インテグリンリンクドキナーゼ不活化遺伝子導入による神経原線維変化モデルの開発

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研究成果報告書

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はしがき

Integrin-linked kinase（以下 ILK）は、β1 インテグリンの細胞質内ドメインに結合し、インテグリン接着班を構成するセリン/スレオニンキナーゼとしてヒト胎盤よりクローニングされた。ILK は、直接インテグリンと相互作用することで細胞内外の情報を二方向性に橋渡しするキーエンザイムと考えられており、インテグリンを介した細胞外マトリックスへの細胞接着、細胞増殖、あるいは癌細胞の転移に関与することで着目されている。ILK は、脳・神経系細胞においても豊富に発現しており、神経細胞の分化や増殖、分化後の生存は、細胞外マトリックスの種類とそれらとの接着特性に大きく影響されることから、神経細胞における ILK の機能はたいへん興味深い。また、脳神経系は、複雑な神経回路網を形成しているが、その基本回路は個々の神経細胞が種々の細胞外ガイダンス因子に誘導されて正しい標的細胞へ神経軸索が伸長することにより形成される。これら一連の反応には細胞外マトリックスとの相互作用や細胞間の接着に依存した細胞内情報伝達機構の関与が必要不可欠である。

研究代表者は、以前、インテグリンシグナルの中でも ILK シグナルに焦点を絞り、脳・神経細胞の分化、生存における ILK シグナルの役割について調べた。神経未分化細胞の N1E-115 細胞は、血清非存在下でラミニンをコートしたプレート上で培養する（分化条件）と神経突起を形成し神経細胞へと分化する。この N1E-115 細胞の神経突起形成は、β1 インテグリン抗体の処置や、モルモット大脳からクローニングした ILKcDNA(WILK)より作製した ILK 不活化変異体 cDNA（DN-ILK）の導入発現により阻

害された。一方、WILK をトランジエントに高発現した細胞は、非分化条件下においても神経突起を形成し神経へと分化した。これらの結果は神経突起形成に ILK の機能が必要不可欠であることを示している。

神経細胞における微小管は、神経分化時の軸索や神経突起の形成、細胞骨格として細胞形態の構築、細胞内輸送などに重要に機能しており、微小管ならびにその関連タンパク質の異常は、種々の異なる神経変性疾患において認められている。また、微小管関連タンパク質であるタウタンパク質は、中枢ならびに末梢神経系神経細胞の軸策や樹状突起に多く発現しており、その機能は主にチュブリンと結合することで微小管形成を促進、あるいは安定化することにある。最近、研究代表者は、DN-ILK を培養神経細胞にステイブルに高発現させ、神経細胞内在の ILK を阻害した場合に生じる微小管ならびに微小管調節タンパク質であるタウタンパク質の変化について調べた。その結果、タウタンパク質が高度に異常リン酸化されていることがわかった。中でも、Ser^{199} ならびに Ser^{202} の異常リン酸化部位は神経原線維変化の異常タウタンパク質でも確認されており、アルツハイマー病の発生機構を理解する上でも大変興味深い。さらに、タウタンパク質の Ser^{199} と Ser^{202} の異常リン酸化を触媒する glycogen synthase kinase-3β（GSK-3β）の活性について調べた結果、DN-ILK 発現細胞は、コントロール細胞に比べて GSK-3β の活性型(Tyr ^{216}-p)が増加し、その酵素活性はコントロール細胞に比べて約３倍程度の高い活性を示した。一方、GSK-3β の不活性型(Ser^{9}-p)は分化、非分化条件にかかわらずコントロール細胞においてのみ認められた。これらの結

果から、ILK は直接、あるいは protein kinase B (PKB/Akt)を介して間接的に glycogen synthase kinase-3βの Ser^9 をリン酸化することでそれを不活化し、タウタンパク質が異常にリン酸化されないように保護していると考えられる。一方、アルツハイマー病などの神経変性疾患で認められる神経原線維変化は、タウタンパク質の高度異常リン酸化が原因するとの報告があり、ILK が神経原線維変化における病態形成に関与している可能性を強く示唆している。そこで、本研究は、ILK の遺伝子操作により神経原線維変化をマウス脳に形成させることで、アルツハイマー病などの神経変性疾患で確認される神経脱落像ならびにそれに基づく神経活動の異常に即した病態を再現した動物モデルの作製を試みた。

まず、最初に、野生型 ILK および DN-ILK 遺伝子をマウス脳・神経細胞に導入する方法を選択するための様々な予備試験を行った。遺伝子導入発現の指標となるマーカー遺伝子としては、green fluorescent protein (GFP)とβ-galactosidase (β-gal) の両者を用いた。脳神経細胞への導入には、目的遺伝子をマウス白血病ウイルス(MLV)由来のウイルスベクターに構築し、ecotropic ウイルスの env を導入したパッケージング細胞に導入後、回収したウイルス粒子をマウスの第三脳室に投与するレトロバイラスシステム発現系と、直鎖型 polyethylenimine を利用した *in vivo* 用の遺伝子導入試薬に目的遺伝子を混和後、それらをマウスの第三脳室に投与し、発現ベクターの CMV プロモーターを利用する非バイラスシステム発現系を用い、各々の遺伝子発現効率について調べた。方法としては、実際に GFP ならびにβ-gal マーカー遺伝子

をマウス脳に導入し、それらのマーカー遺伝子のタンパク質発現量を指標に導入発現の効率を比較した。その結果、非バイラスシステムの発現系は、約 12 週間にわたりマーカー遺伝子の安定した発現を可能にしたが、レトロバイラスシステムを利用した発現系は、安定した発現効率を得ることが出来ず、再現性に問題が認められた。そこで、直鎖型 polyethylenimine を利用した非バイラスシステム発現系がレトロバイラスシステム発現系よりも優れていると判断し、本研究の遺伝子発現系として前者を採用した。

非バイラスシステム発現法を用いて、野生型 ILK ならびに DN-ILK 遺伝子をマウス脳に導入発現し、遺伝子導入 2 週間後の急性期でホールボード試験を行い自発運動と情動に対する影響を評価した。その結果、野生型 ILK を発現したマウスの自発運動は対照群と比べて変化が認められなかったが、DN-ILK 発現したマウスは不安・抑うつなどの情動性変化を生じる個体(~ 60%)が出現した。

モーリス水迷路試験を行い、海馬機能を反映する空間記憶能力に対する影響を評価した結果、野生型 ILK を発現したマウスは flag test において高い学習効率が認められ、さらに probe test においても記憶力向上の傾向が認められた。一方、DN-ILK を発現したマウスの学習効率と記憶能力は、対照群と比べて有意な変化は認められなかった。

行動試験終了後(遺伝子導入３週後の急性期) のマウス脳ならびに遺伝子導入 12 週後の慢性期のマウスを用いて病理学的評価を行った。遺伝子導入後の急性期なら

びに慢性期のマウスは、野生型 ILK ならびに DN-ILK いずれの遺伝子発現群においてもコントロール群（ベクターのみ発現）と比べ顕著な変化が認められなかったが、一部の DN-ILK を発現したマウスにおいて、脳室の拡張傾向が認められた。

野生型 ILK を発現したマウスは学習効率ならびに記憶力が向上する傾向が認められたので、記憶学習能力に関与する海馬領域に焦点を絞り、遺伝子導入３週後の急性期マウスの脳を用いて生化学的評価を行った。野生型 ILK を発現したマウス海馬領域の ILK 酵素活性は、コントロール群と比べて有意に高い活性が認められ、逆に DN-ILK を発現したマウスでは ILK 酵素活性の有意な低下が認められた。DN-ILK を発現した培養神経細胞で認められたタウタンパク質の Ser^{199} ならびに Ser^{202} の異常リン酸化は、遺伝子導入３週間後の DN-ILK 発現群の海馬領域において、増加の傾向が認められ、さらに、Ser^{199} と Ser^{202} の異常リン酸化を触媒する GSK-3β の活性型(Tyr ^{216}-p)はコントロール群に比べて有意に増加した。一方、野生型 ILK 発現群においてはそれらに変化は認められなかった。

遺伝子導入 12 週後の慢性期で生化学的評価を行ったところ、野生型 ILK を発現したマウス海馬領域の ILK 酵素活性は、コントロール群と比べて、遺伝子導入３週後の場合と同様にいぜん高い活性が認められた。ところが、DN-ILK を発現したマウスの ILK 酵素活性は、遺伝子導入３週後で認められた ILK 活性の低下が、導入 12 週後ではコントロール群のレベルにまで回復した。さらに、同様に、遺伝子導入 12 週後の DN-ILK 発現群は、導入３週後の急性期で認められたタウタンパク質の異常リン酸

化量の増加ならびに GSK-3βの活性型(Tyr ^{216}-p)量の増加においても、コントロール群のレベルにまで減少し、それらの回復が認められた。

DN-ILK 発現群の遺伝子導入３週後の急性期と 12 週後の慢性期の異なる時間経過で、ILK の酵素活性、タウタンパク質の異常リン酸化量、ならびに GSK-3βの活性型(Tyr ^{216}-p)量に変化が生じた原因を解明する目的で、内在 ILK タンパク質発現量の変化を、免疫組織学的ならびに生化学的解析により調べた。その結果、DN-ILK を発現したマウス海馬領域の内在 ILK タンパク質の発現量は、遺伝子導入 12 週後の慢性期において顕著な増加が認められた。これらの結果は、DN-ILK を発現したマウス海馬領域では、DN-ILK の導入発現により内在 ILK の酵素活性が低下した結果、海馬の生理機能を維持するため、内在 ILK の発現誘導を促進することで順応する生理的適応機構が生じていることが示唆された。一方、野生型 ILK 発現群の内在 ILK 発現量は遺伝子投与３週後の急性期と 12 週後の慢性期でその両者に変化は認められなかった。以上の結果から、DN-ILK をマウス脳に発現することにより内在 ILK の酵素活性を阻害すると、一過性に ILK の酵素活性が低下し、その結果、タウタンパク質の異常リン酸化量と GSK-3βの活性型(Tyr ^{216}-p)量が増加する。ところが、その後、脳の生理機能を維持するための適応機構が生じ、内在 ILK の発現を誘導することで、ILK ならび GSK-3βの酵素活性を正常のレベルにまで回復させ、タウタンパク質の機能を維持していることが示唆された。

本研究では、ILK の遺伝子操作により神経原線維変化をマウス脳に形成させる

ことで、アルツハイマー病などの神経変性疾患で確認される神経脱落像ならびにそれに基づく神経活動の異常に即した病態を忠実に再現した動物モデルの作製を試みたが、培養細胞の場合とは異なり in vivo の動物個体では、長期間 ILK の酵素活性を阻害すると、その時間経過に伴い生理機能を維持するための適応機構が出現するため、神経原線維変化の発生には至らず、病態モデルを完成することが出来なかった。しかし、今後、ILK 酵素活性を阻害した場合に生じる適応機構の詳細を解明し、それら適応機序を部分的に阻止する方法を見出すことが出来れば、ILK の遺伝子操作により神経原線維変化の動物モデルを作製することが十分可能でると考える。一方、逆の視点から見れば、アルツハイマー病の初期では、ILK の活性を高める薬物の投与やその処置により、神経原線維変化の進行を阻止できる可能性を強く示唆している。

モーリス水迷路試験で認められた野生型ILK遺伝子発現マウスの空間記憶能力の向上や、DN-ILK 遺伝子発現マウスで生理機能を維持するために非常に早期に適応機構が出現したことを鑑みると、脳・神経機能の維持には ILK が必要不可欠であることが理解できる。

本研究で得られた成果が、脳・神経系における ILK の更なる役割の解明や神経原線維変化の予防・治療薬の開発に寄与できることを期待する。

研究組織

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Role of integrin-linked kinase in neuronal cells.

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Abstract

Integrin-linked kinase (ILK) is a focal adhesion serine/threonine protein kinase that binds to the cytoplasmic domain of β1 integrin and has an important role in integrin and growth factor signaling pathways. Clustering of integrins on the cell surface in contact with the extracellular matrix induces focal adhesion that recruits numerous mitogenic signaling proteins other than ILK, such as growth factor receptors, mitogen-activated protein kinase, and small GTP-binding proteins, to integrin receptors and forms signaling centers where adhesive and mitogenic pathways can integrate. ILK is highly expressed in neuronal cells and its enzyme activity is activated by cell adhesion on the extracellular matrix in a phosphatidylinositol 3-kinase-dependent manner. Recent studies demonstrated that ILK interacts with and regulates many different signaling pathways in neuronal cells, which implies an important role for ILK in a variety of neuronal functions. This article discusses the role of ILK in neuronal cells and also the possible involvement of ILK in neuronal disorders.

Keywords: Integrin-linked kinase, Neurite, Tau, GSK-3β, Protein kinase B/Akt, p38 MAP kinase, PI3-kinase.

Introduction

Integrin-linked kinase (ILK) is a cytoplasmic protein serine/threonine kinase that can interact with integrin β1 and β3 cytoplasmic domains [1] and has an important role in various cellular processes, such as cell adhesion, migration, growth, differentiation, and survival [2-4]. Clustering of integrins on the cell surface in contact with the extracellular matrix (ECM) induces focal adhesion [5]. ILK functions as a molecular scaffold to interact with other focal adhesion proteins such as PINCH [6], Nck-2 [7], paxillin [8], affixin [9], and calponin homology-ILK binding protein [10] at cell-ECM adhesion sites and transduces signals derived from cell- ECM interactions or other stimuli, leading to diverse cellular responses to proper signaling pathways via the formation of specific complexes with those focal adhesion signaling molecules. Moreover, ILK is also a critical effector in the phosphatidylinositol (PI) 3-kinase-dependent signaling pathway that is downstream from growth factor receptor activation [2]. ILK has a pleckstrin-homology (PH) domain that binds the PI3-kinase product, PI (3,4,5) triphosphate (PI(3,4,5)P3), and its enzyme activity is regulated in a PI3-kinase-dependent manner [11]. The protein-protein interactions at the focal adhesion site provide a framework for the formation of ILK signaling complexes that couple integrins and growth-factor receptors to the cytoskeleton and downstream kinases regulating various cellular processes [12, 13]. ILK, however, does not always transmit signals derived from extracellular events such as cell-cell and/or cell-ECM adhesion to the inside of the cells via integrin. Alterations in the level of ILK activity and/or its expression induced by various cellular responses also have a great influence on cell-cell and/or cell-ECM adhesion [3], suggesting that ILK can transmit signals derived from intracellular events to the

outside of the cells. Thus, ILK is involved in signal transmission of bi-directional (i.e., "from inside to outside" and "outside to inside") transmembrane signaling pathways.

The intracellular signaling cascades that are activated when integrins bind to their ECM ligands vary depending on the cell-type [5]. The specific intracellular pathways differ depending on the specific integrin/ECM ligand interaction and/or on the specific integrin/intracellular signal proteins coupling in different cell types. In neuronal cells, neurite formation in response to differentiation signals is strongly promoted by ECM ligands such as laminin, fibronectin, and collagen [14, 15]. ILK is expressed in neuronal cells and is involved in neuronal differentiation [16, 17]. In addition, ILK is expressed in glial cells in various brain regions and those glial ILK proteins are involved in myelination in the central nervous system (CNS) [18]. In this review, I discuss the role of ILK in neuronal cells, focusing on intracellular downstream signaling of ILK, and propose the possible involvement of ILK in neuronal disorders.

Functional domains of ILK

The functional domains of ILK are shown in Fig.1. ILK contains four ankyrin repeats at the N-terminus followed by a PH -like domain. The N-terminal ankyrin repeats interact with the five LIM domains-only adaptor protein, PINCH, through the first LIM domain [3, 6]. The LIM domain of PINCH is a protein-binding motif consisting of a cysteine-rich consensus sequence of approximately 50 residues that form 2 separate zinc fingers [19]. PINCH also interacts with the Src-homology (SH) 2 /SH3 containing adaptor protein, Nck-2, through the fourth LIM domain [7]. Nck-2, via PINCH, couples ILK to growth factor receptor kinase or intracellular components of growth factor signaling pathways, such as insulin receptor substrate 1 (IRS-1)[4,6,7]. The PH-like

domain of ILK, through an interaction with PI (3, 4, 5) P3, is involved in growth factor and integrin receptor-induced PI3-kinase-dependent activation of ILK [11]. ILK interacts with the cytoplasmic domains of β1 and β3 integrins via its C-terminal domain [1]. The ILK C-terminal domain exhibits significant homology to other kinase catalytic domains and catalyzes serine/threonine phosphorylation of downstream target molecules, including β integrins, protein kinase B (PKB)/Akt, and glycogen synthase kinase (GSK)-3, which leads to modulation of cell-ECM interactions, gene expression, or cell survival [1,11,20-25]. The calponin homology domain-containing ILK binding protein (CH-ILKBP; also known as α-parvin and actopaxin), which interacts with the C-terminus of ILK, connects to the actin cytoskeleton [10]. CH-ILKBP localizes to focal adhesions and the cytoskeleton, and regulates cell adhesion, cell spreading, cell survival, and the localization of ILK to focal adhesions [10,12,13,27]. A close homology of CH-ILKBP, affixin (also known as β-parvin), and the focal adhesion protein, Paxillin, also interact with the C-terminus of ILK, connect to the actin cytoskeleton, and regulate cell spreading [8, 9].

ILK activity in neuronal cells.

N1E-115 neuronal cells exhibit neurite outgrowth in response to serum deprivation [28,29]. Neurite outgrowth in serum-starved N1E-115 cells is enhanced when cells are grown on a laminin matrix [30]. On the other hand, neurite outgrowth of the pheochromocytoma (PC)12 cells, which are induced to differentiate into sympathetic neuron-like cells by growth factors such as nerve growth factor (NGF), is also affected by cell adhesion to the ECM [31,32]. Thus, the ECM is importantly involved in neurite outgrowth. Recent studies demonstrated that ILK is highly expressed in neuronal cells and also that the expression level of ILK proteins remains constant

during neuronal differentiation [16]. ILK activity in N1E-115 cells is transiently activated after seeding on a laminin matrix under serum-free conditions [16]. Moreover, transient ILK activation is also observed in PC12 cells grown on laminin and/or collagen after NGF treatment [17]. Thus, transient activation of ILK occurs in both integrin- and growth factor-mediated neuronal differentiation. ILK activation under neuron-differentiating conditions is prevented by treatment of the cells with LY294002, a specific PI3-kinase inhibitor [16], suggesting that ILK activation is PI3-kinase-dependent in neuronal cells, similar to other cells [11,33]. On the other hand, weak basal ILK activity is also detected in those neuronal cells, even under non-differentiating conditions [16,17]. Such weak basal activity of ILK, therefore, might be necessary for maintaining neuronal activity. The mechanism that maintains basal ILK activity, however, remains unknown. A recent study using integrin-activating peptide indicated basal activities of both ILK and PI3 kinase in cultured hippocampal neurons and transient upregulation of ILK following integrin activation without further increase in PI3-kinase activity [24]. The data suggest that basal activity of PI3-kinase is necessary for basal ILK activity, which probably facilitates the full activation of ILK after integrin activation.

ILK controls both integrin- and growth factor-dependent neurite outgrowth.

A stable transfection of a kinase-deficient mutant of ILK (DN-ILK) results in inhibition of integrin-dependent neurite outgrowth in N1E-115 neuroblastoma cells [16]. ILK activity in the parental cells was transiently activated after seeding on the laminin matrix under serum-free conditions, whereas that in the DN-ILK-transfected cells was not [16]. These results suggest that transient activation of ILK is required for integrin-dependent neurite outgrowth. Under the same

conditions, p38 mitogen-activated protein (MAP) kinase, but not MAP kinase/extracellular signal-regulated kinase kinase or extracellular signal-regulated kinases, was transiently activated after N1E-115 cell attachment to laminin, but not in the DN-ILK-expressed cells [16]. The time course of p38 MAP kinase activation was very similar to that of ILK activation. Furthermore, a p38 MAP kinase inhibitor, SB203580, blocked neurite outgrowth. Thus, activation of p38 MAP kinase is involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth in N1E-115 cells. p38 MAP kinase is important for neurite outgrowth [34-37]. The mechanism by which ILK triggers p38 MAP kinase activation after adhesion to laminin under serum-free conditions, however, is unknown. ILK interacts with PINCH, an adaptor protein comprising five LIM domains, through the amino-terminal ankyrin repeat domain [6]. PINCH interacts with the SH2/SH3 domain-containing adaptor protein Nck-2, which associates with ligand-activated growth factor receptor kinases and intracellular components of growth factor signaling pathways such as insulin receptor substrate [4,7]. Thus, the ILK-PINCH interaction seems to be critical for th formation of signal complex coupling integrins and growth factor receptors. On the other hand, p21-activated kinase (Pak), a family of Ser/Thr kinases, activates p38 MAP kinase [38]. Pak also potentially mediates Rac/Cdc42 signaling, which can lead to activation of p38 MAP kinase via Pak [39]. Nck-2 recognizes and activates Pak via the SH2/SH3 domain [40]. Therefore, a possible mechanism by which ILK activates p38 MAP kinase is that Pak is activated by direct interaction with Nck-2, which probably occurs following the ILK-PINCH interaction, and leads to p38 MAP kinase activation via its phosphorylation (Fig.2).

PC12 cells exhibit neurite outgrowth in response to NGF [41,42]. Activation of the extracellular signal-regulated kinases and p38 MAP kinase pathways is required for NGF-induced neuronal

differentiation in PC12 cells [34,43]. Recent study of the role of ILK in NGF-induced neurite outgrowth demonstrated that ILK is an important effector in NGF-mediated neurite outgrowth in PC12 cells and also in dorsal root ganglion neurons using a pharmacologic inhibitor of ILK (KP-392) and a DN-ILK overexpression system [17]. ILK activity was stimulated after treatment of cells with NGF and inhibition of ILK activity by overexpression of DN-ILK or treatment with KP-392 blocked NGF-induced neurite outgrowth. Moreover, NGF-induced stimulation of both Akt and GSK-3β, which reside in downstream signaling pathways of ILK, was inhibited in the presence of KP-392 or afterDN-ILK transfection, suggesting that those kinases are involved in NGF-induced neurite outgrowth [17].

NGF-induced activation of MAP kinase pathways is critical for neurite outgrowth of PC12 cells [34,43]. The relation between the ILK- and MAP kinase-signaling pathways in NGF-induced neurite outgrowth, therefore, is very intriguing. Recently, Huang et al. demonstrated that paxillin, a focal adhesion adaptor protein involved in adhesion dynamics, is required for neurite outgrowth [44]. They also demonstrated that paxillin is phosphorylated by p38 MAP kinase and phosphorylated paxillin is involved in NGF-induced neurite outgrowth [44]. Moreover, activation of p38 MAP kinase is involved in ILK-mediated signal transduction and leads to integrin-dependent neurite outgrowth in N1E-115 cells [16]. Therefore, these reports suggest that phosphorylation of paxillin via p38 MAP kinase, which is activated by ILK activation, is involved in both NGF- and integrin-dependent neurite outgrowth.

ILK is involved in neuronal cell survival.

Activation of integrin receptors in neurons promotes cell survival and synaptic plasticity [45-49]. Integrin signaling activates the PI3-kinase-Akt pathway [50-52], which is one of the anti-apoptotic pathways utilized by many cell types [24,52,53]. This PI3-kinase-Akt pathway is also the cell-survival signaling pathway activated by neurotropic factors and cytokines [54,55]. The kinase activity of Akt is dependent on the generation of PI (3,4,5) P3 and on activation of phosphatidylinositol-dependent kinase 1 (PDK1) and the putative phosphatidylinositol-dependent kinase 2 (PDK2), which phosphorylate Akt on Thr^{308} and Ser^{473}, respectively [56]. Phosphorylation of Akt on these residues leads to Akt activation [57] and, once activated, Akt acts to either inhibit pro-apoptotic or activate anti-apoptotic processes [54,57,58]. On the other hand, ILK can phosphorylate Akt on Ser^{473} [59] and promotes the survival of some non-neuronal cells in an Akt-dependent manner [11,26]. A recent study by Gray et al. [24] demonstrated that ILK has an important role in integrin-mediated cell survival in neurons. This study using hippocampal neurons demonstrated that ILK is upregulated following integrin activation and then activates Akt, which is essential for integrin-mediated cell-survival signaling in hippocampal neurons [24]. On the other hand, although PI3-kinase activity was necessary for Akt activation in neurons, it was not upregulated following integrin activation [24]. Thus, ILK functions as a transducer of survival signaling initiated by integrin receptor activation mainly via Akt activation, which is necessary for basal PI3-kinase activity, but not for its upregulation.

Role of ILK in oligodendrocytes.

