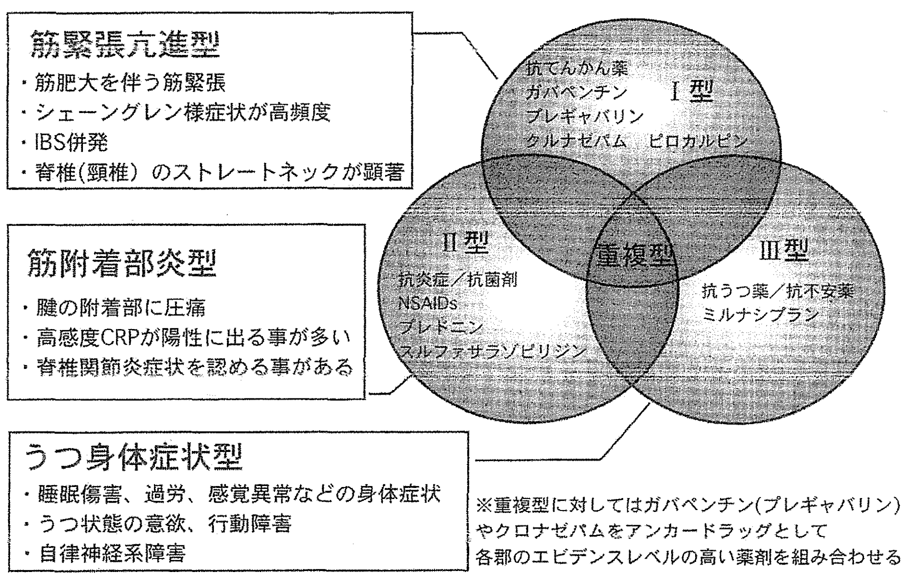

図　日本纖維筋痛症診療ガイドライン2009より改変

て、全身性に痛みを生じるタイプの多くが情動由来のⅢ型に含まれる。線維筋痛症では、発症の背景の多くに幼少時の生活環境に大きな精神ストレスのトラウマを抱えているケースが多く報告され発症のリスクファクターとして注目されている。また上記で示した筋痛病態のような筋肉に異常も認められず、診断そのものが困難である事から、潜在的患者は報告されている罹患率よりも多い事が推定される。Ⅲ型には抗うつ薬が有効な例が多いが治療の程度は個人差が大きく、安定した特効薬というよりはむしろ個人に効果的な種を探索しながら慎重に用いられる事が多い。またこの他に、精神を安定化する目的で抗不安薬や睡眠薬が用いられる。

4） 重複型

上記の分類１～３それぞれを重複している患者は全体の25％と言われている。重症度レベルが高い症例が多い。ガバペンチン、プレガバリンをアンカードラッグとして上記の薬物と組み合わせて処方されるのが一般的なようである。

2．鎮痛薬の基礎メカニズム

上記１で示したように同じ線維筋痛症の診断名であっても病態的には様々な分類に分けられる事から、患者個人に対応した薬物治療が重要となる。近年では、鎮痛薬に分類されていないが、鎮痛補助薬として効果を示すものが多く使用されている。これらの薬物を処方する時は、患者に使用目的が疼痛緩和である旨を十分に説明する必要性が求められる。

1） 抗炎症薬

抗炎症薬とは、浮腫・紅斑・疼痛・発熱などの炎症症状を抑制する薬物でステロイド性と非ステロイド性に大きく分けられる。ステロイド性抗炎症薬は生体膜を通過後にグルココルチコイド受容体蛋白質と結合し、様々な遺伝子発現を調節する。抗炎症作用は、プロスタグランジン類やロイコトルエン類の合成に重要なホスホリパーゼＡ２の直接的・間接的抑制誘導の他、免疫機能抑制などによる。非ステロイド性抗炎症薬は一般的な筋骨格系の痛みに対する第一選択薬として処方される事が多い。シクロオキシゲナーゼを阻害し、痛みの生理活性物質であるプロスタグランジン類の産生を抑制する他、直接抗炎症作用を有するとの報告もある。天井効果があるために、増量しても作用持続は延長するが一定以上の鎮痛効果は認められない場合がある。線維筋痛症では炎症を伴わない症例が多い事から、有用性が低い事が懸念される。

2） オピオイド

オピオイドは一般的にがん性疼痛に使用され、慢性の非がん性疼痛に対しては使用される事は少ない。その理由としては、薬物乱用の誤った知識による偏見や、適切な使用法や副作用に関する理解度が低い事があげられる。しかしながら、正確な知識を

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もって使用すれば最も強力な鎮痛薬である。ただし線維筋痛症では、有効であった例も一部報告されているものの効果が低い事が懸念される報告が多いようである[1-4]。オピオイド作用と抗うつ作用を有するトラマドール（商品名：トラマール）には有効例が報告されているが個人差が大きく、いずれにせよ詳細は明らかでない[5]。

３） 抗てんかん薬、抗けいれん薬

Ⅰ型に多く使用される抗てんかん薬は、筋弛緩作用および鎮痛作用を目的に使用される[6]。カルバマゼピン（商品名：テグレトール）は三環系抗うつ薬に構造が近似した化合物であり、筋弛緩作用、抗躁作用、鎮痛作用を有する。三叉神経痛に適応のある唯一の抗てんかん薬である事から、疼痛目的に使用される頻度が最も多い。主な作用機序は、電位依存性のナトリウムチャネル遮断作用で、知覚神経のAδ線維、C線維の過剰興奮に対して自発性発火を抑制（発火頻度依存性遮断）する。クロナゼパム（商品名：リボトリール、ランドセン）は、抑制系GABA神経伝達の増強と考えられており、構造がベンゾジアゼピン系に類似する事から抗不安作用、筋弛緩作用も有する。近年、神経因性疼痛においてバルプロ酸（商品名：デパケン）のHDAC（ヒストン脱アセチル化酵素）阻害による鎮痛効果が注目されているが線維筋痛症における効果は不明である。

４） ガバペンチン、プレギャバリン

ガバペンチン（商品名：ガバペン）はGABAと類似構造をもつ抗てんかん薬の一種であるが、GABA神経を介する神経伝達系よりむしろカルシウムチャネルα2δ-サブユニットにおける効果が主である。近年では帯状疱疹後神経痛や糖尿病性ニューロパシーのような神経障害性疼痛の第一選択薬として鎮痛効果が期待されている。類似薬としてのプレギャバリン（商品名：リリカ）は2010年に神経障害性疼痛に対する保険適応薬として処方が可能となった。線維筋痛症においても高い鎮痛効果を示した例が比較的多く報告されている[7-10]。これらをアンカードラッグとして他の薬物を併用する事が多い。

５） 抗うつ薬

抗うつ薬は、主にⅢ型疾患に多く用いられる。うつや不安症状を併発している症例が多いため、これらを軽減する目的もあるが、抗うつ薬自身のもつ鎮痛効果が線維筋痛症患者においては有効な例が報告されているためであると考えられる[11-13]。抗うつ薬は三環系、四環系、SNRI（セロトニン・ノルアドレナリン再取り込み阻害薬）、SSRI（選択的セロトニン再取り込み阻害薬）と、海外では新たにNaSSA（ノルアドレナリン作動性・特異的セロトニン作動性）が発売されており国内でもあらたな治療手段の一つとして期待されている。一般的には副作用を最小限にするために少量から投薬を開始し、増量していく。アミトリプチリン（商品名：トリプタノール）やイミプラミン（商品名：トフラニール）のような三環系あるいは四環系ミアンセリン（商品名：テトラミド）などの鎮痛効果は、セロトニン・ノルアドレナリンの再取り込み阻害作用の他、α1受容体遮断作用、ナトリウムチャネル遮断作用、カルシウムチャネル遮断作用、NMDA受容体拮抗作用などが関与する可能性が報告されている。

これに対し、ミルナシプラン（商品名：トレドミン）に代表されるSNRIやSSRI（パロキセチン（商品名：パキシル）、フルボキサミン（商品：ルボックス））は神経接合部のセロトニン・ノルアドレナリンの再取り込みを阻害する事により効果を示すが、シナプス後膜の受容体には直接親和性をもたないため、抗コリン作用などの副作用が少ない。抗うつ薬はそれぞれ特徴ある薬理作用を持つため、効果を認めない場合には他の抗うつ薬で治療にあたる。効果のある薬剤には個人差があるため、どの薬理作用が効果を示したのかは不明であるが、臨床経験的に治療には積極的に用いられ効果のあった例も多数報告されている。

６） 抗不安薬・睡眠薬

抗不安薬および睡眠薬は直接的な鎮痛効果というより主に鎮静目的で用いられる事が多い。線維筋痛症の場合、特にⅢ型患者に不眠症状によるQOL（生活の質）の低下が知られている。不安やうつ症状は痛みと密接に関連しているため、軽度の線維筋痛症患者には、この不快な情動を緩和する事で痛みが軽減する例もあるようである。

７） その他

ノイロトロピン（商品名：ノイロトロピン）は、明確な作用機序は不明であるが疼痛制御機構である

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表　線維筋痛症に使用される薬物一覧

	一般名	商品名
抗うつ薬	イミプラミン	トフラニール
	アミトリプチリン	トリプタノール
	セルトラリン	ジェイゾロフト
	ミルナシプラン	トレドミン
	フルボキサミン	ルボックス
	パロキセチン	パキシル
抗てんかん薬	ガバペンチン	ガバペン
	プレガバリン	リリカ
	カルバマゼピン	テグレトール
	クロナゼパム	リボトリール
	バルプロ酸	デパケン
オピオイド関連薬	モルヒネ	モルヒネ
	トラマドール	トラマール
	ナルトレキサン	

	一般名	商品名
抗不安薬・睡眠薬	アルプラゾラム	ソラナックス
	エチゾラム	デパス
	ゾルピデム	マイスリー
	フルニトラゼパム	ロヒプノール
	ゾピクロン	アモバン
	トリアゾラム	ハルシオン
	ロラゼパム	ワイパックス
抗炎症薬	アスピリン	アスピリン
	イブプロフェン	イブプロフェン
	ナプロキセン	ナイキサン
	ロキソプロフェン	ロキソニン
	インドメタシン	インフリー
	ジクロフェナク	ボルタレン
抗リウマチ薬	サラゾスルファピリジン	アザルフィジン
その他	ノイロトロピン	ノイロトロピン
	ケタミン	ケタミン

