の前処置は、アンフェタミンおよびメタンフェタミンによる行動感作（behavioral sensitization）の形成を抑制する17-19)。これらの事実から、ベンゾジアゼピンは側坐核を含む中脳辺縁ドパミン神経系を直接抑制する作用を持つと考えられる。しかしながら、ベンゾジアゼピンの精神依存性は実験計画における薬物動態、ベンゾジアゼピンの薬理作用、実験動物の種差などの相違などにより反応性が異なって生じる可能性があり、その評価には十分留意する必要があると考えられる。

ゾルピデムやザレプロンは GABA$_A$ 受容体のベンゾジアゼピン結合部位に作用するが、α_2 あるいは α_3 サブユニットを含む GABA$_A$ 受容体よりも α_1 サブユニットを含む GABA$_A$ 受容体において Cl^- 流入量を増加させる20)。ゾルピデムは一般的なベンゾジアゼピンよりも強い自己投与を誘導し、バルビツール酸誘導体に匹敵する強化効果を有することが報告されている21,22)。ザレプロンもゾルピデム同程度の作用を示すことが報告されている23)。また、α_1GABA$_A$ 受容体に対する内因性活性を持たないL-838417やTPA023などの薬物を用いてGABA$_A$ 受容体の精神依存形成における役割も検討されている。TPA123 は GABA$_A$ 受容体の部分作動薬であり α_1GABA$_A$ 受容体に対しても弱い活性を示すのに対し、TPA023 は GABA$_A$ 受容体の部分作動薬であるが α_1GABA$_A$ 受容体に対し作用しない24)。ヒヒを用いたコカインによる自己投与法による検討では、TPA023 は自己投与に影響を与えない25)が、TPA023 と類似の作用を有する L-838417 は、アカゲザルを用いた検討により、弱いながらも強化効果を示す。このように、ベンゾジアゼピンによる精神依存性の増強は、α_1GABA$_A$ 受容体を介している可能性が考えられるが、α_2、α_3、α_5 サブユニットも含む他の GABA$_A$ 受容体の関与の有無、GABA$_A$ 受容体の脳内局在の相違による異なる関与の様式、などの関連性についてはほとんど解明されていない。

2．身体依存性

ベンゾジアゼピン連用後の休薬による退薬症候についての特徴は多くの報告により明らかにされているが、身体依存の形成機序については多くの研究がなされているにもかかわらず未だ明らかでない。α_1GABA$_A$ 受容体に高い選択性を持つゾルピデムには身体依存は認められるが、古典的なベンゾジアゼピンに比べ弱いとの報告もある26)。また、GABA$_A$ 受容体作動薬連続投与後、逆作動薬である FG-7142 により誘発する痙攣を指標に身体依存性を評価した報告27)によれば、トリアゾラム、クロナゼパム、およびジアゼパムの連続投与により、高頻度で FG-7142 誘発性痙攣が観察され、ロラゼパム、ミダゾラム、アルプラゾラムでは約半数のマウスに FG-7142 誘発性痙攣が認められた。また、非選択的部分作動薬であるブレタゼニル（Ro 16-6028）や NS2710 では FG-7142 誘発性痙攣が少数のマウスに見られた。一方、選択的部分作動薬 L-838417 や SL651498（α_2GABA$_A$、α_3GABA$_A$ 完全作動薬および α_1GABA$_A$、α_5GABA$_A$ 部分作動薬）では、FG-7142 誘発性痙攣は認められなかった。また、TPA023 を慢性処置マウスにおいても FG-7142 誘発性痙攣は認められないとの報告もある24)。これらの結果を考え合わせると、ベンゾジアゼピンによる身体依存形成には特定のサブタイプというよりは、全ての GABA$_A$ 受容体サブタイプが関与している可能性が考えられる。

3．耐性

薬物の繰り返し処置は受容体数に変化を及ぼし神経細胞の薬物に対する反応性を減弱させる。すなわち、作動薬の慢性処置による持続的な刺激はその受容

体の down-regulation を引き起こし、逆に拮抗薬を慢性処置するとその受容体の up-regulation が引き起こされると考えられている。この考えを基盤にしてベンゾジアゼピン処置による受容体数や親和性の変化について数多くの受容体結合実験による検討が行われたが、ベンゾジアゼピンを慢性処置しても受容体数に変化は認められず28,29)、受容体数の減少が認められても、これらは治療用量より推測される血中濃度に比して著しく高濃度において生じている30)。したがって、アロステリックモジュレーターであるベンゾジアゼピンはその慢性処置により極めて緩徐に $GABA_A$ 受容体の立体構造を変化させるものと考えられる。事実、ジアゼパムの慢性処置により、$GABA_A$ 受容体の親和性が変化し脱感作状態に転換されることが報告されている31)。

ベンゾジアゼピンによる細胞内情報伝達系の変化

薬物混入飼料法によるジアゼパム身体依存モデルを用いた検討では、休薬約45時間をピークとする顕著な退薬症候が観察され、これらの退薬症候は MK-801 の処置により著明に抑制される。この身体依存モデルでは NR1 ならびに NR2B サブユニットの増加を伴った NMDA 受容体発現量の増加が認められることから、ベンゾジアゼピンの身体依存には、NR1 と NR2B で構築された NMDA 受容体が重要な役割を果たすものと考えられる32-34)。また、ジアゼパム慢性処置により AMPA 受容体 GluR1 サブユニットが増加するとの報告もある35)。このようなグルタミン酸神経系の変化は、覚せい剤や麻薬などの薬物依存においても認められる。

また、著者らはジアゼパム、ブロチゾラム、クロバザムを慢性投与したマウスにおける高電位開口性L型カルシウムチャネルの変化について報告しており、大脳皮質領域においてカルシウムチャネルを構成するα1C、α1D、α2/δ1 サブユニットの有意な増加が認められることを確認している36)。また、メタンフェタミン、コカイン、モルヒネの報酬効果獲得マウスの大脳皮質領域、側坐核領域において、同様の変化が認められることから37,38)、ベンゾジアゼピンも上述のような高電位開口性 L 型カルシウムチャネルの機能変化を共通の機構として精神依存形成あるいは依存性薬物に対する耽溺を引き起こす可能性が考えられる。

ジアゼパム処置により、神経可塑性に関与すると考えられる calcium/calmodulin-dependent kinase II (CaMKII) や brain derived neurotrophic factor (BDNF) の変化が認められることから、ベンゾジアゼピンによる薬物依存症も $GABA_A$ 受容体を介した神経系の変化により、形成・発現・維持など神経の可塑的変化を引き起こしている可能性が考えられる。

おわりに

$GABA_A$ 受容体は中枢神経系の機能制御に深く関わる神経伝達物質受容体であり、その機能解析の多くが各サブユニットの点変異の導入による動物モデルの作製とその行動薬理学的あるいは薬理学的、神経科学的、生化学的検討などで行われている。本稿で概述した各サブユニットの生理機能や病態生理学的役割が現在徐々に明らかになりつつあることから、今後薬物依存を含む有害作用の少ないサブユニット選択的作用を有するベンゾジアゼピンの開発につながり、ベンゾジアゼピンによる薬物依存を顧慮せずに治療への応用が可能となることが期待される。

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図 1　GABA$_A$受容体の構造模式図。GABA$_A$受容体は五量体を形成しており、αおよびβサブユニットの境界領域に高親和性 GABA 結合部位が存在する。GABA の結合により受容体が活性化されると、5 個のサブユニットの中心部に形成されるチャネルを通して Cl$^-$の細胞内流入が増加し、その結果細胞膜には過分極が誘発され、神経細胞興奮が抑制される。ベンゾジアゼピンはαおよびγサブユニットとの境界部位に結合し、GABA の反応を亢進させる。(文献 1) より引用)

図 2　GABA$_A$受容体の細胞膜上の存在部位。GABA$_A$受容体はシナプス直下に存在するシナプス受容体とシナプス直下以外の樹状突起棘基底部やその周辺部位に存在するシナプス外受容体とに分類される。GABA$_A$受容体の GABA に対する EC$_{50}$ 値は 50μM 以下であり、通常シナプス間隙における GABA 濃度は最も高い時には 0.3～3.0mM になる。(文献 1) より引用)

図 3　GABA$_A$受容体サブユニットの脳内部位における染色性の相違の実例。図中の星印などのマーキングの説明は表 2 の説明を参照のこと。A:大脳皮質第 III 層、B：小脳、C:扁桃体基底外側核、D:視床下部前部、E:嗅球顆粒層。スケールバー＝25mm (文献 5) より引用改変)

表 1　GABA$_A$受容体サブユニットの薬理学的特性と脳内分布 (文献 1) より引用)

表 2　GABA$_A$受容体サブユニットの脳内分布と各部位における細胞内局在の様式。白星 (☆) :強い染色が瀰漫性に神経網に見られるが、神経細胞体はほとんど染色されていない。黒星 (★) :神経細胞体と樹状突起に染色がみられ、細胞一つ一つが確認できる。点付き星 (☆) : 神経網に散在的に弱い染色が認められる。

アステリスク（*）：全体的に染色性が弱い領域で、強く染色されている細胞がみられる。 シンボル数は免疫強度を示す：4；非常に強い、3；強い、2；中程度、1；弱い、「ー」；染色が認められない。（文献 5）より引用改変）

