with either DSAP or SAP control solution. The dose of centrally administered ghrelin (5 μg/kg) is often used as a standard while investigating the effect of i.c.v.-administered ghrelin on food intake under various conditions (Nakazato et al., 2001; Kamegai et al., 2000; Toshinai et al., 2003). Thus, this dosage is recognized as the most appropriate in constantly inducing food intake when administered i.c.v. Therefore, this dosage was also selected as a standard to evaluate i.c.v. administered ghrelin induced feeding. After ghrelin injection, rats were immediately returned to their cages. Two hour food intake was then measured.

Quantitative RT-PCR

Two hours after intravenous administration of ghrelin (15 μg/kg) or saline to rats, total RNA was extracted from the NTS using TRIZOL Reagent (Invitrogen Corp.). Quantitative RT-PCR for DBH was conducted with a LightCycler system (Roche Diagnostics) using a LightCycler-Fast Start DNA Master SYBR Green I kit (Roche) and the following primer set for rat DBH: 5′-CTAGGGCCCTGGGCGCCAAGGCATT-3′ and 5′-GCCAGAGGAGTCGCG CCGGCCTT-3′. Known amounts of DBH cDNA were used to obtain a standard curve. Rat rRNA levels were also measured as an internal control.

Microdialysis

One week after midbrain transection, the rats were lightly anesthetized with isoflurane, and the dummy cannula was replaced with a microdialysis probe. The tip of the microdialysis probe, covered with hollow fibers (1.0 mm in length, 220 μm external diameter, regenerated cellulose membrane with a molecular weight cutoff of 48 kDa; Eicom), was set to extend 1 mm beyond the guide cannula to reach the ARC. Microdialysis was performed under free-moving conditions. A microinfusion pump was used to continually perfuse the probe with modified physiological Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$ [pH 6.5]) at a constant flow rate of 1 μl/min. To measure NA, chromatographic analysis of dialysates was carried out by HPLC with electrochemical detection as described previously (Ishizuka et al., 2000). The perfusate from the ARC was automatically injected into the HPLC every 20 min. After a 3 hr stabilization period, baseline NA levels were assessed in four consecutive dialysate samples. At the end of each experiment, rats were sacrificed with an overdose of pentobarbital sodium; the brains were then fixed in 10% neutral buffered formalin. Placement of the microdialysis probe was verified histologically in 40-μm cresyl violet-stained coronal sections (Figure S3B).

Effect of adrenoceptor blockers on ghrelin-induced feeding

At 0930 hr, rats were i.c.v. administered either vehicle alone (saline, n = 6) or one of the specified adrenergic receptor antagonists: prazosin (selective α_1 antagonist: 0.6 or 1.2 μg/kg, n = 6 each) (Sigma Chemical Co.), yohimbine (selective α_2 antagonist: 1.0 μg/kg, n = 6) (Sigma), atenolol (selective β_1 antagonist: 0.8 μg/kg, n = 10) (Sigma), or ICI 118 (selective β_2 antagonist: 0.5 or 1.0 μg/kg, n = 6 each) (Sigma). Thirty minutes after adrenergic receptor antagonist injection, ghrelin (50 μg/kg) was administered intraperitoneally to rats; 2 hr food intake was measured. We also tested the orexigenic effect of centrally administered ghrelin in rats that had been injected with prazosin or ICI 118. Thirty minutes after prazosin (1.2 μg/kg, n = 6) or ICI 118 (1.0 μg/kg, n = 6) injection, ghrelin (5 μg/kg) was administered i.c.v. to rats; 2 hr food intake was measured. The rats fasted between the two injections.

Immunohistochemistry

Ghrelin (15 μg/kg) or saline was injected i.v. into rats 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde (n = 5 per group). The brains of animals were then cut into 20-μm thick sections. The sections were first incubated with anti-c-Fos antiserum (1:500, Santa Cruz Biotechnology), and then with Alexa Flour 350-conjugated donkey anti-goat IgG (Molecular Probes, Inc.). Next, samples were incubated with anti-NPY antiserum (1:500, ImmunoStar, Inc.), then with Alexa Flour 488-conjugated chicken anti-rabbit IgG (Molecular Probes, Inc.). Finally, the samples were incubated with anti-DBH antiserum (1:1,000, Chemicon International, Inc.), then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). Samples were then observed under a BH2-RFC microscope (Olympus Corp.). We counted the number of Fos-immunoreactive cells in the bilateral ARCs (bregma: −2.30 to −3.30 from Paxinos and Watson's rat brain atlas). Sections from unilaterally transected rats were also incubated with anti-DBH antiserum, and then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). A significant (>60%) depletion of DBH-immunoreactive fibers was determined by semi-quantitative comparison of the strength of the DBH-positive innervation of the ARC ipsilateral and contralateral to the lesion by two independent observers (Sawchenko, 1988). Sections from unilaterally transected rats were incubated with anti-c-Fos antiserum (Santa Cruz Biotechnology), and then stained by the avidin-biotin complex method (Date et al., 1999). The number of Fos-immunoreactive cells was compared in the ARC ipsilateral and contralateral to the letion. Fos-expressing cells of the ARC in a 0.7-mm right triangle (0.245 mm^2) were counted in every fifth section (ten tissue sections per rat) using a cell-counting program written for NIH Image (v1.62; NIH).

Electron microscope immunohistochemistry

Three Wistar rats were perfused as described above. The brain was cut into 30–40 μm thick sections using an Oxford vibratome (Oxford Instruments). Electron microscope immunohistochemistry was performed using anti-NPY antiserum and anti-DBH antiserum as described previously (Toshinai et al., 2003).

Statistical analysis

We analyzed groups of data (means ± SEM) using analysis of variance (ANOVA) and post hoc Fisher tests. p values less than 0.05 were considered to be significant (two-tailed tests).

Supplemental data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/4/323/DC1/.

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