下行性抑制系を賦活化する事で鎮痛作用を示すと考えられている[14]。錠剤で処方される事もあるが、多くの場合静脈注射あるいはトリガーポイント注射が行われる事があるようである。その他に、海外の報告であるがNMDA拮抗作用のもつケタミンが有効であった例が報告されている[15,16]。神経興奮に関与するNMDA受容体に作用し、痛みにともなう神経興奮を抑制するためと考えられているが、詳細は不明である。しかしケタミンは副作用や乱用の恐れから使用頻度は低い。同様にNMDA拮抗作用をもつ薬物にデキストロメトルファン（商品名：メジコン）なども知られている。デキストロメトルファンをオイピオドと併用する事で、少量のオピオイドが有効である事が報告されている[17]。

３．科学的根拠に基づく新たな治療戦略

これまで線維筋痛症における治療は、経験的な薬物療法で得られた情報によるものであった。一般的に新薬の開発や治療薬のスクリーニングではヒトを対象とする前に細胞や実験動物における検証がなされる。しかしながら線維筋痛症は組織損傷や炎症とは異なる特殊な病態であり、それらの動物モデルでは応用できなかった。線維筋痛症病態モデルとしては、筋痛モデル[18]やストレスを繰り返し曝露する事で痛みを発症する亅型様モデル（ICSモデル）が提案されている[19]。

薬理学的解析としてこれらのモデルに対してモルヒネを投与した場合、筋痛モデルでは高用量のモルヒネに有効な鎮痛効果が報告されている[2]。一方、ストレス性のICSモデルではモルヒネの効果は無効で[20]、臨床効果を反映している。この事によりモルヒネの作用機序に及ぼす影響が発症要因の違いにより異なる可能性が考えられる。ICSモデルにおいては特に中枢（脳室内）における鎮痛効果が極端に低く、異常機構として下降性抑制系に関連するセロトニン神経系の減弱を認めている。近年、臨床研究において標的因子としてのセロトニンの重要性を裏付ける科学的根拠になりうると考えられる。

また、治療効果が高いとされるガバペンチンは筋痛モデルおよびICSモデルともに有効な鎮痛効果を示す事が明らかにされている[21]。ICSモデルでは少用量でも鎮痛効果を示し、特に中枢に投与すると鎮

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痛効果の持続性を認めている。中枢移行性の高い類似薬の開発へ応用するだけでなく、中枢性機構および作用点のカルシウムチャネルに着目する事で、線維筋痛症の原因解明に対する手掛かりになりうるのではないかと考えられる。

線維筋痛症はその病態の特異性が今まで不明な点が多かったが、上記２に示すような薬物療法が積極的に行われている。しかし、その個体差が大きいため慎重な治療計画の設計が必要である。また、オピオイドや抗うつ薬などの特殊な薬物をはじめとして多剤併用療法をおこなう際には、各専門医との連携によって的確な治療をおこなう事が重要である。効果的と考えられる薬物の中に線維筋痛症に適応とされる薬物はなく、それぞれの薬物を保険適応内で服用するためには併発症状に対する治療という名目を用いるのが現状であるようだが、動物モデルを用いた科学的エビデンスを臨床にフィードバックする事で今後線維筋痛症適応の薬物が増える可能性期待される。また、臨床研究で遺伝子多型解析や脳内fMRI、SPECT画像解析などによる中枢機構の異常が報告されているようにこれらのターゲット領域をさらに分析し基礎研究と連携する事で、より有効性の高い治療方針の確立が期待される。

【編集】日本ペインクリニック学会神経障害性疼痛
薬物療法ガイドライン作成ワーキンググループ
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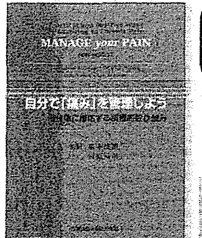
痛みの診療に携わるすべての医療従事者を対象とした、日本ペインクリニック学会編集の本邦発の神経障害性疼痛に対する薬物療法ガイドライン



自分で「痛み」を管理しよう
慢性痛に順応する積極的取り組み

【監訳】坂本篤裕　日本医科大学麻酔科学講座 主任教授
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疼痛関連医療従事者の道標となるとともに、患者自身の痛みへの理解と患者自ら痛みを管理しようとするモチベーションを高めるための書

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The Selective Serotonin Reuptake Inhibitor Paroxetine, but not Fluvoxamine, Decreases Methamphetamine Conditioned Place Preference in Mice

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Abstract: Monoamine transporters are the main targets of methamphetamine (METH). Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), decreased METH conditioned place preference (CPP), suggesting that serotonin transporter (SERT) inhibition reduces the rewarding effects of METH. To further test this hypothesis, in the present study we investigated the effects of additional SSRIs, paroxetine and fluvoxamine, on METH CPP in C57BL/6J mice. In the CPP test, pretreatment with 20 mg/kg paroxetine abolished the CPP for METH, whereas pretreatment with 100 mg/kg fluvoxamine prior to administration of METH failed to inhibit METH CPP. These results suggest that paroxetine, a medication widely used to treat depression, may be a useful tool for treating METH dependence. Further, these data suggest that molecules other than the SERT [such as G protein-activated inwardly rectifying K^+ (GIRK) channels] whose activities are modulated by paroxetine and fluoxetine, but not by fluvoxamine, are involved in reducing METH CPP by paroxetine and fluoxetine.

Keywords: Conditioned place preference, Fluvoxamine, Methamphetamine, Mice, Paroxetine, Serotonin transporter.

INTRODUCTION

Methamphetamine (METH) is abused in worldwide [1]. In Japan, the number of people arrested for METH possession or use is approximately 100 times higher than those arrested for cocaine, opioids, or cannabis. Further, METH frequently induces psychotic states with symptoms similar to those seen in paranoid schizophrenia [2]. Such psychotic states are treated primarily in hospitals resulting in high medical costs. Thus, there is great need for the discovery of new medications for METH abuse [3] because the current treatments are mostly oriented toward the treatment of psychosis with no treatments available to prevent relapse to METH abuse.

The dopamine transporter (DAT) is the main target for METH and cocaine. However, mice lacking the DAT show conditioned place preference (CPP) to cocaine [4] and self-administer cocaine [5]. Interestingly, heterozygous and homozygous serotonin transporter (SERT) knockout mice that also have a homozygous knockout of the DAT do not exhibit cocaine CPP [6]. Cocaine administration leads to increases in extracellular dopamine concentration in the striatum of DAT knockout mice but not of DAT/SERT double knockout mice [7]. Taken together, these reports suggest that SERT inhibition may decrease METH and cocaine CPP.

Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), abolished METH CPP when METH was administered during both the development and expression phases of the CPP procedure, supporting the hypothesis that SERT inhibition decreased the rewarding effects of METH [8]. To further test this hypothesis, in the present study we investigated the effects of the SSRIs paroxetine (Paxil®) and fluvoxamine (Lubox® or Depromel®) on METH CPP.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (8-10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed for 1-2 weeks before the experiments began in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12/12 h light/dark cycle with lights on at 8:00 am. Food and water were available *ad libitum*. All behavioral testing was conducted during the light phase. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Conditioned Place Preference (CPP) Test

The CPP test was performed according to the method of Hoffman and Beninger [9] with some modifications. We used a two-compartment Plexiglas chamber (Neuroscience Inc., Osaka, Japan). One compartment (17.5 × 15 × 17.5 cm: width × length × height) was black with a smooth floor, and the other compartment was of the same dimensions, but with a white textured floor. This two-compartment chamber was located in a sound- and light-attenuated box under conditions

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of dim illumination (approximately 40 lux) to reduce bias toward either compartment [10]. Mice were assigned randomly to the treatment groups (see below).

On Day 1, the mice (n = 14-26 per group) were allowed to freely explore the two compartments for 15 min. On Day 2, the mice again were allowed to explore the two compartments freely for 15 min, and the time spent in each compartment and the number of transitions between compartments were measured. Conditioning sessions then were conducted once daily for 4 consecutive days (Days 5-8). For the Day 5 conditioning session, mice were i.p. injected with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine) 60 min before injection with METH (2 mg/kg, i.p.). Immediately after METH administration, mice were confined to the black or white compartment for 50 min. On Day 6, the mice were pretreated with the same solution (saline or SSRI, i.p.) 60 min before a saline injection. Immediately after the saline injection, mice were confined to the opposite compartment for 50 min. On Days 7 and 8, the same conditioning as on Days 5 and 6 was repeated. On Day 9, the mice were pretreated with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine, i.p.), and 60 min later were allowed to freely explore the two compartments for 15 min without METH injection. The time spent in each compartment and the number of transitions between compartments were measured. In summary, there were a total of eight groups in this experiment corresponding to the four pretreatments (paroxetine, fluvoxamine, saline; there were two saline groups that were run concurrently with the paroxetine and fluvoxamine groups) and the two phases of the experiment during which they were pretreated with the drug (conditioning days 5-8 or test day 9). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning exploratory phase (Day 2). The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning exploratory phase (Day 2).

Drugs

Methamphetamine hydrochloride was purchased from Dainippon Pharmaceutical (Osaka, Japan). Paroxetine maleate and fluvoxamine maleate were purchased from Sigma (St. Louis, MO, USA) and TOCRIS (Hung Road, Bristol, UK), respectively. All drugs were dissolved in saline. Drugs and vehicle were administered i.p. in a volume of 0.1 ml/10 g body weight. All drug doses are reported as salt.