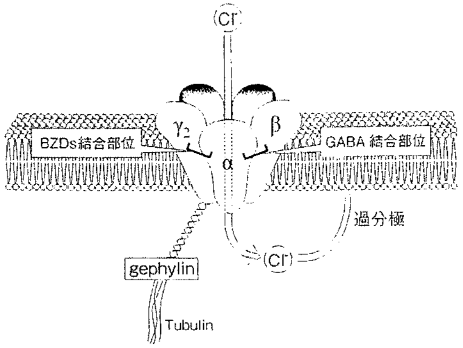

図1　GABA$_A$受容体の構造模式図

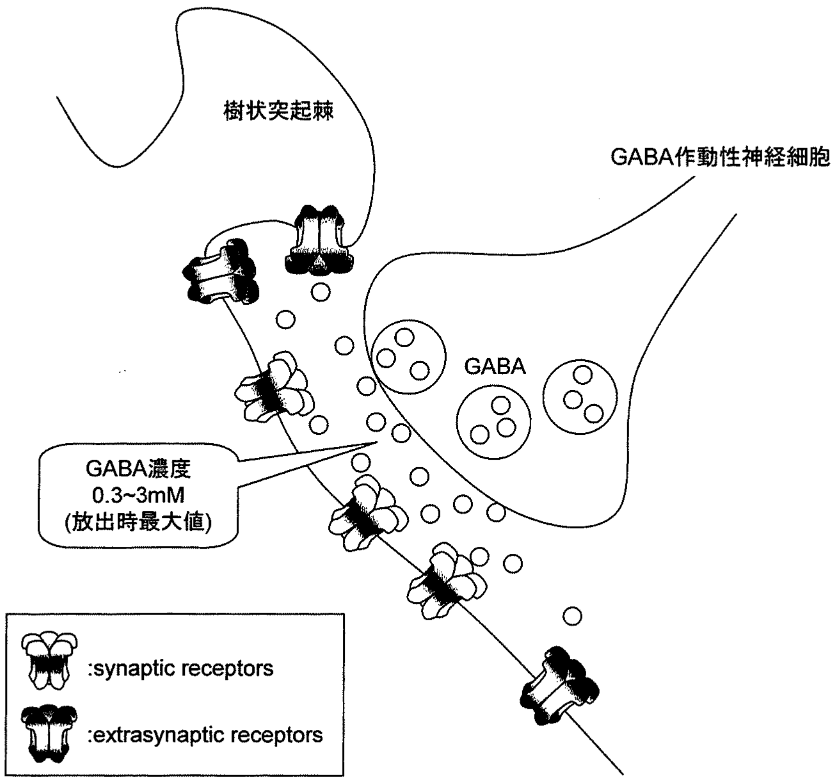

図2 GABA$_A$受容体の細胞膜上の存在部位

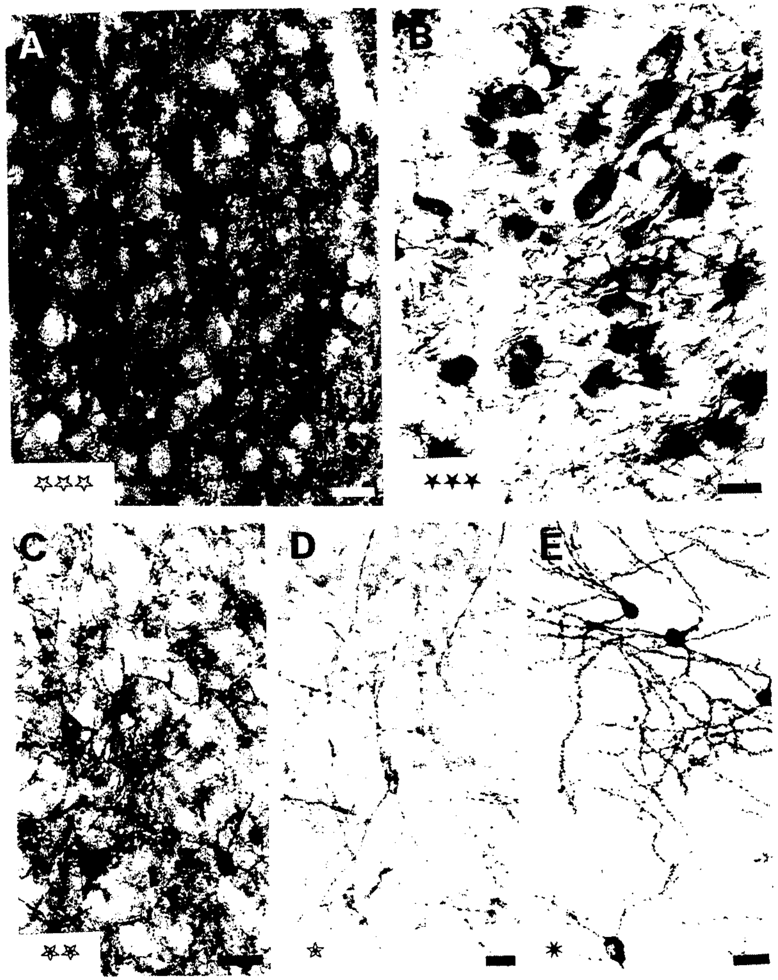

図3 表2中に示すシンボルのGABA$_A$受容体細胞内局在の様子

サブユニット	多くみられるサブユニットの組み合せ	神経細胞における局在部位	脳内分布	薬理学的特性
α_1				BZDs＋
	$\alpha_1\beta_2\gamma_2$	synaptic extrasynaptic	広汎に分布	最も多く分布（60%）鎮静，健忘， 抗けいれん作用
α_2				BZDs＋
	$\alpha_2\beta_2\gamma_2$	synaptic		15-20% 抗不安作用
	$\alpha_2\beta_x\gamma_1$	synaptic		
α_3				BZDs＋，ドパミン神経系機能の調節
	$\alpha_3\beta_x\gamma_{2/3}$	synaptic extrasynaptic		10-15%
α_4			視床，歯状回	BZDs－
	$\alpha_4\beta_{2/3}\gamma$	extrasynaptic		5% 以下
	$\alpha_4\beta_{2/3}\delta$	extrasynaptic		5% 以下
α_5			視床，嗅球，大脳皮質	BZDs＋
	$\alpha_5\beta_3\gamma_2$	extrasynaptic		5% 以下，記憶，学習
α_6				BZDs－
	$\alpha_6\beta_{2/3}\gamma_2$	extrasynaptic	小脳	
	$\alpha_4\beta_{2/3}\delta$	extrasynaptic	小脳	5% 以下
β_1	$\alpha_3\beta_1\gamma_{2/3}$	?		BZDs＋
β_2	$\alpha_{1/4/6}\beta_{2/}\gamma_{1/2}$	synaptic, extrasynaptic		静脈麻酔時の鎮静
	$\alpha_{4/6}\beta_2\delta$	extrasynaptic		
β_3	$\alpha_2\beta_3\gamma_2$	synaptic, extrasynaptic		静脈麻酔時の不安化，無痛
γ_1	$\alpha_2\beta_3\gamma_1$	?		BZDs＋
γ_2	$\alpha_x\beta_x\gamma_2$	synaptic		BZDs＋
γ_3	$\alpha_3\beta_x\gamma_3$ $\alpha_5\beta_3\gamma_3$	?		BZDs＋
δ	$\alpha_4\beta_{2/3}\delta$ $\alpha_6\beta_{2/3/}\delta$	extrasynaptic	小脳，歯状回	BZDs－
ε		?		BZDs－
θ		?		BZDs－

BZDs＋：ベンゾジアゼピン系薬物に感受性あり，BZDs－：ベンゾジアゼピン系薬物に感受性なし，？：不明

表1 GABA$_A$受容体サブユニットの薬理学的特性

Nucleus	α1	α2	α3	α5	β2,3	γ2	δ
Cerebral cortex							
Neocortex							
Layer 1	☆☆☆	☆☆	☆	☆	☆☆☆	☆☆☆	☆
Layers 2–3	☆☆☆	☆☆	☆	☆	☆☆☆	☆☆☆	☆
Layer 4	☆☆☆	☆☆	☆	—	☆☆☆	☆☆☆	☆
Layer 5	☆☆	☆	★★	★★	☆☆	☆☆	☆
Layer 6	☆☆☆	☆	★★★	★★★	☆☆☆	☆☆☆	☆
Interneurons	★★★	★	—	—	★★	★★	★
Hippocampus							
CA1, stratum pyramidale	—	★★	—	★★	★★	★★	—
CA1, stratum oriens and radiatum	☆☆	☆☆☆	☆	☆☆☆	☆☆☆	☆☆☆☆	—
CA1, stratum lacunosum-modeculare	☆	☆☆☆	—	☆☆☆	☆☆☆	☆☆☆	—
CA3, stratum pyramidale	—	★★	★	★★	★★	★★	—
CA3, stratum oriens and radiatum	☆☆	☆☆☆	☆	☆☆☆	☆☆☆	☆☆☆☆	—
Interneurons	★★★	—	—	—	★★	★★	★
Dentate gyrus							
Granule cell layer	—	★★	—	★	★★	★★	—
Molecular layer	☆☆	☆☆☆☆	☆	☆	☆☆☆	☆☆☆☆	☆
Hilus	★	☆☆	☆☆	—	☆	☆	—
Interneurons	★★★	★	—	—	★★	★★	★
Amygdala							
Anterior amygdaloid area	★★	★	★	★	★★	★★	—
Anterior cortical nucleus	★★	★★	★★	★	★★	☆☆	—
Central nucleus, medial part	★	★★	★	★	★★★	★★	—
Central nucleus, lateral part	★	★★★	★★	★	★★★	★★★	
Basomedial nucleus, anterior part	★★	☆☆	★★★	★	★★	★★	—
Basomedial nucleus, posterior part	★★	☆☆	☆	☆☆	★★	★★	—
Basolateral nucleus	★★	☆☆☆	☆☆☆	★★★	★★★	★★★	—
Basolateral nucleus, ventral part	★★	☆☆	☆☆☆	★	★★	★★	★
Lateral nucleus	★★★	☆☆☆	★	★	★★★	★★★	—
Intercalated nuclei	★	☆☆☆	★★★	☆	★★★	★★★	—
Medial nucleus	★★★	☆☆	★	☆	★★★	★★	—
Posterior cortical nucleus	★★★	☆☆	★	☆	★★★	★★	—
Amygdalohippocampal area	★★★	☆☆	☆☆	★	★★	★★	—
Cerebral nucleu							
Caudatoputamen (striatum)	☆	☆☆☆	☆	☆☆	☆☆☆	☆☆☆	☆
Nucleus accumbens	☆	☆☆☆	☆	☆☆	☆☆☆	☆☆☆	—
Claustrum	☆☆	☆	☆☆☆	—	☆☆☆	☆☆☆	—
Globus pallidus	★★★	★	★	—	☆☆	☆☆	—
Ventral pallidum	★★★★	★	★	—	★★★	★★★	—
Entopeduncular nucleus	★★★★	★	★	—	★★★★	★★★★	—
idbrain and pons	★★★★	★	★	—	★★★★	★★★★	—
Ventral tegmental area	★★	—	★★	—	★★	★★	—
Substantia nigra, pars compacta		★	★★★	—		★★★	—
Substantia nigra, pars reticulata	★★★★	—	—	—	★★★★	★★★	—
Dorsal raphe nucleus	★★	★	★★	—	★★	★★	—
Locus coeruleus	—	★★	★★★	—	—	★★	—
Medulla							
Raphe magnus nucleus	★★★	★★	★★	—	★★★	★★★	—

表2 GABA$_A$受容体サブユニットの脳内分布

An Inducer for Glial Cell Line-Derived Neurotrophic Factor and Tumor Necrosis Factor-α Protects Against Methamphetamine-Induced Rewarding Effects and Sensitization

Minae Niwa, Atsumi Nitta, Yuichiro Yamada, Akira Nakajima, Kuniaki Saito, Mitsuru Seishima, Liya Shen, Yukihiro Noda, Shoei Furukawa, and Toshitaka Nabeshima

Background: There are few efficacious medications for drug dependence. We investigated the potential of Leu-Ile, which induces the expression of glial cell line-derived neurotrophic factor (GDNF) and tumor necrosis factor-α (TNF-α), as a novel therapeutic agent for methamphetamine (METH)-induced dependence.

Methods: The levels of GDNF and TNF-α messenger RNA (mRNA) were determined by real-time reverse transcription polymerase chain reaction. Enzyme immunoassays and immunohistochemistry were employed to determine levels of these proteins. Effects of Leu-Ile on METH-induced rewarding effects and sensitization were investigated with conditioned place preference and locomotor activity tests. Extracellular dopamine (DA) levels and DA uptake into synaptosomes were examined with an in vivo microdialysis and trititated thymidine ([^{3}H]) DA uptake assay.

Results: Leu-Ile induced the expression of not only GDNF but also TNF-α. Pretreatment with Leu-Ile blocked the acquisition of METH-induced place preference and sensitization. Interestingly, post-treatment with Leu-Ile attenuated them even after their development. An inhibitory effect of Leu-Ile on METH-induced place preference was observed in neither GDNF heterozygous nor TNF-α knockout mice. Leu-Ile inhibited DA release in the nucleus accumbens and the decrease in synaptosomal DA uptake in the midbrain induced by repeated METH treatment.

Conclusions: These results suggest that Leu-Ile inhibits METH-induced rewarding effects and sensitization by regulating extracellular DA levels via the induction of GDNF and TNF-α expression.