In the brain, ILK is expressed not only in neuronal cells, but also in glial cells and Purkinje cells [18]. Oligodendrocytes in theCNS, similar to Schwann cells in the peripheral nervous system,

have an important role in the formation of myelin through extending large lamellae to spiral around the adjacent axons [18,60-62]. Recent studies demonstrated that laminin-2 (LN-2; also called merosin) stimulates oligodendrocytes to extend elaborate membrane sheets through integrin α6β1 in vitro [61]. LN-2 is expressed on the axonal surface in the developing CNS [63] and congenital LN-2 deficiency is associated with abnormal CNS white matter [64]. Examination and analysis of the role of LN-2 in CNS myelination using LN-2-deficient mice demonstrated that ILK is necessary for the LN-2-induced cell spreading and myelin formation in oligodendrocytes [18]. ILK is expressed in oligodendrocytes and LN-2 stimulates ILK activity [18]. Moreover, ILK expression during early myelination in vivo is restricted to interfascicular oligodendrocytes and is only detected from postnatal day 9, which is when myelination occurs [18]. These studies suggest that ILK activation is an initial step in the intracellular signaling cascade of LN-2-induced oligodendrocyte cell spreading and myelination after cell adhesion on LN-2 via integrin. LN-2 activated Akt, activation of which is regulated via phosphorylation at two sites, Thr-308 and Ser-473, by PDK-1 [56] and ILK [59] or PDK-2 [56], respectively, continuously up to 6 h during LN-2-induced cell spreading [18]. On the other hand, DN-Akt-transfected cells had decreased LN-2-induced cell spreading, suggesting that Akt activation is involved in LN-2-enhanced cell spreading in oligodendrocytes [18]. Moreover, DN-ILK blocked phosphorylation of Akt Ser^{473} and also of GSK-3β Ser^{9}, which is a downstream substrate of ILK and Akt [11,20,65], and inhibited LN-2-induced cell spreading and myelination [18]. These results suggest that ILK-downstream signaling molecules, Akt and GSK-3β, are critical for LN-2-induced cell spreading and myelination in oligodendrocytes.

Relevance of ILK to neuronal disorders.

Tau is a microtubule-associated protein that stabilizes microtubules within neurites and axons [66]. It is hypothesized that tau hyperphosphorylation leads to the destabilization of microtubules and aggregation of tau proteins, which impairs axonal transport and eventually results in neuronal cell death [66-68]. Indeed, tau hyperphosphorylation appears to be an early event preceding the formation of paired helical filaments (PHF) in the brains of patients with Alzheimer's disease [69]. On the other hand, tau phosphorylation also seems to control microtubule dynamics during neurite outgrowth and neuronal maturation, because embryonic and neonatal tau is much more heavily phosphorylated than adult tau [70-73]. Recently, Ishii et al. [74] demonstrated that stable transfection of DN-ILK to inactivate endogenous ILK results in aberrant tau phosphorylation in N1E-115 cells at sites recognized by the Tau-1 antibody (recognizes tau dephosphorylated at Ser^{195}, Ser^{198}, Ser^{199}, Ser^{202}, and Thr^{205} [74,75]) and also the anti-phospho(Ser^{199}, Ser^{202})-Tau antibody (recognizes tau aberrantly phosphorylated at Ser^{199} and Ser^{202}), which are identical to some of the phosphorylation sites in PHF-tau in the brains of patients with Alzheimer's disease [76,77]. These results suggest that inactivation of endogenous ILK results in aberrant hyperphosphorylation of tau. On the other hand, tau in the non-transfected parental cells was not phosphorylated at sites recognized by those antibodies under both normal and differentiating conditions, suggesting that endogenous ILK prevents aberrant tau phosphorylation [74]. Similarly, treatment of PC12 cells with KP-393, a selective ILK inhibitor, resulted in aberrant hyperphosphorylation of tau, which was recognized by antibody PHF-1 (recognizes tau phosphorylated at Ser^{396} and Ser^{404}) [17]. Immunofluorescence staining of cell monolayers with the antibody against aberrantly phosphorylated tau revealed that

DN-ILK-transfected cells, but not parental cells, were strongly stained with the antibody against phosphorylated tau under normal and differentiating conditions [74]. Aberrantly phosphorylated tau at Ser^{199} and Ser^{202} was also observed in the intracellular cytoplasm, but not in the nucleus, and participates in the formation of microtubule-like structures in the DN-ILK-transfected cells [74]. Further analysis of the DN-ILK-transfected cells using confocal laser scanning microscopy demonstrated that microtubule-like structures spread immediately under the entire plasma membrane of the cells and form basket-like structures [74](Fig. 3).

Recent studies of signal pathways involved in aberrant tau phosphorylation indicated that ILK inactivation leads to activation of GSK-3β [17,74] and inhibition of Akt [17]. GSK-3β is one of the candidate kinases that phosphorylates tau at both Ser^{199} and Ser^{202} [78] and also has an important role in the ILK-mediated signal pathway [11,20,65]. Activation of GSK-3β is dependent on Tyr^{216} phosphorylation [79]. On the other hand, GSK-3β activity is inhibited by direct phosphorylation at Ser^{9} by ILK [20,21] and by PKB/Akt, which is also activated via phosphorylation at Ser^{473} by ILK [11,80]. Interestingly, Tyr^{216} in GSK-3β is highly phosphorylated in DN-ILK-transfected neuronal cells, but very weakly phosphorylated in parental cells [74]. In contrast, Ser^{9} in GSK-3β was highly phosphorylated in parental cells but not in DN-ILK transfected neuronal cells [74]. Moreover, these phosphorylation levels were not significantly different between non-differentiating and differentiating conditions. These studies suggest that ILK inactivation results in Ser^{9} dephosphorylation and increased Tyr^{216} phosphorylation in GSK-3β, thereby activating the enzyme. The specific tyrosine kinase, which might be activated by ILK inactivation, however, is not known. To understand the ILK-mediated

regulatory mechanisms of GSK-3β, the specific tyrosine kinase involved in GSK-3β Tyr^{216} phosphorylation must be determined.

Lithium is an un-competitive GSK-3β inhibitor [81]. Treatment of DN-ILK-transfected cells with LiCl prevents aberrant tau phosphorylation in a dose-dependent manner [74]. This result suggests that GSK-3β activation induced by ILK inactivation is directly involved in aberrant tau phosphorylation. Moreover, treatment of DN-ILK-transfected cells with LiCl partially recovered neurite outgrowth from its inhibition caused by inactivation of endogenous ILK [74]. These results suggest that aberrant tau phosphorylation is partly involved in the inhibition of neurite outgrowth in DN-ILK-transfected neuronal cells. Tau hyperphosphorylation decreases the association of tau with microtubules [82] and inhibits total neurite number [83-85]. Therefore, the aberrant tau phosphorylation induced by ILK inactivation might affect microtubule stability or dynamics and lead to the inhibition of neurite outgrowth. On the other hand, endogenous ILK protects tau from aberrant phosphorylation and probably maintains a kind of equilibrium status responsible for microtubule reorganization (Fig.4).

ILK-deficient mice die at the peri-implantation stage due to abnormal accumulation of actin at sites of integrin attachment at the basement membrane zone [86]. Analysis of ILK-deficient mice embryos had neuronal defects caused by impaired actin dynamics, such as shorter axonal extensions [86]. The results imply that ILK is critical for axonal extension rather than axon formation. The roles of ILK in neurite and axonal extension suggest that ILK also must have an important role in brain development and plasticity, which is closely implicated in various brain functions, such as learning and memory, in addition to involvement in neuronal disorders.

Glutamate has an important role in regulating neurite outgrowth, synaptogenesis, and cell survival in the developing CNS [87] and is a mediator of synaptic transmission and synaptic plasticity in the mature CNS [88]. Over-activation of glutamate receptors, however, is considered to be involved in the neuronal death caused by stroke, Alzheimer's disease, amyotrophic lateral sclerosis, and other neurodegenerative disorders [49,89-91]. A recent study examining integrin-mediated cell survival in hippocampal neurons demonstrated that activation of ILK and its downstream signal molecule Akt promotes neuroprotection from glutamate-induced apoptosis [24]. These reports suggest that ILK has a key role in integrin-mediated cell- survival signaling in the pathogenesis of neurodegenerative conditions in which glutamate receptors are over-activated.

Concluding remarks.

This review focuses on the role of ILK in neuronal cells, especially with regard to the intracellular signal pathways, and discusses recent biochemical evidence of various ILK-mediating cellular processes to understand the potential role of ILK in neuronal cells and/or the CNS. The recent evidence suggests that ILK regulates both integrin- and growth factor-mediated signaling in neurons and regulates many cellular events: regulation of microtubule stability and the rearrangement necessary for integrin- and growth factor-mediated neurite outgrowth, regulation of Akt-mediated integrin and growth factor survival signals, regulation of cell adhesion and proliferation in neurons, and regulation of integrin-mediated cell spreading and myelination in oligodendrocytes. All these functions of ILK are critical for neuronal differentiation and formation and contribute to maintenance of neuronal activity and survival, which indicates that ILK is involved in brain development and plasticity. On the other hand,

because ILK has such important roles in neuronal cells, ILK dysfunction leads to serious neuronal disorders. Although we proposed the possible involvement of ILK in neuronal disorders in this review, it might be a little early to predict neuronal diseases in which ILK is involved because there are not enough studies available. More knowledge of the ILK-signaling pathways that control neuronal activity is important for further understanding of the neuronal abnormalities caused by ILK dysfunction. Moreover, further studies to fully elucidate all of the factors that regulate ILK-signaling pathways in neuronal cells might lead to the identification of potential molecular targets for therapy of neurodegenerative diseases.

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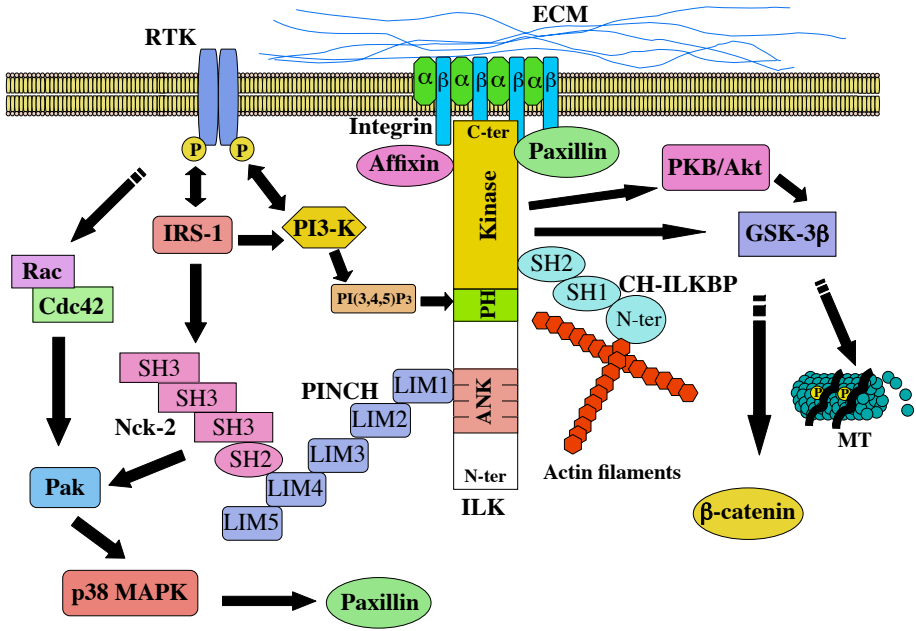
Figure legends.

Figure 1. **Functional domains of ILK and hypothetical ILK-mediated signal pathways.** ECM, extracellular matrix; RTK, receptor tyrosine kinase; IRS-1, insulin receptor substrate 1; ILK, integrin-linked kinase; PKB, protein kinase B; PI3-K, phosphatidylinositol 3-kinase; PH, pleckstrin homology-like motif; GSK-3β, glycogen synthase kinase 3β; ANK, ankyrin-like repeat; SH, Src homology; LIM, LIM motif; MT, microtubule; CH-ILKBP, calponin homology domain-containing ILK binding protein; PINCH, five LIM domains-only adaptor protein; Pak, p21-activated kinase; Nck-2, Src-homology (SH)2/SH3 containing adaptor protein.

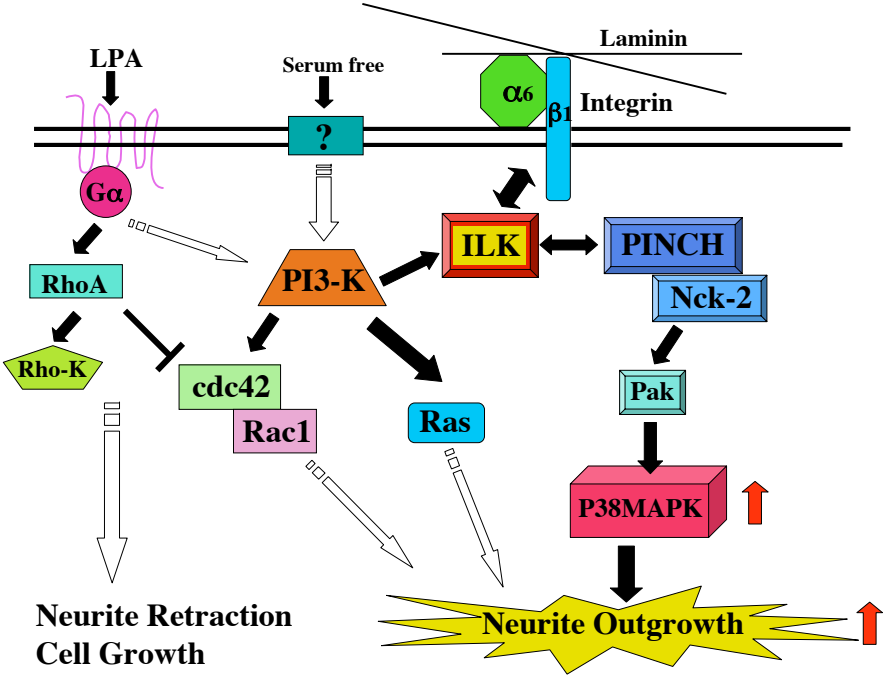
Figure 2. **P38 MAP kinase activation is involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth in N1E-115 cells.** ILK is activated after cell adhesion on laminin in serum-free conditions in a phosphatidylinositol 3-kinase-dependent manner. Stimulation of ILK activity results in activation of p38 MAP kinase, which is important for ILK-dependent neurite outgrowth in N1E-115 cells (74). The mechanism by which ILK triggers p38 MAP kinase activation, however, is not known. ILK can activate Nck-2 via ILK-PINCH interaction. On the other hand, Nck-2 can recognize and activate Pak. Therefore, a possible mechanism by which ILK activates p38 MAP kinase is that Pak activation by direct interaction with Nck-2, which probably occurs following the ILK-PINCH interaction, leads to p38 MAP kinase activation via its phosphorylation.

Figure 3. **Subcellular localization of aberrantly phosphorylated tau at Ser^{199} and Ser^{202}**. After cells were cultured for 16 h under either differentiating (a and b) or non-differentiating (c and d) conditions, cells were stained with anti-phospho (Ser^{199}, Ser^{202})-Tau antibody. In DN-ILK-transfected cells, the intracellular cytoplasm, except for the nucleus, was strongly stained with the antibody and microtubule-like structures were observed under normal and differentiating conditions (b and d). On the other hand, parental cells were not significantly stained under differentiating conditions (c), but very weak dot-like structures were observed in the non-differentiated cells (a). Further analysis of the DN-ILK-transfected cells using the confocal laser scanning microscopy. Microtubule-like structures spread immediately under the entire plasma membrane of the cells, and formed basket-like structures (left).

Figure 4. **Schematic model of aberrant tau phosphorylation in DN-ILK-transfected N1E-115 cells.** ILK phosphorylates GSK-3β at Ser^{9}, leading to GSK-3β inactivation. GSK-3β inactivation results in the inhibition of aberrant tau phosphorylation, an increase in microtubule stability, and induces neurite outgrowth in combination with p38 MAP kinase activation. On the other hand, ILK inactivation by DN-ILK induces GSK-3β phosphorylation at Tyr216 via activation of an unidentified tyrosine kinase, leading to GSK-3β activation. GSK-3β activation results in aberrant tau phosphorylation, microtubule instability, and decreased neurite outgrowth.



Ishii, Figure 1



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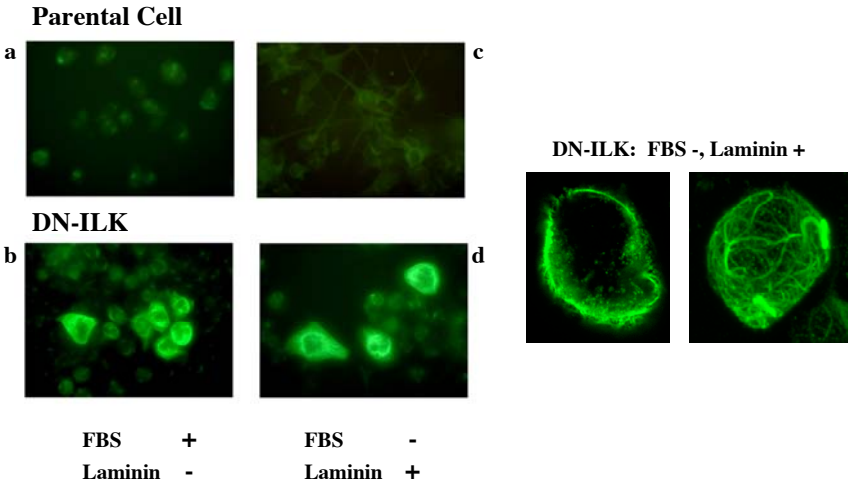

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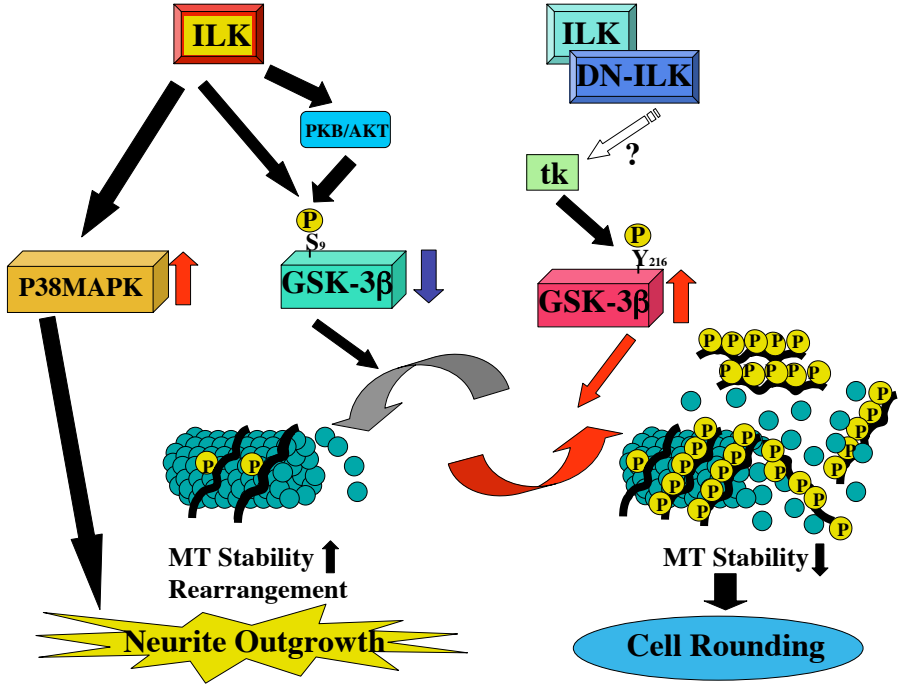

Ishii, Figure 4

The mesencephalic trigeminal sensory nucleus is involved in the control of feeding and exploratory behavior in mice

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Abstract

The mesencephalic trigeminal nucleus (Me5), which receives input from oral proprioceptors and projects to higher brain regions, is involved in mastication-induced modulation of satiation. To investigate how the Me5 is involved in the control of feeding and exploratory behavior, we examined the effect of bilateral electrolytic lesions of the Me5 on feeding and exploratory behavior in mice. Mouse feeding and exploratory behaviors were analyzed using a food-search-compulsion-apparatus (FSCA), which was designed to distinguish between the two behaviors under standard living conditions. To assess anxiety in mice in an unfamiliar environment, exploratory activity was analyzed in an automated hole-board apparatus. Mice with bilateral Me5 lesions had unique feeding and exploratory behavior profiles in the FSCA compared with sham-operated mice. Me5-lesioned mice spent more time in the food chamber during each trial in the FSCA, but the number of entries into the food chamber was decreased by 40% compared to sham-operated mice. Moreover, Me5 lesions markedly inhibited exploratory behavior, manifested as low-frequency exploration. In spite of the low-frequency exploration in the FSCA, Me5 lesions had no effect on various exploratory activities analyzed in the hole-board apparatus, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping. These results suggest that the Me5 is involved in the control of feeding and exploratory behavior through its ascending neuronal pathways in mice without modulating the emotional state.

Theme: Neural Basis of Behavior
Topic: Neuroethology
Keywords: feeding, exploratory behavior, Me5 lesions

Introduction

The mesencephalic trigeminal nucleus (Me5) receives proprioceptive sensory afferents of the trigeminal nerve from the jaw-closing muscle spindles and the periodontal ligaments, and also innervates the motor trigeminal nucleus, relating to the jaw-jerk reflex [2,7]. The Me5 fibers also project into the tuberomammillary nucleus (TMN) of the posterior hypothalamus where the cell bodies of histamine (HA) neurons are localized [3]. HA neurons in the TMN project to the ventromedial hypothalamus (VMH) and to the Me5 [8,9,14]. Fujise et al. (1993) [5] suggested that the oral proprioceptive signals induced by mastication might modulate hypothalamic HA neurons through the ascending pathway from the Me5. In fact, the HA nerve systems in the VMH and Me5 are activated in response to feeding, although the time necessary for activation of both regions differs [6].

Fujise et al. (1998) [6] demonstrated that the Me5 is involved in mastication-induced modulation of satiation and eating parameters. Their study suggests that the Me5 receives signals relating to mastication-induced proprioceptive sensation and modulates satiation via a satiety center in the VMH. Their role of the Me5 in feeding and related behaviors, however, remains unknown. In the present study, to investigate how the Me5 is involved in the control of feeding and exploratory behavior, we examined the effect of bilateral electrolytic lesions of the Me5 on feeding and exploratory behavior in mice using a food-search-compulsion-apparatus (FSCA), which was designed to distinguish between feeding and exploratory behaviors under standard living conditions. Moreover, we analyzed various exploratory activities using an automated hole-board apparatus to evaluate whether Me5 lesions affect the emotional state and spontaneous activity of mice.

Methods

Animals and FSCA

Male *ddY* mice were maintained under controlled temperature and lighting conditions with a 12-h light/dark cycle (lights on at 0600). Feeding and exploratory behavior were monitored using an FSCA (Fig. 1A). The FSCA was an acrylic cage equipped with two separate vertical cylinders (180 cm high) of stainless steel wire, the tops of which had a chamber containing either food or nothing. Mice had to climb up to the chamber to seek and obtain food. The number of entries and the duration of time spent in each chamber were monitored using detectors attached to the chambers. Mice could obtain water *ad libitum* without climbing the cylinders.

Me5 lesions

Bilateral electrolytic Me5 lesions were produced under avertin anesthesia (0.36 g kg^{-1}) with 0.2-mm-diameter stainless steel electrodes at 6 wk of age. Using a stereotaxic apparatus, the electrode was positioned 5.3 mm posterior to bregma, 0.9 mm lateral to the midsagittal suture, and 3.2 mm below the surface of the skull. Anodal electrolytic lesions were produced by passing a 1.3-mA current for 1 s, three times. Out of 16 mice that received lesions, 7 had successful bilateral Me5 lesions, 7 had unilateral Me5 lesions, and 2 had lesions in peri-Me5 regions. All successful Me5 lesions were restricted to the caudal level of the Me5. Sham-operated mice (7 mice) underwent an identical operation, except no current was passed. Histologic analysis was performed by light-microscopic examination. After fixation with 10% neutral buffered paraformaldehyde solution and embedding in paraffin, serial brain sections (4 μm thick) were

stained with hematoxylin-eosin to reveal the extent of damage to the Me5. Only the data from mice with successful Me5 lesions were used.

Feeding and exploratory behavior under standard living conditions

The training task was conducted by placing mice in the FSCA for 2 wk (from 4 - 6 wk of age). A food chamber containing pellets and an empty chamber were connected to the left and right cylinders, respectively. Bilateral electrolytic Me5 lesions were produced at 6 wk of age. The mice were returned to normal plastic cages and maintained for 2 wk. After a 2-wk recovery period, mice were transferred back to the FSCA and maintained for 2 wk. Entries into the food chamber were designated as feeding. Entries into the empty chamber were designated as exploratory behavior, although whether mice explored their territory with a specific aim was not determined. Feeding and exploratory behavior were recorded at 10 wk of age.