Statistical Analyses

The CPP and transition scores of mice pretreated with saline or SSRI during the conditioning and CPP test phases were subjected to a two-way analysis of variance (ANOVA). The ANOVA had two between-subjects factors, each with two levels (saline/SSRI pretreatment in the conditioning phase and saline/SSRI pretreatment in the CPP test phase). Two separate ANOVAs were conducted on the paroxetine and fluvoxamine data. Similar ANOVAs were conducted on the transition scores. The CPP scores from the paroxetine experiment were subjected to a one-way ANOVA followed by *post hoc* comparisons with the Scheffe test. In this ANOVA, there were four levels corresponding to the four treatment conditions (saline in both the conditioning and the CPP test phases, pretreatment with paroxetine only in the conditioning phase, pretreatment with paroxetine only in the CPP test phase, pretreatment with paroxetine in both the conditioning and the CPP test phases). For the CPP data, the durations of time that the mice spent in the METH-paired compartment before and after conditioning were compared using paired t-tests for each group. For the transition data, the number of transitions between the METH-paired compartment and the saline-paired compartment before and after conditioning were compared using paired t-tests for each group. The level of significance was set at 0.05.

RESULTS

Effects of Paroxetine on METH CPP

The two-way ANOVA revealed that mice treated with paroxetine during the test phase exhibited decreased CPP scores compared to mice treated with saline during the test phase ($F_{1,72}$ = 7.888, $P < 0.01$), whereas mice treated with paroxetine during the conditioning phase did not differ significantly from mice treated with saline during the test phase in the CPP score [$F_{1,72}$ = 1.704, not significant (n.s.); Fig. (**1A**)]. There was no statistically significant interaction between the factor saline/paroxetine during the conditioning phase and the factor saline/paroxetine during the CPP test phase ($F_{1,72}$ = 0.1690, n.s.), indicating that the important factor was treatment with paroxetine during the expression phase of the experiment. In addition, a one-way ANOVA on the CPP scores was conducted on data for all four groups. The ANOVA showed a significant difference in the CPP scores among these four groups ($F_{3,72}$ = 3.940, $P < 0.05$). The Scheffe *post hoc* test showed that the CPP score of the paroxetine/paroxetine group was significantly lower than that of the saline/saline group ($P < 0.05$). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups (Fig. (**1B**)). Whereas the saline/saline and paroxetine/saline groups spent significantly more time in the METH-paired compartment after conditioning than before conditioning (saline/saline: n = 23, df = 22, t = -6.050, $P < 0.001$; paroxetine/saline: n = 15, df = 14, t = -2.884, $P < 0.05$), the saline/paroxetine and paroxetine/paroxetine groups did not show METH CPP (saline/paroxetine: n = 15, df = 14, t = -2.033, n.s.; paroxetine/paroxetine: n = 23, df = 22, t = -0.908, n.s.). Paroxetine pretreatment had no significant effects on the transition scores compared to the saline/saline treatment group (data not shown).

Effects of Fluvoxamine on the METH CPP

The two-way ANOVA revealed that both the factor saline/fluvoxamine pretreatment during the conditioning phase and the factor saline/fluvoxamine pretreatment during the CPP test phase had no effects on CPP scores (conditioning phase: $F_{1,68}$ = 0.045, n.s.; CPP test phase: $F_{1,68}$ = 3.016, n.s.; Fig. (**2A**)). There was no statistically significant interaction between the two factors ($F_{1,68}$ = 0.066, n.s.). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups. All four groups spent significantly more time in the METH-paired

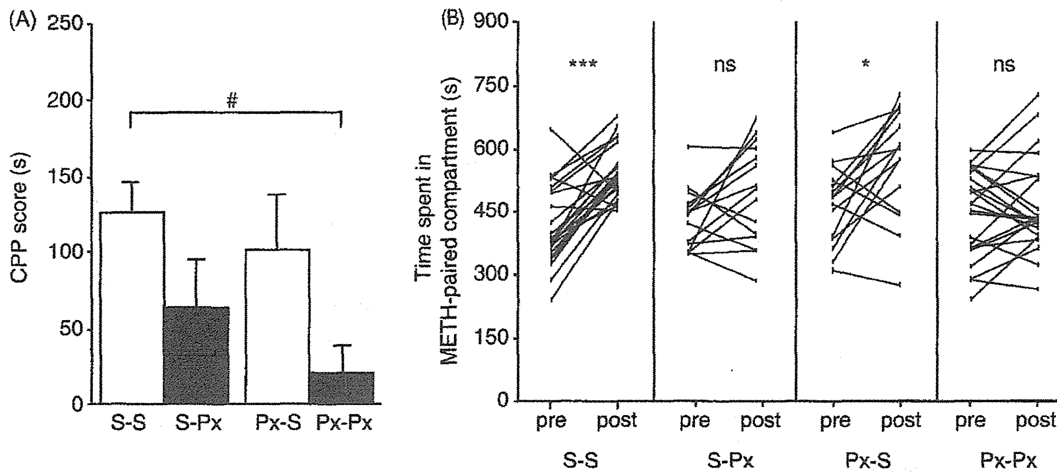

Fig. (1). Effects of paroxetine on CPP for METH in mice. (**A**) Reduction of METH CPP by paroxetine (Px) pretreatment. Mice were pretreated with saline (S) in both the conditioning and CPP test phases (S-S), paroxetine only in the CPP test phase (S-Px), paroxetine only in the conditioning phase (Px-S), and paroxetine in both the conditioning and the CPP test phases (Px-Px). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning phase (Day 2). The CPP score of the Px-Px group was significantly lower than that of the S-S group ($^{\#}P < 0.05$). (**B**) Comparison of time spent in the conditioned compartment before and after conditioning in the four groups. There was a significant CPP in the S-S and Px-S groups, but not in the S-Px and Px-Px groups (when paroxetine was administered in the CPP test phase). $^{***}P < 0.001$, $^{*}P < 0.05$, ns: not significant ($P > 0.05$).

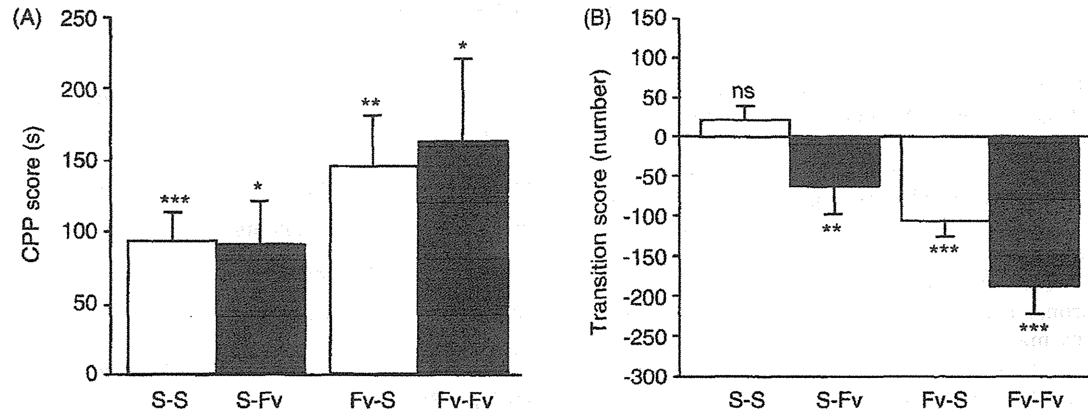

Fig. (2). Effects of fluvoxamine on CPP for METH and on transitions between compartments. (**A**) Lack of a significant effect of fluvoxamine (Fv) on METH CPP. Mice were pretreated with saline in both the conditioning and the CPP test phases (S-S), fluvoxamine only in the CPP test phase (S-Fv), fluvoxamine only in the conditioning phase (Fv-S), and fluvoxamine in both the conditioning and the CPP test phases (Fv-Fv). There was a significant CPP in all groups. Fluvoxamine pretreatment in the conditioning phase and/or the CPP test phase failed to inhibit METH CPP (pre- and post-conditioning preference test results were analyzed with paired t-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). (**B**) Decreases in transitions between the compartments by fluvoxamine pretreatment. There were significant decreases in transitions in the S-Fv, Fv-S, and Fv-Fv groups, but not in the S-S group [number of transitions in the pre- and post-conditioning phases was analyzed with paired t-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, ns: not significant ($P > 0.05$)]. The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning phase (Day 2).

compartment after conditioning than before conditioning (saline/saline: $n = 26$, $df = 25$, $t = -4.541$, $P < 0.001$; saline/fluvoxamine: $n = 14$, $df = 13$, $t = -2.983$, $P < 0.05$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = -3.949$, $P < 0.01$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = -2.757$, $P < 0.05$).

The two-way ANOVA revealed that both fluvoxamine pretreatment during the conditioning phase and during the CPP test phase significantly decreased transition scores (conditioning phase: $F_{1,68} = 24.321$, $P < 0.001$; CPP test phase: $F_{1,68} = 10.292$, $P < 0.01$; Fig. (**2B**)). There was no statistically significant interaction between the two factors ($F_{1,68} = 0.007$, n.s.). Paired t-tests were conducted to compare the number of transitions before and after conditioning for each of the four groups. The S-S group showed no significant differences in the number of transitions before and after conditioning ($n = 26$, $df = 25$, $t = -1.213$, n.s.). However, mice pretreated with fluvoxamine (saline/fluvoxamine, fluvoxamine/saline, fluvoxamine/fluvoxamine) showed significant decreases in the number of transitions after conditioning (saline/fluvoxamine: $n = 14$, $df = 13$, $t = 3.829$, $P < 0.01$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = 5.520$, $P < 0.001$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = 6.025$, $P < 0.001$).

DISCUSSION

In the present study, we showed that paroxetine, a widely used medication for treating depression, inhibited METH CPP in mice, similar to the results we reported previously with fluoxetine [8]. No significant effects of paroxetine on transition scores suggest that the effects of paroxetine on METH CPP are not due to changes in locomotor activity but due to reduction of METH reward and conditioned reward by paroxetine. Based on these findings, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. By contrast, the other SSRI tested here, fluvoxamine, did not affect METH CPP. These data demonstrate that there are differences in the effects of SSRIs on METH CPP, suggesting the possibility that molecules other than the SERT are involved in the inhibition of METH CPP by paroxetine and fluoxetine reported here and in our previous study [8].