Key Words: Dopamine (DA), glial cell line-derived neurotrophic factor (GDNF), methamphetamine (METH), rewarding effects, sensitization, tumor necrosis factor-α (TNF-α)

The abuse of substances such as psychostimulants, opiates, nicotine, and alcohol has become a significant social and public health concern worldwide. Activation of the mesocorticolimbic dopamine (DA) system has been implicated in the positive reinforcing (rewarding) effects of drugs of abuse (Robbins and Everitt 1999; Yamada and Nabeshima 2004). The psychostimulant effects of methamphetamine (METH), a typical drug of abuse, are associated with an increase in extracellular DA levels in the brain, by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Giros *et al.* 1996; Heikkila *et al.* 1975; Seiden *et al.* 1993).

Neurotropic factors and cytokines, which are known to influence synaptic transmission and neuronal morphology (Boulanger and Poo 1999; Connor and Dragunow 1998; Neumann *et al.* 2002), might be involved in alterations of the morphology of dendrites and dendritic spines in the nucleus accumbens (NAc) and prefrontal cortex after repeated injection of psychostimulants (Robinson and Kolb 1997, 1999; Yamada *et al.* 2000). Glial cell line-derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine (Messer *et al.* 2000). Furthermore, we have previously demonstrated that tumor necrosis factor-α (TNF-α) inhibits METH-induced dependence (Nakajima *et al.* 2004; Yamada and Nabeshima 2004). Taken together, GDNF and TNF-α would be candidates for therapeutic agents against drug dependence. However, there are serious obstacles to their therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, because it is a macromolecule that cannot penetrate the blood-brain barrier (BBB) (Lin *et al.* 1993) and is easily broken down by proteases in the blood stream. In addition, TNF-α, an inflammatory cytokine, damages the peripheral tissues, because it triggers the expression of other cytokines (Bluthe *et al.* 1994). Therefore, GDNF and TNF-α cannot be used directly as therapeutic tools for drug dependence. We hypothesized that a low-molecular-weight compound that induces production of GDNF and TNF-α in the brain could be a novel therapeutic agent for drug dependence. Previous study has demonstrated that inflammatory stimuli such as TNF-α and lipopolysaccharide induces the synthesis of GDNF in cultured astrocytes from mouse brain (Appel *et al.* 1997). Furthermore, Leu-Ile, a hydrophobic dipeptide, induces GDNF synthesis both in vivo and in vitro (Nitta *et al.* 2004). Taken together, Leu-Ile is expected to induce the production of not only GDNF but also TNF-α and to inhibit drug dependence.

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In the present study, we examined: 1) whether Leu-Ile induces production of TNF-α, and 2) the effects of Leu-Ile on the rewarding actions and the sensitization to the locomotor-stimulating effects of METH and on the increase in extracellular DA levels and the decrease in DA uptake induced by METH.

Methods and Materials

Reagents

Glial cell line-derived neurotrophic factor and TNF-α were donated by Amgen (Thousand Oaks, California) and Dainippon Pharmaceutical (Osaka, Japan), respectively. Leu-Ile was purchased from Kokusan Chemical (Tokyo, Japan). All other materials used were of reagent grade.

Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23° ± 1°C; 50% ± 5% humidity; 12-hour light/dark cycle starting at 8:00 AM) and had ad libitum access to food and water, except during behavioral experiments. Animal care and use was in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-123, 1983) and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. The behavioral experiment's schedule is shown in Figure 1.

The wild-type C57BL/6 mice were obtained from Slc Japan (Hamamatsu, Japan).

Male C57BL/6-GDNF heterozygous [GDNF-(±)] mice and C57BL/6-TNF-α knockout (TNF-α-[−/−]) mice, 8–12 weeks of age, were used in the experiments. The GDNF-(±) and TNF-α-(−/−) mice were generated as described previously (Nakajima *et al.* 2004; Pichel *et al.* 1996; Taniguchi *et al.* 1997); GDNF (−/−) homozygous knockout mice die shortly after birth (postnatal 7 days), but GDNF (±) mice are viable. Glial cell line-derived neurotrophic factor levels in the frontal cortex (Fc), NAc, striatum, and hippocampus (Hip) of GDNF-(±) mice are 54.8%, 65.4%, 59.0%, and 66.8 %, respectively, of those in littermate GDNF-(+/+) mice (Table 1). Littermate GDNF-(+/+) mice were used as control subjects in the behavioral experiments.

Drug Treatment

Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) once/day 1 hour before METH (1 mg/kg, SC) treatment for 9 days. In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH after 9 successive days of METH administration. To determine tritiated thymidine ([^{3}H]) DA uptake, messenger RNA (mRNA) expression, and protein levels, mice were decapitated 1, 2, and 24 hours after the last METH injection, respectively.

Enzyme Immunoassay of GDNF

Glial cell line-derived neurotrophic factor levels were measured with an enzyme immunoassay (EIA) with a minor modification (Nitta *et al.* 1999a, 1999b, 2004). Homogenate buffer (.1 mol/L Tris-HCl [pH 7.4] containing 1 mol/L sodium chloride (NaCl), 2% bovine serum albumin, 2 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), and .2% sodium nitride [Na3N]) was added to brain tissue at a ratio of 1 g wet weight / 19 mL of buffer, pulse-sonicated for 100 sec, and centrifuged at 100,000 *g* for 30 min. The supernatant was collected and used for the EIA.

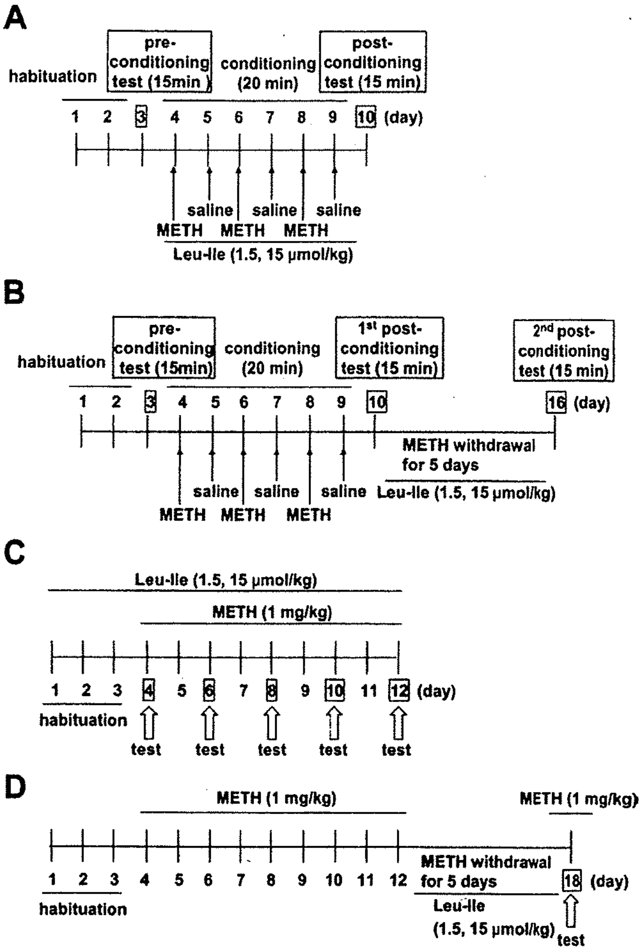

Figure 1. Experimental schedules. **(A)** Experimental schedule for the conditioned place preference task. Mice were co-treated with Leu-Ile and methamphetamine (METH) in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving METH (1 mg/kg, SC) or saline. Closed arrows indicate the days of METH or saline injection. **(B)** Experimental schedule for the conditioned place preference task to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were not treated with Leu-Ile in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from METH. **(C)** Experimental schedule for measurement of locomotor activity. Mice were treated with Leu-Ile during the habituation period for 3 days and then co-treated with Leu-Ile and METH for 9 days. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the METH (1 mg/kg, SC) injection. Locomotor activity was measured for 2 hours after the METH treatment. Open arrows indicate the days when locomotor activity was measured. **(D)** Experimental schedule for measurement of locomotor activity to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were treated with Leu-Ile after the establishment of METH (1 mg/kg, SC)-induced sensitization: mice were treated with METH for 9 days and then with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days without METH. On day 18, mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours after the METH treatment.

Semi-Quantative mRNA Analysis by Real-Time Reverse Transcription Polymerase Chain Reaction

Corti-hippocampal neurons from 18-day-old rat embryos were cultured as previously described (Nitta *et al.* 1999a, 1999b). More than 95% of the cells were positive for microtuble-associated protein-2 (MAP2) immunoreactivity. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free

Table 1. The Difference of GDNF Levels in the Brain (Fc, NAc, Str, Hip) Between GDNF-(+/+) and GDNF-(+/−) Mice

Brain Region	GDNF Levels (pg/g Wet Tissue)		
	GDNF-(+/+) Mice	GDNF-(+/−) Mice	% of GDNF-(+/+) Mice
Fc	1285.0 ± 254.3	704.0 ± 26.4*	54.8
NAc	1253.4 ± 58.5	820.2 ± 58.4*	65.4
Str	2083.1 ± 231.0	1229.4 ± 178.2*	59.0
Hip	782.2 ± 99.9	522.5 ± 20.9*	66.8

Mice were decapitated without any treatment, and the brains were quickly removed. Glial cell line-derived neurotrophic factor (GDNF) levels were measured using an enzyme immunoassay. Values are means ± SE ($n = 10$).

Fc, frontal cortex; Hip, hippocampus; NAc, nucleus accumbens; Str, striatum.

*$p < .05$ versus GDNF-(+/+) mice.

defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL) or TNF-α (2, 20, 100, and 200 ng/mL). Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted into complementary DNA with a SuperScriptTM First-Strand System for RT-PCR Kit (Invitrogen Life Technologies, Carlsbad, California). The rat GDNF primers used were as follows: 5'-AGCTGCCAGCCCAGAGAATT-3' (forward) and 5'-GCACCCCCGATTTTTGC-3' (reverse), and TaqMan probe: 5'-CAGAGGGAAAGGTCGCAGAGGCC-3'. The rat TNF-α primers used were as follows: 5'-ATTTGGCCCCATCCTTCC-3' (forward) and 5'-GCCTCCATGGCAGAGCC-3' (reverse), and TaqMan probe: 5'-TCCCAGGACATCAGGACTCTGTTCCC-3'. The 18S ribosomal RNA Kit was used as the internal control (PE Applied Biosystems, Foster, California). The amplification consisted of an initial step (50°C for 2 min and 95°C for 2 min) and then 40 cycles of denaturation for 15 sec at 95°C and annealing for 1 min at 60°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Tokyo, Japan). The expression levels were calculated as described previously (Wada *et al.* 2000).

Immunohistochemical Analysis

Polyclonal rabbit anti-GDNF (1:50; sc-328; Santa Cruz Biotechnology, Santa Cruz, California), polyclonal goat anti-TNF-α (1:100; R&D Systems, Minneapolis, Minnesota), monoclonal mouse anti-MAP2 (1:200; Sigma-Aldrich, Saint Louis, Missouri), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Chemicon International, Temecula, California) served as primary antibodies. Goat anti-rabbit Alexa Fluor 546 (1:1000, Molecular Probes, Eugene, Oregon) and goat anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for GDNF immunostaining. Donkey anti-goat Alexa Fluor 546 (1:1000, Molecular Probes) and rabbit anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for TNF-α immunostaining. Each stained tissue was observed under a fluorescence microscope (Axioskoop 2 plus: Carl Zeiss, Jena, Germany) and analyzed with Axiovision 3.0 systems (Carl Zeiss). The area with TNF-α-positive cells in the defined NAc region of mice was determined with the software WinROOF (Mitani, Fukui, Japan) (Kuwahara *et al.* 1999; Tsuji *et al.* 1999). We employed an immunostaining method with which one can analyze the distribution and levels of TNF-α protein in the present investigation, because it is too difficult to use Western blotting or an enzyme immunoassay to quantify the amount of TNF-α protein in brain tissue.