Exploratory activities in an unfamiliar environment

General exploratory activities, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping, were recorded using an automated hole-board apparatus (model ST-1, Muromachi Kikai Co., Ltd., Japan), which consisted of a gray wooden box (50 x 50 x 50 cm) with four equidistant holes (3 cm diameter) in the floor. An infrared beam sensor was installed in the wall of each hole to detect the frequency and duration of rearing and head-dipping behaviors and the latency to the first head-dipping. Other behavioral parameters such as location, distance, and speed of movement of mice in the hole-board were recorded by an overhead color CCD camera; the heads of the mice were painted yellow and the color CCD camera

tracked their centre of gravity. Data from the infrared beam sensor and the CCD camera were collected through a custom-designed interface (CAT-10, Muromachi Kikai Co., Ltd.) as a reflection signal. Head-dipping behaviors were double-checked via the infrared sensor and the overhead color CCD camera. Thus, only when both the head intercepted the infrared beam and the head was detected at the hole by the CCD camera was head-dipping behavior counted. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS, Muromachi Kikai Co., Ltd.). Mice were placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min.

Statistical methods

Statistical significance was determined using Student's unpaired *t* test. A P value of less than 0.05 was considered statistically significant.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee in Obihiro University of Agriculture and Veterinary Medicine and were conducted under the Guidelines for Animal Experiments in Obihiro University of Agriculture and Veterinary Medicine and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989. The animals were humanely killed by an overdose of anesthetic ether at the end of the experiment.

Results

Profiles of feeding and exploratory behavior in Me5-lesioned and sham-operated mice analyzed in an FSCA under standard living conditions

To investigate how the Me5 is involved in the control of feeding and exploratory behavior, we analyzed those behaviors in Me5-lesioned and sham-operated mice in an FSCA under standard living conditions. Bilateral Me5-lesioned and sham-operated mice had different feeding and exploratory behavior. Sham-operated mice displayed high-frequency exploratory behavior and repeatedly entered the empty chamber (Fig. 2A). In contrast, bilateral Me5-lesioned mice seldom re-entered the empty chamber, and some never entered it at all (Fig. 2B); this was termed low-frequency exploratory behavior. On the other hand, bilaterally Me5-lesioned mice spent more time in the food chamber during each trial, but the number of entries into the food-containing chamber was lower compared to sham-operated mice (Fig. 2A & B). The number of entries into the empty chamber (exploratory behavior) in both bilaterally and unilaterally Me5-lesioned mice was significantly lower compared to sham-operated mice (Fig. 3A). Thus, Me5 lesions markedly inhibited exploratory behavior in the home cage. On the other hand, the number of entries into the food-containing chamber (feeding behavior) in bilaterally Me5-lesioned mice was significantly decreased by 40% compared to sham-operated mice (Fig. 3B). The total time spent in the food chamber per 24 h, however, was not significantly different between the Me5-lesioned and sham-operated mice (Fig. 3C). There was no significant difference in the mean body weight between the Me5-lesioned and sham-operated mice (Fig. 1C).

Various exploratory activities in Me5-lesioned and sham-operated mice analyzed by an automated hole-board apparatus in an unfamiliar environment

Me5 lesions markedly inhibited exploratory behavior in the FSCA (Fig. 2B). This low-frequency exploratory behavior in Me5-lesioned mice might be due to increased anxiety induced by Me5 lesions. We, therefore, analyzed various exploratory activities in Me5-lesioned and sham-operated mice in an unfamiliar environment using an automated hole-board apparatus and compared the activity between them. The hole-board system is a simple method for measuring the response of an animal to an unfamiliar environment [1,13] and is a useful tool for objectively estimating the level of anxiety in mice [15,16,17]. The effect of Me5 lesions on various exploratory activities, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping, is shown in Fig. 4. These exploratory activities in Me5-lesioned mice, however, were not significantly different from those in sham-operated mice. Thus, Me5 lesions influence neither spontaneous activity nor exploratory activity in mice in an unfamiliar environment.

Discussion

In the present study, we demonstrated that Me5 lesions change feeding behaviour profiles and inhibit exploratory behavior without affecting the emotional state of mice. The Me5 receives input from oral proprioceptors [7] and projects its fibers into the TMN of the posterior hypothalamus [4]. HA neurons in the TMN project to the VMH and also to the Me5 [8,9,14]. The depletion of neuronal HA from the Me5 or the VMH affects eating parameters and meal duration [6]. Thus, the oral proprioceptive signals induced by mastication modulate hypothalamic HA

neurons through the ascending pathway from the Me5, and the hypothalamic feeding center controls masticatory function via the Me5.

Recent studies demonstrated that the Me5 is involved in mastication-induced modulation of satiation via the satiety center of the VMH [6,14]. The Me5 is thought to transfer proprioceptive signals to the VMH through HA neurons in the TMN [14]. In the FSCA in the present study, bilaterally Me5-lesioned mice spent more time in the food chamber during each trial, but the number of entries into the food-containing chamber was lower, compared to sham-operated mice (Fig. 2). The total time spent in the food chamber per 24 h, however, was not significantly different between the Me5-lesioned and sham-operated mice (Fig.3C). Because meal duration is prolonged when neuronal HA is depleted in the VMH [6], Me5-lesions might affect the activity of HA neurons in the VMH and result in a longer stay in the food chamber during each trial to eat food. Alternatively, Me5- lesions might change the feeding profile itself by affecting the satiety center of the VMH. In either case, these results suggest that the Me5 is involved in the control of feeding behavior, which can be caused by transmitting oral proprioceptive signals to the VMH through the TMN as well as mastication-induced modulation of satiation.

Sham-operated mice displayed high-frequency exploratory behavior and repeatedly entered the empty chamber (Fig. 2A). In contrast, bilaterally Me5-lesioned mice seldom re-entered the empty chamber, and some never entered it at all (Fig. 2B). Thus, Me5 lesions markedly inhibited exploratory behavior in the FSCA. The low-frequency exploratory behavior in Me5-lesioned mice might be due to increased anxiety induced by Me5 lesions. We, therefore, analyzed exploratory activity in an unfamiliar environment using an automated hole-board apparatus, which is a useful tool for objectively estimating the level of anxiety in mice [15,16,17]. There were no significant

differences in any exploratory activities, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping, however, between Me5-lesioned and sham-operated mice. Thus, Me5 lesions did not affect the emotional state of mice. These results strongly suggest that the Me5 is involved in the control of feeding and exploratory behavior without modulation of the emotional state of mice.

The development and excitability of the central nervous system are modulated by signals derived from dietary properties via sensory afferent neurons [6,10,11,14]. Moreover, a soft-diet after the weaning period reduces synaptic formation in the cerebral cortex and impairs spatial learning ability in adulthood [18]. There is a possible link between reduced mastication and hippocampal neuron loss in senile impairment of spatial memory [12,18]. Thus, it seems that sensory signals from oral proprioceptors transmit to higher brain regions via the Me5 and affect brain function and development. Moreover, the Me5 might modulate signal transmission of bi-directional signal pathways (i.e., "from hypothalamic regions to motor trigeminal nucleus" and "from oral proprioceptors to hypothalamic regions") necessary for control of feeding and exploratory behavior other than satiation.

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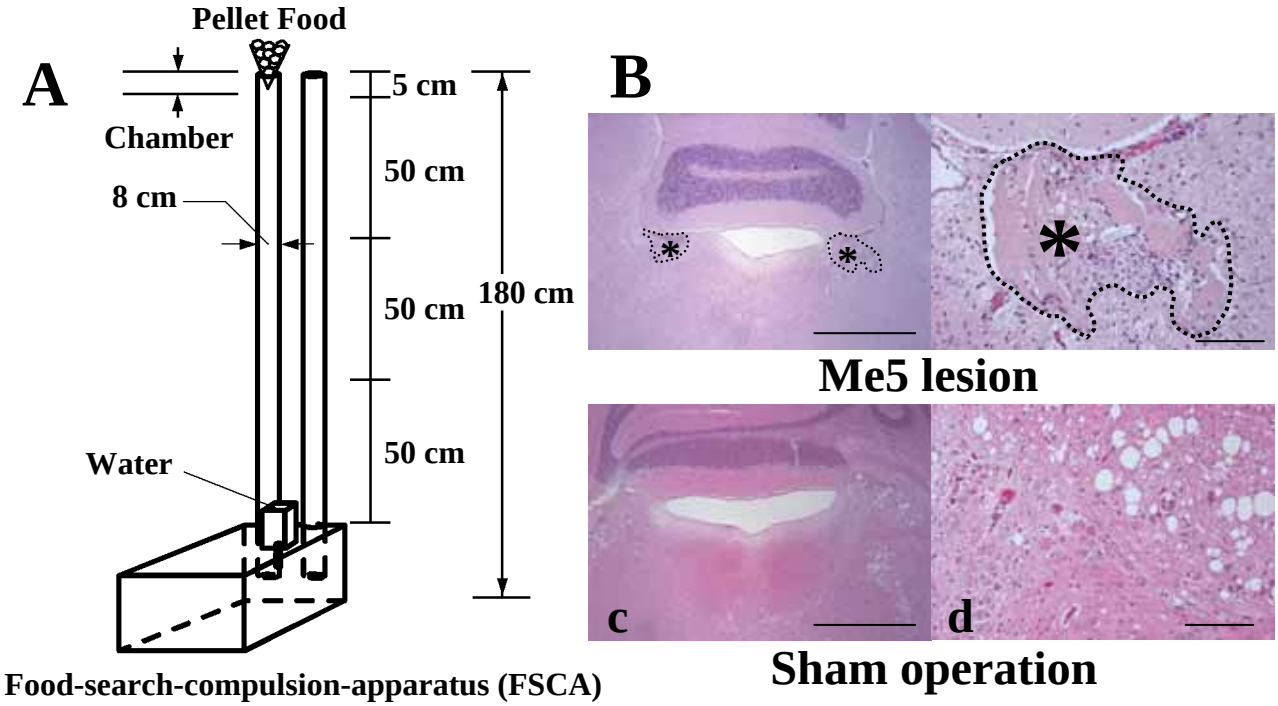
Figure legends

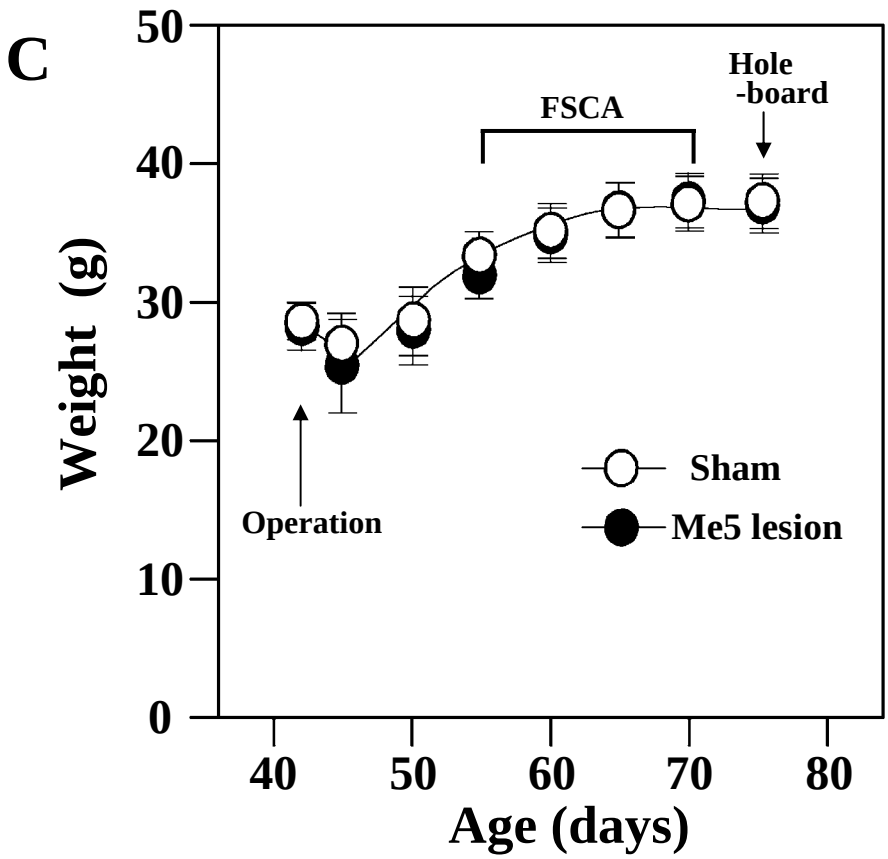
Figure 1. Food-search-compulsion-apparatus (FSCA) and changes in body weight after bilateral Me5 lesions. (A) Illustration of the food-search-compulsion-apparatus (FSCA). The FSCA is an acrylic cage equipped with two separate vertical stainless steel wire cylinders, the top of which has a chamber containing either food (food pellets) or nothing. The number of entries into the empty and the food containing chambers was termed exploratory and feeding behavior, respectively. (B) Histologic analysis of hematoxylin & eosin-stained brain sections in Me5-lesioned (a, b) and sham-operated mice (c, d). Scale bar, 1 mm (a, c) and 100 μm (b, d). Asterisks show the sites of the electrolytic lesions of Me5 and the area of lesion is bordered by broken line. (C) Changes in body weight of bilaterally Me5-lesioned mice (n=7) and sham-operated mice (n=7) after surgery at 6 wk of age. Except for the analysis of feeding and exploratory behaviors in the FSCA, the mice were maintained in normal plastic cages and fed food and water *ad libitum*. Feeding and exploratory behaviors were recorded in an FSCA at 10 wk of age, and general exploratory activities using a hole-board apparatus were recorded at 11 wk. The mean body weight of Me5-lesioned mice was not significantly different from that of sham-operated mice. Vertical bars represent SD.

Figure 2. Feeding and exploratory profiles in bilaterally Me5-lesioned and sham-operated mice housed in an FSCA. (A and B) Feeding and exploratory profiles in sham-operated mice (A) and bilaterally Me5-lesioned mice (B) at 10 wk of age. Sham 1-5 and Lesion 1-6 represent the profiles of different mice in each group.

Figure 3. Comparison of feeding and exploratory behavior between sham-operated and Me5-lesioned mice. (A) Exploratory behavior in unilaterally (n=7) and bilaterally Me5-lesioned mice (n=7) was significantly lower than that in sham-operated mice (n=7). (B and C) Comparison of the number of entries into the food chamber (B) and time spent in the food chamber (C) between sham-operated mice and Me5-lesioned mice. The number of entries into the food chamber, but not time spent in the chamber in bilaterally Me5-lesioned mice (n=7), was significantly lower than that in sham-operated mice (n=7). Vertical bars represent SD. Asterisk indicates $p<0.05$. Double asterisk indicates $p<0.01$. Statistical significance was determined using the unpaired Student's *t* test.

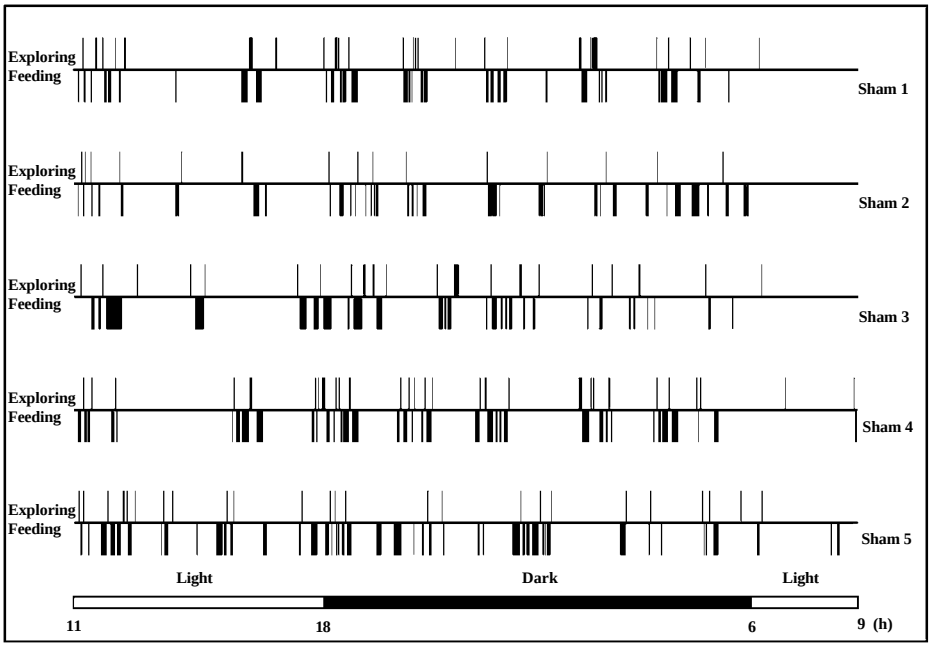
Figure 4. Effect of bilateral Me5 lesions on general exploratory activities in the automated hole-board test. Exploratory activities on the hole-board, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping, were measured for 5 min. Vertical bars represent SD.



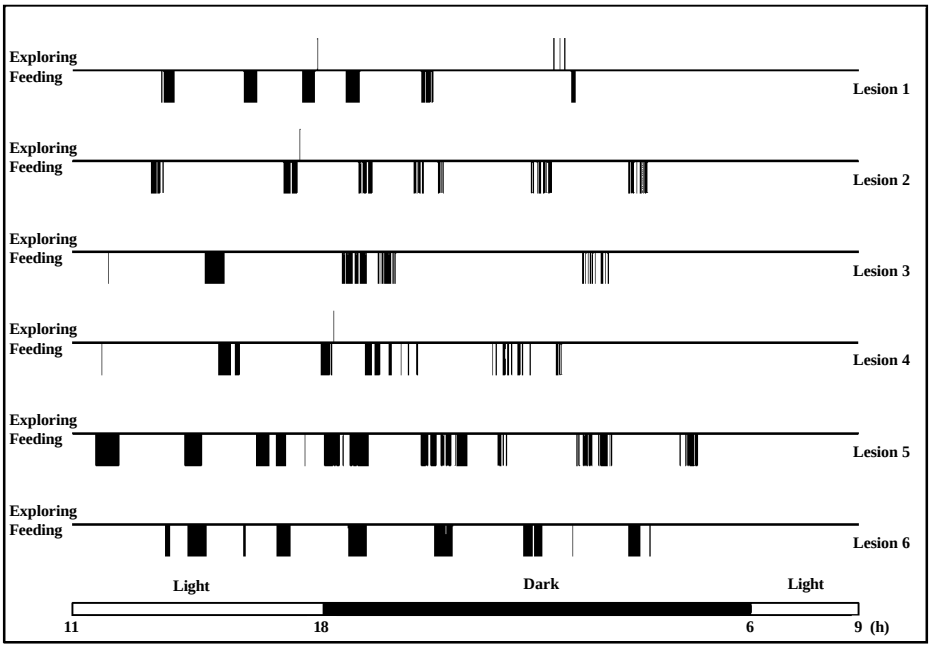


Ishii et al. Figure 1

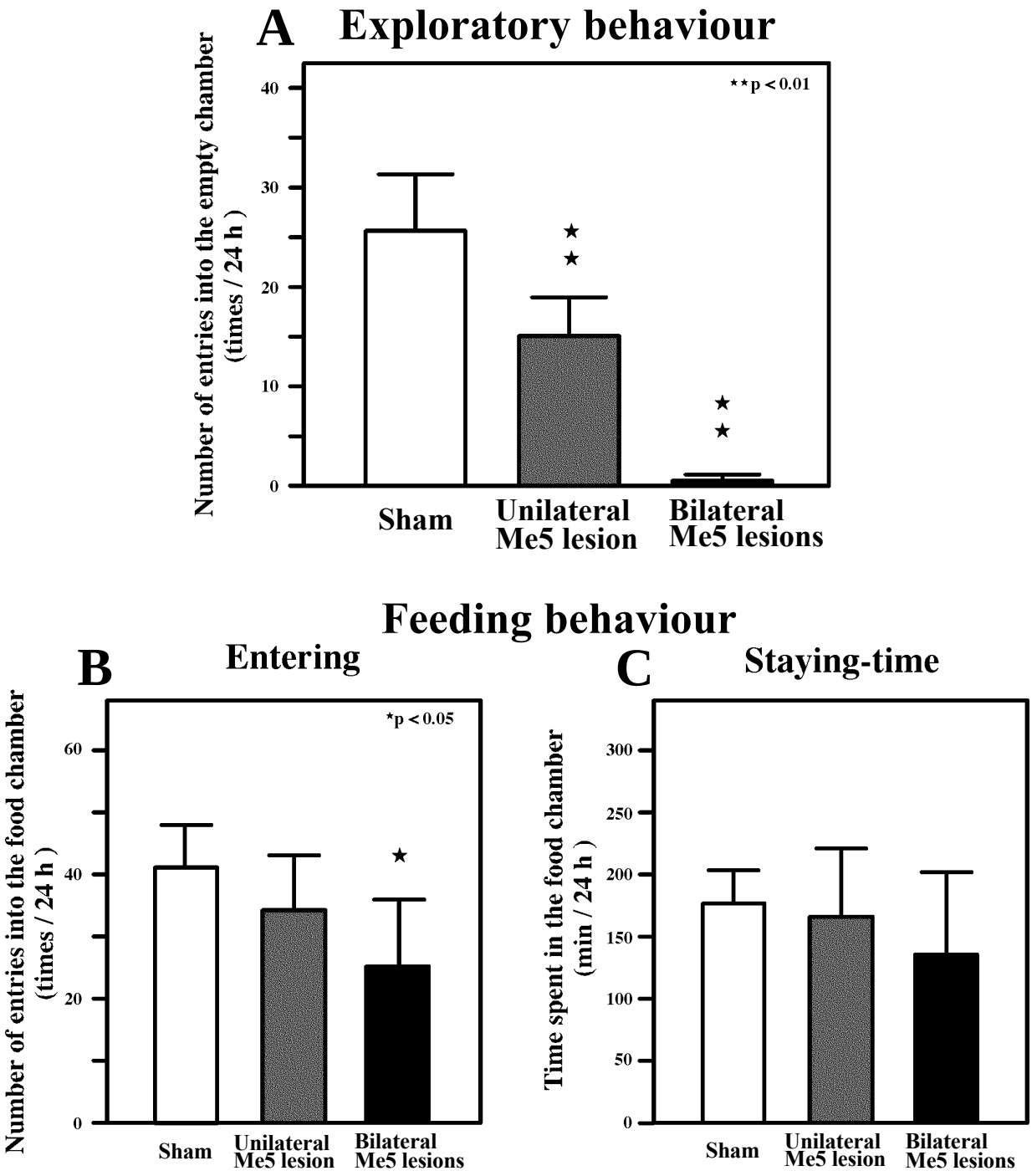
A **Feeding and exploring profiles in sham-operated mice**



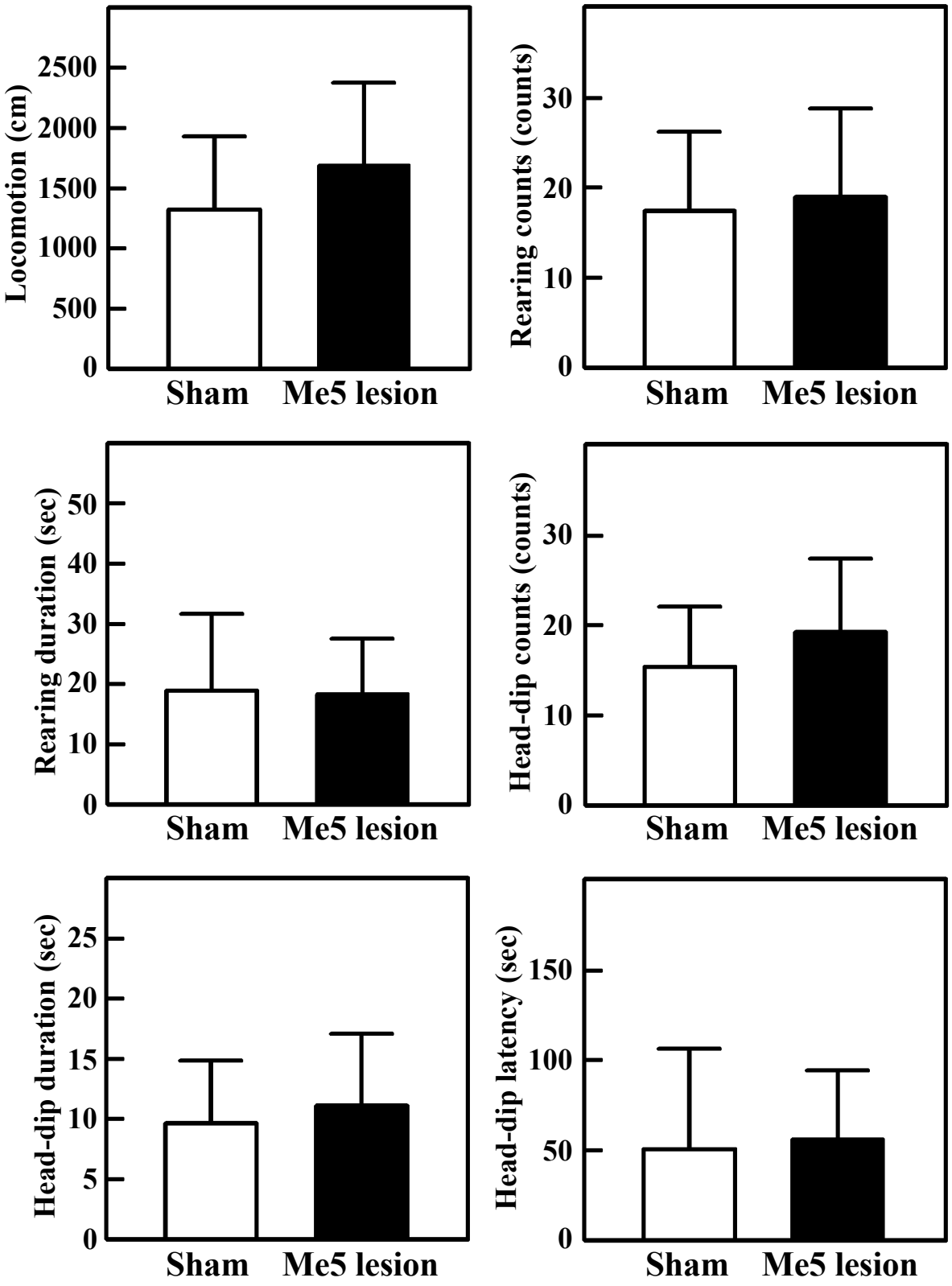
B **Feeding and exploring profiles in mice with Me5 lesions**



Ishii et al. Figure 2



Ishii et al. Figure 3



Ishii et al. Fig.4

Comparison of growth and exploratory behavior in mice fed an exclusively milk formula diet and mice fed a food-pellet diet post weaning

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Abstract

An exclusively milk formula diet stunted the growth of mice immediately following weaning. Milk-fed mice displayed a low-frequency profile of exploratory behavior, while pellet-fed mice showed high-frequency exploration. In contrast to exploratory behavior, feeding behavior did not differ significantly between milk- and pellet-fed mice. Despite showing low-frequency exploratory behavior, mice on an exclusively milk formula diet showed no difference in behavioral activities analyzed by an automatic hole-board apparatus compared to pellet-fed mice. These results suggest that the growth stunt caused by an exclusively milk formula diet retards the acquisition of active exploratory behavior without affecting the emotional state of mice.