In addition to SERT inhibition, paroxetine inhibits the function of muscarinic cholinergic receptors [11], nicotinic acetylcholine receptors [12], volume-related anion channels [13], membrane steroid transporters [14], and nitric oxide synthase [15]. Recently, Kobayashi and colleagues [16] reported that paroxetine also inhibits the function of G protein-activated inwardly rectifying K^+ (GIRK) channels. It is intriguing that paroxetine and fluoxetine, but not fluvoxamine, inhibit GIRK channels [16-18]. Various G protein-coupled receptors (such as M2 muscarinic, α2 adrenergic, D_2 dopaminergic, 5-HT_{1A}, opioid, nociceptin/orphanin FQ, and A_1 adenosine) activate GIRK channels [19-22] through the direct action of G protein subunits [23]. In addition, GIRK channels are activated by ethanol independently of G protein-coupled signaling pathways [24, 25]. Activation of GIRK channels leads to membrane hyperpolarization [22]. These channels play an important role in the inhibitory regulation of neuronal excitability. Thus, modulators of GIRK channel activity may affect many brain functions. Kobayashi and colleagues [26] also have reported that ifenprodil, a cerebral vasodilator which inhibits morphine CPP [27], also inhibits the function of GIRK channels. Morgan and colleagues [28] demonstrated that GIRK channel knockout mice exhibited dramatically reduced intravenous self-administration of cocaine. In the present study, we found that paroxetine and fluoxetine, but not fluvoxamine, inhibited METH CPP. These findings, together with the previous findings, suggest that the inhibition of GIRK channels by paroxetine or fluoxetine may be involved in the inhibition of METH CPP by these drugs.

Fluvoxamine administration (60 mg/kg) leads to a significant decrease in spontaneous locomotor activity [29]. Consistent with this observation, significant decreases in transition scores were observed in all of the 100 mg/kg fluvoxamine-treated groups compared to the saline/saline-treated group in the present study. The number of transitions of the fluvoxamine/fluvoxamine treated group during the CPP test phase (101.4 ± 85.3, mean ± SEM) was the smallest among the four groups in this experiment, but more than 100 transitions indicated adequate locomotion to reveal potential differences in CPP. The lack of effect of fluvoxamine on CPP for methamphetamine is likely to reflect a lack of effect of fluvoxamine on the rewarding effects of METH rather than being a nonspecific effect of fluvoxamine.

In conclusion, we found that paroxetine, but not fluvoxamine, inhibited METH CPP in mice. Although further preclinical studies are needed to elucidate the mechanisms underlying these inhibitory effects of paroxetine on processes relating to METH dependence, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. The present results suggest that molecules other than the SERT (such as GIRK channels) are involved in the inhibition of METH CPP by paroxetine and fluoxetine.

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ABBREVIATIONS

ANOVA	=	Analysis of variance
CPP	=	Conditioned place preference
DAT	=	Dopamine transporter
GIRK	=	G protein-activated inwardly rectifying K^+
METH	=	Methamphetamine
n.s.	=	Not significant
SERT	=	Serotonin transporter
SSRI	=	Selective serotonin reuptake inhibitor

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Effects of MDMA on Extracellular Dopamine and Serotonin Levels in Mice Lacking Dopamine and/or Serotonin Transporters

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Abstract: 3,4-Methylendioxymethamphetamine (MDMA) has both stimulatory and hallucinogenic properties which make its psychoactive effects unique and different from those of typical psychostimulant and hallucinogenic agents. The present study investigated the effects of MDMA on extracellular dopamine (DA_{ex}) and serotonin ($5\text{-}HT_{ex}$) levels in the striatum and prefrontal cortex (PFC) using *in vivo* microdialysis techniques in mice lacking DA transporters (DAT) and/or 5-HT transporters (SERT). Subcutaneous injection of MDMA (3, 10 mg/kg) significantly increased striatal DA_{ex} in wildtype mice, SERT knockout mice, and DAT knockout mice, but not in DAT/SERT double-knockout mice. The MDMA-induced increase in striatal DA_{ex} in SERT knockout mice was significantly less than in wildtype mice. In the PFC, MDMA dose-dependently increased DA_{ex} levels in wildtype, DAT knockout, SERT knockout and DAT/SERT double-knockout mice to a similar extent. In contrast, MDMA markedly increased $5\text{-}HT_{ex}$ in wildtype and DAT knockout mice and slightly increased $5\text{-}HT_{ex}$ in SERT-KO and DAT/SERT double-knockout mice. The results confirm that MDMA acts at both DAT and SERT and increases DA_{ex} and $5\text{-}HT_{ex}$.

Keywords: MDMA, serotonin transporter, dopamine transporter, knockout, microdialysis.

INTRODUCTION

3,4-Methylendioxymethamphetamine (MDMA, street name: ecstasy) exhibits both stimulatory and hallucinogenic properties which make its psychoactive effects unique and different from those of typical hallucinogens or psychostimulants. Under *in vitro* conditions, MDMA has been shown to increase the release of dopamine (DA), serotonin (5-HT), and norepinephrine (NE) from brain slices and prevent the reuptake of DA, 5-HT, and NE into brain synaptosomes [1-4]. MDMA binds with higher affinity to the 5-HT transporter (SERT) than to the DA transporter (DAT) [5, 6] and produces a greater release of 5-HT than DA [7-9].

In vivo microdialysis studies have revealed that systemic injection of MDMA increases extracellular levels of DA and 5-HT in the striatum and prefrontal cortex (PFC) [7, 10-13]. MDMA induces DA release, at least in the striatum, through several mechanisms. For example, the release of DA elicited by MDMA is hypothesized to involve both transporter- [14, 15] and impulse-dependent processes [8]. Additionally, the increased 5-HT function resulting from MDMA-induced 5-HT release has been suggested to stimulate $5\text{-}HT_2$ receptors, thereby further enhancing DA release [11, 16, 17].

Monoamine transporter knockout (KO) mice provide useful *in vivo* models to analyze the effects of psychoactive drugs. In SERT-KO mice, Begels *et al.* (1998) reported a lack of locomotor-stimulating effects of MDMA [18]. MDMA self-administration is also absent in SERT-KO mice [13]. Moreover, the ability of MDMA administration to induce γ-aminobutyric acid transporter 1 expression in the frontal cortex and midbrain was reduced in SERT-KO mice [19]. In contrast, DAT-KO mice are hyperactive [20, 21] and display perseverative locomotor patterns [22]. MDMA decreases hyperactivity and potentiates the perseverative pattern of locomotor activity in DAT-KO mice [23]. However, the mechanisms underlying these MDMA effects have not been sufficiently elucidated.

To clarify the action of MDMA on the DAT or SERT in the striatum and PFC, we investigated the effects of MDMA on extracellular levels of DA (DA_{ex}) and 5-HT ($5\text{-}HT_{ex}$) using *in vivo* microdialysis in mice lacking the DAT and/or SERT.

METHODS

Animals

Wildtype and DAT-KO mouse littermates from crosses of heterozygous/heterozygous DAT-KO mice on a 129/C57 mixed genetic background served as subjects. SERT-KO and DAT/SERT double-KO mouse littermates from crosses of heterozygous DAT/homozygous SERT knockout mice on a 129/C57 mixed genetic background also served as subjects.

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The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Psychiatry, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines. Naive adult mice were housed in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12 h light/dark cycle with lights on at 8:00 a.m. and off at 8:00 p.m. Food and water were available *ad libitum*. In microdialysis experiments, male and female mice from 10 to 24 weeks old were examined.

Surgery and Microdialysis Procedure

Mice were stereotaxically implanted, under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally), with microdialysis probes in the striatum (anterior +0.6 mm, lateral +1.8 mm, ventral -4.0 mm from bregma) or PFC (anterior +2.0 mm, lateral +0.5 mm, ventral -3.0 mm from bregma), according to the atlas of Franklin and Paxinos [24]. Twenty-four hours after implantation, the dialysis experiments were performed in freely-moving animals. Evaluation of DA_{ex} and $5\text{-}HT_{ex}$ has been previously described [25]. Basal levels of DA_{ex} and $5\text{-}HT_{ex}$ were obtained from average concentrations of three consecutive samples when they were stable.

Drugs

Drugs were dissolved in saline and administered subcutaneously (s.c.) in a volume of 10 ml/kg. MDMA (3 and 10 mg/kg) was administered after establishment of stable baseline, and the dialysate was continuously collected for 180 min.

Statistical Analysis

DA_{ex} and $5\text{-}HT_{ex}$ responses to drugs were expressed as a percentage of basal levels. Areas under the curve (AUC) of DA_{ex} and $5\text{-}HT_{ex}$ during the 180 min period after drug administration were calculated as the effects of drugs. AUC values of all groups were analyzed using a two-way analysis of variance (ANOVA). Individual *post hoc* comparisons were performed with Fisher's protected least significant difference (PLSD) test. In all cases, the PLSD test was applied for multiple comparisons, and values of $p < 0.05$ were considered statistically significant. Data were analyzed with Statview J5.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baselines of DA_{ex} and $5\text{-}HT_{ex}$ in the Striatum and PFC

The baselines of DA_{ex} and $5\text{-}HT_{ex}$ in the striatum and PFC are shown in Table 1. As previously reported [25], baselines of DA_{ex} in the striatum were significantly higher in DAT-KO and DAT/SERT-double KO mice than in wildtype mice (one-way ANOVA; $F_{3,66} = 37.708$, $p < 0.001$). Baselines of DA_{ex} in the PFC were not different between wildtype, DAT-KO, SERT-KO, and DAT/SERT double-KO mice (one-way ANOVA; $F_{3,76} = 0.291$, $p = 0.832$). Baselines of $5\text{-}HT_{ex}$ were significantly higher in SERT-KO and DAT/SERT double-KO mice than in wildtype mice in both the striatum (one-way ANOVA; $F_{3,66} = 37.716$, $p < 0.001$) and PFC (one-way ANOVA; $F_{3,76} = 47.715$, $p < 0.001$).