Measurement of the TNF-α Concentration in Peripheral Blood

Blood was collected into tubes containing 5% EDTA 1, 2, and 4 hours after the injection of TNF-α (4 μg/body, IP). Mice were treated with Leu-Ile (1.5 μmol/kg, IP), and their blood was collected into tubes containing 5% EDTA 0, 1, 2, 4, and 8 hours after the injection. The blood samples were centrifuged at 2000 × *g* for 20 min at 4°C. The supernatants were taken as the samples. The TNF-α concentration was assessed by using a specific human (QuantiGlo QTA00, R&D Systems) or mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Quantikine MTA00, R&D Systems), according to the instructions provided.

Behavioral Tests

Conditioned Place Preference. The place conditioning paradigm was performed by using previously established procedure with a minor modification (Nagai *et al.* 2004; Nakajima *et al.* 2004; Noda *et al.* 1998). The experimental schedule for the conditioned place preference (CPP) task is shown in Figures 1A and 1B. The mouse was allowed to move freely between transparent and black boxes for 15 min once/day for 3 days (days 1–3) in the preconditioning. On day 3, the time the mouse spent in each box was measured. On days 4, 6, and 8, the mouse was treated with METH and confined in either the transparent or black box for 20 min. On days 5, 7, and 9, the mouse was given saline and placed opposite the METH-conditioning box for 20 min. On day 10, the postconditioning test was performed without drug treatment, and the time the mouse spent in each box was measured for 15 min.

Locomotor Activity. Locomotor activity was measured with an infrared detector (Neuroscience, Tokyo, Japan) in a plastic box (32 × 22 × 15 cm high). Mice were administered METH (1 mg/kg, SC) or saline 1 hour after the Leu-Ile treatment, and the locomotor activity was measured for 2 hours immediately after the METH or saline administration (Figure 1C). In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH (days 13–17) after 9 successive days of METH administration. On day 18, the mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours immediately after the administration (Figure 1D).

In Vivo Microdialysis

Mice were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto, Japan) was implanted into the NAc (+1.1 mm anteroposterior, +1.0 mm mediolateral from bregma, and −4.0 mm dorsoventral to dura) according to the atlas of Franklin and Paxinos (1997) and secured to the skull with stainless steel screws and dental acrylic cement. One day after the operation, a dialysis probe (AI-8-1; 1-mm membrane length, EICOM) was inserted through the guide cannula and perfused continuously with artificial cerebrospinal fluid (aCSF; 147 mmol/L NaCl, 4 mmol/L potassium chloride, and 2.3 mmol/L calcium dichloride) at a rate of 1.0 μL/min. Dialysate was collected in 20-min fractions and injected into the HPLC system (EICOM) for the measurement of DA levels. Three samples were used to establish baseline levels of extracellular DA before the administration of Leu-Ile and METH.

Synaptosomal $[^3H]$ DA Uptake

Midbrain synaptosomal $[^3H]$ DA uptake was determined as previously described (Fleckenstein *et al.* 1997; Nakajima *et al.* 2004). Samples were incubated at 37°C for 4 min, and then ice-cold Krebs-Ringer's solution containing 10 μmol/L GBR12909, a specific DA uptake inhibitor, was added. Nonspecific values were determined in the presence of 100 μmol/L GBR12909. The radioactivity trapped on Whatman GF/B filters (Brandel, Gaithersburg, Maryland) was measured with a liquid scintillation counter.

Statistical Analysis

All data were expressed as means ± SEM. Statistical differences between two groups were determined with Student *t* test. Statistical differences among more than three groups were determined with a one-way analysis of variance (ANOVA) or a repeated ANOVA followed by the Bonferroni multiple comparison test; $p < .05$ was regarded as statistically significant.

Results

Effect of Leu-Ile on METH-Induced Increase in GDNF Levels

Levels of GDNF expression induced by Leu-Ile (.037, .37, 3.7, and 37 μg/mL) were determined in the cultured neurons with the EIA method. The GDNF levels were significantly increased 24 hours after the addition of Leu-Ile (.37 μg/mL) resulting in a bell-shaped dose response curve compared with the control group [$F(4,25) = 8.895$, $p < .05$, one-way ANOVA] (Figure 2A). Therefore, a dose of .37 μg/mL was used in this experiment. The time course of GDNF mRNA expression was determined 6, 12, and 24 hours after Leu-Ile (.37 μg/mL) treatment in the cultured neurons. The GDNF mRNA levels were significantly elevated 24 hours after Leu-Ile treatment in the cultured neurons compared with the control group [$F(5,42) = 7.627$, $p < .05$, one-way ANOVA] (Figure 2B).

Glial cell line-derived neurotrophic factor–positive cells were found among the neurons, which were immunopositive for MAP2, in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC); GDNF-positive cells were also found among astro glial cells, which were immunopositive for GFAP, in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC) (Figure 2C).

The GDNF levels in the NAc were determined after the co-administration of Leu-Ile and METH with the EIA method. Methamphetamine (1 mg/kg) increased GDNF levels in the NAc compared with those in the vehicle/saline-treated mice. The GDNF levels after the co-administration of Leu-Ile (1.5 and 15 μmol/kg, IP) and METH (1 mg/kg) were much more increased compared with those in the vehicle/METH-treated mice [$F(5,38) = 16.971$, $p < .05$, one-way ANOVA] (Figures 2C and 2D). Moreover, we determined GDNF levels in the NAc after Leu-Ile treatment during the withdrawal from METH after 9 successive days of METH administration. The schedule is described in Figure 1D. An acute challenge of METH in mice treated with vehicle for 5 days after the development of METH-induced sensitization increased GDNF levels in the NAc compared with levels in the saline/vehicle/METH-treated mice. An acute challenge of METH in mice treated with Leu-Ile (1.5 and 15 μmol/kg) for 5 days after the development of METH-induced sensitization resulted in a much greater increase compared with levels in the METH/vehicle/METH-treated mice [$F(3,20) = 23.777$, $p < .05$, one-way ANOVA] (Figure 2E).

Effect of Leu-Ile on METH-Induced Increase in TNF-α Levels

Expression levels of TNF-α mRNA induced by Leu-Ile (.037, .37, 3.7, and 37 μg/mL) were determined in the cultured neurons with the real-time reverse transcription polymerase chain reaction (RT-PCR) method, because TNF-α induces the synthesis of GDNF as described in the introductory section of this report. Levels of TNF-α mRNA were significantly increased 24 hours after the addition of Leu-Ile (.37 μg/mL), resulting in a bell-shaped dose response curve compared with the control group [$F(4,30) = 2.572$, $p < .05$, one-way ANOVA] (Figure 3A). The time course of TNF-α mRNA expression was determined 6, 12, and 24 hours after Leu-Ile (.37 μg/mL) treatment in the cultured neurons. The TNF-α mRNA levels were significantly elevated 24 hours after the Leu-Ile (.37 μg/mL) treatment in the cultured neurons compared with the control group [$F(5,42) = 6.067$, $p < .05$, one-way ANOVA] (Figure 3B).

Tumor necrosis factor-α–positive cells were found among the neurons that were immunopositive for MAP2 but not for GFAP in the NAc of mice co-treated with Leu-Ile (1.5 μmol/kg, IP) and METH (1 mg/kg, SC) (Figure 3C).

The areas occupied by TNF-α-immunopositive cells were measured to estimate the effects of Leu-Ile on the production of TNF-α protein. The area with TNF-α immunoreactive cells was determined in the NAc by using the software WinROOF (Mitani Shoji, Fukui, Japan). Methamphetamine (1 mg/kg, SC) potentiated the immunoreactivity to TNF-α in the NAc. After the co-administration of Leu-Ile (1.5 and 15 μmol/kg, IP) and METH (1 mg/kg, SC), immunoreactivity was much more increased in the NAc [$F(5,32) = 26.836$, $p < .05$, one-way ANOVA] (Figures 3C and 3D). Moreover, we determined levels of TNF-α protein in the NAc after Leu-Ile treatment during the withdrawal from METH after 9 successive days of METH administration. The schedule is described in Figure 1D. An acute challenge of METH in mice treated with vehicle for 5 days after the development of METH-induced sensitization increased TNF-α levels in the NAc compared with those in the saline/vehicle/METH-treated mice. An acute challenge of METH in mice treated with Leu-Ile (1.5 and 15 μmol/kg) for 5 days after the development of METH-induced sensitization markedly increased TNF-α levels compared with those in the METH/vehicle/METH-treated mice [$F(3,22) = 26.800$, $p < .05$, one-way ANOVA] (Figure 3E).

We checked whether the concentration of TNF-α in venous blood was changed after the intraperitoneal injection of Leu-Ile in mice. In venous blood, the TNF-α concentration was not changed 1, 2, 4, and 8 hours after the Leu-Ile treatment (1.5 μmol/kg, IP). In contrast, the concentration was dramatically increased 1, 2, and 4 hours after the TNF-α treatment (4 μg/body, IP) (Figure 3F).

Regulatory Effect of TNF-α on GDNF Expression in Cultured Neurons

With the real-time RT-PCR method, levels of GDNF mRNA stimulated by TNF-α (20 and 100 ng/mL) were determined in the cultured neurons. The mRNA levels were significantly elevated by TNF-α (100 ng/mL) compared with the control group [$F(2,11) = 7.826$, $p < .05$, one-way ANOVA] (Figure 4A). Next, the time course of GDNF mRNA expression was determined 6, 12, and 24 hours after TNF-α (100 ng/mL) treatment. The mRNA levels were significantly elevated 24 hours after the treatment with TNF-α (100 ng/mL) compared with the control group [$F(5,46) = 6.114$, $p < .05$, one-way ANOVA] (Figure 4B). The GDNF levels showed a significant increase 24 hours after the addition of TNF-α (100 and 200 ng/mL) compared with the control group [$F(4,25) = 12.372$, $p < .05$, one-way ANOVA] (Figure 4C).

Effects of Leu-Ile on METH-Induced Place Preference and Hyperlocomotion/Sensitization in Wild-Type Mice

We investigated the effects of Leu-Ile on the behavioral responses to METH. First, the effects of Leu-Ile on the rewarding effects of METH were examined in CPP paradigm, in which animals learn the association of an environment paired with drug exposure; CPP is, therefore, considered a measure of the rewarding properties of drugs of abuse. The experimental schedule is described in Figure 1A. As shown in Figure 5A, METH (1 mg/kg, SC) produced place preference in mice. When mice were treated with Leu-Ile (1.5 μmol/kg, IP) 1 hour before receiving METH, the METH-induced place preference was significantly attenuated [$F(5,53) = 5.338$, $p < .05$, one-way ANOVA] (Figure 5A). Leu-Ile (1.5 and 15 μmol/kg, IP) itself failed to induce place preference in mice (Figure 5A second and third columns in saline-treated group).

We next examined the effects of Leu-Ile on METH-induced hyperlocomotion and sensitization. The sensitization to the locomotor-stimulating effects is argued to reflect one neuroadaptive process associated with dependence. The experimental schedule is described in Figure 1C. As shown in Figure 5B, acute METH treatment at a dose of 1 mg/kg caused a marked increase in locomotor activity, and repeated administration for 9 days resulted in an enhancement of the locomotor-stimulating effect of METH (sensitization) [$F(4,45)=2.919$, $p < .05$, one-way ANOVA]. Leu-Ile (1.5 and 15 μmol/kg, IP) did not inhibit acute METH-induced hyperlocomotion on day 4, although it inhibited METH-induced hyperlocomotion and sensitization on days 8–12 [$F(5,54) = 24.374$ at day 8, $F(5,54) = 20.738$ at day 10, $F(5,54) = 30.956$ at day 12, $p < .05$, one-way ANOVA] (Figure 5B). Leu-Ile (1.5 and 15 μmol/kg, IP) did not affect spontaneous locomotor activity in saline-treated mice (Figure 5B).