Key words: feeding, exploratory behavior, growth, weaning, milk formula

Introduction

Milk formula is widely utilized as a substitute for mother's milk in all mammals. The influence of milk formula on growth and development during the suckling period has been studied using artificially reared rat pups (Smart et al., 1983, 1984; Auested et al., 1990; Kanno et al., 1997). Some differences in behavior and brain development were observed between artificially reared and mother-reared rat pups (Diaz et al., 1982; Smart et al., 1984; Moore et al., 1990; Kaneko et al., 1996). These studies suggested that the nutritional composition of milk formula and/or maternal separation strongly affects behavior and brain development. No information is currently available concerning the effect of a prolonged post-weaning milk formula diet on brain development. The present study investigates the effects of an exclusively milk formula post-weaning diet on the feeding and exploratory behavior of mice. Feeding and exploratory behavior were analyzed using a food-search-compulsion-apparatus (FSCA), which was designed to distinguish these two behaviors under the living conditions of mice. We further analyzed various exploratory activities using an automatic hole-board apparatus to examine emotionality and anxiety of mice in an unfamiliar environment. We demonstrated that an exclusively milk formula diet stunts the growth of mice immediately following weaning and prevents the acquisition of active exploratory behavior without affecting the emotional state of the mice.

Materials and Methods

Animals, diet, and food-search-compulsion-apparatus (FSCA)

Male *ddY* mice were maintained under controlled temperature and lighting conditions with a 12 h light/dark cycle (lights on at 0600). Mice were isolated from the mother at 20 d of

age and fed either milk or food pellets until 10.5 wk of age. Constituents of milk formula and food pellets used in this study are shown in Table 1. The milk formula has lower protein and higher carbohydrate content, but is isoenergetic with mouse milk (Sensui et al., 1996). Feeding and exploratory behavior were monitored using an FSCA (Fig. 1A). The FSCA was an acrylic cage equipped with two separate vertical cylinders (180 cm high) of stainless steel wire, the tops of which had a chamber containing either food (milk or pellets) or nothing. Mice needed to climb up to the chamber to seek and obtain food. The number of entries and the duration of time spent in each chamber were monitored using detectors attached to the chambers. Mice had access to water *ad libitum* without climbing the cylinders.

Feeding and exploratory behavior under mice's living conditions

The training task was conducted by placing mice in the FSCA for 2 wk (from 5 - 7 wk of age). A food chamber containing milk or pellets and an empty chamber were connected to the left and right cylinders, respectively. Following training, the mice were returned to normal plastic cages and maintained for 2 wk. After a 2-wk recovery period, mice were transferred back to the FSCA. We designated entries into the food chamber as feeding behavior. Entries into the empty chamber were designated exploratory behavior, although whether mice explored their territory with a specific aim was not determined. Feeding and exploratory behavior were recorded at 10 wk of age.

Behavioral activities in unfamiliar environments

Exploratory activities, i.e., total locomotor activity, numbers and duration of rearing and head-dipping, and latency to the first head-dipping, were recorded using the automatic hole-board apparatus (model ST-1 Muromachi Kika, Japan) consisting of a gray wooden box

(50 x 50 x 50 cm) with four 3 cm equidistant holes in the floor. An infrared beam sensor was installed on the wall to detect the number and duration of rearing and head-dipping behaviors and the latency to the first head-dipping. Other behavioral parameters such as locus, distance and speed of movement of mice in the hole-board were recorded by an overhead color CCD camera; the heads of the mice were painted yellow and the color CCD camera followed their center of gravity. Data from the infrared beam sensor and the CCD camera were collected through a custom-designed interface (CAT-10, Muromachi Kika, Japan) as a reflection signal. Head-dipping behaviors were double-checked via an infrared sensor and the overhead color CCD camera. Thus, only when both the head intercepted the infrared beam and the head was detected at the hole by the CCD camera was head-dipping behavior counted. All data were analyzed and stored on a personal computer using analytical software (Comp ACT HBS, Muromachi Kika, Japan). Mice were placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min while exploratory activities were automatically recorded.

Statistical methods

Statistical significance was determined using Student's unpaired *t* test.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee at Obihiro University of Agriculture and Veterinary Medicine and were conducted under the Guidelines for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989. The animals were humanely killed by an overdose of anesthetic ether at the end of the experiment.

Results

Profiles of feeding and exploratory behavior in milk- and pellet-fed mice

Milk-fed mice had lower body weight gain than pellet-fed mice during the first 2 wk post weaning. The body weight of milk-fed mice increased shortly afterward and reached that of pellet-fed mice at 8 wk of age. The mean body weight of milk-fed mice was significantly lower than that of pellet-fed mice between 4 and 7 wk of age; however, after 8 wk of age the difference was no longer statistically significant (Fig. 1B). Thus, an exclusively milk formula diet stunted the growth of mice only immediately following weaning.

We analyzed both feeding and exploratory behavior in milk-and pellet-fed mice at 10 wk of age. Milk-fed and pellet-fed mice had different exploratory behavior profiles. Pellet-fed mice displayed high-frequency exploratory behavior and repeatedly entered the empty chamber (Fig. 2A). In contrast, milk-fed mice displayed low-frequency exploratory behavior; either seldom re-entering the empty chamber or never entering it at all (Fig. 2B). The number of entries into the empty chamber (exploratory behavior) in milk-fed mice was significantly lower than pellet-fed mice, whereas the number of entries into the food-containing chamber (feeding behavior) was not significantly different (Fig. 2C, D).

Various exploratory activities in milk- and pellet-fed mice analyzed by an automatic hole-board apparatus in unfamiliar environments

An exclusively milk formula diet markedly inhibited exploratory behavior in an FSCA (Fig. 2B&C). This low-frequency exploratory behavior in milk-fed mice may have been caused by emotional depression; therefore, analysis and comparison of various exploratory activities in milk- and pellet-fed mice under unfamiliar environments were conducted. An automatic hole-board apparatus offers a simple method for measuring the response of an

animal to an unfamiliar environment (Boisser and Simon, 1964; Rodriguez Echandia et al., 1987) and is a useful tool for objectively estimating various emotional states of animals (Takeda et al., 1998; Tsuji et al., 2000; Tsuji et al., 2001). The effect of an exclusively milk formula diet on various exploratory activities, i.e., total locomotor activity, number and duration of rearing and head-dipping, and latency to the first head-dipping, is shown in Fig. 3. Results of these exploratory activities in milk-fed mice, however, were not significantly different from those of pellet-fed mice, suggesting that an exclusively milk formula diet after weaning does not affect the emotional state of mice.

Discussion

In the present study, we demonstrated that an exclusively milk formula diet stunts the growth of mice immediately following weaning and prevents the acquisition of active exploratory behavior without affecting emotional state. In contrast to exploratory behavior, feeding behavior did not differ significantly between milk- and pellet-fed mice. These results suggest that exclusively milk-fed mice fail specifically in the expression of active exploratory behavior. The stunting of growth, caused by an exclusively milk formula diet after weaning, might affect brain development or function and lead to the failure in acquisition of active exploratory behavior.

Feeding and exploratory behavior were recorded at 10 wk of age. Body weight gain of milk-fed mice was significantly lower than that of pellet-fed mice for the first 2 wk post weaning, which is likely to be caused by the different nutritional compositions and/or different diet quality between milk formula and pellet food. As a consequence of the lower body weight gain, the mean body weight of milk-fed mice was significantly lower than that of pellet-fed mice between 4 and 7 wk of age, but the difference was no longer statistically significant after

8 wk of age (Fig. 1B), indicating that an exclusively milk formula diet stunted the growth of mice only immediately following weaning. Therefore, growth and/or brain development in the weeks following weaning appear to be the most important for the acquisition of active exploratory behavior. Conversely, feeding behavior and other exploratory activities, analyzed using the automatic hole-board apparatus, did not differ significantly between milk- and pellet-fed mice (Fig. 2D & 3). These results suggest that low-frequency exploratory behavior in milk-fed mice is not caused by emotional depression or a decrease in spontaneous activity, and also appears to be unrelated to the motivation to obtain food.

The influence of milk formula during the suckling period on growth and development has been studied (Smart et al., 1983, 1984; Auested et al., 1990; Kanno et al., 1997). Several investigators have reported the importance of the nutritional composition of milk formula and/or maternal separation in behavior and brain development (Diaz et al., 1982; Smart et al., 1984; Moore et al., 1990; Kaneko et al., 1996). The present report is the first to demonstrate the effect of a prolonged post-weaning milk formula diet on behavior and brain function. After physiologic weaning periods, mice begin to seek food independent of their mother. The acquisition mechanisms of this self-sufficiency in obtaining food, however, remain unknown. The acquisition of active exploratory behavior, which was not observed in mice fed an exclusively milk formula diet, might relate to the acquisition of the self-sufficiency in obtaining food. Thus, the growth stunt caused by an exclusively milk formula diet may retard the development of brain function involved in the motivation to explore their territory, resulting in low-frequency exploration. Our results suggest that growth and/or development of the brain in the several weeks following weaning are critical for the acquisition of active exploratory behavior. Further studies are required to elucidate what nutritional compositions

are lacking in a prolonged milk formula diet and to determine the brain area involved in the acquisition of active exploratory behavior.

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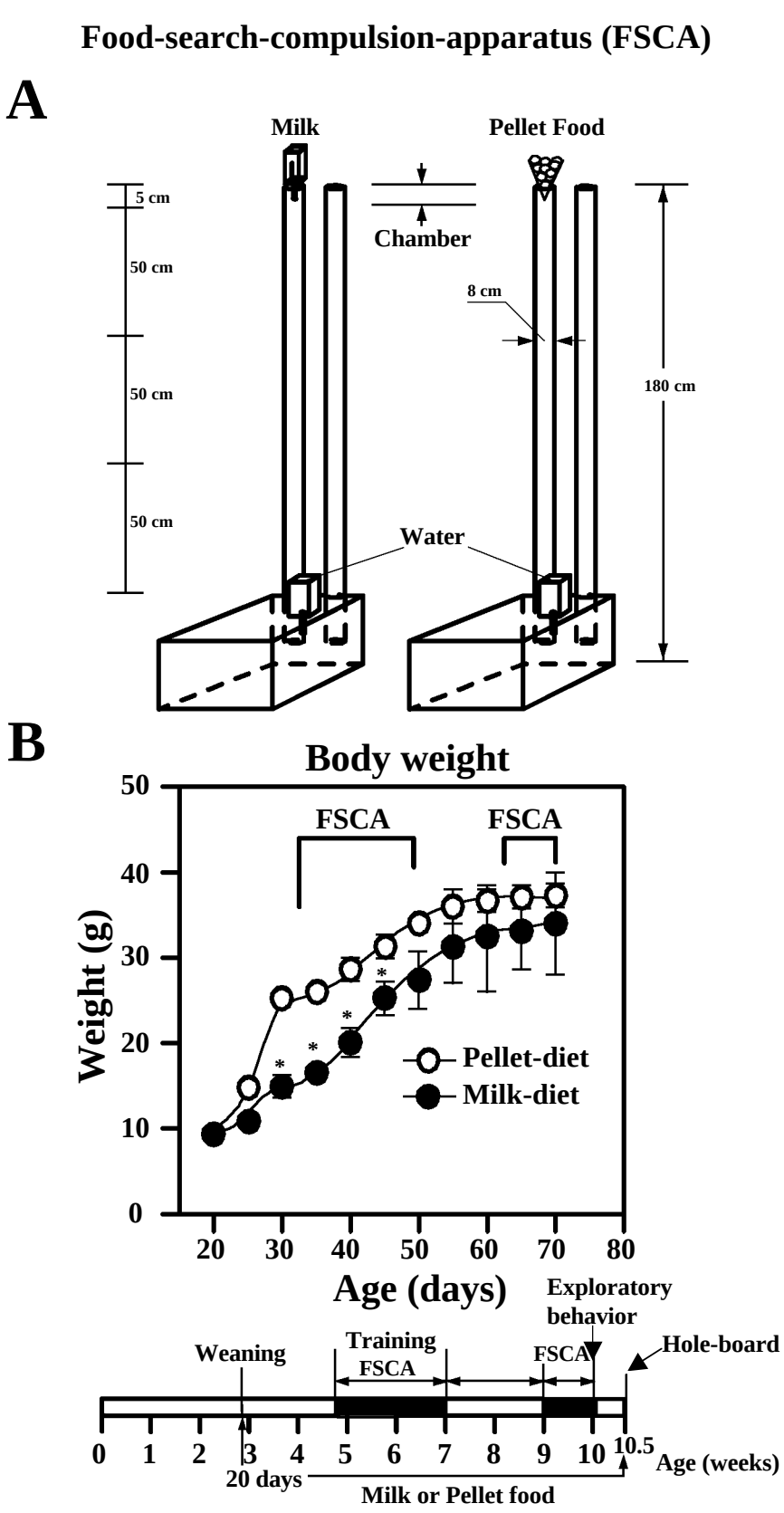
Figure legends

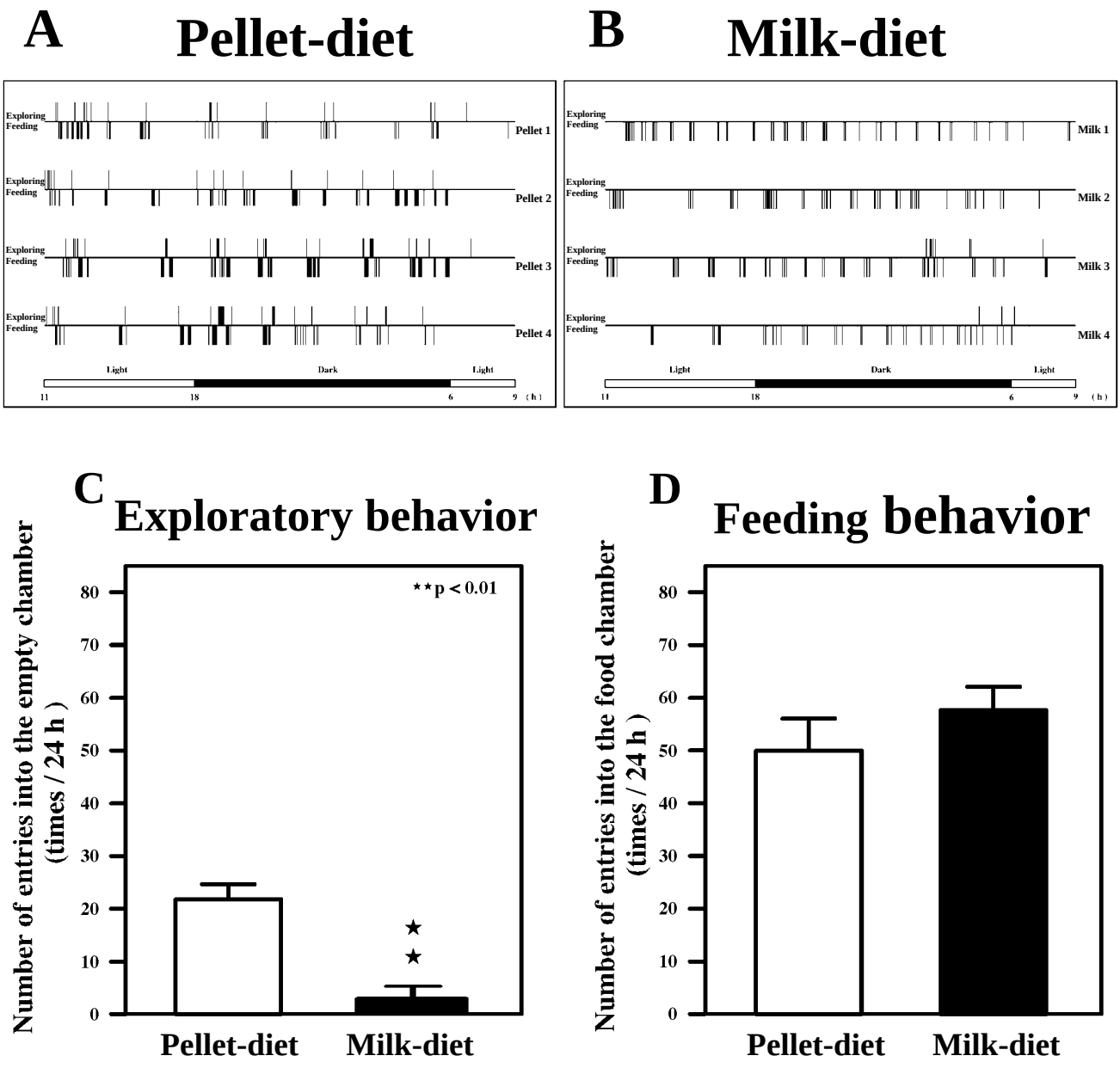
Fig. 1. Experimental apparatus and changes in body weight. (A) Illustration of the food-search-compulsion-apparatus (FSCA). The FSCA is an acrylic cage equipped with two separate vertical stainless steel wire cylinders, the top of which has a chamber containing either food (milk or pellets) or nothing. Entries into the empty chamber were termed exploratory behavior and those into the food chamber were termed feeding behavior. (B) Changes in body weight of milk-fed mice (n = 7) and pellet-fed mice (n = 7) for 7 wk after weaning at 20 d of age. Except for training and the analysis of exploratory behavior, the mice were maintained in normal plastic cages and fed milk or food pellets and water *ad libitum*. The mean body weight of milk-fed mice was significantly less compared to pellet-fed mice between 4 and 7 wk of age. After 8 wk of age, however, there was no significant difference between groups. Vertical bars represent SD. Asterisk indicates $p < 0.05$. Statistical significance was determined using Student's *t* test.

Fig. 2. Profiles of feeding and exploratory behavior of pellet- and milk-fed mice housed in an FSCA. (A and B) Profiles of feeding and exploratory behavior in pellet-fed mice (A) and milk-fed mice (B) at 10 wk of age. Pellet 1-4 and Milk 1-4 represent the profiles of four different mice in each group (n = 7). (C) Comparison of exploratory behavior between milk- and pellet-fed mice. Exploratory behavior in milk-fed mice (n = 7) was significantly lower compared to pellet-fed mice (n = 7). (D) Comparison of feeding behavior between milk- and pellet-fed mice. Feeding behavior was not significantly different. Vertical bars represent SD. Double asterisk indicates $p < 0.01$. Statistical significance was determined using Student's *t* test.

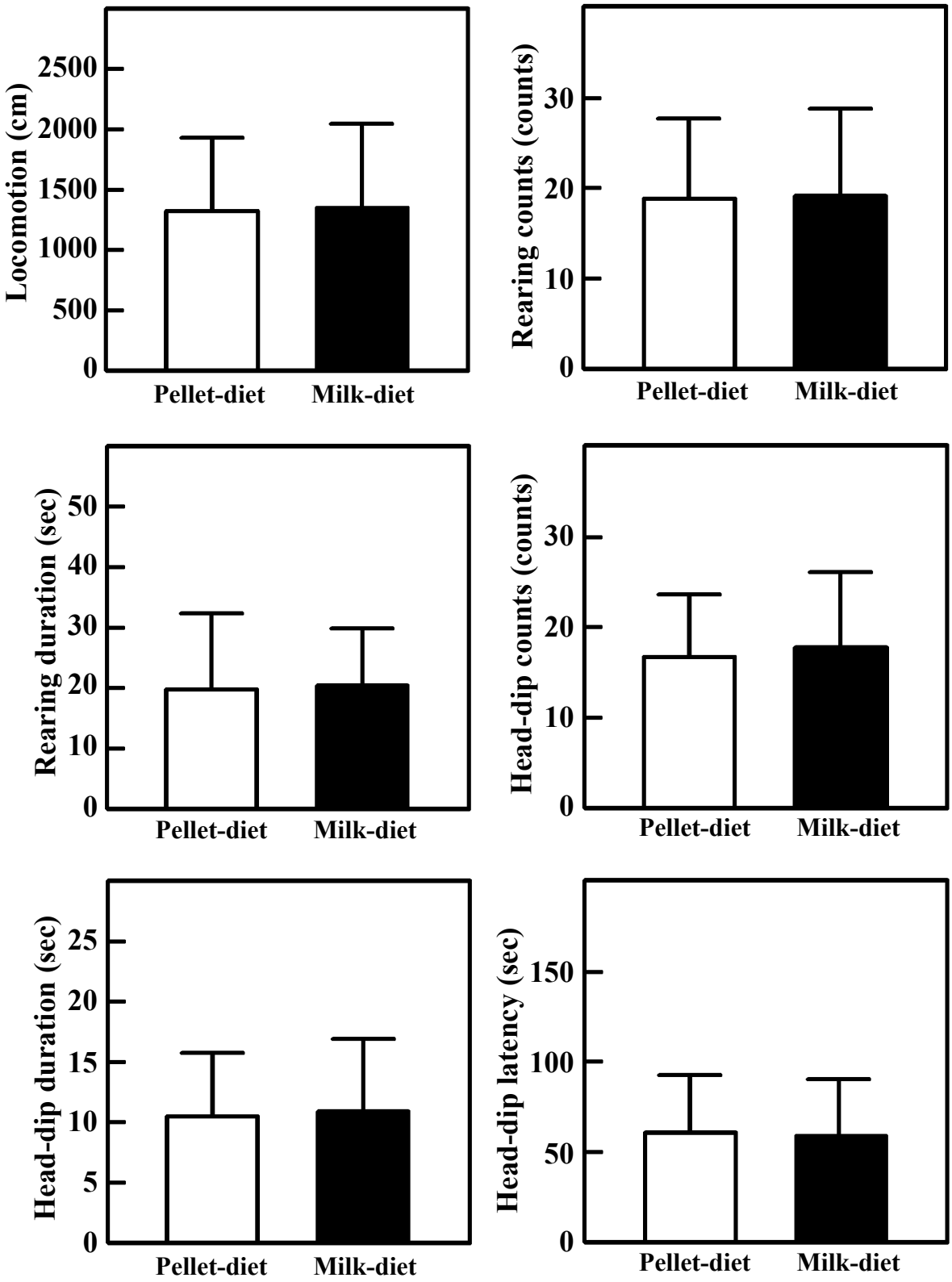
Fig. 3. Exploratory activities in milk-fed and pellet-fed mice analyzed by the automatic hole-board test under unfamiliar environments. Exploratory activities on the hole-board, i.e., total locomotor activity, number and duration of rearing and head-dipping, and latency to the first head-dipping were measured for 5 min. Vertical bars represent SD.



Ishii et al. Figure 1



Ishii et al. Figure 2



Ishii et al. Fig.3

Table 1 Constituents of formula and food pellets (%)

	Water	Protein	Lipid	Carbohydrate	Ash	Crude fiber
Food pellets	8.9	25.4	4.4	50.3	6.9	4.1
Formula	87.6	1.6	3.5	7.0	0.3	—

The mesencephalic trigeminal sensory nucleus is involved in acquisition of active exploratory behavior induced by changing from a diet of exclusively milk formula to food pellets in mice

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Abbreviations

Me5, mesencephalic trigeminal nucleus; VMH, ventromedial hypothalamus; FSCA, food search compulsion apparatus.