Effects of MDMA on DA_{ex} and $5\text{-}HT_{ex}$ in the Striatum

MDMA (3 and 10 mg/kg) dose-dependently increased DA_{ex} in wildtype and SERT-KO mice, but not in DAT/SERT double-KO mice (Fig. **1A**, **B**). Two-way ANOVA (drug × genotype) of the DA_{ex} AUC calculated during the 180 min posttreatment period revealed significant effects of drug ($F_{2,58} = 94.751$, $p < 0.001$) and genotype ($F_{3,58} = 26.775$, $p < 0.001$) and a significant drug × genotype interaction ($F_{6,58} = 21.352$, $p < 0.001$). *Post hoc* comparisons revealed that the effects of MDMA (10 mg/kg) on DA_{ex} in SERT-KO mice was significantly less than in wildtype mice ($p < 0.001$; Fisher's PLSD *post hoc* test). However, DAT-KO mice exhibited significant MDMA (10 mg/kg)-induced increases in DA_{ex} levels ($p < 0.001$; Fisher's PLSD *post hoc* test), increases that were less than in wildtype mice ($p < 0.001$; Fisher's PLSD *post hoc* test). MDMA (3 and 10 mg/kg) dose-dependently increased $5\text{-}HT_{ex}$ in wildtype and DAT-KO mice (Fig. **1C**, **D**). Two-way ANOVA (drug × genotype) of $5\text{-}HT_{ex}$ revealed significant effects of drug ($F_{2,58} = 23.578$, $p < 0.001$) and genotype ($F_{3,58} = 21.589$, $p < 0.001$) and a significant drug × genotype interaction ($F_{6,58} = 7.769$, $p < 0.001$). The effects of MDMA (3 and 10 mg/kg) on $5\text{-}HT_{ex}$ in DAT-KO mice was significantly higher than in wildtype mice ($p < 0.05$ and $p < 0.01$, respectively; Fisher's PLSD *post hoc* test). When the effects of MDMA were analyzed only in SERT-KO and DAT/SERT double-KO mice, two-way ANOVA (drug × genotype) of $5\text{-}HT_{ex}$ revealed a significant effect of drug ($F_{2,25} = 11.858$, $p < 0.001$) but no effect of genotype ($F_{1,25} = 0.492$, $p = 0.489$) and no drug × genotype interaction ($F_{2,25} = 2.773$, $p = 0.082$). The effects of

Table 1. The Baselines (fmol/10 min) of DA_{ex} and $5\text{-}HT_{ex}$ in the Striatum and PFC

	Striatum			PFC		
Genotype	n	DA_{ex}	$5\text{-}HT_{ex}$	n	DA_{ex}	$5\text{-}HT_{ex}$
Wildtype	20	43.00 ± 5.15	1.24 ± 0.17	24	1.24 ± 0.18	1.87 ± 0.20
DAT-KO	19	486.26 ± 62.00***	1.01 ± 0.13	21	1.16 ± 0.16	1.87 ± 0.24
SERT-KO	16	56.18 ± 7.44	13.07 ± 1.97***	16	1.32 ± 0.28	15.09 ± 1.73***
DAT/SERT-KO	15	596.18 ± 73.38***	15.13 ± 1.91***	19	1.42 ± 0.22	12.21 ± 1.43***

Data presented are means ± S.E.M. ***P < 0.001 compared to the corresponding wildtype datum.

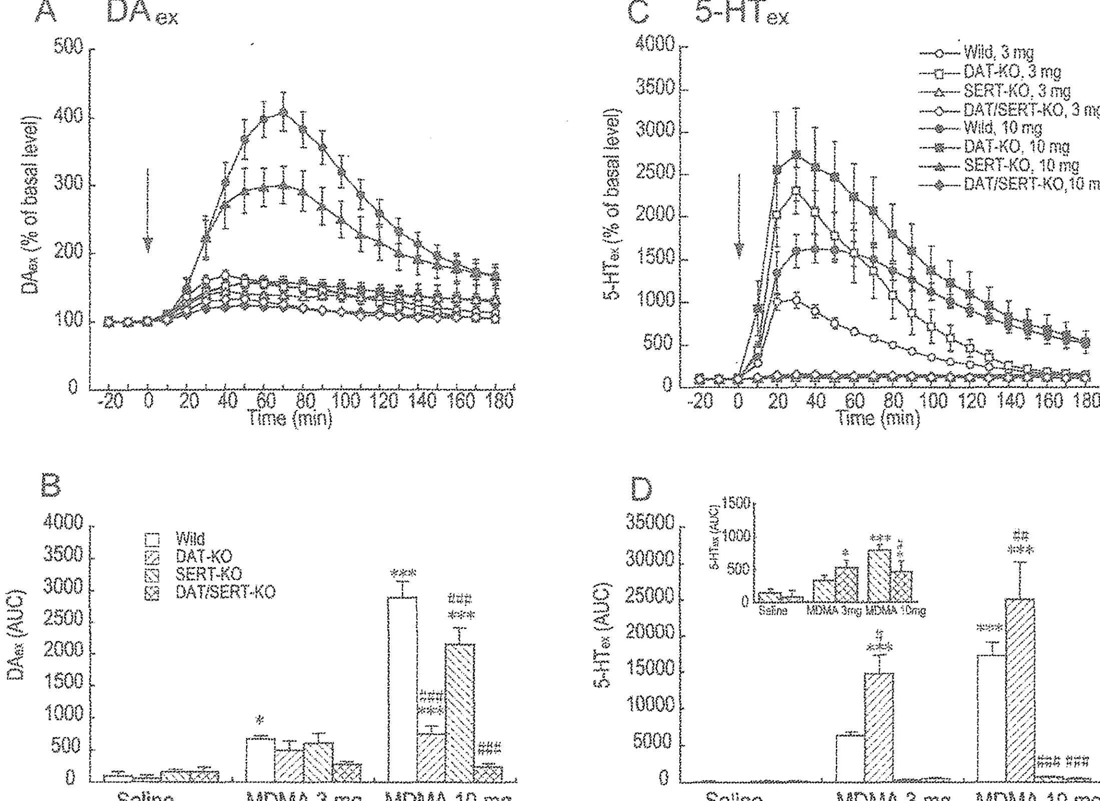

Fig. (1). Effects of MDMA on DA_{ex} and $5\text{-}HT_{ex}$ in the striatum in wildtype, DAT-KO, SERT-KO, and DAT/SERT double-KO mice. (A, C) Temporal pattern of DA_{ex} and $5\text{-}HT_{ex}$ before and after injection with saline or MDMA (3 and 10 mg/kg, s.c.). The arrows indicate drug injection time. Each point represents the mean ± SEM of the percentage of DA_{ex} or $5\text{-}HT_{ex}$ baselines. (B, D) Histogram representing the mean AUC (± SEM) of DA_{ex} or $5\text{-}HT_{ex}$ during the 180 min period after injection with saline or MDMA (n = 4-8). $^*p < 0.05$, $^{***}p < 0.001$, compared with saline group of the same genotype; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with corresponding wildtype data in the same drug treatment (two-way ANOVA followed by Fisher's PLSD *post hoc* test).

MDMA (10 mg/kg) on $5\text{-}HT_{ex}$ in DAT/SERT double-KO mice was significantly less than in SERT-KO mice ($p < 0.05$; Fisher's PLSD *post hoc* test).

Effects of MDMA on DA_{ex} and $5\text{-}HT_{ex}$ in the PFC

MDMA (3 and 10 mg/kg) dose-dependently increased DA_{ex} in wildtype, DAT-KO, SERT-KO, and DAT/SERT double-KO mice (Fig. **2A**, **B**). Two-way ANOVA (drug × genotype) of DA_{ex} revealed a significant effect of drug ($F_{2,68}$ = 53.368, $p < 0.001$) but no effect of genotype ($F_{3,68}$ = 0.203, p = 0.894) and no drug × genotype interaction ($F_{6,68}$ = 0.408, p = 0.871). MDMA (3 and 10 mg/kg) dose-dependently increased $5\text{-}HT_{ex}$ in wildtype and DAT-KO mice (Fig. **2C**, **D**). Two-way ANOVA (drug × genotype) of $5\text{-}HT_{ex}$ revealed significant effects of drug ($F_{2,68}$ = 32.357, $p < 0.001$) and genotype ($F_{3,68}$ = 19.078, $p < 0.001$) and a significant drug × genotype interaction ($F_{6,68}$ = 10.596, $p < 0.001$). The effect of MDMA (10 mg/kg) on $5\text{-}HT_{ex}$ in DAT-KO mice was significantly less than in wildtype mice ($p < 0.01$; Fisher's PLSD *post hoc* test). When the effects of MDMA were analyzed only in SERT-KO and DAT/SERT double-KO mice, two-way ANOVA (drug × genotype) of $5\text{-}HT_{ex}$ revealed a significant effect of drug ($F_{2,29}$ = 28.906, $p < 0.001$) but no significant effect of genotype ($F_{1,29}$ = 0.236, p = 0.631) and no drug × genotype interaction ($F_{2,29}$ = 0.609, p =0.551).

DISCUSSION

MDMA increased DA_{ex} and $5\text{-}HT_{ex}$ in the striatum and PFC, consistent with several previous microdialysis studies [7, 10-13]. In DAT/SERT double-KO mice, MDMA did not increase DA_{ex} in the striatum, and the increases in $5\text{-}HT_{ex}$ were minimal in the striatum and PFC. These results confirm that MDMA acts at both the DAT and SERT.