Effects of Leu-Ile Treatment After the Development of Place Preference and Sensitization Induced by METH in Wild-Type Mice

The therapeutic effects of Leu-Ile on METH-induced place preference were examined. The experimental schedule is described in Figure 1B. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) after the development of METH-induced place preference for 5 days without METH treatment. In this experiment, the second post-conditioning was carried out 5 days after the first post-conditioning. Although METH (1 mg/kg)-induced place preference was maintained for 5 days after the first post-conditioning in wild-type mice, it was attenuated by Leu-Ile treatment (1.5 μmol/kg, IP) for 5 days between the first and second post-conditionings [$F(5,58) = 14.407$, $p < .05$, one-way ANOVA] (Figure 5C).

Next, the therapeutic effects of Leu-Ile on METH-induced sensitization were examined. The experimental schedule is described in Figure 1D. The repeated administration of METH (1 mg/kg, SC) for 9 days resulted in sensitization of METH. Sensitization was observed on day 18 of the administration of METH (1 mg/kg, SC) 5 days after withdrawal from METH [$F(5,154) = 23.107$, $p < .05$, one-way ANOVA]. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days during the withdrawal period. Leu-Ile (1.5 and 15 μmol/kg, IP) inhibited METH (1 mg/kg, SC)-induced sensitization on day 18 [$F(3,36) = 21.840$, $p < .05$, one-way ANOVA] (Figure 5D).

We confirmed that Leu-Ile at the low dose, 1.5 μmol/kg, which could inhibit METH-induced rewarding effects, increased GDNF and TNF-α both in combination with METH and after withdrawal from repeated treatment with METH in CPP paradigm. In contrast, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit METH-induced rewarding effects, failed to increase them [data not shown; $F(5, 30) = 12.387$ and 19.764 for GDNF and TNF-α levels, respectively, after co-treatment of Leu-Ile and METH in CPP paradigm; $F(3,20) = 12.260$ and 16.670 for GDNF and TNF-α levels, respectively, after Leu-Ile treatment during withdrawal from METH in CPP paradigm]. The results of GDNF and TNF-α contents in the NAc after locomotor test are described in Figures 2D, 2E, 3D, and 3E.

Effects of Leu-Ile on the Rewarding Effects of METH in GDNF-(±) and TNF-α-(−/−) Mice

To confirm the involvement of GDNF and TNF-α in the rewarding effects of METH, the effect of Leu-Ile on METH-induced place preference was examined in GDNF-(±) and TNF-α-(−/−) mice. The experimental schedule is described in Figure 1A. As shown in Figure 6, although at a low dose of METH (.3 mg/kg), GDNF-(+/+) and wild-type mice did not develop place preference, GDNF-(±) and TNF-α-(−/−) mice did [GDNF-(±): $F(7,64) = 6.493$; TNF-α-(−/−): $F(7,56) = 9.514$, $p < .05$, one-way ANOVA]. When Leu-Ile (1.5 and 15 μmol/kg, IP) was administered 1 hour before METH, it failed to exhibit a significant effect on the action of METH in GDNF-(±) and TNF-α-(−/−) mice (Figure 6). These results suggest that GDNF and TNF-α act to negate the rewarding effects of METH and are involved in the effects of Leu-Ile on these rewarding effects.

Effects of Leu-Ile on METH-Induced DA Responses

To explore the mechanisms of the inhibitory effects of Leu-Ile on METH-induced rewarding effects and sensitization, the effects of Leu-Ile on the increase in extracellular DA levels induced by repeated or single METH treatment were examined in the NAc of mice, by using an in vivo microdialysis technique. Repeated and single METH (1 mg/kg, SC) treatment caused a marked increase in extracellular DA levels in the NAc on day 9 and day 1, respectively (Figures 7A and 7B). Peaks of extracellular DA levels were 2.5- and 2.0-fold the baseline, respectively. Treatment with Leu-Ile (1.5 μmol/kg, IP) for 9 days significantly inhibited the repeated METH-induced increase in extracellular DA levels [$F(1,7) = 5.227$, $p < .05$, repeated ANOVA] (Figure 7A). In contrast, pretreatment with Leu-Ile (1.5 μmol/kg, IP) 1 hour before the single METH treatment failed to inhibit the increase in extracellular DA levels (Figure 7B).

We examined the therapeutic effect of Leu-Ile on the METH-induced increase in extracellular DA levels in the NAc of mice after the repeated treatment with METH. As shown in Figure 7C, an acute challenge with METH (1 mg/kg, SC) 5 days after withdrawal caused a marked increase in extracellular DA levels in the NAc of mice treated with vehicle for 5 days during the withdrawal from METH (1 mg/kg, SC). The peak of extracellular DA levels was 2.0-fold the baseline. Leu-Ile (1.5 μmol/kg, IP) treatment for 5 days during the withdrawal from METH significantly attenuated the METH-induced increase in extracellular DA levels [$F(1,14) = 1.689$, $p < .05$, repeated ANOVA] (Figure 7C).

Next, we examined the effect of Leu-Ile on the METH-induced decrease in synaptosomal DA uptake in the midbrain. Repeated METH treatment (1 mg/kg, SC) caused a decrease in $[^3H]$ DA uptake by 38% compared with the vehicle/saline-treated mice. Leu-Ile (1.5 μmol/kg, IP) inhibited the METH-induced decrease in synaptosomal $[^3H]$ DA uptake [$F(3,28) = 12.477$, $p < .05$, one-way ANOVA] (Figure 7D).

Finally, we examined the therapeutic effect of Leu-Ile on the METH-induced decrease in synaptosomal DA uptake in the midbrain. As shown in Figure 7E, an acute challenge with METH (1 mg/kg, SC) after treatment with vehicle for 5 days during withdrawal from METH caused a decrease in $[^3H]$ DA uptake by 51% in the midbrain compared with the saline/vehicle/METH-treated mice. Leu-Ile (1.5 μmol/kg, IP) treatment for 5 days

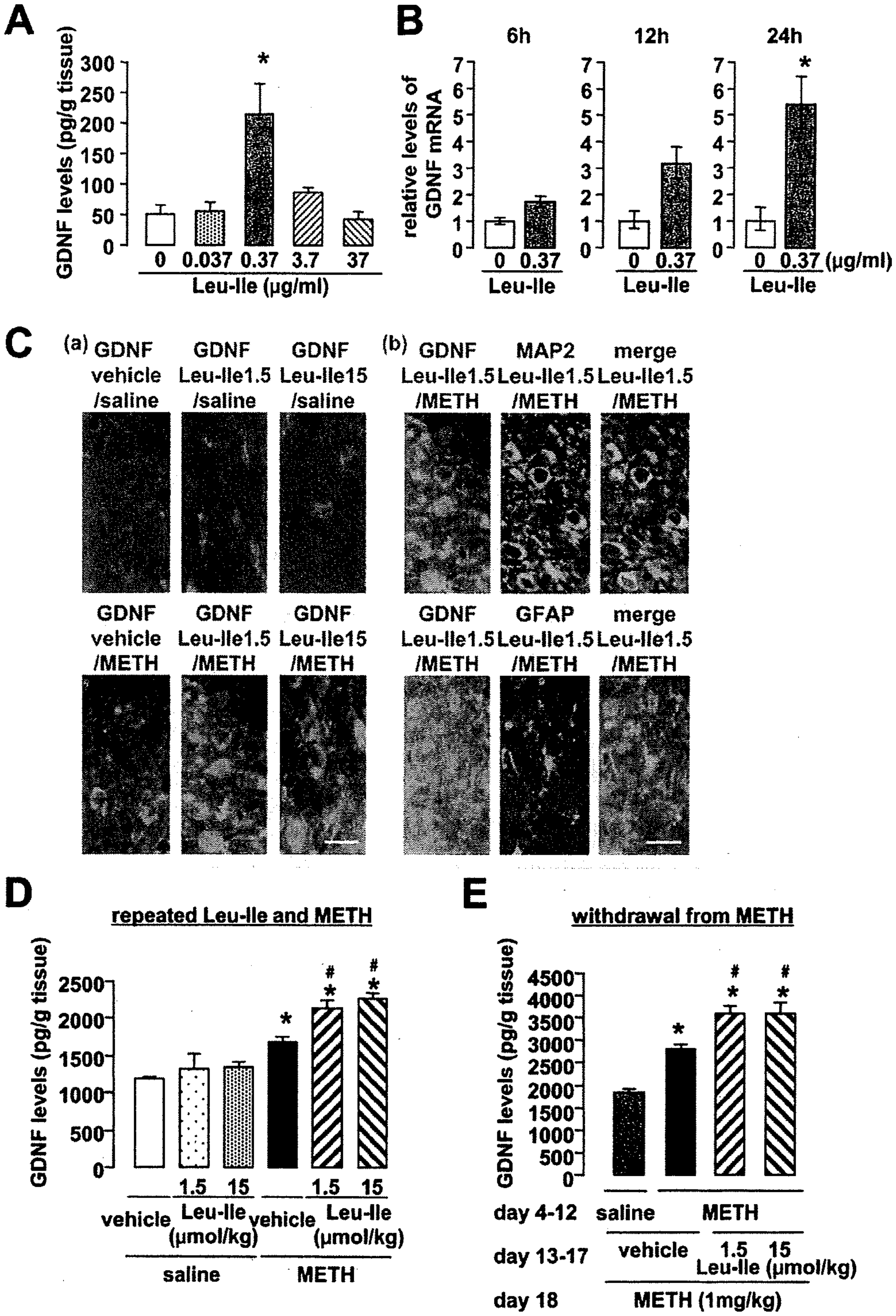

Figure 2. Effect of Leu-Ile on methamphetamine (METH)-induced increase in glial cell line-derived neurotrophic factor (GDNF) levels. **(A)** The dose-response stimulatory effects of Leu-Ile on GDNF levels in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL). The GDNF levels were determined 24 hours after the addition of Leu-Ile by enzyme immunoassay. Values are means ± SEM ($n = 5–10$). *$p < .05$ versus control subjects. **(B)** The time-course stimulatory effects of Leu-Ile on the expression of GDNF messenger RNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.37 μg/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 8$). *$p < .05$ versus control subjects. **(C)** Immunostaining of GDNF in the nucleus accumbens (NAc) after the administration of Leu-Ile and/or METH **(a)**. Double-labeling fluorescence photomicrographs for GDNF and microtuble-associated protein-2 (MAP2) or glial fibrillary acidic protein (GFAP) **(b)**. The GDNF-immunoreactive cells (red) were colocalized with MAP2-positive and GFAP-positive cells (green) in the NAc. Double immunostaining for GDNF and MAP2 or GFAP in the NAc reveals GDNF expression in neuronal and astro glial cells. Scale bars, 20 μm. **(D)** Change of GDNF levels in the NAc after the administration of Leu-Ile and/or METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days and decapitated 24 hours after the last METH injection. Values are means ± SEM ($n = 6–8$). *$p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus vehicle/METH-treated mice. **(E)** Change of GDNF levels in the NAc after Leu-Ile treatment during the withdrawal from METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 24 hours after the administration. Values are means ± SEM ($n = 6$). *$p < .05$ versus saline/vehicle/METH-treated mice. $^{\#}p < .05$ versus METH/vehicle/METH-treated mice.