Abstract

Post-weaning mice fed exclusively milk display low-frequency exploratory behavior (Ishii, T., Itou, T., and Nishimura, M. (2005) Life Sci. 78, 174-179) compared to mice fed a food pellet diet. This low-frequency exploratory behavior switched to high-frequency exploration after a switch from exclusively milk formula to a food pellet diet. Acquisition of the high-frequency exploratory behavior was irreversible. Recently, we demonstrated that the mesencephalic trigeminal nucleus (Me5) is involved in the control of feeding and exploratory behavior in mice without modulating the emotional state (Ishii, T., Furuoka, H., Itou, T., Kitamura, N., and Nishimura, M. (2005) Brain Res. 1048, 80-86). We therefore investigated whether the Me5 is involved in acquisition of high-frequency exploratory behavior induced by the switch in diet from an exclusively milk formula to food pellets. Mouse feeding and exploratory behaviors were analyzed using a food search compulsion apparatus, which was designed to distinguish between the two behaviors under standard living conditions. Immunohistochemical analysis of immediate early genes indicated that the Me5, which receives signals from oral proprioceptors, is transiently activated after the diet change. The change from low-frequency to high-frequency exploratory behavior was prevented in milk-fed mice by bilateral lesion of the Me5. These results suggest that the Me5 is activated by signals associated with mastication-induced proprioception and contributes to the acquisition of active exploratory behavior.

Section: Cognitive and Behavioral Neuroscience
Themes: Neural Basis of Behavior
Topics: Neuroethology
Keywords: exploratory behavior, Me5 lesions, weaning

1. Introduction

The mesencephalic trigeminal nucleus (Me5) receives proprioceptive sensory afferents of the trigeminal nerve from the jaw-closing muscle spindles and the periodontal ligaments, and it innervates the motor trigeminal nucleus, which participates in the jaw-jerk reflex (Corbin and Harrison, 1940; Harrison and Corbin, 1942). The Me5 fibers also project into the tuberomammillary nucleus of the posterior hypothalamus, where the cell bodies of histamine neurons are localized (Ericson, 1989). Histamine neurons in the tuberomammillary nucleus project to the ventromedial hypothalamus (VMH) and to the Me5 (Inagaki et al., 1987; Inagaki et al., 1988; Sakata et al., 2003). Fujise et al. (1993) suggested that the oral proprioceptive signals induced by mastication might modulate hypothalamic histamine neurons through the ascending pathway from the Me5. Moreover, Fujise et al. (1998) demonstrated that the Me5 is involved in mastication-induced modulation of satiation and eating parameters. Their study suggests that the Me5 receives signals relating to mastication-induced proprioception and modulates satiation via a satiety center in the VMH. To investigate how the Me5 is involved in the control of feeding and exploratory behavior, we previously examined the effect of bilateral electrolytic lesions of the Me5 on feeding and exploratory behavior in mice using a food search compulsion apparatus (FSCA) designed to distinguish between the two behaviors under standard living conditions (Ishii et al., 2005a; Ishii et al., 2005b). We found that mice with bilateral Me5 lesions have unique feeding and exploratory behavior profiles in the FSCA compared with sham-operated mice. Me5-lesioned mice spent more time in the food chamber during each trial in the FSCA, but the number of entries into the food chamber was decreased by 40% compared with sham-operated mice. Moreover, Me5 lesions markedly inhibited exploratory behavior, manifested as low-frequency exploration (Ishii et al., 2005b). In spite of the low-frequency exploration in the FSCA, Me5 lesions had no effect on various behavioral

activities analyzed in an automatic hole-board apparatus (Ishii et al., 2005b), the apparatus of which offers a simple method for measuring the response of an animal to an unfamiliar environment (Boisser and Simon, 1964; Rodriguez Echandia et al., 1987) and is a useful tool for objectively estimating various emotional states of animals (Takeda et al., 1998; Tsuji et al., 2000; Tsuji et al., 2001). The Me5, therefore, controls exploratory behavior other than feeding behavior in mice through its ascending neuronal pathways without modulating the emotional state.

Milk formula is widely utilized as a substitute for mother's milk in all mammals. The influence of milk formula on growth and development during the suckling period has been studied using artificially reared rat pups (Smart et al., 1983, 1984; Auested et al., 1990; Kanno et al., 1997). Some differences in behavior and brain development were observed between artificially reared and mother-reared rat pups (Diaz et al., 1982; Smart et al., 1984; Moore et al., 1990; Kaneko et al., 1996). To investigate the effect of a prolonged post-weaning milk formula diet on brain development, we previously analyzed the feeding and exploratory behavior of mice fed either milk or food pellets until 10 wk of age and compared them between exclusively milk- and pellet-fed mice (Ishii et al., 2005a). Milk-fed mice displayed a low-frequency profile of exploratory behavior, while pellet-fed mice showed high-frequency exploration. In contrast to exploratory behavior, feeding behavior did not differ significantly between milk- and pellet-fed mice. Despite showing low-frequency exploratory behavior, mice on an exclusively milk formula diet showed no difference in behavioral activities analyzed by the hole-board apparatus compared to pellet-fed mice (Ishii et al., 2005a). Thus, a prolonged post-weaning milk formula diet prevents the acquisition of active exploratory behavior in mice without affecting the emotional state.

In the present study, we examined the effect of changing mice from an exclusively milk formula diet to food pellets at 10 wk of age on feeding and exploratory behavior using an FSCA. To determine whether signals induced by a diet change from milk formula to food pellets are transmitted via the Me5, we examined the expression of Fos B and c-Fos in the Me5 neurons of milk-fed mice after switching to the pellet diet. Moreover, to investigate whether the Me5 is involved in acquisition of active exploratory behavior, we examined the effect of bilateral electrolytic lesions of the Me5 on feeding and exploratory behavior in milk-fed mice.

2. Results

Acquisition of active exploratory behavior after a diet change from an exclusively milk formula to food pellets

Recently, we demonstrated that an exclusively milk formula diet prevents the acquisition of active exploratory behavior in mice without affecting their feeding behavior or their emotional state (Ishii et al., 2005a). Namely, milk-fed mice displayed low-frequency exploratory behavior, either seldom re-entering the empty chamber or never entering it at all. In contrast, pellet-fed mice showed high-frequency exploratory behavior and repeatedly entered the empty chamber. The number of entries into the empty chamber (exploratory behavior) in milk-fed mice was significantly lower than pellet-fed mice, whereas the number of entries into the food-containing chamber (feeding behavior) was not significantly different. In spite of the low-frequency exploration, an exclusively milk formula diet had no effect on various behavioral activities analyzed in the hole-board apparatus, i.e., total locomotor activity, frequency and duration of rearing and head-dipping, and latency to the first head-dipping (Ishii et al., 2005a). In the present study, to investigate whether a diet change from exclusively milk formula to food pellets causes a switch from low-frequency to high-frequency exploratory

behavior, we examined the effect of changing mice from an exclusively milk formula diet to food pellets at 10 wk of age on feeding and exploratory behavior using an FSCA. We found that the diet change caused a switch from low-frequency to high-frequency exploratory behavior. This change to an active high-frequency exploratory behavior occurred at least 3 d after the diet change (Fig. 2A). The number of entries into the empty chamber (exploratory behavior) in milk-fed mice significantly increased 5 days after a diet change to food pellets ($p < 0.01$, by Wilcoxon signed-ranks test) (Fig. 2B, left). The increased number of entries into the empty chamber, which corresponds to that in mice fed a food-pellet diet post weaning (Ishii et al., 2005a), did not change even after the diet was switched back to milk formula ($p > 0.05$ vs. before switching back to milk formula, by paired *t*-test; $p < 0.01$ vs. before the diet change to food pellets, by Wilcoxon signed-ranks test) (Fig. 2B, left). Moreover, Welch's *t*-test indicated that significant differences in exploratory behavior between milk-fed mice after a series of diet change and age-matched milk-fed control mice (between 5 d after a diet change to pellet food, 27.4 ± 5.2, and age-matched milk-fed control mice at 10 wk + 5 d of age, 2.1 ± 1.6 times / 24 h, $p < 0.01$; between 5 d after a return to a milk-diet, 30.6 ± 9.0, and age-matched milk-fed control mice at 10 wk + 10 d of age, 3.5 ± 1.4 times / 24 h, $p < 0.01$). On the other hand, Student's *t*-test indicated that no significant differences in feeding behavior between milk-fed mice after a series of diet change and age-matched milk-fed control mice (between 5 d after a diet change to pellet food, 52.3 ± 6.4, and age-matched milk-fed control mice at 10 wk + 5 d of age, 49.1 ± 5.3 times / 24 h, $p > 0.05$; between 5 d after a return to a milk-diet, 57.7 ± 6.7, and age-matched milk-fed control mice at 10 wk + 10 d of age, 50.3 ± 7.3 times / 24 h, $p > 0.05$). Thus, once the mice acquired high-frequency exploratory behavior, they maintained it, even if the diet was switched back to milk formula. Feeding behavior did not change after altering the diet in either direction ($p > 0.05$, by paired *t*-test) (Fig. 2B, right).

Changes in Fos expression in the Me5 following diet change from an exclusively milk formula to food pellets

The Me5 receives signals from oral proprioceptors (Harrison and Corbin, 1942) and projects to higher brain regions (Ericson et al., 1991). Moreover, the Me5 is involved in mastication-induced modulation of satiation and feeding (Fujise et al., 1998; Sakata et al., 2003). We hypothesized that the Me5 receives signals related to a diet change from an exclusively milk formula to food pellets and transmits these signals to higher brain regions associated with exploratory and feeding-related behaviors. We therefore investigated whether the expression of the immediate early genes Fos B and c-Fos (Sagar et al., 1988; Zerial et al., 1989) in the Me5 is enhanced during acquisition of high-frequency exploratory behavior following a change from an exclusively milk formula diet to a food pellet diet. We found that the number of both Fos B- and c-Fos-expressing cells in the Me5 significantly increased 1, 2, and 3 d after the diet change [c-Fos, $F(4,15) = 125.67$, $p < 0.001$; Fos B, $F(4,15) = 316.37$, $p < 0.001$, one-way ANOVA] ($p < 0.05$, by Tukey-Kramer post hoc test) (Fig. 3A, B). Thus, Fos B and c-Fos were transiently expressed in Me5 neurons 1 to 3 d after switching to the pellet diet. Maximal expression of c-Fos was observed 2 d after the diet change and then rapidly declined (Fig. 3A, B). The time course of Fos B expression in the Me5 was similar to c-Fos. Moreover, the time course of Fos B and c-Fos expression in the Me5 preceded the change from low-frequency to high-frequency exploratory behavior (Fig. 2 and 3).

Feeding and exploratory behavior in milk-fed mice with Me5 lesions

To investigate whether the Me5 is involved in the transmission of signals related to diet change and whether it is required for acquisition of active exploratory behavior, we examined

the effect of bilateral electrolytic lesions of the Me5 on exploratory behavior after changing the diet. There was no significant difference in exploratory behavior between bilateral Me5-lesioned and sham-operated milk-fed mice prior to the change to pellet food ($p > 0.05$, by Student's *t*-test) (Fig. 4B). After the switch to pellet food, however, the exploratory behavior of sham-operated mice changed from low-frequency to high-frequency exploration ($p < 0.01$, by Wilcoxon signed-ranks test) (Fig. 4B, left). In contrast, Me5-lesioned milk-fed mice maintained low-frequency exploratory behavior following the change in diet ($p > 0.05$, by paired *t*-test) (Fig. 4A, B, left). Thus, Me5 lesions prevented the acquisition of active exploratory behavior in milk-fed mice following a change from an exclusively milk formula to a food pellet diet. In contrast, the feeding behavior of both Me5-lesioned and sham-operated mice did not change after the switch to the food pellet diet ($p > 0.05$, by paired *t*-test) (Fig. 4B, right). Moreover, there was no significant difference in the mean body weight between the Me5-lesioned and sham-operated mice ($p > 0.05$, by Student's *t*-test) (Fig. 1B).

3. Discussion

In the present study, we demonstrated that the Me5 contributes to the acquisition of active exploratory behavior in mice after a diet change from exclusively milk formula to food pellets. Exclusively milk-fed mice display a low-frequency of exploratory behavior, whereas pellet-fed mice show high-frequency exploration. This low-frequency of exploratory behavior in exclusively milk-fed mice is maintained without regard to age (Ishii et al., 2005a). However, the low-frequency exploratory behavior in milk-fed mice changed to high-frequency exploration after switching them to a food pellet diet. The change in exploratory behavior occurred at least 3 d after the diet change, and mice continued the high-frequency behavior even if their diet was switched back to milk. Thus, the change from low-frequency to

high-frequency exploratory behavior is irreversible under these conditions. On the other hand, age-matched milk-fed control mice without the diet change kept the low-frequency exploratory behavior. These results suggest that signals induced by a diet change from milk formula to food pellets are transmitted to the brain, triggering a permanent change from low-frequency to high-frequency exploratory behavior.

Recently, we demonstrated that the Me5 is involved in the control of feeding and exploratory behavior in mice without modulating the emotional state (Ishii et al., 2005b). The Me5 receives proprioceptive sensory input from periodontal ligaments (Harrison and Corbin, 1942) and from the masseteric muscle spindles through the trigeminal sensory nerve (Corbin and Harrison, 1940). Fujise et al. (1993, 1998) suggest that the Me5 receives signals relating to mastication-induced proprioception and modulates satiation via a satiety center in the VMH. To determine whether signals induced by a diet change from milk formula to food pellets are transmitted via the Me5, we examined the expression of Fos B and c-Fos in Me5 neurons of milk-fed mice after a diet change at 10 wk of age. We found that Fos B and c-Fos in Me5 neurons are transiently expressed 1 to 3 d after the switch to the food pellet diet. Maximal expression of Fos B and c-Fos was observed 2 d after the diet change and then rapidly declined. Thus, the time-course of Fos B and c-Fos expression in the Me5 preceded the change in exploratory behavior. These results suggest that a signal from oral proprioceptors after the diet change activates the Me5.

Neurons express c-Fos following synaptic excitation by sensory stimulation (Friauf, 1992). Immunohistochemical analysis indicates that Fos B and c-Fos immunoreactivity is rapidly increased several hours after the onset of stimulation (Miyata et al., 2001; Morgan and Curran, 1990; Sagar et al., 2003). Therefore, the induction of immediate early genes is considered an early and sensitive marker of neuronal activation (Herrera and Robertson, 1996;

Liu et al., 1998; Miyata et al., 2001; Nakazato et al., 2001; Sagar et al., 1998). In the present study, however, Fos B and c-Fos expression in the Me5 reached maximal levels 2 d after a diet change from milk formula to food pellets. Compared to other studies, Fos B and c-Fos expression in the Me5 took substantially longer to reach maximal levels after the onset of stimulation. It is not clear why Fos activation is delayed under these conditions.

To further examine whether the Me5 is involved in acquisition of active exploratory behavior, we produced bilateral Me5 lesions in milk-fed mice. Sham-operated milk-fed mice underwent a change from low-frequency to high-frequency exploratory behavior after a diet change to food pellets. In contrast, Me5-lesioned mice retained low-frequency exploratory behavior after changing to a food pellet diet. These results suggest that the Me5 is involved in the transmission of signals to the brain relating to diet change and that activation of the Me5 is required for acquisition of high-frequency exploratory behavior. On the other hand, there was no difference in the feeding behavior between Me5-lesioned and sham-operated milk-fed mice. We previously reported that, in pellet-fed mice, Me5 lesions inhibit not only exploratory but also feeding behavior (Ishii et al., 2005b). The reason for this different effect of Me5 lesions on feeding behavior between milk-fed and pellet-fed mice, however, remains unclear.

The development and excitability of the central nervous system are modulated by signals from sensory afferent neurons that are generated in response to dietary composition (Fujise et al., 1998; Liu et al., 1998; Masumoto et al., 1998; Sakata et al., 2003). Moreover, a soft diet after the weaning period reduces synaptic formation in the cerebral cortex and impairs spatial learning ability in adulthood (Yamamoto and Hirayama, 2001). Furthermore, there is a possible link between reduced mastication and hippocampal neuron loss in senile impairment of spatial memory (Onozuka et al., 1999; Yamamoto and Hirayama, 2001). The Me5 projects to the posterior hypothalamus (Ericson et al., 1991) and is involved in mastication-induced

modulation of satiation (Fujise et al., 1998; Sakata et al., 2003). Thus, it seems that sensory signals from oral proprioceptors transmit to higher brain regions via the Me5 and affect brain function and development. A liquid diet, such as milk formula, results in decreased mastication compared to a food pellet diet (Liu et al., 1998). Therefore, we suspect that changes in mastication induced by a diet change from milk formula to food pellets is transmitted to higher brain regions via the Me5 through its ascending neuronal pathways, resulting in the high-frequency exploratory behavior. Further studies are required to elucidate which neuronal pathways are involved in the acquisition of active exploratory behavior and to determine the physiologic significance of high-frequency exploratory behavior.

4. Experimental Procedures

Animals and FSCA

Male *ddY* mice were maintained under controlled temperature and lighting conditions with a 12-h light/12-h dark cycle (lights on at 0600). Mice were isolated from the mother at 20 d of age and fed milk formula until 10 wk of age (Ishii et al., 2005a). The diet was then changed from exclusively milk formula to food pellets. The milk formula has lower protein and higher carbohydrate content but is isoenergetic with mouse milk (Sensui et al., 1996). Feeding and exploratory behavior were monitored using an FSCA (Fig. 1A) (Ishii et al., 2005a; Ishii et al., 2005b). The FSCA was an acrylic cage equipped with two separate vertical cylinders (180 cm high) of stainless steel wire, the tops of which had a chamber containing either food or left empty. Mice had to climb up to the chamber through the intra-cylinders to seek and obtain food. The number of entries and the duration of time spent in each chamber were monitored using detectors attached to the chambers. Mice could obtain water *ad libitum* without climbing the cylinders.

Feeding and exploratory behavior in milk-fed mice before and after diet change

The training task was conducted by placing mice in the FSCA for 2 wk (from 4 to 6 wk of age). A food chamber containing milk and an empty chamber were connected to the left and right cylinders, respectively. Following training, the mice were returned to normal plastic cages and maintained for 2 wk. Mice were then transferred back to the FSCA. We designated entries into the food chamber as feeding behavior. Entries into the empty chamber were designated exploratory behavior, although whether mice explored their territory with a specific aim was not determined (Ishii et al., 2005a; Ishii et al., 2005b). General feeding and exploratory behavior were recorded at 10 wk of age. After this first recording of these behaviors, the diet was changed from exclusively milk formula to food pellets. A second recording of feeding and exploratory behavior was conducted 5 d after the diet change from milk formula to food pellets. After the second recording of these behaviors, the diet was changed back to milk formula, and a third recording of feeding and exploratory behavior was conducted after 5 d.

Me5 lesions

Bilateral electrolytic Me5 lesions were produced in 6-wk-old mice anesthetized with Avertin® (0.36 g kg^{-1}). Using a stereotaxic apparatus, a 0.2-mm-diameter stainless steel electrode was positioned 5.3 mm posterior to the bregma, 0.9 mm lateral to the midsagittal suture, and 3.2 mm below the surface of the skull. As described in our previous report (Ishii et al., 2005b), anodal electrolytic lesions were produced by passing a 1.3-mA current through the electrode three times for 1 s each. Out of 12 mice that received lesions, 6 had successful bilateral Me5 lesions, 3 had unilateral Me5 lesions, and 3 had lesions in peri-Me5 regions. All

successful Me5 lesions were restricted to the caudal level of the Me5. Sham-operated mice (6 mice) underwent an identical operation but without application of a current. Histologic analysis was performed by light microscopy. After fixation with 10% neutral-buffered paraformaldehyde solution and embedding in paraffin, serial brain sections (4 μm thick) were stained with hematoxylin-eosin to reveal the extent of damage to the Me5. Only data from the mice with successful Me5 lesions were used.

Feeding and exploratory behavior in Me5-lesioned mice

The training task was conducted by placing mice in the FSCA for 2 wk (from 4 to 6 wk of age). Bilateral electrolytic Me5 lesions or sham operation was carried out at 6 wk of age. The mice were returned to normal plastic cages and maintained for 2 wk. After this 2-wk recovery period, mice were transferred back to the FSCA. General feeding and exploratory behaviors were recorded at 10 wk of age. After this first recording of these behaviors, the diet was changed from exclusively milk formula to food pellets. A second recording of feeding and exploratory behavior was conducted 5 d after the diet change from milk formula to food pellets.

Immunohistochemistry

Milk-fed mice before, and 24, 48, 72, and 96 h after a diet change to food pellets were anesthetized with Avertin® (0.36 g kg^{-1}) and transcardially perfused with 15 ml of 4% neutral-buffered paraformaldehyde solution following heparinized phosphate-buffered saline. All mice were maintained in the same condition as the mice analyzed feeding and exploratory behavior in the FSCA. The brains were dissected out and postfixed with 10% neutral-buffered paraformaldehyde solution. After embedding in paraffin, serial brain sections (4 μm thick) were prepared. Brain sections were pretreated with pepsin and then incubated for 2 h with

1:1000 rabbit anti-c-Fos polyclonal antibody (Ab-2; Oncogene Research Products, Boston, MA) or with 1:1000 rabbit anti-Fos B polyclonal antibody (sc-48; Santa Cruz Biotechnology, Santa Cruz, CA), and bound antibodies were detected using an ABC kit (Vector Laboratories, Burlingame, CA). Stereologic analysis of the total number of c-Fos- expressing cells in the caudal level of the Me5 was conducted using a series of sections obtained from the region as follows: 5 sets of 8 sections in each mouse were obtained from every tenth section picked from a series of 75 sections (4 μm thick) from the Me5 region, and the total number of cells expressing Fos B and c-Fos in each set was counted and averaged.

Statistical methods

To evaluate the changes of Fos expression in the Me5 following diet change, data were analyzed by one-way ANOVA after Bartlett test. Tukey-Kramer was used as a post hoc test. In comparison of feeding and exploratory behavior in milk-fed mice before and after a series of diet change and also in milk-fed mice with and without Me5 lesions before and after diet change, data were analyzed by either paired *t*-test or Wilcoxon signed-ranks test after F-test. In comparison of feeding and exploratory behavior in milk-fed mice after a series of diet change with those in age-matched milk-fed control mice, data were analyzed by either Student's *t*-test or Welch's *t*-test after F-test. A p value of less than 0.05 was considered statistically significant.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee in Obihiro University of Agriculture and Veterinary Medicine and were conducted under the Guidelines for Animal Experiments in Obihiro University of

Agriculture and Veterinary Medicine and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989. The animals were humanely killed by an overdose of anesthetic ether at the end of the experiment.

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Figure legends

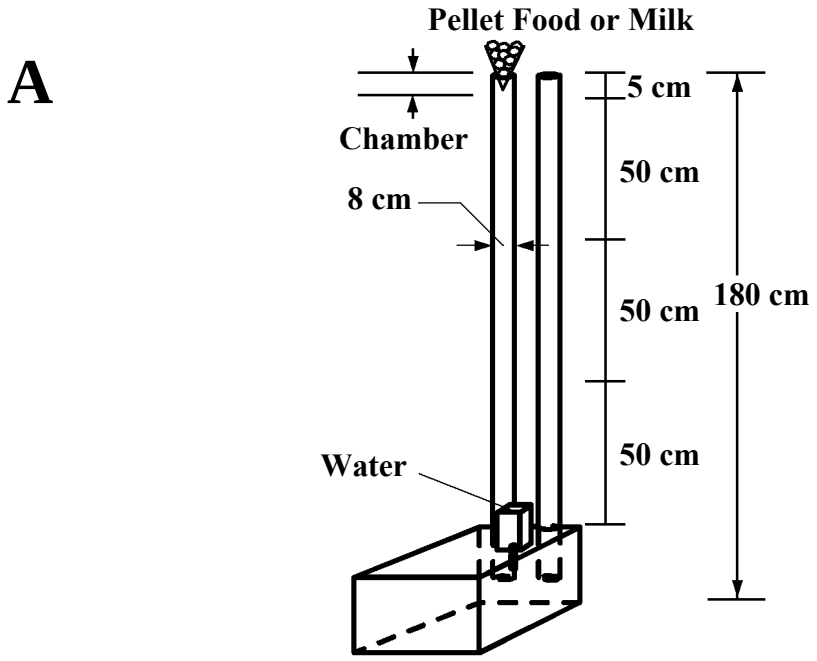
Figure 1. FSCA and changes in body weight after bilateral Me5 lesions. (A) Illustration of the FSCA. The FSCA is an acrylic cage equipped with two separate vertical stainless steel wire cylinders, the top of which has a chamber containing food (milk formula or food pellets) or left empty. Entries into the empty and the food-containing chambers were termed exploratory and feeding behaviors, respectively. (B) Changes in body weight of bilaterally Me5-lesioned mice (n=6) and sham-operated mice (n=6) after surgery at 6 wk of age. Except for the analysis of feeding and exploratory behaviors in the FSCA, the mice were maintained in normal plastic cages and fed food and water *ad libitum*. The mean body weight of Me5-lesioned mice was not significantly different from that of sham-operated mice. Results represent the means ± SD.