MDMA increased DA_{ex} in wildtype and SERT-KO mice, but not in DAT/SERT double-KO mice. In the absence of the DAT, MDMA-induced changes in DA_{ex} were smaller than in wildtype mice. Therefore, the DAT is likely mainly involved in the changes in DA_{ex} induced by MDMA. Although DAT-KO mice exhibited significant MDMA-induced increases in DA_{ex} levels, these increases were less than in wildtype mice. The increase in DA_{ex} produced by MDMA in DAT-KO mice may have two possible explanations. One possibility is that elevated $5\text{-}HT_{ex}$ levels produced by MDMA may influence DA release. Microdialysis studies have shown that MDMA, by increasing $5\text{-}HT_{ex}$, indirectly increases DA_{ex} *via* an action at $5\text{-}HT_2$ receptors [7, 8, 17]. Another possibility is that MDMA inhibits DA uptake into 5-HT axon terminals and increases DA_{ex}. The SERT is able to transport DA into 5-HT cells [26, 27], and the selective SERT blocker fluoxetine increases DA_{ex} in the striatum of DAT-KO mice [25].

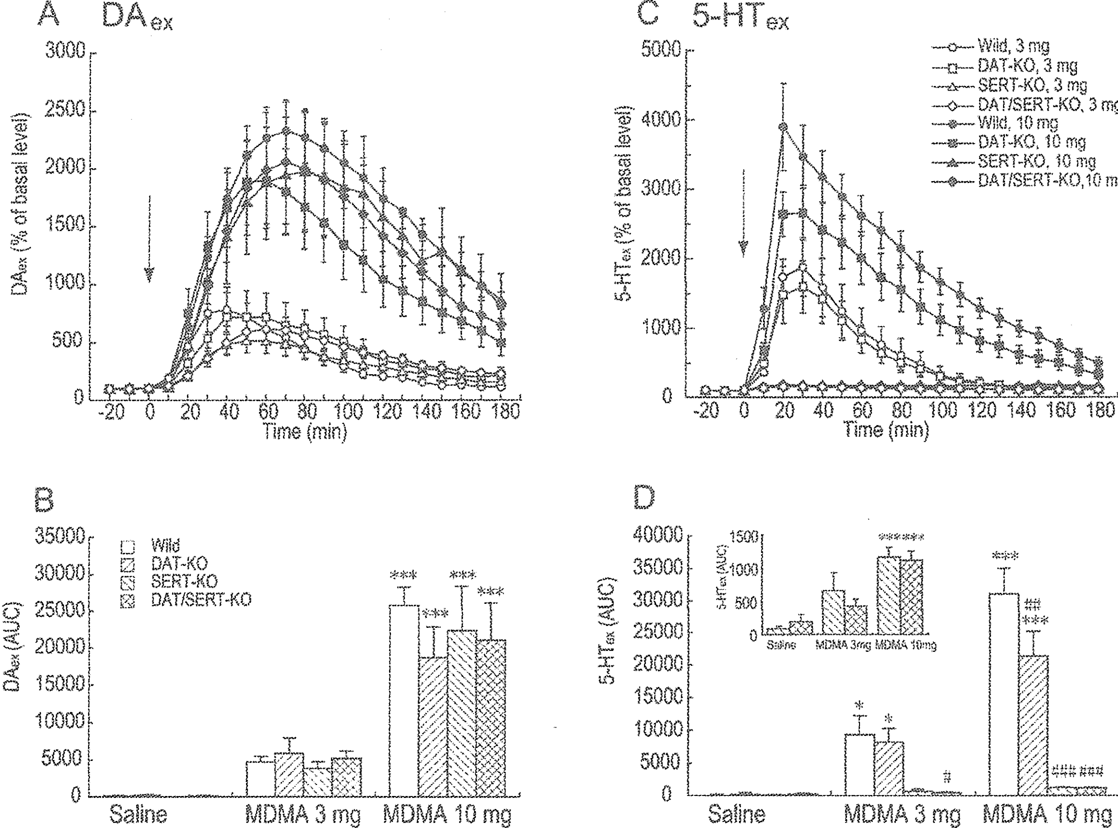

Fig. (2). Effects of MDMA on DA_{ex} and $5\text{-}HT_{ex}$ in the PFC in wildtype, DAT-KO, SERT-KO, and DAT/SERT double-KO mice. (A, C) Temporal pattern of DA_{ex} or $5\text{-}HT_{ex}$ before and after injection with saline or MDMA (3 and 10 mg/kg, s.c.). The arrows indicate drug injection time. Each point represents the mean ± SEM of the percentage of DA_{ex} or $5\text{-}HT_{ex}$ baselines. (B, D) Histogram representing the mean AUC (± SEM) of DA_{ex} or $5\text{-}HT_{ex}$ during the 180 min period after injection with saline or MDMA (n = 4-10). $^{*}p < 0.05$, $^{***}p < 0.001$, compared with saline group of the same genotype; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with corresponding wildtype data in the same drug treatment (two-way ANOVA followed by Fisher's PLSD *post hoc* test).

Microdialysis studies have shown that NET inhibitors increased DA_{ex} in the PFC [28, 29], suggesting that NET can influence DA neurotransmission. Moron *et al.* (2002) reported that DA uptake in the PFC depends primarily on the NET [30]. This study showed a similar basal extracellular DA concentration in the PFC in DAT-KO and wildtype mice. DA_{ex} in the PFC is regulated by the NET. MDMA dose-dependently increased DA_{ex} in wildtype, DAT-KO, SERT-KO, and DAT/SERT double-KO mice. Therefore, MDMA may act at the NET and increase DA_{ex} levels in the PFC.

MDMA slightly increased $5\text{-}HT_{ex}$ in the striatum and PFC in mice lacking the SERT. The selective DAT blocker GBR12909 produced a substantial increase in dialysate 5-HT in SERT-KO mice that was not found in wildtype mice [25]. When the SERT is disrupted in SERT-KO mice, 5-HT is found in DA neurons in the substantia nigra and ventral tegmental area [31]. The DAT appears to play a compensatory role in 5-HT uptake in SERT-KO mice. Therefore, MDMA may act at the DAT and increase $5\text{-}HT_{ex}$ levels in the striatum in SERT-KO mice. The NET also appears to be able to play a role in 5-HT uptake [32]. In the PFC, MDMA may increase $5\text{-}HT_{ex}$ levels by acting at the NET in SERT-KO mice.

MDMA markedly increased $5\text{-}HT_{ex}$ in wildtype and DAT-KO mice. MDMA binds with higher affinity to the SERT than to the DAT [5, 6]. Consistent with *in vitro* results, MDMA produced greater elevations in 5-HT than DA. Relevant studies have shown that many of the subjective effects of MDMA in human volunteers are reduced after administration of a $5\text{-}HT_2$ receptor antagonist or 5-HT reuptake inhibitors, suggesting that these effects are dependent on SERT-mediated enhancement of serotonergic transmission [33, 34].

In conclusion, the present microdialysis study using DAT- and/or SERT-KO mice demonstrated that MDMA targets monoamine transporters and stimulates predominantly serotonergic transmission.

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Enhanced Hyperthermia Induced by MDMA in Parkin Knockout Mice

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Abstract: MDMA (3,4-methylenedioxymethamphetamine) is reportedly severely toxic to both dopamine (DA) and serotonin neurons. MDMA significantly reduces the number of DA neurons in the substantia nigra, but not in the nucleus accumbens, indicating that MDMA causes selective destruction of DA neurons in the nigrostriatal pathway, sparing the mesolimbic pathway. Parkinson's disease (PD) is a neurodegenerative disorder of multifactorial origin. The pathological hallmark of PD is the degeneration of DA neurons in the nigrostriatal pathway. Mutations in the parkin gene are frequently observed in autosomal recessive parkinsonism in humans. Parkin is hypothesized to protect against neurotoxic insult, and we attempted to clarify the role of parkin in MDMA-induced hyperthermia, one of the causal factors of neuronal damage, using parkin knockout mice. Body temperature was measured rectally before and 15, 30, 45, and 60 min after intraperitoneal injection of MDMA (30 mg/kg) at an ambient temperature of 22 ± 2°C. Significantly enhanced hyperthermia after MDMA injection was observed in heterozygous and homozygous parkin knockout mice compared with wildtype mice, suggesting that parkin plays a protective role in MDMA neurotoxicity.

Keywords: Hyperthermia, knockout, mice, MDMA, parkin.

INTRODUCTION

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA) is abused by young adults despite its potentially neurotoxic effects and psychiatric complications. MDMA produces a rapid enhancement of serotonin and dopamine (DA) release in the brain [1, 2]. Administration of MDMA in mice is well known to produce acute hyperthermia and degeneration of striatal DA nerve terminals [3]. Recently, Granado and colleagues [4] reported that MDMA produces a significant decrease in the number of tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra. This decrease was accompanied by a dose-dependent decrease in TH- and DA transporter (DAT)-immunoreactivity in the striatum. MDMA significantly reduces TH- and DAT-immunoreactivity in the striatum, but not in the nucleus accumbens, indicating that MDMA causes selective destruction of DA neurons in the nigrostriatal pathway, sparing the mesolimbic pathway. The degree of long-term neurodegeneration produced by MDMA appears to be closely related to the magnitude of the hyperthermic response [5]. Attenuation of the hyperthermia alleviates MDMA-induced loss of striatal dopamine [3].

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The major pathological hallmark of PD is the degeneration of DAergic neurons in the substantia nigra that innervate the striatum. The major symptoms of PD include tremor, bradykinesia, cogwheel rigidity, and postural instability, which arise from the degeneration of DAergic neurons in the substantia nigra. PD is a neurodegenerative disorder of multifactorial origin, and mutations in the gene encoding parkin, an E3 ubiquitin-protein ligase [6], are frequently observed in autosomal recessive parkinsonism in humans. The loss of parkin function has been suggested to result in aberrant accumulation of parkin substrate proteins [6]. Accumulation of these proteins has been postulated to confer toxicity to DAergic neurons in the substantia nigra [7].

In the present study, we hypothesized that parkin protects against neurotoxic insult, and we attempted to clarify the role of parkin in MDMA-induced hyperthermia, one of the causal factors of neuronal damage, using parkin knockout mice.

MATERIALS AND METHODS

Mice

Wildtype, heterozygous, and homozygous parkin knockout mice were prepared from heterozygous/heterozygous parkin knockout mouse crosses (21-37 g, 12-29 weeks of age). Mice were housed in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12/12 h light/dark cycle with lights on at 8:00 a.m. Food and water were available *ad libitum*. All behavioral testing was conducted during the light cycle. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry, and all animals were treated humanely in accordance with our institutional animal experimentation guidelines.