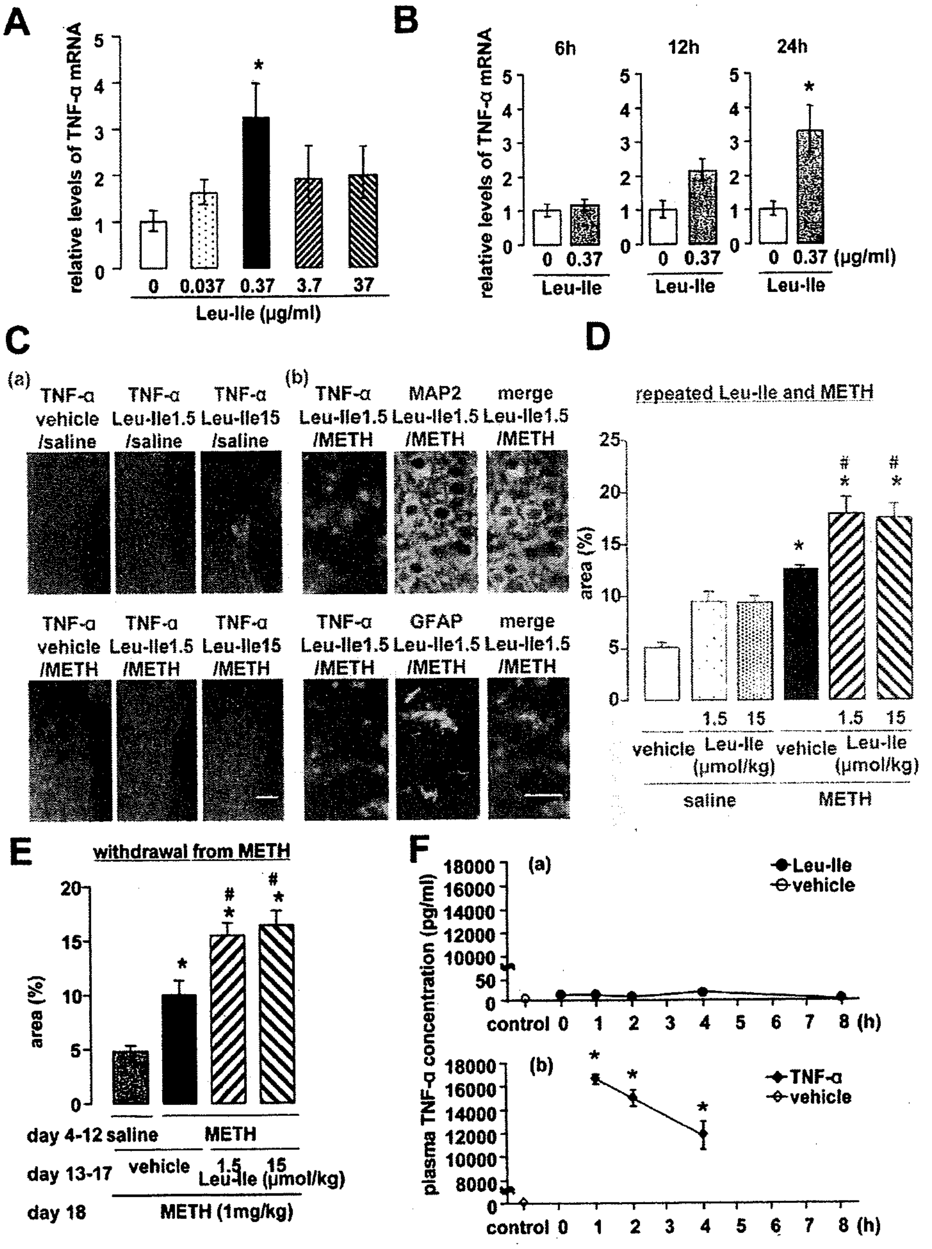

Figure 3. Effect of Leu-Ile on METH-induced increase in tumor necrosis factor-α (TNF-α) levels. **(A)** The dose-response stimulatory effects of Leu-Ile on the expression of TNF-α messenger RNA (mRNA) in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL). Total RNA was prepared 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 7$). $^*p < .05$ versus control subjects. **(B)** The time-course stimulatory effects of Leu-Ile on the expression of TNF-α mRNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing Leu-Ile (.37 μg/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of Leu-Ile. Values are means ± SEM ($n = 8$). $^*p < .05$ versus control subjects. **(C)** Immunostaining of TNF-α in the NAc after the administration of Leu-Ile and/or METH **(a)**. Double-labeling fluorescence photomicrographs for TNF-α and MAP2 or GFAP **(b)**. The TNF-α-immunoreactive cells (red) were colocalized with MAP2-positive cells (green) in the NAc. Double immunostaining for TNF-α and MAP2 in the NAc reveals TNF-α expression in neuronal cells. Scale bars, 100 **(a)** and 20 **(b)** μm. **(D)** Change of TNF-α expression in the NAc after the administration of Leu-Ile and/or METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before METH (1 mg/kg, SC) for 9 days and decapitated 24 hours after the last METH injection. The area of TNF-α positive cells in 3.8×10^4 μm^2 was estimated with the software WinRoof. Values are means ± SEM ($n = 6–8$). $^*p < .05$ versus vehicle/saline-treated mice. $^\#p < .05$ versus vehicle/METH-treated mice. **(E)** Change of TNF-α expression in the NAc after Leu-Ile treatment during the withdrawal from METH. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 24 hours after the administration. Values are means ± SEM ($n = 6–8$). $^*p < .05$ versus saline/vehicle/METH-treated mice. $^\#p < .05$ versus METH/vehicle/METH-treated mice. **(F)** Tumor necrosis factor-α concentration in peripheral blood after the treatment with Leu-Ile. Mice were treated with Leu-Ile (1.5 μmol/kg, IP), and venous blood was collected 0, 1, 2, 4, and 8 hours after the injection **(a)**. Values are means ± SEM ($n = 4$) (upper panel). Mice were treated with TNF-α (4 μg/body, IP), and venous blood was collected 1, 2, and 4 hours after the injection **(b)**. Values are means ± SEM (n = 3–4) (lower panel). The concentration of TNF-α was measured with a mouse TNF-α (upper panel) or human TNF-α enzyme-linked immunosorbent assay (ELISA) kit (lower panel). $^*p < .05$ versus vehicle-treated mice. Other abbreviations as in Figure 2.

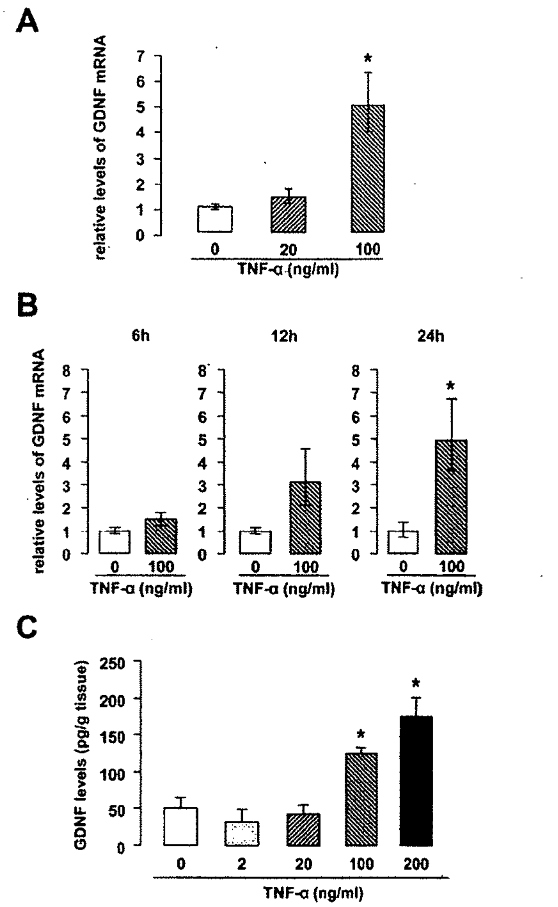

Figure 4. Regulatory effect of TNF-α on GDNF expression in cultured neurons. **(A)** The dose-response stimulatory effects of TNF-α on the expression of GDNF messenger RNA (mRNA) in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (20 and 100 ng/mL). Total RNA was prepared 24 hours after the addition of TNF-α. Values are means ± SEM ($n = 4$–5). $*p < .05$ versus control subjects. **(B)** The time-course stimulatory effects of TNF-α on the expression of GDNF mRNA in cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (100 ng/mL). Total RNA was prepared 6, 12, and 24 hours after the addition of TNF-α. Values are means ± SEM ($n = 8$–12). $*p < .05$ versus control subjects. **(C)** The dose-response stimulatory effects of TNF-α on GDNF levels in the cultured neurons. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free defined medium containing TNF-α (2, 20, 100, and 200 ng/mL). The GDNF levels were determined 24 hours after the addition of TNF-α by enzyme immunoassay. Values are means ± SEM ($n = 5$–10). $*p < .05$ versus control subjects. Other abbreviations as in Figure 2.

during the withdrawal from METH inhibited the METH-induced decrease in synaptosomal [^{3}H] DA uptake [$F(2,21) = 7.544$, $p < .05$, one-way ANOVA] (Figure 7E).

Discussion

There are currently few efficacious medications for drug dependence. Recently, it has been reported that an opioid κ receptor agonist, TRK-820, inhibits not only the rewarding effects of morphine and cocaine but also a mecamylamine-precipitated nicotine-withdrawal aversive effect (Mori *et al.* 2002; Tsuji *et al.* 2001). A DA D3 receptor partial agonist, BP897affects cocaine-associated stimulus-induced drug-seeking behavior in rats (Cervo *et al.* 2003). These medications should be effective even when they are administered after the development of drugs of abuse. In this study, the METH-induced place preference and sensitization that formed before Leu-Ile treatment were attenuated by Leu-Ile (Figures 5C and 5D), by regulating extracellular DA levels (Figures 7C and 7E). These results suggest that Leu-Ile could be a novel therapeutic agent for dependence on METH.

Leu-Ile increased GDNF levels in the cultured neurons (Figures 2A and 2B). In vivo, Leu-Ile treatment both in combination with METH and after withdrawal from repeated treatment with METH also increased GDNF levels (Figures 2D and 2E). We have previously demonstrated that Leu-Ile targets the Hsc70/Hsp90 cochaperone and, thus, triggers Akt/CREB signaling, resulting in an upregulation of GDNF expression (Cen *et al.* 2006). In addition, Leu-Ile treatment, both in combination with METH and after withdrawal from repeated treatment with METH, inhibited place preference and sensitization to METH (Figure 5). Glial cell line-derived neurotrophic factor blocks the ability of cocaine and morphine to increase levels of tyrosine hydroxylase in the VTA and blunts the biochemical and behavioral responses to chronic cocaine or morphine exposure (Messer *et al.* 2000). Glial cell line-derived neurotrophic factor decreases tyrosine hydroxylase levels in normal animals, suggesting an active down-regulation of the synthesis of this enzyme (Lu and Hagg 1997). These results suggest that Leu-Ile plays an inhibitory role in the rewarding effects and sensitization induced by METH via the induction of GDNF expression.