Figure 2. Time course of the change from low-frequency to high-frequency exploratory behavior and comparison of feeding and exploratory behavior after a series of diet changes. (A) Representative example of the change in exploratory behavior of a milk-fed mouse after changing to a pellet diet at 10 wk of age (n = 8). (B) Comparisons of feeding and exploratory behavior after a series of diet changes. Feeding and exploratory behavior of milk-fed mice (n = 8) were examined before and 5 d after a diet change to pellet food. The diet of the mice was returned to milk, and feeding and exploratory behavior were examined after 5 d. Results represent means ± SD. $^{**}p < 0.01$ *vs.* exclusively milk-fed mice.

Figure 3. Fos B and c-Fos expression in the Me5 after a diet change from exclusively milk to food pellets. (A) Time course of Fos B and c-Fos expression in the Me5 of milk-fed mice after changing to a food pellet diet at 10 wk of age. Fos B- and c-Fos-immunostained sections before (0 h), and 24, 48, and 72 h after changing to a pellet diet. Controls lacking the primary antibody

are labeled as "- 1st Ab control". Scale bar, 100 μm. (B) The number of Fos B- and c-Fos-expressing cells in the Me5. Stereologic analysis of the total number of Fos B- and c-Fos-expressing cells in the caudal level of the Me5 was conducted using a series of brain sections of milk-fed mice before (0 h) (n = 4), and 24 (n = 4), 48 (n = 4), 72 (n = 4), and 96 h (n = 4) after changing to a pellet diet. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post hoc test. Results represent means ± SD. *$p < 0.05$.

Figure 4. Effect of bilateral Me5 lesions on feeding and exploratory behavior in milk-fed mice. (A) Representative profile of feeding and exploratory behavior in a bilateral Me5-lesioned milk-fed mouse (top) and in the same mouse 5 d after changing to a food pellet diet (bottom). *Inset*, histologic analysis of a hematoxylin-eosin-stained brain section from a Me5-lesioned mouse. Scale bars, 1 mm (left) and 100 μm (right). Asterisks (*) show the sites of the electrolytic lesions in the Me5. (B) Comparison of feeding and exploratory behavior of sham-operated (n = 6) and Me5-lesioned milk-fed mice (n = 6) before and 5 d after a diet change to food pellets. Low-frequency exploratory behavior of sham-operated milk-fed mice (n = 6) but not of Me5-lesioned milk-fed mice (n = 6) changed to active high-frequency exploration 5 d after switching to a food pellet diet (left). Feeding behavior of both Me5-lesioned (n = 6) and sham-operated milk-fed mice (n = 6) did not change after a switch to a food pellet diet (right). Results represent means ± SD. **$p < 0.01$ *vs.* before diet change.



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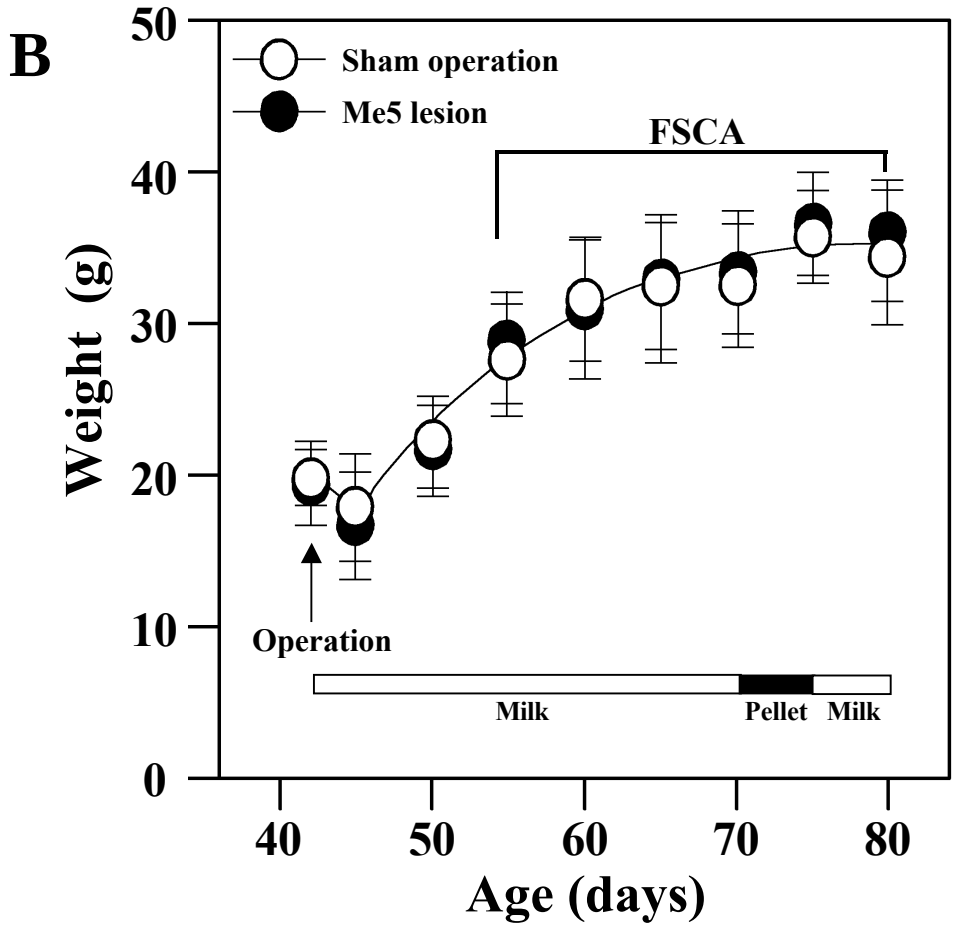

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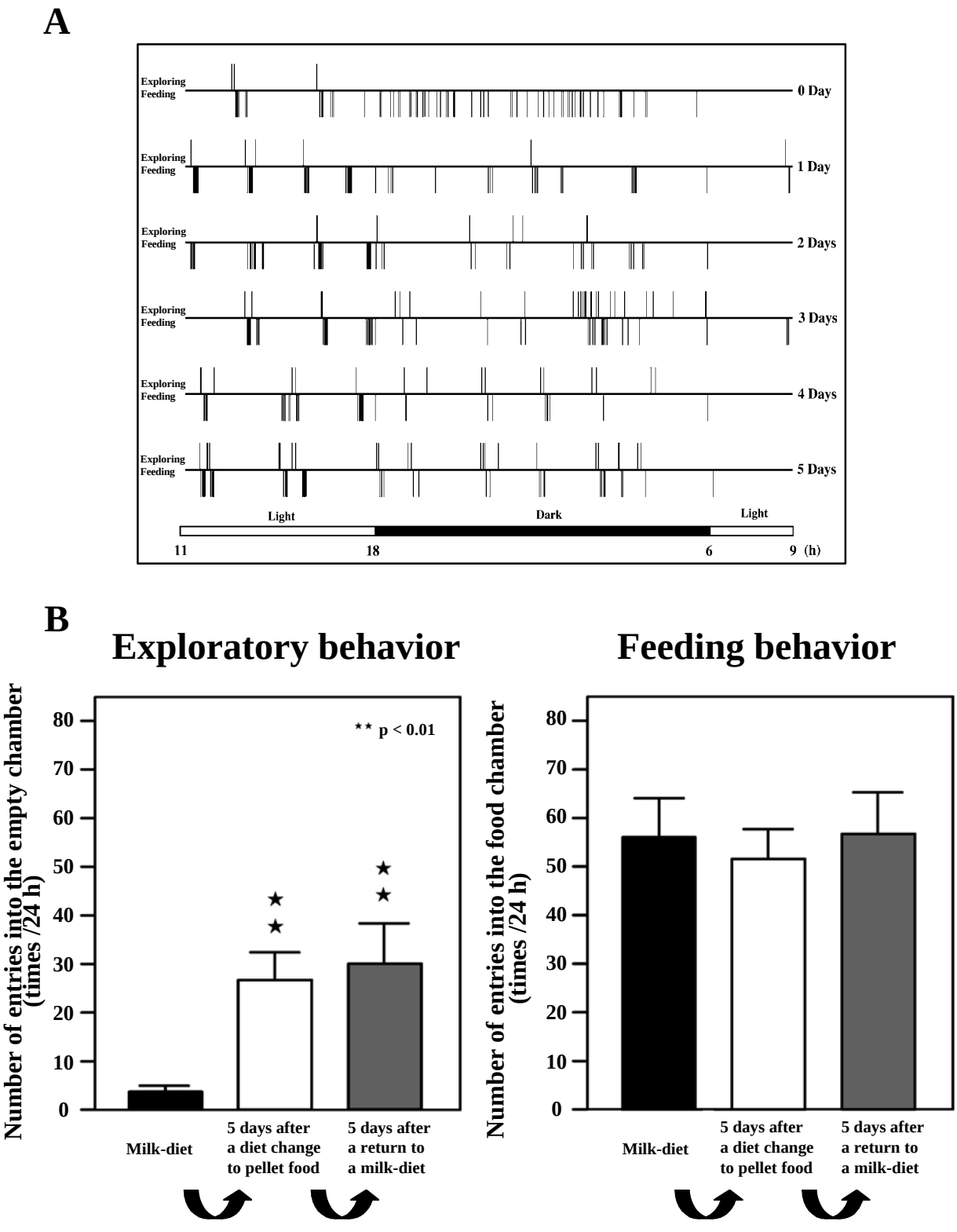

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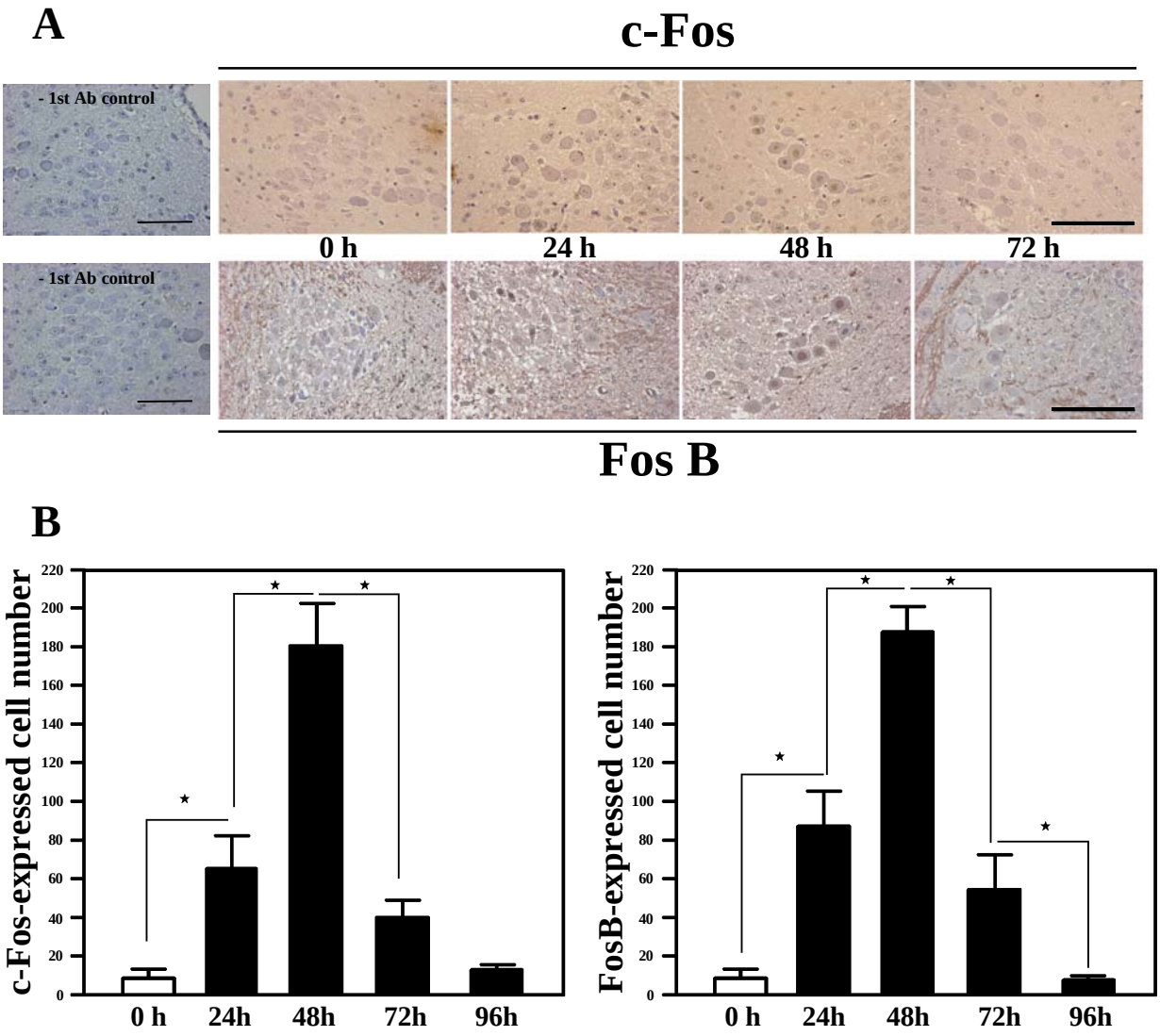

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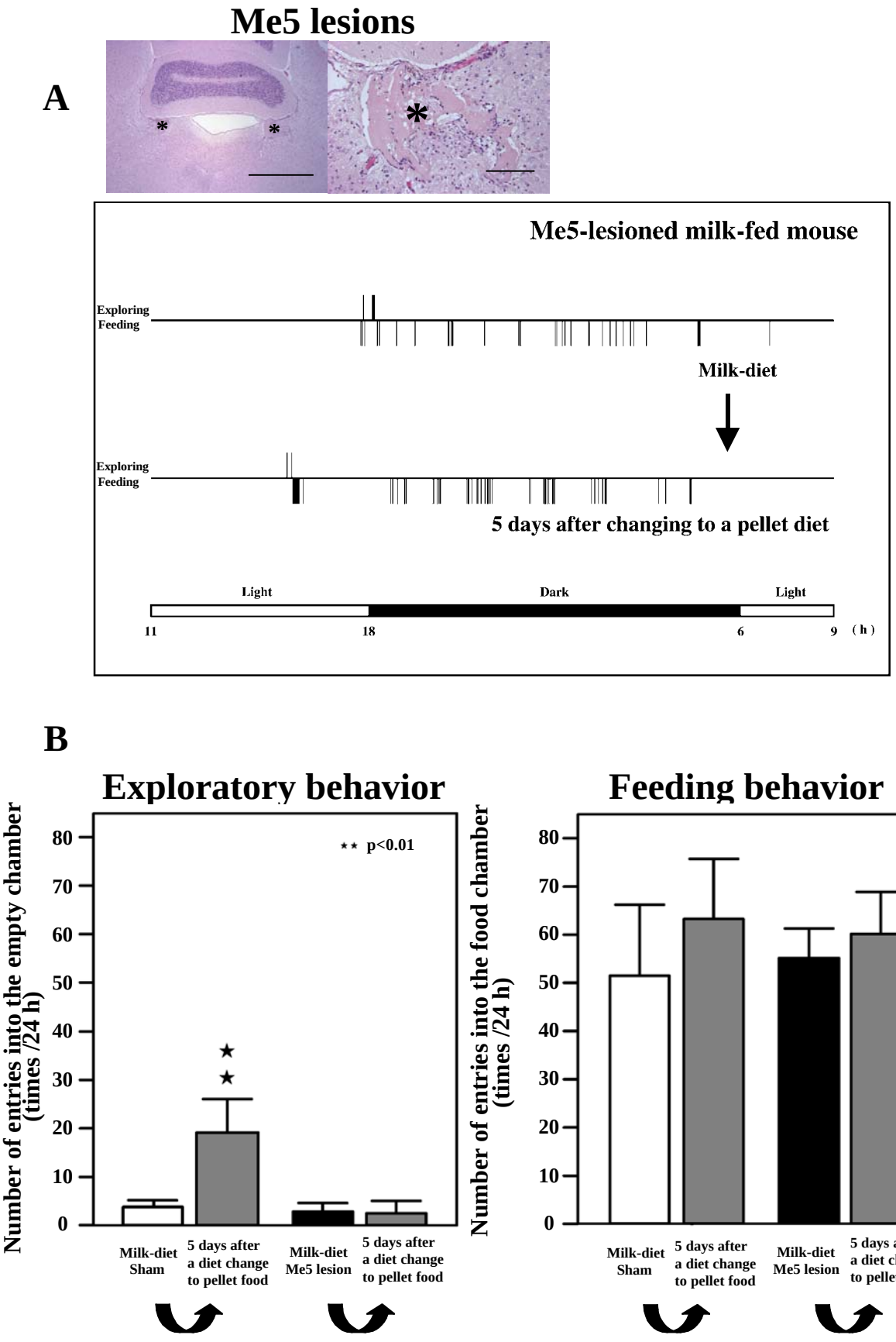

Ishii et al. Fig. 4.

Chronic intracerebroventricular administration of anti-neuropeptide Y antibody stimulates starvation-induced feeding via compensatory responses in the hypothalamus

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***Abbreviations*:** NPY, neuropeptide Y; NPY-ab, neutralizing NPY antibodies; ICV, intracerebroventricular; AGRP, agouti-related protein; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; LHA, lateral hypothalamic area; α-MSH, α -melanocyto-stimulating hormone; MC4Rs, melanocortin-4 receptors; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; TPBS, Triton X-100 in PBS.

Abstract

To investigate how compensatory responses develop after the onset of inhibition of NPY signaling, we examined the effect of continuous intracerebroventricular (ICV) injection of neutralizing NPY antibodies (NPY-ab) on daily and fast-induced food intake in mice. A single ICV injection of NPY-ab reduced food intake in fasted mice. In contrast to a single injection, continuous ICV injection of NPY-ab for 13 d increased fast-induced food intake, although daily food intake was unaffected by continuous administration of NPY-ab. Immunohistochemistry indicated that the expression of NPY protein increases in the arcuate nucleus, lateral hypothalamic area, and paraventricular nucleus 7 d after onset of continuous NPY-ab infusion and remains at an elevated level, whereas the expression of the NPY Y1 receptor transiently increases in the same areas for 3 d and then gradually decreases. Similar results were obtained for the expression of NPY and NPY Y1 receptor mRNA. The mRNA level of agouti-related protein, another orexigenic neuropeptide, also increased in parallel with NPY, whereas that of pro-opiomelanocortin did not change over the 13 d of the NPY-ab administration. These results suggest that chronic central inhibition of NPY immediately activates orexigenic signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes the regulation of feeding and energy expenditure to maintain energy homeostasis. On the other hand, in mice that have acquired this compensation, fast-induced food intake further increases even after the energy deficit is corrected because of the dominant orexigenic signal.

Section: Regulatory Systems.

Keywords: NPY, feeding, food intake, compensation, hypothalamus, central NYP deficiency.

1. Introduction

The arcuate nucleus (Arc) of the hypothalamus, adjacent to the third ventricle, receives central and peripheral signals related to energy stores and contains at least two distinct populations of neurons involved in the regulation of feeding and body weight (Clark et al., 1984; Woods et al., 1998; Marsh et al., 1998). Orexigenic neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons in the Arc are first-order neurons involved in the hypothalamic response to circulating satiety and hunger signals, including glucose, leptin, insulin, ghrelin, and peptide YY (Schwartz et al., 2000; Schwarz and Porte, 2005). Populations of these first-order neurons in the Arc are regulated by leptin and insulin and project to other hypothalamic areas, including the paraventricular nucleus (PVN), perifornical area, and the lateral hypothalamic area (LHA) (Elmquist et al., 1998, 1999), which contain second-order neuropeptide neurons involved in the regulation of food intake and energy homeostasis (Schwartz et al., 2000).

Activation of POMC neurons reduces food intake via the release α-melanocyte-stimulating hormone (α-MSH), a degradation product of POMC, which activates melanocortin-4 receptors (MC4Rs) (Cone et al., 1996). NPY/AGRP neurons have direct inhibitory inputs onto POMC cells (Cowley et al., 2001) and also antagonize the action of α-MSH on MC4R-bearing neurons via the release of AGRP, a natural antagonist of α-MSH receptor (Ollmann et al., 1997). Moreover, both POMC and NPY/AGRP neurons express autoreceptors for some of their respective neuropeptides (Cowley et al., 2001). Thus, these two types of neurons in the ARC exert an inhibitory factor on each other.

Although intracerebroventricular (ICV) injection of NPY stimulates robust feeding (Stanley et al., 1986), surprisingly, genetic disruption of NPY signaling was found to have little effect on feeding and body weight (Marsh et al., 1998; Erickson et al., 1996; Kushi et al.,

1998). It was also reported that neonatal ablation of NPY/AgRP neurons had minimal effects on feeding, whereas that their ablation in adults caused rapid starvation (Luquet et al., 2005). The chronic absence of NPY, therefore, may elicit compensatory mechanisms that mask the effect of its deficiency in NPY-null mice. In this study, to investigate how the compensatory responses develop after the onset of inhibition of NPY signaling, we analyzed the change in expression of neuropeptides in first-order hypothalamic neurons and daily and fast-induced food intake in mice during the continuous ICV administration of NPY-neutralizing antibodies (NPY-ab). NPY We found that compensatory responses to the inhibition of NPY signaling develop in first-order hypothalamic neurons via activation of orexigenic signaling rather than inhibition of anorexigenic signaling. In this way, the regulation of food intake and energy expenditure is normalized in the absence of NPY.

2. Results

A single ICV injection of NPY-ab inhibits fast-induced but not non-fast-induced food intake

Stanley et al. (1992) reported that a single ICV injection of NPY antisera suppresses the eating induced by mild food deprivation in rat. We also examined the effect of a single ICV injection of NPY-ab on fast-induced food intake using varying NPY-ab dilutions. The increase in food intake caused by a 24-h fast was significantly inhibited by a single ICV injection of NPY-ab diluted 1:1500 (52% inhibition), 1:4000 (48% inhibition), and 1:8000 (33% inhibition) but not 1:12000 (Fig.1A). Thus, immunoneutralization of endogenous NPY by NPY-ab markedly inhibits fast-induced food intake in a dose-dependent fashion and the inhibitory effect by a 1:4000 NPY-ab dilution seems to be sub-maximal. Moreover, significant inhibition of fast-induced food intake was observed first 2 h after NPY-ab

(1:4000) injection (Fig.1B). On the other hand, mice that received a single ICV injection of NPY (0.5 nmol) showed a significant increase in food intake compared to saline-treated control mice. This NPY-induced increase in food intake was blocked by pretreatment with NPY-ab (1:4000), but there was no significant difference in *ad libitum* food intake for up to 4 h following the injection (Fig. 1C). These results suggest that NPY is involved in fast-induced hyperphagia but has less of a role in normal spontaneous food intake.

Effect of continuous ICV infusion of NPY-ab on food intake and body weight

We examined the effect of continuous ICV infusion of NPY-ab on daily food intake and body weight. Neither daily food intake nor body weight in NPY-ab-infused mice were significantly different from those in saline-infused control mice (Figs. 2A and B). We next examined the effect of chronic NPY-ab infusion on fasting-induced food intake. Food intake was measured for 3 h after a 24-h fast. In contrast to a single injection of NPY-ab, fasting-induced food intake was significantly increased in mice infused for 13 d with NPY-ab compared to mice infused with saline (Fig. 3). The different effects of a single and chronic ICV injection of NPY-ab on fasting-induced food intake may be due to compensatory mechanisms that are engaged upon the chronic inhibition of NPY signaling. On the other hand, this further stimulation of fasting-induced food intake in chronic NPY-ab-infused mice was significantly blocked by a single peritoneal injection of leptin (Fig. 3). Furthermore, 90 min after refeeding, the amount of food intake in chronic NPY-ab-infused mice was much less after treatment with leptin than with saline. This may be due to an increase in plasma leptin to nearly normal levels because fasting lowers the plasma leptin concentration.

Analysis of mRNA expression levels for NPY, NPY Y1 receptor, AGRP, and PONC during continuous infusion of NPY-ab

Seven and 13 d after the onset of continuous NPY-ab infusion, the level of Arc NPY mRNA was higher in NPY-ab infused mice than control mice (Fig. 4A). The mRNA level for AGRP, another orexigenic neuropeptide in the Arc, was also higher in NPY-ab-infused mice than in control mice after 13 d of infusion with NPY-ab (Fig. 4B). In contrast, the mRNA level of Arc POMC, a precursor of melanocortins such as α-MSH that promotes a negative energy balance, did not change during the 13 d of administration of NPY-ab (Fig. 4C). The level of NPY Y1 receptor mRNA showed a different pattern: its level transiently increased in the Arc and PVN and then decreased over time (Fig. 5A and B). Similar results, although not statistically significant, were obtained for the mRNA levels of the LHA NPY Y1 receptor (Fig. 5C).