Body Temperature Measurement

Rectal temperature measurement was performed using a digital thermometer (BAT-12; Physitemp Instruments Inc.,

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Clifton, NJ, USA) with 0.1°C accuracy and a rectal probe for mice (RET-3, Physitemp Instrument Inc.). Each mouse was lightly restrained by hand for approximately 20 s while the probe was inserted approximately 2 cm into the rectum and a steady reading was obtained. Body temperature was measured rectally before and 15, 30, 45, and 60 min after intraperitoneal (i.p.) injection of MDMA (30 mg/kg) at an ambient temperature of 22 ± 2°C.

Drugs

MDMA was synthesized at Matsuyama University College of Pharmaceutical Sciences and freshly dissolved in saline. MDMA and vehicle were administered in a volume of 0.1 ml/10 g body weight.

Statistical Analysis

Mean and standard error were calculated from the values of 12-17 subjects. Changes in body temperature were analyzed by repeated-measures analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Baseline temperature and changes in body temperature areas-under-the-curve (AUC) were analyzed by one-way ANOVA and Scheffe's *post hoc* test.

RESULTS

Baseline Body Temperature in Parkin Knockout Mice

Baseline body temperature was measured before MDMA injection at room temperature (22 ± 2°C). No significant difference in baseline body temperature was observed among wildtype, heterozygous, and homozygous parkin knockout mice (Fig. **1**).

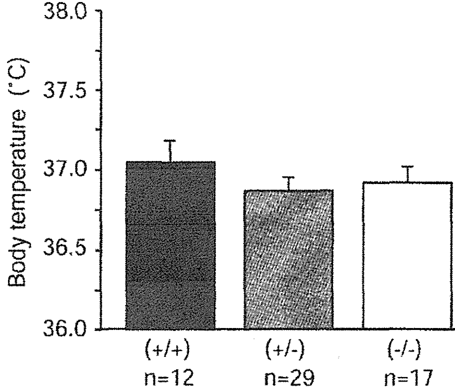
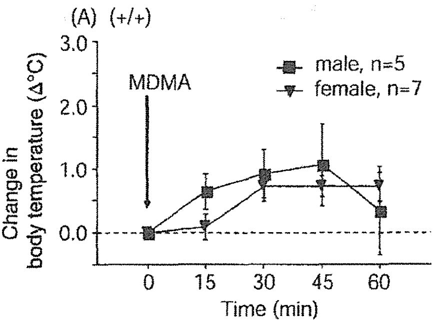
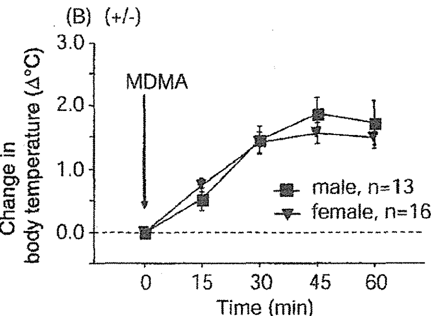


Fig. (1). No significant differences in baseline body temperature were observed among wildtype, heterozygous, and homozygous parkin knockout mice. Body temperature prior to MDMA injection did not significantly differ among genotypes. Baseline body temperature was analyzed by one-way ANOVA ($F_{2,55}$ = 0.629, p = 0.5369) at an ambient temperature of 22 ± 2°C.

No Sex Differences in MDMA-Induced Hyperthermia

Body temperature was measured 15, 30, 45, and 60 min after i.p. injection of MDMA (30 mg/kg) at an ambient temperature of 22 ± 2°C. No significant differences in MDMA-induced hyperthermia were observed between males and females within each genotype (Fig. 2).





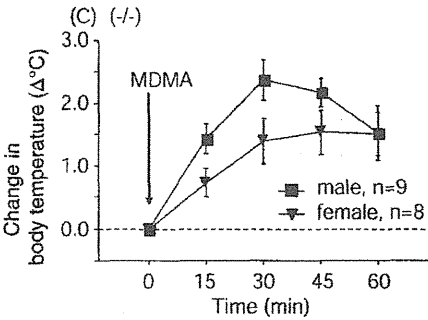
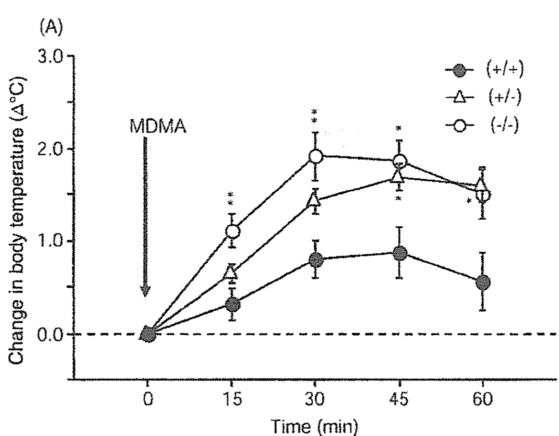


Fig. (2). Similar MDMA-induced (30 mg/kg, i.p.) hyperthermia was observed in male and female mice within each genotype. Body temperature areas-under-the-curve were analyzed by repeated-measures ANOVA. (**A**) Sex, $F_{1,10}$ = 0.181, p = 0.6796; Time, $F_{4,40}$ = 4.741, p = 0.0032; Sex × Time interaction, $F_{4,40}$ = 1.124, p = 0.3587. (**B**) Sex, $F_{1,27}$ = 0.134, p = 0.7170; Time, $F_{4,108}$ = 62.705, p < 0.0001; Sex × Time interaction, $F_{4,108}$ = 1.231, p = 0.3021. (**C**) Sex, $F_{1,15}$ = 2.350, p = 0.1461; Time, $F_{4,60}$ = 26.22, p < 0.0001; Sex × Time interaction, $F_{4,60}$ = 2.059, p = 0.0974.

Enhancement of MDMA-Induced Hyperthermia in Parkin Knockout and Heterozygous Mice

Body temperature gradually increased from baseline after MDMA injection in all genotype groups. MDMA significantly enhanced hyperthermia from 15 to 45 min after injection in parkin knockout mice and from 45 to 60 min after injection in heterozygous mice compared with wildtype mice (Fig. **3A**). MDMA produced hyperthermia, with a maximum increase of 0.9°C (37.9°C) 45 min after injection in wildtype mice, 1.7°C (38.6°C) 45 min after injection in heterozygous mice, and 1.9°C (38.8°C) 30 min after injection in parkin knockout mice. Body temperature AUC values reflected significantly enhanced hyperthermia in parkin knockout and heterozygous mice compared with wildtype mice (Fig. **3B**).



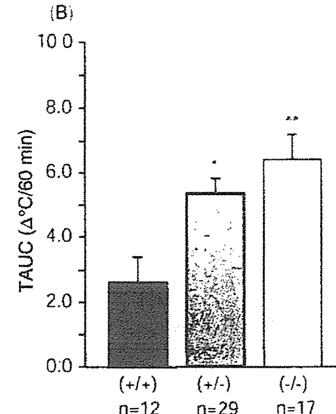


Fig. (3). Enhanced MDMA-induced hyperthermia in heterozygous and homozygous parkin knockout mice compared with wildtype mice. (**A**) Change in body temperature in mice injected with MDMA (30 mg/kg, i.p.). Body temperature areas-under-the-curve were analyzed by repeated-measures ANOVA (Genotype, $F_{2,55} = 6.746$, $p = 0.0024$; Time, $F_{4,220} = 61.267$, $p < 0.0001$; Genotype × Time interaction, $F_{8,220} = 3.664$, $p = 0.0005$) followed by Scheffe's *post hoc* test (*$p < 0.05$, **$p < 0.01$). (**B**) Change in body temperature areas-under-the-curve (TAUC) shown as an integration of the temperature *vs.* time curve shown in panel A. TAUC values were analyzed by one-way ANOVA ($F_{2,55} = 6.746$, $p = 0.0024$) followed by Scheffe's *post hoc* test (*$p < 0.05$, **$p < 0.01$).

DISCUSSION

In the present study, significantly enhanced MDMA-induced hyperthermia was observed in parkin knockout and heterozygous mice compared with wildtype mice (Fig. **3B**). The enhanced MDMA-induced hyperthermia in parkin knockout mice supports the hypothesis that parkin protects against MDMA-induced neurotoxic insult.

Hyperthermia is one of the major symptoms of acute MDMA-induced toxicity, which has been shown to be affected by body temperature [3]. MDMA produces a rapid enhancement of DA release in the striatum [1] and preoptic anterior hypothalamus [8]. MDMA-induced hyperthermia was blocked by a DA D_1 receptor antagonist [9]. Moreover, both the hyperthermia and augmented DA levels in the preoptic anterior hypothalamus after i.p. MDMA injection were significantly reduced by pretreatment with a D_1 antagonist [8]. Interestingly, Sato *et al.* (2006) [10] reported that D_1 receptor levels in the striatum in parkin knockout mice was higher than in wildtype mice, although no change in TH-positive substantia nigra neurons was found in parkin knockout mice, and no significant decrease in DAT levels was observed in the striatum. Therefore, the enhanced MDMA-induced hyperthermia observed in the present study may be attributable to increased levels of D_1 receptors in parkin knockout mice.

Sato *et al.* (2006) [10] also suggested that presynaptic neurons (i.e., DAergic neurons) are functionally impaired in parkin knockout mice. DA synthesis is significantly decreased and methamphetamine-induced DA release is reduced in parkin knockout mice. Considering that DAergic neurons in the substantia nigra are severely damaged in PD patients, the enhanced MDMA-induced hyperthermia in parkin knockout mice may be attributable to functional impairment of DAergic neurons, although the relationship between hyperthermia and DAergic neuron dysfunction remains to be elucidated.

Additionally, we found no significant difference in baseline body temperature among wildtype, heterozygous, and homozygous parkin knockout mice (Fig. **1**). These data suggest that parkin does not play a crucial role in the system that maintains basal body temperature.