As described at the beginning of this article, TNF-α induces GDNF expression and blocks METH-induced dependence. Therefore, we investigated whether Leu-Ile induces the expression of TNF-α. Leu-Ile increased TNF-α mRNA levels in the cultured neurons (Figures 3A and 3B). In vivo, Leu-Ile treatment, both in combination with METH and after withdrawal from repeated treatment with METH, also increased TNF-α levels (Figures 3D and 3E) in the brain but not in the peripheral blood stream (Figure 3F). Leu-Ile can penetrate the BBB and initiate the synthesis of GDNF in the brain (Nitta *et al.* 2004). Therefore, we suggest that Leu-Ile penetrates the BBB and induces TNF-α expression only in the brain. The expression of TNF-α is induced through the activation of transcription factors such as activator protein-1 and nuclear factor-κB (NF-κB) (Guha *et al.* 2000; Rahman and MacNee 2000). Changes in transcription factors might result in long-term changes in gene expression, thereby contributing to neuronal adaptations that underlie behavioral sensitization to chronic psychostimulant treatment (Nestler 2001). Furthermore, TNF-α influences synaptic strength and transmission (Albensi and Mattson 2000; Beattie *et al.* 2002). Collectively, these observations lead to the hypothesis that Leu-Ile might have inhibitory effects on long-lasting behavioral changes induced by repeated METH treatment via the induction of TNF-α expression.

Tumor necrosis factor-α was expressed in the neurons of the NAc after the co-administration of Leu-Ile with METH (Figure 3C), whereas GDNF was expressed in the neuronal and astro glial cells (Figure 2C). Therefore, TNF-α expression induced by Leu-Ile might regulate GDNF expression in neuronal cells, although little is known about the regulation of GDNF synthesis in the brain. It has been reported that GDNF expression in astrocytes can be induced by inflammatory stimuli such as TNF-α and lipopolysaccharide (Appel *et al.* 1997). These previous reports have suggested that the induction of GDNF expression might be regulated through production of TNF-α. In the present study, TNF-α increased GDNF expression in the cultured neu-

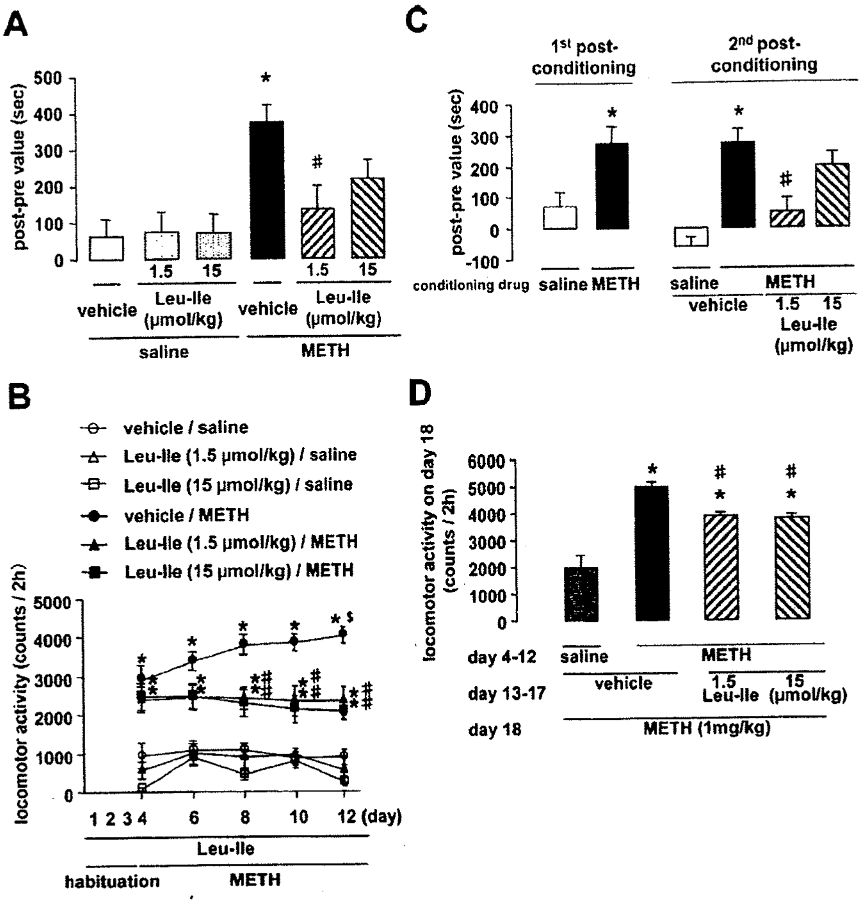

Figure 5. Effects of Leu-Ile on methamphetamine (METH)-induced place preference and hyperlocomotion/sensitization in wild-type mice. **(A)** Effect of Leu-Ile on METH-induced place preference in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving METH (1 mg/kg, SC) or saline during the conditioning. Values are means ± SEM ($n = 9–10$). $^*p < .05$ versus vehicle/saline-treated mice. $^\#p < .05$ versus vehicle/METH-treated mice. **(B)** Effect of Leu-Ile on METH-induced hyperlocomotion and sensitization in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the METH (1 mg/kg, SC) injection. Values are means ± SEM ($n = 10$). Locomotor activity was measured for 2 hours after the METH treatment. Analysis of variance with repeated measures revealed significant differences in the locomotor activity [$F(5,54) = 59.1278$, $p < .05$]. $^*p < .05$ versus vehicle/saline-treated mice. $^\#p < .05$ versus vehicle/METH-treated mice. $^\$p < .05$ versus vehicle/METH-treated mice on day 4. **(C)** Effect of Leu-Ile treatment after the development of place preference induced by METH in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from METH. Values are means ± SEM ($n = 7–10$). $^*p < .05$ versus saline/vehicle-treated mice. $^\#p < .05$ versus METH/vehicle-treated mice. **(D)** Effect of Leu-Ile treatment after the development of METH-induced sensitization in wild-type mice. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. Values are means ± SEM ($n = 10$). Locomotor activity was measured for 2 hours after the METH treatment on day 18. $^*p < .05$ versus saline/vehicle/METH-treated mice. $^\#p < .05$ versus METH/vehicle/METH-treated mice.

rons (Figure 4). Furthermore, Leu-Ile induced GDNF and TNF-α expression (Figures 2 and 3). Therefore, Leu-Ile might induce GDNF as a result of TNF-α expression to inhibit METH-induced rewarding effects and sensitization, although another signal pathway should be considered—that Leu-Ile upregulates GDNF expression by activating Hsp90/Akt/CREB signaling (Cen *et al.* 2006).

Leu-Ile increased GDNF and TNF-α expression in the cultured neurons (Figures 2A and 3A) and inhibited METH-induced place preference (Figures 5A and 5C) in bell-shaped response curves. It has been reported that TNF-α, reactive oxygen species (H_2O_2), and β-amyloid activate NF-κB in bell-shaped dose-response curves (Kaltschmidt *et al.* 1999). Rasagiline, an anti-Parkinson drug, activates NF-κB and increases GDNF in bell-shaped dose-response curves (Maruyama *et al.* 2004). We confirmed that Leu-Ile at the lower dose, 1.5 μmol/kg, which could inhibit the rewarding effects of METH, increased GDNF and TNF-α expression both in combination with METH and after withdrawal from repeated METH treatment in the CPP paradigm. Conversely, Leu-Ile at the higher dose, 15 μmol/kg, which could not inhibit the rewarding effects of METH, failed to increase GDNF and TNF-α levels (data not shown). These results suggest involvement of induction of GDNF and TNF-α expression in inhibitory effect of Leu-Ile on the rewarding effects and sensitization of METH.

Leu-Ile attenuated the METH-induced place preference (Figure 5A). Glial cell line-derived neurotrophic factor and TNF-α could be involved in the inhibitory effects of Leu-Ile on the rewarding effects of METH, because no effects of Leu-Ile were observed in the GDNF-(±) and TNF-α-(−/−) mice (Figure 6). These findings support that GDNF and TNF-α play important roles in METH-induced behavioral changes and suggest that Leu-Ile attenuates rewarding effects via the induction of GDNF and TNF-α expression.

The mesolimbic dopaminergic pathway projecting from the VTA to NAc is considered to play a major role in mediating the rewarding effects of electrical stimulation of the brain and drugs of abuse (Everitt and Wolf 2002; Koob 1992; Koob *et al.* 1998). This pathway is important not only for the rewarding effects but also for the locomotor-stimulating effects of METH (Mizoguchi *et al.* 2004; Nagai *et al.* 2005b; Nakajima *et al.* 2004). Leu-Ile inhibited METH-induced hyperlocomotion and sensitization (Figures 5B and 5D), at least in part, through the NAc, because it showed inhibitory effects on the increase in extracellular DA levels induced by repeated METH treatment (Figures 7A and 7C) and the decrease in synaptosomal DA uptake (Figures 7D and 7E) in the NAc. Although Leu-Ile failed to inhibit the hyperlocomotion induced by single METH treatment, it inhibited repeated METH-induced sensitization (Figure 5B). These results suggest that Leu-Ile has inhibitory effects on neuronal plasticity induced by repeated METH treatment but not on single METH-induced hyperlocomotion or the increase in extracellular DA levels (Figure 7B). Because acute treatment of Leu-Ile failed to inhibit single METH-induced hyperlocomotion and the increase in extracellular DA levels, the induction of GDNF and TNF-α expression requires repeated treatment of Leu-Ile.

We have previously reported that TNF-α attenuates the METH-induced increase in extracellular DA levels and potentiates DA uptake into synaptic vesicles and negates METH-induced inhibition of DA uptake in the striatum (Nakajima *et al.* 2004). We have also demonstrated that the tissue plasminogen activator-plasmin system accelerates the release of DA, which is involved in the rewarding effects of METH and morphine (Nagai *et al.* 2004, 2005a, 2005b). In the present study, we demonstrated that Leu-Ile attenuated the increase in extracellular DA levels in the NAc induced by repeated METH treatment (Figures 7A and 7C) and negated METH-induced inhibition of DA uptake in the midbrain (Figures 7D and 7E). The inhibition of the METH-induced increase in extracellular DA levels and decrease of DA uptake by repeated Leu-Ile treatment might be one plausible mechanism by which Leu-Ile inhibits METH-induced chronic behavioral changes via the induction of TNF-α expression. One might consider that TNF-α could attenuate the rewarding effects and sensitization of other drugs of abuse if it activates DA uptake and thereby attenuates the METH-induced increase in extracellular DA levels. Our results have shown that TNF-α or Leu-Ile inhibits morphine-induced place preference and sensitization by regulating extracellular DA levels (Niwa M, Nitta A, Yamada Y, Nakajima A, Saito K, Seishima M, Noda Y, and Nabeshima T, unpublished observations). These observations support our hypothesis about effect of Leu-Ile on drug abuse.