Changes in NPY and NPY Y1 receptor protein levels during continuous infusion of NPY-ab

We used immunohistochemistry to assess how NPY and NPY Y1 receptor protein levels are affected by continuous infusion by NPY-ab. The level of NPY protein was significantly higher in the Arc at 7 d and 13 d, and in both the LHA and PVN at 13 d after onset of continuous NPY-ab infusion, whereas the level of NPY Y1 receptor protein transiently increased in the same areas after 3 d and then gradually decreased (Fig. 6A and B). Interestingly, the number of NPY-positive cells in the Arc increased, but the number of NPY fibers was much lower during the first 3 d after the onset of NPY-ab infusion (Fig. 7). In addition, some NPY-positive neuronal cell bodies and fibers in the Arc were also positive for NPY Y1 receptor protein (Fig. 7).

3. Discussion

The results of the present study demonstrate that chronic central inhibition of NPY immediately activates orexigenic signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes feeding and energy expenditure to maintain energy homeostasis. Enhancement of fasting-induced hyperphagia by chronic administration of NPY-ab may therefore be due to activation of a dominant orexigenic signal after acquisition of this compensatory mechanism.

Food intake by the mice was significantly increased by a single ICV injection of NPY compared to injection of saline. Although this increase in food intake by NPY was prevented by pretreatment with NPY-ab, a single ICV injection of NPY-ab did not affect *ad libitum* food intake in non-fasted mice. This suggests that NPY does not play a positive role in spontaneous food intake in mice. On the other hand, a single ICV injection of NPY-ab after a 24-h fast significantly inhibited the fasting-induced increase in food intake. Therefore, it appears that NPY participates in fast-induced hyperphagia but much less to normal spontaneous food intake.

NPY gene expression and secretion of NPY in the hypothalamus are increased during the depletion of body fat stores (Kalra et al., 1991; White and Kershaw, 1990) in conjunction with reduced leptin/insulin signaling to the brain (Wilding et al., 1993). Moreover, leptin inhibits Arc NPY gene expression (Schwartz et al., 1996; Stephens et al., 1995), and the genetic knockout of NPY reduces hyperphagia and obesity in ob/ob mice (Erickson et al., 1996), indicating that the full response to leptin deficiency requires NPY signaling. The hyperphagic response in insulin-deficient diabetes is accompanied by increased hypothalamic synthesis and release of NPY (Williams et al., 1989), and these responses are blocked by either systemic administration or direct injection of insulin into the brain (Sipols et al., 1995).

Therefore, NPY may not have an important role in spontaneous feeding responses when leptin or insulin levels are normal.

We also examined the effect of continuous ICV infusion of NPY-ab on daily food intake and body weight. Both daily food intake and body weight in NPY-ab-infused mice were not significantly different from those in saline-infused control mice. We further examined the effect of chronic NPY-ab infusion on fasting-induced food intake. In contrast to the effects of a single NPY-ab injection, fasting-induced food intake was significantly increased in the mice infused for 13 d with NPY-ab compared to mice treated with saline. This further stimulation of fasting-induced food intake in chronic NPY-ab-infused mice was partially blocked by a single peritoneal injection of leptin. The chronic inhibition of NYP signaling in NPY-ab-infused mice might induce mechanisms to compensate for the decreased NPY signaling, and low leptin and/or insulin levels induced by fasting might therefore result in the activation of anabolic neural pathways, enhancing hyperphagia.

Orexigenic NPY and AGRP are colocalized in Arc neurons (Hahn et al., 1998), and anorexigenic POMC also exist in a distinct, but adjacent, subset of Arc neurons (Elias et al., 1998). A majority of NPY/AGRP and POMC neurons express leptin receptors (Cheung et al., 1997; Baskin et al., 1999), and both types of neurons are regulated by leptin but in an opposing manner: NPY/AGRP neurons are inhibited by leptin and consequently are activated when leptin levels are low (Schwartz et al., 1996; Hahn et al., 1998), whereas POMC neurons are activated by leptin, and are therefore inhibited when leptin levels are low (Schwarz et al., 1996; Thornton et al., 1997). Furthermore, there is a high concentration of insulin receptors in the Arc (Baskin et al., 1988), and a deficiency of insulin seems to activate NPY/AGRP neurons (Williams et al., 1989; Sipols et al., 1995). Compensatory mechanisms to decrease NPY signaling caused by chronic NPY-ab administration seem to occur in the first-order

neurons in the Arc, orexigenic NPY/AGRP neurons, and anorexigenic POMC neurons because they are the neurons that respond to the circulating satiety and hunger signals, including glucose, leptin, and insulin (Schwartz et al., 2000).

To examine this hypothesis, we measured mRNA levels for NPY, NPY Y1 receptor, AGRP, and PONC during continuous infusion of NPY-ab. The levels of Arc NPY and AGRP mRNA increased in a time-dependent manner, and their levels were significantly different between saline- and NPY-ab-infused mice 7 and 13 d after the onset of the NPY-ab infusion, respectively. In contrast, the level of Arc POMC mRNA did not change during 13 d of NPY-Ab infusion. The level of NPY Y1 receptor mRNA showed a different pattern: it transiently increased in the Arc and PVN and then gradually decreased during the infusion of NPY-ab. Although not statistically significant, similar results were obtained for the level of LHA NPY Y1 receptor mRNA. These results suggest that the first compensatory response to normalize the NPY-ab-induced inhibition of NPY signaling is a transient increase in the mRNA level for the NPY Y1 receptor. Thereafter, the mRNA level for the NPY Y1 receptor increases but recovers to the basal level within 7 d. Finally, the levels of both NPY and AGRP mRNAs increase. Such compensatory responses were also observed by immunohistochemical analysis for NPY and the NPY Y1 receptor. On the other hand, although anorexigenic POMC neurons are first-order neurons, the mRNA level for POMC in the Arc did not change over the 13 d of infusion with the NPY-ab. These results suggest that chronic central inhibition of NPY immediately activates orexigenic NPY signaling without affecting anoexigenic POMC signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes feeding and energy expenditure to maintain energy homeostasis. On the other hand, when mice that have acquired this compensation are

compelled to fast, fast-induced food intake is further increased even after the energy deficit is corrected because of the dominant orexigenic signal.

MC4R antagonism leads to hyperphagia, reduced energy expenditure and ultimately obesity (Lu et al., 1994). Thus, it was suggested that α-MSH acts tonically to limit food intake. In the present study, the mRNA level of AGRP, which antagonizes the action of α-MSH on MC4Rs, increased in the Arc after chronic central inhibition of NPY. Therefore, the compensatory change in AGRP expression may lead to not only suppression of anorexigenic signaling but also alternation of set point to limit food-intake, which consequently results in enhancement of fasting-induced hyperphagia. A single ICV injection of NPY-ab inhibited the fasting-induced increase in food intake but did not affect food intake in non-fasted mice. Moreover, continuous ICV infusion of NPY-ab stimulated fasting-induced food intake but did not affect daily *ad libitum* food intake and body weight. However, the stimulation of fasting-induced food intake caused by continuous ICV infusion of NPY-ab was blocked by leptin. These results imply that the circulating satiety and hunger signals such as glucose, leptin, and insulin could be importantly involved in the different effect of NPY-ab on food intake observed in fasted and non-fasted conditions.

4. Experimental Procedures

Animals and ICV infusion of NPY-Ab

Rabbit whole antisera to NPY were purchased from Sigma-Aldrich (Tokyo, Japan). A certificate of analysis obtained from the manufacturer shows that a feeble cross-reactivity (less than 0.01%) is observed with NPY fragments including NPY (18-36), NPY (13-36) and peptide YY (PYY) conjugated to BSA by dot blot analysis. We purified the IgG from the whole antisera by using protein G-Sepharose columns and concentrated it using

Slide-A-Lyzer dialysis kit (PIERCE, Rockford, IL). The IgG fractions from the NPY antisera were reconstituted to original volume with saline and then used as NPY-ab in this experiment.

Male *ddY* mice were maintained under controlled temperature and lighting conditions with a 12-h light/12-h dark cycle (lights on at 06:00). Seven-wk-old mice were anesthetized with Avertin® (0.36 g kg^{-1}), and permanent 30-gauge stainless steel infusion cannulae (Alzet Brain infusion Kit 3; Durect Corp., Cupertino, CA) that had been connected to Alzet osmotic pumps (model 2002; Durect Corp.) filled with either NPY-Ab or saline were stereotactically placed 0.8 mm posterior to the bregma, 0.1 mm lateral to the midsagittal suture, and 3 mm below the surface of the skull. The osmotic pump was implanted into a subcutaneous pocket in the midscapular area of the back of the mice as described by the manufacturer. Using this osmotic pump, solutions were infused continuously at a rate of 0.5 μl/h for 14 d. For a single injection, mice were unilaterally implanted with 26-gauge stainless steel cannulae (Plastics One; Roanoke, VA, USA) into the ICV under anesthesia with Avertin® (0.36 g kg^{-1}) 2 wk before experiments. The NPY-ab was diluted 1:4000 with saline, dilution of which was determined by the dose-dependent data shown in Fig.1A and also considering product information from the manufacturer. A single ICV injection of the diluted NPY-ab (1:4000) successfully blocked the 24-h-fast-induced food intake (Fig.1A and B) and also the ability of NPY to stimulate food intake (Fig.1C).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

After the mice were anesthetized with ether and decapitated, their brains were removed and cut into 1-mm thick coronal sections including the mid-hypothalamus. The isolated sections extended rostrally to just behind the optic chiasma and caudally just anterior to the

mammilary bodies, occupying the central region of the hypothalamus. The tissues were transferred to cold phosphate-buffered saline (PBS) and sectioned into the Arc, LHA, and PVN regions with the aid of prominent landmarks (fornix, third ventricle, and optic tract) under a dissecting microscope. The tissue sections of these hypothalamic areas were frozen individually in liquid nitrogen and used for RT-PCR. Total RNA isolated from the tissue sections using TRIZOL Reagent (Invitrogen, Carlsbad, CA) was quantified by measuring the absorbance at 260 nm, and its integrity was confirmed by denaturing agarose gel electrophoresis. The mRNA expression levels of NPY, NPY Y1 receptor, AGRP, POMC, and the housekeeping gene GAPDH were quantified by RT-PCR. Total RNA (25 ng) was reverse-transcribed using an oligo (dT) primer and AMV reverse transcriptase with a Takara RNA LA PCR^{TM} Kit (AMV) v.1.1 (Takara Shuzo Co., Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA products were amplified using primers for mouse NPY, NPY Y1 receptor, AGRP, POMC, and GAPDH (Table 1). The PCR reaction was carried out in a Bio-Rad I cycler (Bio-Rad, Tokyo, Japan).

Analysis of cDNAs of NPY, NPY Y1 receptor, AGRP, POMC, and GAPDH

Amplified cDNAs were separated on 3.0% agarose gels, stained with SYBR Green (Takara Shuzo Co., Kyoto, Japan), and quantified using an Epi-Light UV FA500 analyzer (Aishin Seiki, Tokyo, Japan) and NIH imaging software. The mRNA levels were determined as the ratio of the fluorescence intensity to that for GAPDH cDNA.

Immunohistochemistry

Mice were anesthetized with Avertin® (0.36 g kg^{-1}) and transcardially perfused with heparinized PBS, followed by 15 ml of 4% neutral-buffered paraformaldehyde solution. The

brains were dissected out and post-fixed with 10% neutral-buffered paraformaldehyde solution. After post-fixation, the brains were cut on an oscillating tissue slicer throughout the mid-hypothalamus into 40-mm thick sections. The sections were permeabilized with 0.5% (v/v) Triton X-100 in PBS (TPBS) for 1 h and then blocked in TPBS containing 2.0% normal goat serum for 1 h. After blocking, the sections were incubated for 24 h at 4°C in rabbit anti-NPY polyclonal antibody (Sigma-Aldrich; 1:3000 in TPBS) and sheep anti-NPY Y1 Receptor polyclonal antibody (Biogenesis; 1:2000 in TPBS). After rinsing in TPBS, the sections were incubated for 24 h in AlexaFluor® 488 goat anti-rabbit IgG (Molecular Probes; 1:1000 in TBST) and AlexaFluor® 594 donkey anti-sheep IgG (Molecular Probes; 1:1000 in TBST). After washing with PBS, sections were mounted onto slides and coverslipped with fluorescent mounting medium (Vectashield; Vector laboratories, Burlingame, CA, USA). Images were obtained by confocal laser scanning microscopy (Nikon, Tokyo, Japan).

Statistical methods

To evaluate the changes of food intake, data were analyzed by repeated measures analysis of variance (ANOVA) after Bartlett test. The Tukey-Kramer test was used as a post hoc test. For comparisons of mRNA expression levels, data were analyzed by either Student's *t*-test or Welch's *t*-test following an *F*-test. A *P* value of less than 0.05 was considered to indicate statistical significance.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee in Obihiro University of Agriculture and Veterinary Medicine and were conducted under the Guidelines for Animal Experiments in Obihiro University of

Agriculture and Veterinary Medicine and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989. The animals were humanely killed at the end of the experiment by an overdose of anesthetic ether.

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Figure legends

Fig. 1. Effects of a single ICV injection of NPY-ab on the stimulation of food intake by fasting (A) (B) and NPY (C). (A) Mice were administered NPY-ab (5 μl/mouse) (1:1500, 1:4000, 1:8000, or 1:12000 antibody dilution) or vehicle (saline) alone (5 μl/mouse) by ICV injection after a 24-h fast and then fed *ad libitum*. After injection, total food consumption for 4 h was measured. Results represent means ± SD (n=3). (B)Mice were administered NPY-ab (1:4000; 5 μl/mouse; n = 4) or vehicle (saline) alone (5 μl/mouse; n = 4) by ICV injection after a 24-h fast and then fed *ad libitum*. After injection, food consumption was measured at 1-h intervals for 4 h. Results represent means ± SEM. (C) Mice were treated with vehicle (saline) alone (5 μl /mouse; n = 3), NPY (0.5 nmol/5 μl /mouse; n = 3), or NPY-ab (1:4000; 5 μl /mouse; n = 3) by ICV injection 5 min after ICV injection of saline (5 μl /mouse) and then fed *ad libitum*. Another group (NPY + NPY-ab) received NPY (0.5 nmol/5 μl /mouse; n = 3) by ICV injection 5 min after ICV injection of NPY-ab (1:4000; 5 μl /mouse) and then fed *ad libitum*. After injection, food consumption was measured at 1-h intervals for 4 h. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. control (saline-treated) mice.

Fig. 2. Effect of chronic ICV administration of NPY-ab on daily food intake (A) and body weight (B). Mice (7-wk-old) were continuously infused (0.5 μl/h) for 13 d with NPY-ab (1:4000; n = 7) or vehicle (saline) alone (n = 7) using Alzet osmotic minipumps that were implanted on day 0. Results represent means ± SEM.

Fig. 3. Effects of chronic ICV administration of NPY-ab on food intake after a 24-h fast. Mice (7-wk-old) were continuously infused (0.5 μl/h) for 13 d with NPY-ab (1:4000; n = 5)

or vehicle (saline) alone (n = 5) using osmotic minipumps. Leptin (50 μg/mouse; n = 3) was injected intraperitoneally to NPY-ab-infused mice 2 h before measuring food consumption. Mice were fed *ad libitum* after a 24-h fast, and food consumption was measured at 0.5-h intervals for 2 h. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. saline-infused mice; ^{+}P<0.05 and ^{++}P<0.01 vs. NPY-ab-infused mice.

Fig. 4. Effect of chronic ICV administration of NPY-ab on the expression of NPY (A), AGRP (B), and POMC mRNA (C) in the Arc. NPY, AGRP, and POMC mRNA levels in NPY-ab- and saline-infused mice were measured by RT-PCR 3 (n = 3), 7 (n = 5), and 13 d (n = 5) after the onset of infusion. The mRNA levels of these peptides are shown as the signal relative to that for GAPDH. Results represent means ± SEM. **P*<0.05 vs. saline-infused control mice.

Fig. 5. Effect of chronic ICV administration of NPY-ab on the expression of NPY Y1 receptor mRNA in the Arc (A), PVN (B), and LH (C). The NPY Y1 receptor mRNA level in the Arc, PVN, and LH in NPY-ab- and saline-infused mice was measured by RT-PCR 3 (n = 5), 7 (n = 5), and 13 d (n = 5) after starting infusion. The mRNA levels of these peptides are shown as the signal relative to that for GAPDH. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. saline control mice.

Fig. 6. Changes in the levels of NPY and NPY Y1 receptor proteins in the hypothalamus during ICV infusion of NPY-ab. (A) NPY and NPY Y1 receptor expression in the hypothalamus was analyzed in sections immunostained for NPY and NPY Y1 receptor before and 3, 7, and 13 d after starting ICV infusion of NPY-ab. Images were obtained by confocal laser scanning microscopy. Confocal images of cells stained for both NPY (green) and NPY

Y1 receptor (red) are shown. As shown in the merged images, these two proteins colocalize. The scale bar represents 1 mm. (B) Expression levels of NPY Y1 receptor (Left) and NPY (Right) protein in the Arc, PVN, and LHA were estimated by quantification of the average fluorescent intensity of those areas using Nikon software (EZ-C1). The data were determined as the ratio of the average fluorescent intensity of the Arc, PVN and LHA areas to that of the ventral posteromedial area with the background fluorescent intensity. Results represent means ± SD. *P<0.05, $^{\#}P$<0.05, and ^{+}P<0.05 vs. before ICV infusion of NPY-ab in the Arc, PVN, and LHA, respectively.

Fig. 7. Higher magnification of fluorescent double-immunocytochemical staining of NPY and NPY Y1 receptor in the Arc. NPY and NPY Y1 receptor expression in the Arc was analyzed in sections stained for NPY and NPY Y1 receptor before and 3 and 13 d after starting ICV infusion of NPY-ab. Images were obtained by confocal laser scanning microscopy. The merged images show the colocalization of NPY (green) and NPY Y1 receptor (red). The scale bar indicates 50 μm.

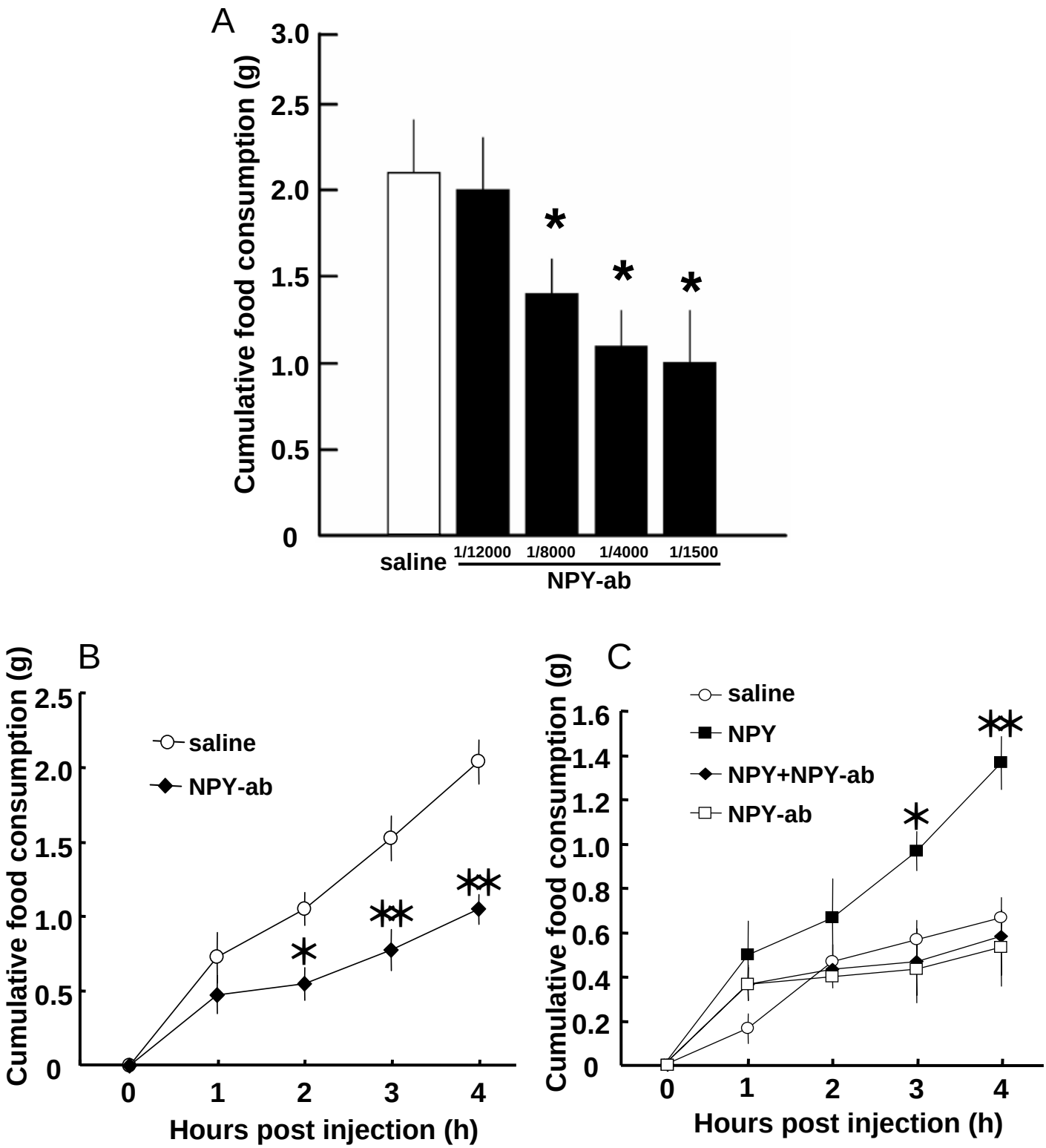

A
Cumulative food consumption (g)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
*
saline
1/12000
1/8000
1/4000
1/1500
NPY-ab
B
Cumulative food consumption (g)
saline
NPY-ab
Hours post injection (h)
C
Cumulative food consumption (g)
saline
NPY
NPY+NPY-ab
NPY-ab
Hours post injection (h)

Fig. 1

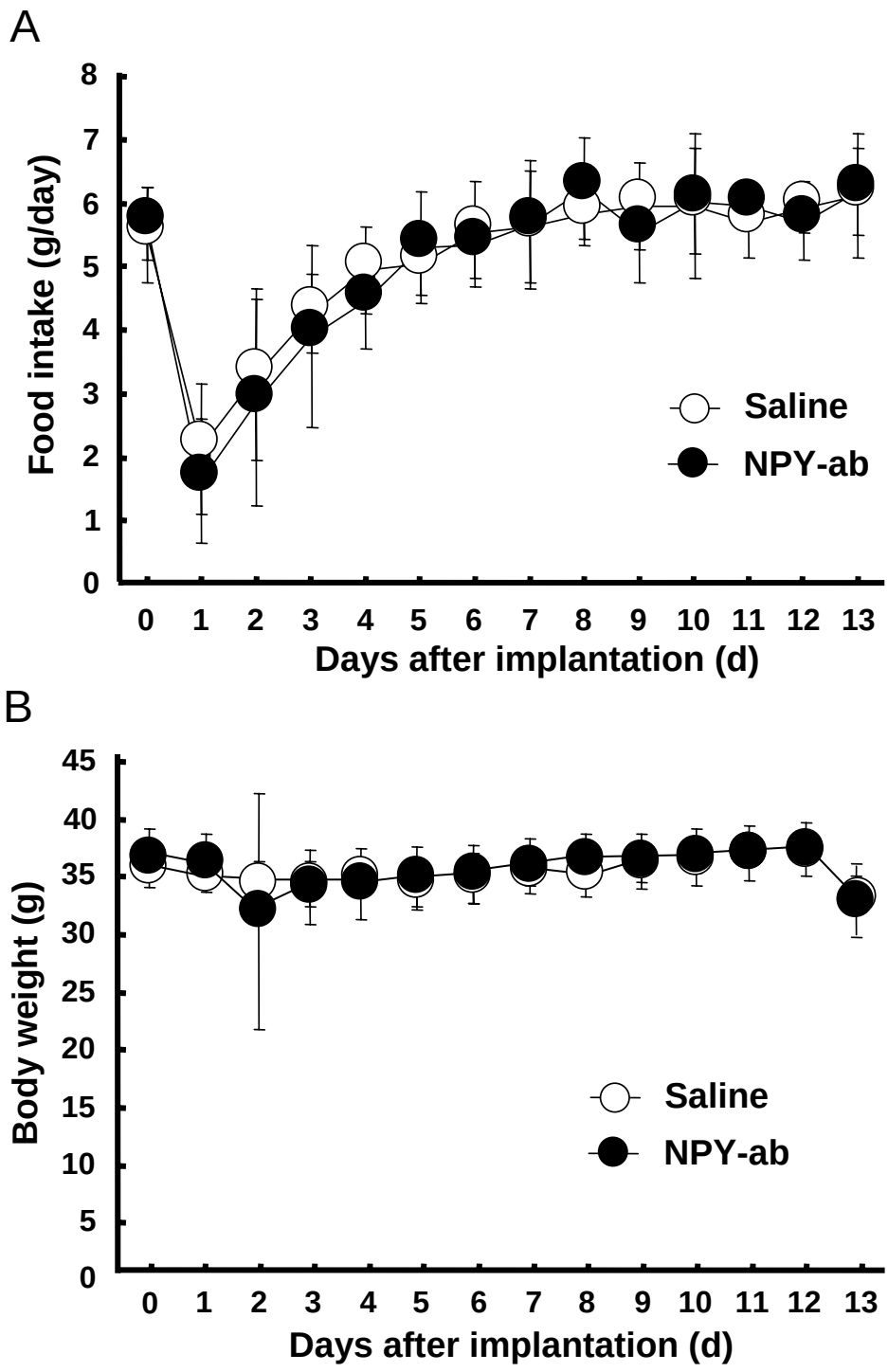

A
Food intake (g/day)
0
1
2
3
4
5
6
7
8
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days after implantation (d)
Saline
NPY-ab
B
Body weight (g)
0
5
10
15
20
25
30
35
40
45
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days after implantation (d)
Saline
NPY-ab