In conclusion, MDMA-induced hyperthermia was enhanced in parkin knockout and heterozygous mice compared with wildtype mice. Parkin is hypothesized to be critical for protecting DAergic neurons from toxic insult, and the present results suggest that parkin plays a protective role against MDMA-induced DAergic neuron neurotoxicity.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ANOVA	=	Analysis of variance
DA	=	Dopamine
DAT	=	Dopamine transporter
MDMA	=	3,4-methylenedioxymethamphetamine
PD	=	Parkinson's disease
TH	=	Tyrosine hydroxylase

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Identification of Selective Agonists and Antagonists to G Protein-Activated Inwardly Rectifying Potassium Channels: Candidate Medicines for Drug Dependence and Pain

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Abstract: G protein-activated inwardly rectifying K^+ (GIRK) channels have been known to play a key role in the rewarding and analgesic effects of opioids. To identify potent agonists and antagonists to GIRK channels, we examined various compounds for their ability to activate or inhibit GIRK channels. A total of 503 possible compounds with low molecular weight were selected from a list of fluoxetine derivatives at Pfizer Japan Inc. We screened these compounds by a *Xenopus* oocyte expression system. GIRK1/2 and GIRK1/4 heteromeric channels were expressed on *Xenopus laevis* oocytes at Stage V or VI. A mouse IRK2 channel, which is another member of inwardly rectifying potassium channels with similarity to GIRK channels, was expressed on the oocytes to examine the selectivity of the identified compounds to GIRK channels. For electrophysiological analyses, a two-electrode voltage clamp method was used. Among the 503 compounds tested, one compound and three compounds were identified as the most effective agonist and antagonists, respectively. All of these compounds induced only negligible current responses in the oocytes expressing the IRK2 channel, suggesting that these compounds were selective to GIRK channels. These effective and GIRK-selective compounds may be useful possible therapeutics for drug dependence and pain.

Keywords: G protein-activated inwardly rectifying K^+ (GIRK, Kir3) channels, Kir channel, agonist, antagonist, Pfizer compounds, *Xenopus* oocyte.

INTRODUCTION

G protein-activated inwardly rectifying K^+ (GIRK) channels, also named as Kir3 channels, are members of the inwardly rectifying potassium channel family. GIRK channels are activated by several $G_{i/o}$ protein-coupled receptors, such as opioid receptors, which causes hyperpolarization of the neurons involved and thus leads to inhibitory regulation. GIRK channels are expressed in many tissues with different subunit compositions [1-3]. In the heart, the GIRK4 subunit is abundantly expressed as a homomultimer or heteromultimer with GIRK1 and is involved in heart rate regulation [4, 5]. In the central nervous system, GIRK channels are mainly expressed as a GIRK1/2 heteromultimer in most regions and as a GIRK2 homomultimer in the substantia nigra and ventral tegmental area. GIRK channels play a key role in analgesia [6], as demonstrated in studies using GIRK channel subunit knockout mice [7-11]. Further, mice lacking the GIRK2 or GIRK3 subunit show decreased cocaine self-administration, suggesting decreased reinforcing effects of cocaine in these mice [12] and hence the involvement of GIRK channels in its rewarding effects.

Therefore, GIRK channel inhibitors may be possible candidates as therapeutic drugs to treat substance dependence. Drugs that selectively open GIRK channels may be expected to exhibit analgesic effects without impacting opioidergic intracellular signaling pathways and $G_{i/o}$ proteins and thus have fewer side effects. It has been known that various compounds inhibit GIRK channels [13-17], but only a few have thus far been shown to activate the GIRK channel [18-20]. To identify more potent GIRK channel agonists and antagonists, we examined the ability of various compounds to activate or inhibit GIRK channels.

METHODS

Compounds

To search for selective GIRK channel agonists and antagonists, a total of 503 possible compounds with low molecular weight were selected from a list of fluoxetine derivatives at Pfizer Japan Inc. The specific names and detailed properties of each compound are not available to the public. For convenience, the compounds were numbered from PF 1 to PF 503. All drugs were dissolved in dimethyl sulfoxide (DMSO).

Electrophysiological Analysis

To screen the PF compounds, a *Xenopus* oocyte expression system was utilized based on a previous report [21]. In this system, murine GIRK1 (Kir3.1), GIRK2 (Kir3.2), and GIRK4 (Kir3.4) subunits were expressed as heteromeric channels of GIRK1/2 and GIRK1/4 in *Xenopus laevis* oocytes at Stage V or VI by coinjection of the cRNAs of mouse GIRK1 and GIRK2 subunits, and GIRK1 and GIRK4 subunits, respectively. The murine IRK2 (Kir2.1) channel,

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which is a member of another inwardly rectifying potassium channel family with similarity to the GIRK channel family, was expressed in the oocytes to examine the selectivity of the identified compounds to GIRK channels. For electrophysiological analyses, a two-electrode voltage clamp (GeneClamp500, Axon Instruments) method was used with the membrane potential kept at -70 mV. A high potassium solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.5mM $CaCl_2$, 5 mM HEPES) served as perfusion solution. Ethanol (100 mM) and $BaCl_2$ (2 mM) were used as positive controls for agonist and antagonist, respectively, and DMSO was used as a negative control. Oocytes without cRNA injection served as controls.

Assay Procedure

The procedure of the assay consisted of three steps. In the first step, among the total of 503 PF compounds, every four compounds were mixed together and dissolved in the high potassium solution to yield a solution containing each compound at 10 μM. Then the total of 126 solutions of pooled PF compounds were applied to the oocytes expressing the GIRK1/2 channel (n = 2), GIRK1/4 channel (n = 2), and oocyte controls (n = 2) without GIRK channel expression. After the first screening step, several pools of compounds were selected based on the following criteria: (i) stronger agonistic or antagonistic effect on GIRK channels, (ii) similar responses between the two oocytes tested, and (iii) substantial difference in the effect of activation or inhibition between GIRK1/2 and GIRK1/4 channels.

In the second step, PF compounds in the selected pools (10 μM) were separately applied to the oocytes expressing the GIRK1/2 channel (n = 2), GIRK1/4 channel (n = 2), and oocyte controls (n = 2) without GIRK channel expression. Several compounds were selected based on the same criteria as those in the first step, in which their magnitude of inhibition/activation and the selectivity for the GIRK1/2 or GIRK1/4 channel were considered.

In the third step, each selected PF compound was applied to the oocytes expressing the GIRK1/2 channel (n = 5) and GIRK1/4 channel (n = 5) at various concentrations to examine concentration-response relationships. Selectivity of the compounds for GIRK channels was tested by applying the compounds to the oocytes expressing IRK2 (n = 2) and oocyte controls (n = 2) without GIRK channel expression. Data were fitted to a standard regression equation by using KaleidaGraph 3.5J (HULINKS, Inc.) for analysis of concentration-response relationships.

Statistical Analyses

The current responses to PF compounds were normalized by those to ethanol or $BaCl_2$, which was applied to each oocyte like PF compounds. For statistical analyses, two-way analysis of variance (ANOVA) or Student's t-test was performed with the significance level set at $P < 0.05$. SPSS v.12.0J for Windows (LEAD Technologies, Inc.) was used for analyses.

RESULTS

In the first screening step, some pools of PF compounds showed agonistic effects on GIRK channels while most others showed antagonistic effects on GIRK1/2 and GIRK1/4 channels with various efficacies. All of the pools of PF compounds induced negligible responses in the oocytes without cRNA injection (data not shown), suggesting the current responses by PF compounds were caused by exogenously expressed GIRK channels. Based on the criteria described above, PF 9 – PF 12, PF 401 – PF 404, and PF 409 – PF 412 pools were selected as candidate agonists with relatively low percentage inhibition of GIRK currents compared to $BaCl_2$ responses, while PF 37 – PF 40, PF 157 – PF 160, PF 185 – PF 188, PF 233 – PF 236, and PF 245 – PF 248 pools were selected as candidate antagonists with relatively high percentage inhibition of GIRK currents (Fig. 1). In addition, the PF 417 – PF 420 pool was selected as candidate agonist or antagonist because it induced moderate inhibition of GIRK1/2 channels and almost no effect on GIRK1/4 channels (Fig. 1).

In the second screening step, the PF compounds in the nine pools selected above were separately applied to the oo-

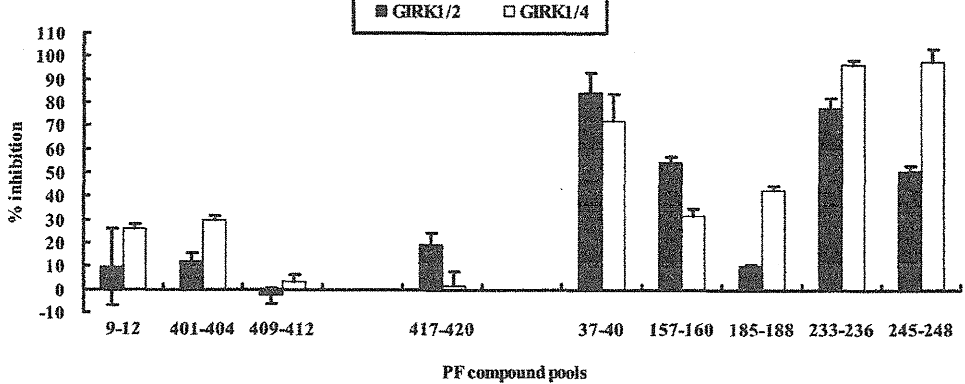

Fig. (1). Current Responses Induced by the Selected PF Compound Pools in the First Screening Step. Normalized current responses to the pools of PF compounds by the response to $BaCl_2$. PF 9 – PF 12, PF 401 – PF 404, and PF 409 – PF 412 pools were selected as candidate agonists, and PF 37 – PF 40, PF 157 – PF 160, PF 185 – PF 188, PF 233 – PF 236, and PF 245 – PF 248 pools were selected as candidate antagonists. The PF 417 – PF 420 pool was selected as a candidate agonist or antagonist in the first screening step.