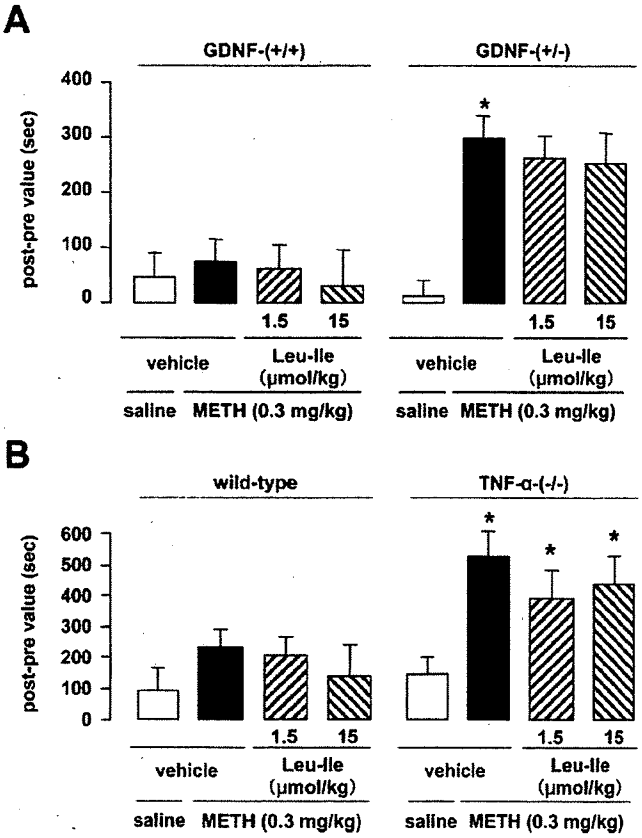

Figure 6. Effect of Leu-Ile on METH-induced place preference in GDNF-(±) and TNF-α-(−/−) mice. **(A)** Effect of Leu-Ile treatment on METH-induced place preference in GDNF-(±) mice. Mice were treated with METH (.3 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 7–12). *$p < .05$ versus vehicle/METH-treated GDNF-(+/+) mice. **(B)** Effect of Leu-Ile treatment on METH-induced place preference in TNF-α-(−/−) mice. Mice were treated with METH (.3 mg/kg, SC) or saline during the conditioning. Values are means ± SEM (n = 8). *$p < .05$ versus vehicle/METH-treated wild-type mice. Abbreviations as in Figure 2.

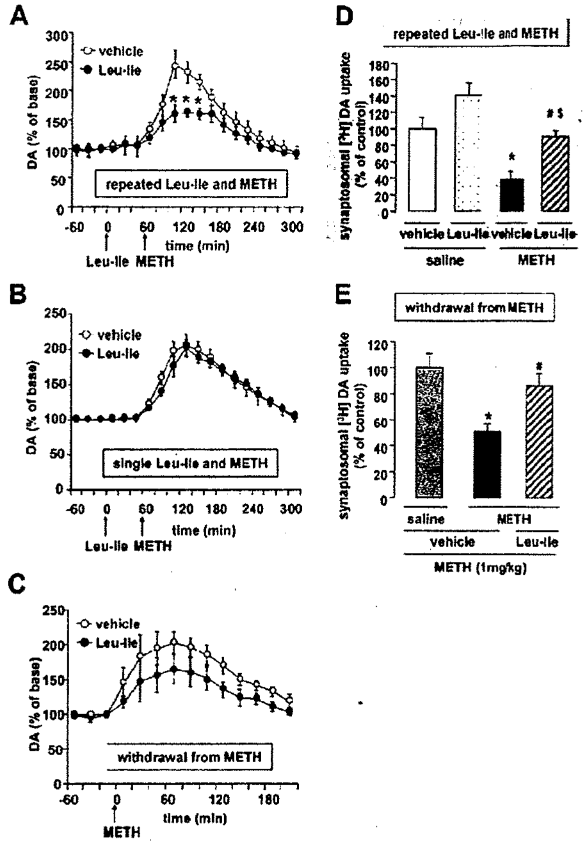

Figure 7. Effect of Leu-Ile on methamphetamine (METH)-induced dopamine (DA) responses. **(A)** In vivo effect of Leu-Ile on the increase in extracellular DA levels induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 µmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days. Basal extracellular DA levels were .50 ± .10 and .81 ± .35 nmol/L for the vehicle/METH- and Leu-Ile/METH-treated mice, respectively. Values are means ± SEM (n = 4–5). *$p < .05$ versus vehicle/METH-treated mice. **(B)** In vivo effect of Leu-Ile on the increase in extracellular DA levels induced by single METH treatment. Mice were treated with Leu-Ile (1.5 µmol/kg, IP) once, 1 hour before receiving METH (1 mg/kg, SC). Basal extracellular DA levels were .81 ± .09 and .80 ± .12 nmol/L for the vehicle/METH- and Leu-Ile/METH-treated mice, respectively. Values are means ± SEM (n = 5). **(C)** In vivo effect of Leu-Ile treatment during the withdrawal from METH on the METH-induced increase in extracellular DA levels. Mice were treated with Leu-Ile (1.5 µmol/kg, IP) for 5 days during the withdrawal from METH (1 mg/kg, SC). Basal extracellular DA levels were .27 ± .02 and .24 ± .01 nmol/L for the METH/vehicle- and METH/Leu-Ile-treated mice, respectively. Values are means ± SEM (n = 8). **(D)** Effect of Leu-Ile on the decrease in synaptosomal DA uptake induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 µmol/kg, IP) 1 hour before METH (1 mg/kg, SC) once/day for 9 days and decapitated 1 hour after the last METH treatment. The synaptosomal [^{3}H] DA uptake was .47 ± .10, .61 ± .10, .13 ± .03, and .39 ± .05 pmol/4-min/mg protein for vehicle/saline-treated, Leu-Ile/saline-treated, vehicle/METH-treated, and Leu-Ile/METH-treated mice, respectively. The final concentration of [^{3}H] DA was 5 nmol/L. Values are means ± SEM (n = 8). *$p < .05$ versus vehicle/saline-treated mice. $^{\#}p < .05$ versus Leu-Ile/saline-treated mice. $^{\$}p < .05$ versus vehicle/METH-treated mice. **(E)** The therapeutic effect of Leu-Ile on the decrease in synaptosomal DA uptake induced by repeated METH treatment. Mice were treated with Leu-Ile (1.5 µmol/kg, IP) for 5 days after the development of METH (1 mg/kg, SC)-induced sensitization. The next day, the mice were administered only METH (1 mg/kg, SC) and decapitated 1 hour after the administration. The synaptosomal [^{3}H] DA uptake was .50 ± .06, .25 ± .03, and .43 ± .05 pmol/4-min/mg protein for saline/vehicle/METH-treated, METH/vehicle/METH-treated, and METH/Leu-Ile/METH-treated mice, respectively. The final concentration of [^{3}H] DA was 5 nmol/L. Values are means ± SEM (n = 8). *$p < .05$ versus saline/vehicle/METH-treated mice. $^{\#}p < .05$ versus METH/vehicle/METH-treated mice.

It has been reported that Leu-Ile induces the expression of brain-derived neurotropic factor (BDNF) (Nitta *et al.* 2004) in addition to that of GDNF and TNF-α. Infusion of BDNF into the NAc enhances the stimulation of locomotor activating by cocaine in rats, whereas the development of sensitization and CPP is delayed in heterozygous BDNF knockout mice compared with wild-type littermates (Hall *et al.* 2003; Horger *et al.* 1999). These results suggest a possible role for BDNF in long-term adaptations of the brain to cocaine (Yamada and Nabeshima 2004). In the present study, we targeted anti-addictive factors like GDNF and TNF-α but not pro-addictive factors like BDNF to find a new therapeutic agent for drug dependence. As shown in bell-shaped dose-response curves described previously, the narrow effective dose range of Leu-Ile might be due to the balance of level between anti- and pro-addictive factors induced by Leu-Ile.

Our findings suggest that Leu-Ile has inhibitory effects on METH-induced rewarding effects and sensitization by negating the METH-induced inhibition of DA uptake as well as attenuating the METH-induced increase in extracellular DA levels in the NAc via the induction of GDNF and TNF-α expression. Leu-Ile could be a novel therapeutic agent for METH-induced dependence.

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Tumor Necrosis Factor-α and Its Inducer Inhibit Morphine-Induced Rewarding Effects and Sensitization

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Background: Tumor necrosis factor-α (TNF-α) is emerging as an important modulator of the function of the central nervous system (CNS). We have demonstrated that TNF-α or Leu-Ile, a TNF-α inducer, inhibits methamphetamine-induced rewarding effects and sensitization. In this study, we investigated the effects of TNF-α or Leu-Ile on morphine (MOR)-induced rewarding effects and sensitization.

Methods: Levels of TNF-α messenger RNA (mRNA) and protein were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. Effects of TNF-α or Leu-Ile on MOR-induced rewarding effects and sensitization were investigated by conditioned place preference and locomotor activity tests. Extracellular dopamine levels were examined using in vivo microdialysis. Effects of TNF-α or Leu-Ile on MOR-induced antinociceptive effect and withdrawal symptoms were examined by hot plate test and naloxone-preciptated withdrawal.

Results: Morphine induced TNF-α mRNA expression via dopamine and opioid receptors. Posttreatment with TNF-α or Leu-Ile attenuated the MOR-induced place preference and sensitization even after their development, as well as pretreatment with TNF-α or Leu-Ile blocked them. An inhibitory effect of Leu-Ile on MOR-induced place preference was not observed in TNF-α knockout mice. Tumor necrosis factor-α or Leu-Ile inhibited the increase in extracellular dopamine levels in the nucleus accumbens induced by repeated MOR treatment.

Conclusions: These results suggest that TNF-α inhibits MOR-induced rewarding effect and sensitization by regulating extracellular dopamine levels, and Leu-Ile inhibits them via the induction of TNF-α.

Key Words: Dopamine (DA), Leu-Ile, morphine (MOR), rewarding effect, sensitization, tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) plays an important role in a variety of infectious, inflammatory, and autoimmune conditions (Vassali 1992). Tumor necrosis factor-α also affects the central nervous system (CNS) directly or indirectly through the stimulation of vagal afferents (Maier and Watkins 1998). Thus, this proinflammatory cytokine is emerging as a modulator of CNS function. Regarding the behavioral effects of TNF-α, transgenic mice expressing high levels of TNF-α in the brain showed several changes in exploratory activity and emotional behavior in association with reduced tyrosine hydroxylase (TH) immunoreactivity in the caudate putamen (CPu) without neuronal cell death (Aloe and Fiore 1997). On the other hand, TNF-α knockout mice show anxiogenic-like behavior accompanied by an increase in serotonin metabolism (Yamada *et al.* 2000).

Recently, we have demonstrated that TNF-α plays a neuroprotective role in methamphetamine (METH)-induced drug dependence and neurotoxicity by inhibiting the METH-induced increase in extracellular dopamine (DA) levels through activation of plasmalemmal dopamine transporter (DAT) as well as vesicular monoamine transporter-2 (Nakajima *et al.* 2004). Furthermore, we have demonstrated that Leu-Ile, which induces glial cell line-derived neurotrophic factor (GDNF) production via TNF-α synthesis, inhibits METH-induced rewarding effect and sensitization by regulating extracellular DA levels in the nucleus accumbens (NAc) (Niwa *et al*, in press).

The psychostimulative effects of METH are associated with an increase in extracellular DA levels in the brain by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Giros *et al.* 1996; Heikkila *et al.* 1975; Kalivas and Stewart 1991; Seiden *et al.* 1993). It is well known that drugs of abuse, including METH and morphine (MOR), modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area (VTA) of the midbrain to the NAc (Koob 1992, 1998; Wise 1996). Brain DA systems have also been focused on in histochemical, biochemical, and pharmacological research into psychological dependence on opioids, such as MOR (Funada *et al.* 1993; Narita *et al.* 2001). Morphine increases dopaminergic neurotransmission in the NAc via the activation of DA cells in the VTA, an area with a high density of μ-opioid receptors. This activation results mainly from the disinhibition of inhibitory γ-aminobutyric acid (GABA)ergic interneurons in the VTA (Bonci and Williams 1997; Johnson and North 1992). Various studies have provided substantial evidence to support roles for mesolimbic dopaminergic transmission in the rewarding effects of and behavioral sensitization to opioids (Vezina and Stewart 1984). Further, it has been proposed that activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (Nestler 2001).

We hypothesized that those genes whose expression was altered by repeated administration of METH and MOR could be candidates for drug-dependence-related genes, because both METH and MOR increase dopaminergic neurotransmis-

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