Fig. 2

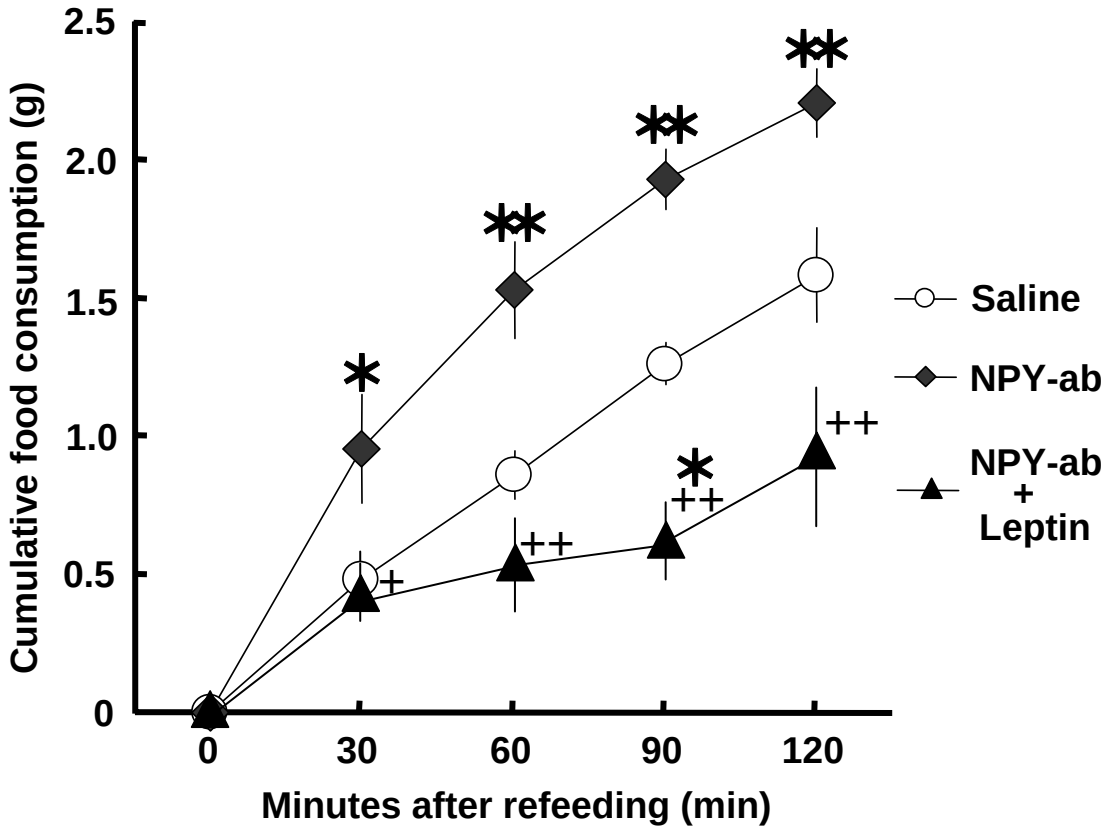

2.5
2.0
1.5
1.0
0.5
0
Cumulative food consumption (g)
0
30
60
90
120
Minutes after refeeding (min)
*
**
**
**
+
++
++
++
Saline
NPY-ab
NPY-ab
+
Leptin

Fig. 3

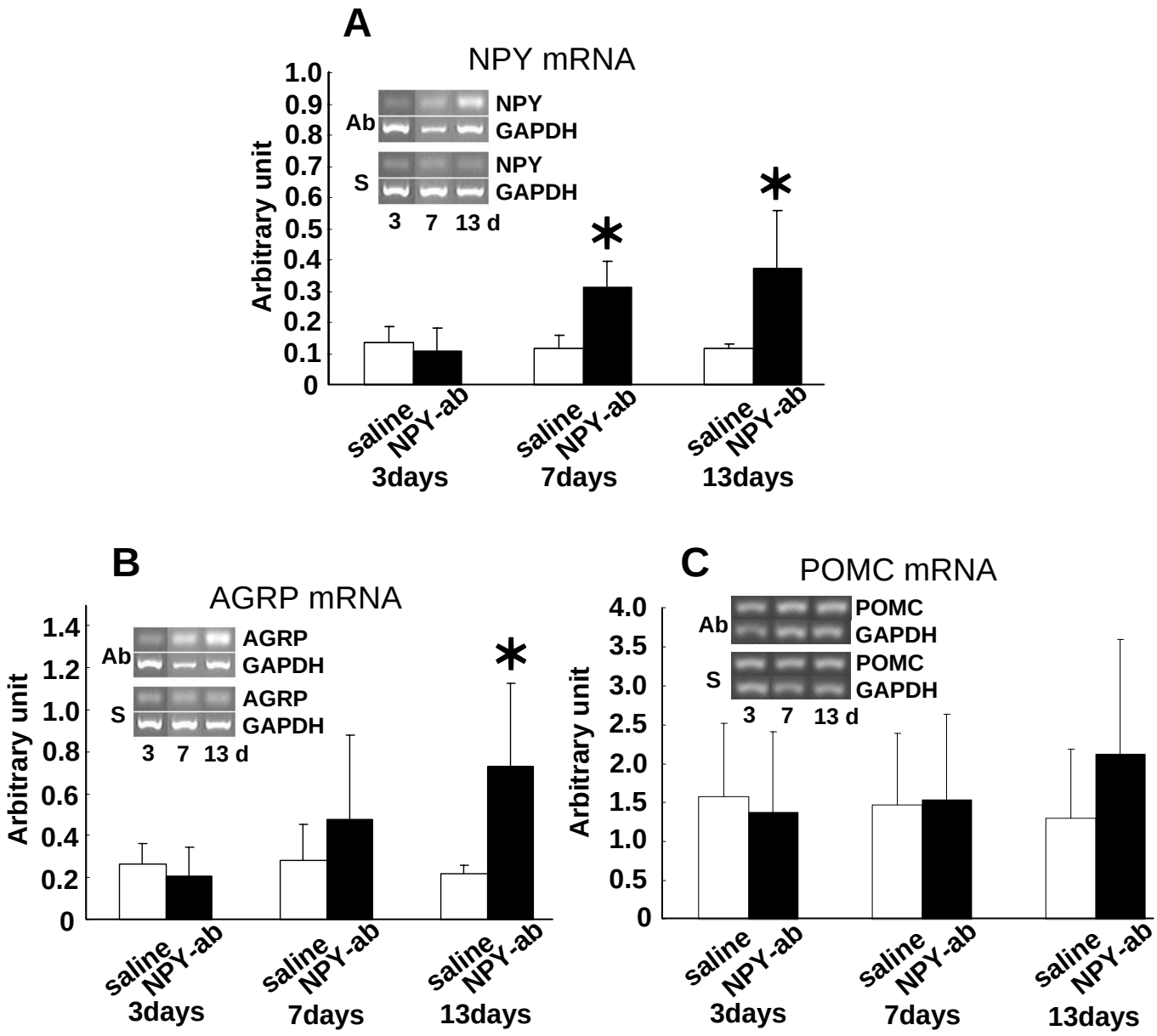

A
NPY mRNA
Arbitrary unit
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ab
NPY
GAPDH
S
NPY
GAPDH
3 7 13 d
saline
NPY-ab
3days
7days
13days
B
AGRP mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
AGRP
GAPDH
C
POMC mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
POMC
GAPDH

Fig. 4

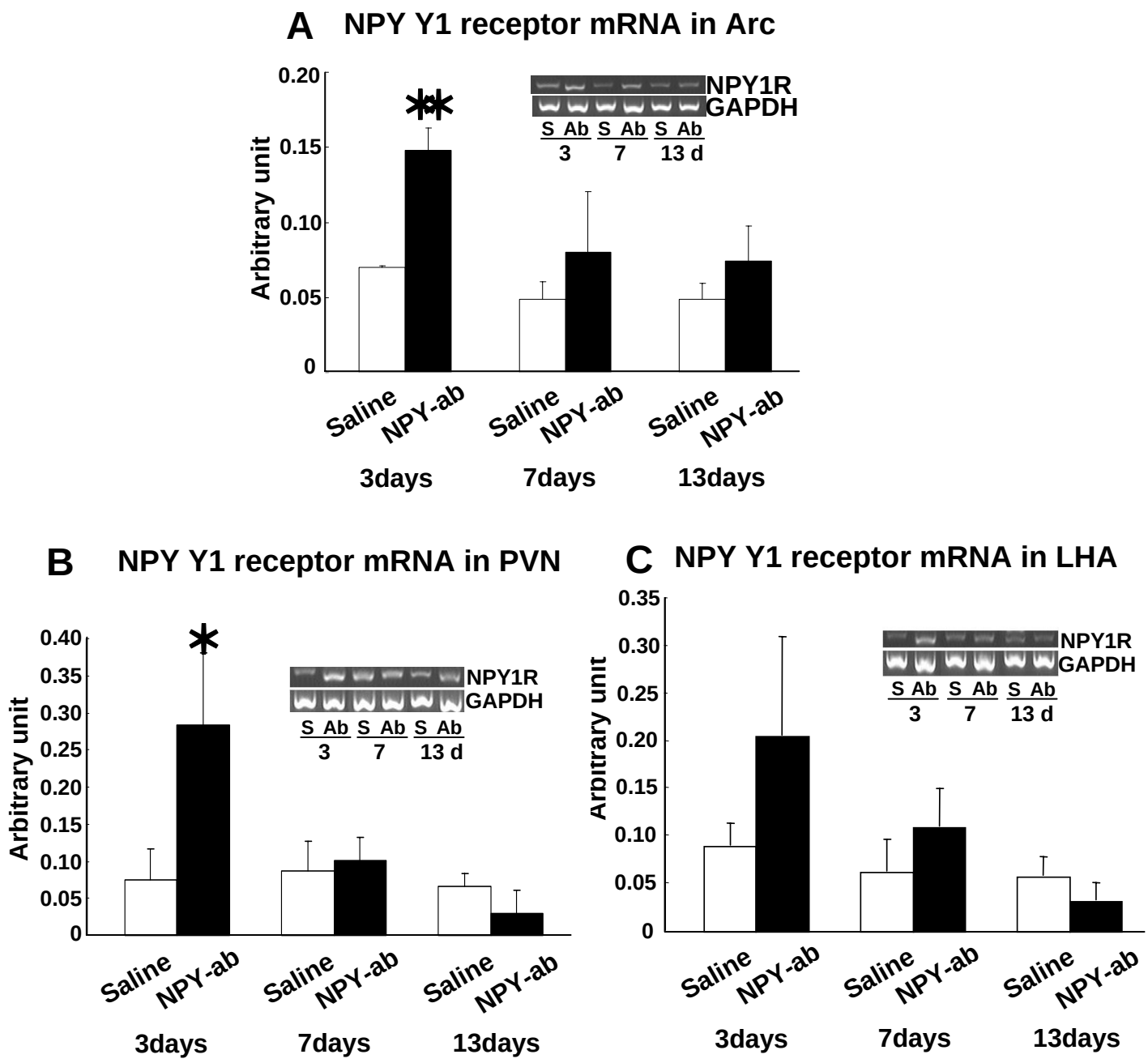
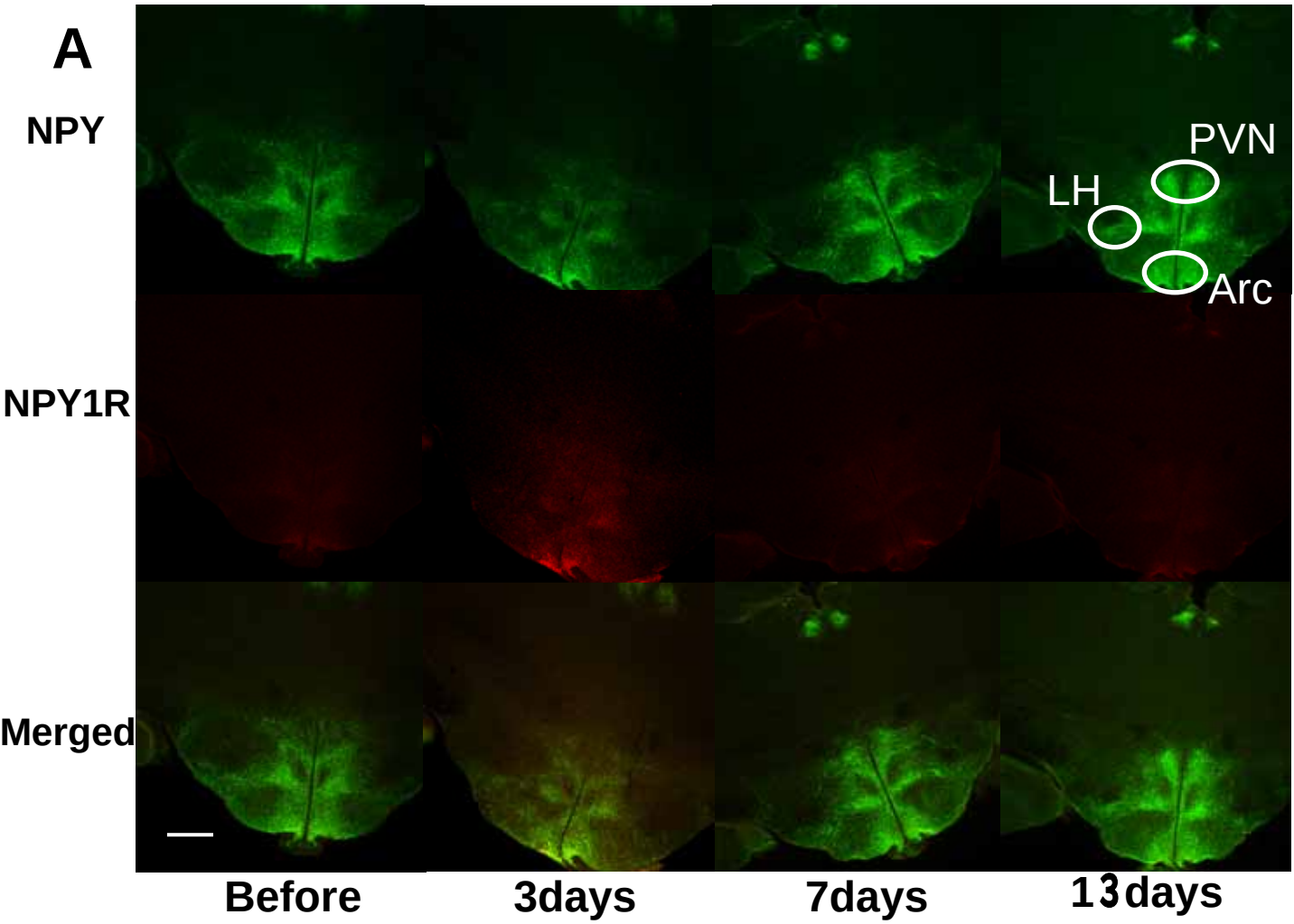

A
NPY Y1 receptor mRNA in Arc
Arbitrary unit
0.20
0.15
0.10
0.05
0
**
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days
B
NPY Y1 receptor mRNA in PVN
Arbitrary unit
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
*
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days
C
NPY Y1 receptor mRNA in LHA
Arbitrary unit
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days

Fig. 5


A
NPY
NPY1R
Merged
PVN
LH
Arc
Before
3days
7days
13days

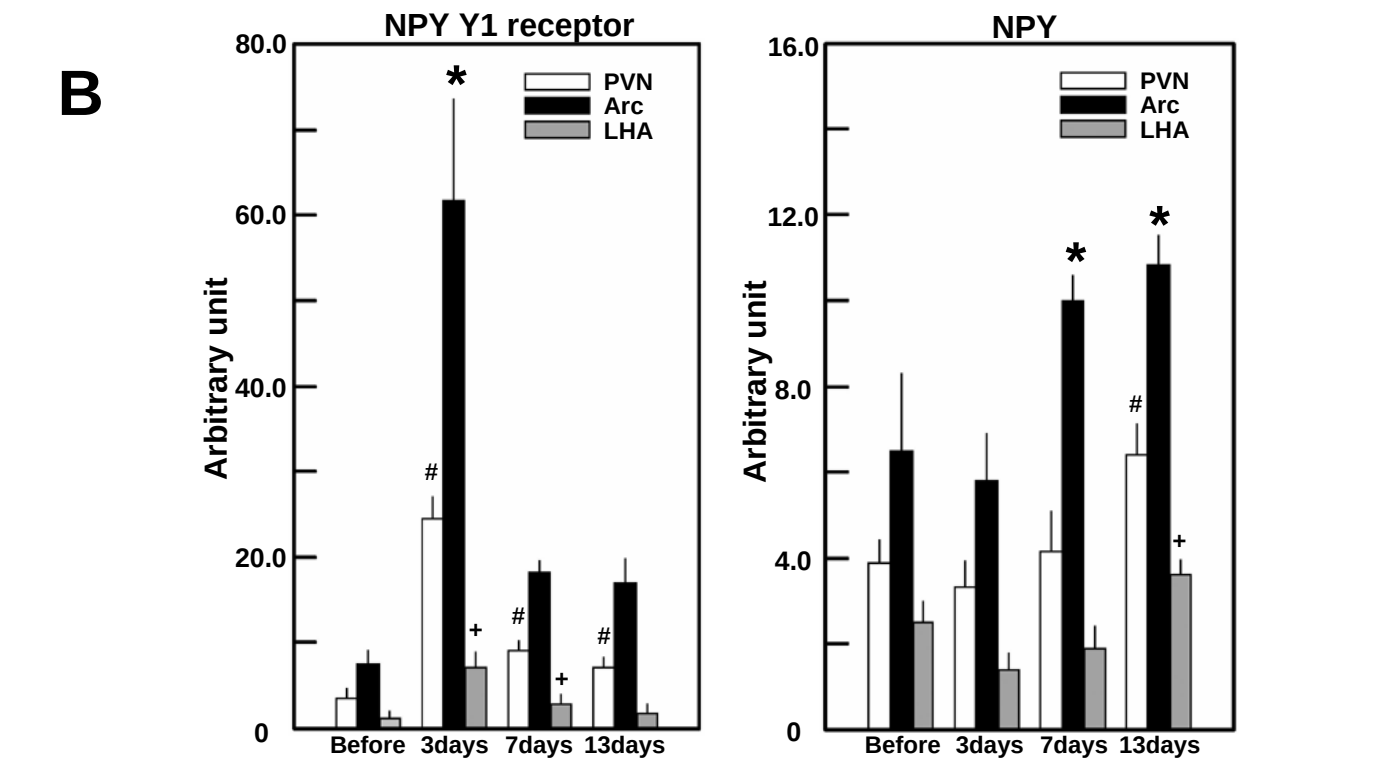

B
NPY Y1 receptor
NPY
PVN
Arc
LHA
Arbitrary unit
80.0
60.0
40.0
20.0
0
16.0
12.0
8.0
4.0
Before
3days
7days
13days
*
#
+

Fig. 6

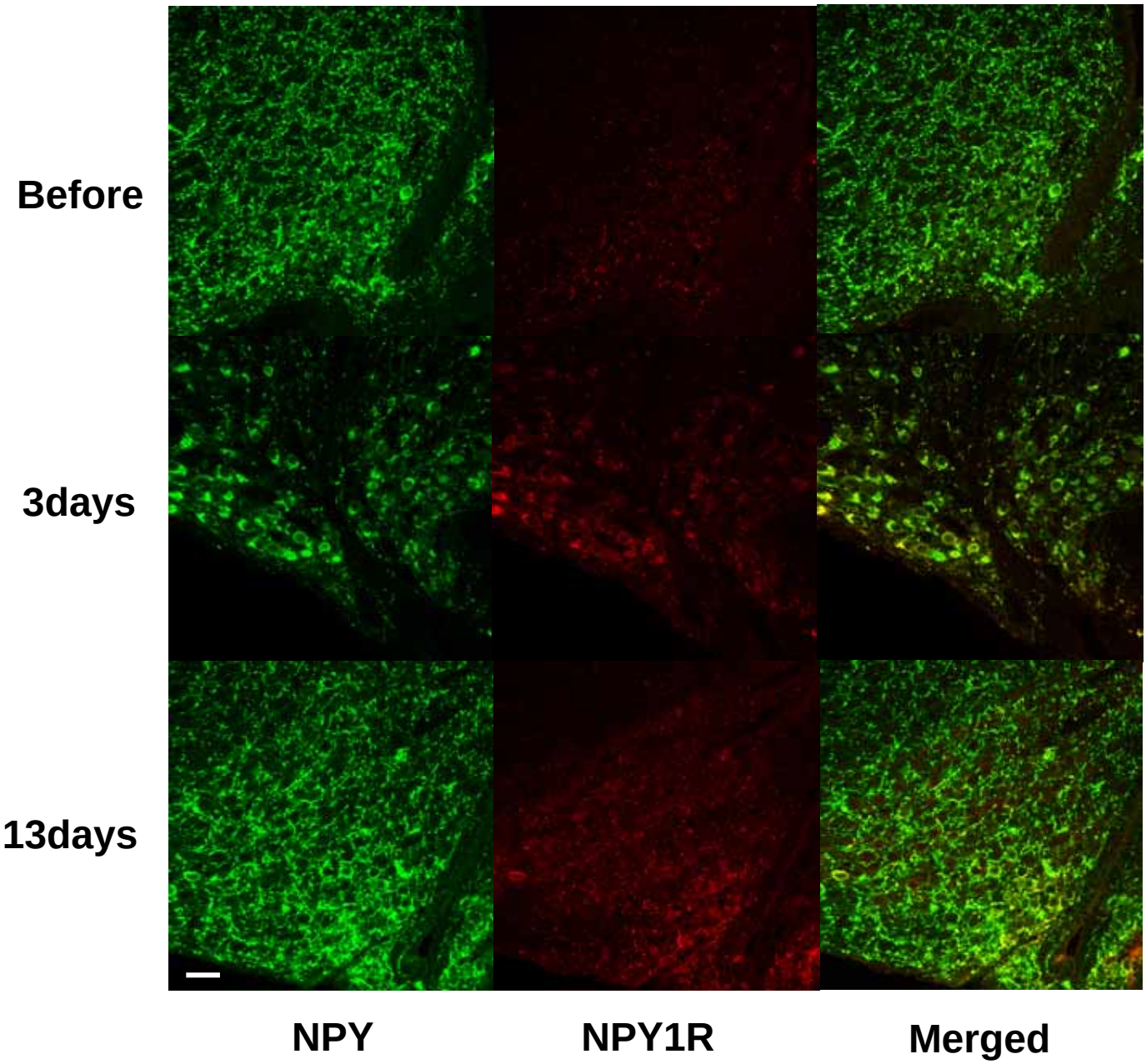


Fig. 7

Table 1. PCR primers in 5'- 3' direction

Transcript	Primers		Product size (bp)
GAPDH	Sense	GGGTGGAGCCAAACGGGTC	**532**
	Antisense	GGAGTTGCTGTTGAAGTCGCA	
NPY	Sense	ACTCTCACAGGCTGTCTTAC	**103**
	Antisense	ATAGTCTCGTAGTCGTCGTC	
NPY1R	Sense	TCAGACCTCTTAATGAAGGAAAGCA	**436**
	Antisense	GAGAACAAGTTTCATTTCCCATCA	
AGRP	Sense	CAGAAGCTTTGGCGGAGGT	**80**
	Antisense	AGGACTCGTGCAGCCTTACAC	
POMC	Sense	CTGCTTCAGACCTCCATAGATGTG	**120**
	Antisense	CAGCGAGAGGCGAGTTTGC	