In conclusion, Leu-Ile could be considered as the dietary supplement for the treatment of Aβ-related memory impairments.

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Overexpression of piccolo C2A domain induces depression-like behavior in mice

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Piccolo is one of the components of the active zone at chemical synapses and regulates the transport of synaptic vesicles. The piccolo C2A domain is an important calcium sensor and binds with phosphatidylinositol or synaptotagmin-1. Recently, clinical studies suggested that a single nucleotide polymorphism in the piccolo C2A domain might be a causal risk factor for major depression. To clarify the association of piccolo with depression, we produced a transgenic mouse overexpressing the C2A domain of piccolo, and investigated the behavior of these mice. The mice exhibited depression-like behavior in both forced swim and tail suspension tests, suggesting that piccolo might regulate the depressive behavior.
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Introduction

The presynaptic cytoskeletal matrix is associated with the active zone of chemical synapses and maintains the neurotransmitter release. Piccolo is a component protein of this matrix and is associated with the active zone of glutamatergic ribbon synapses and conventional λ-aminobutyric acidergic and glycinergic synapses [1].

Earlier, we suggested that piccolo regulates the sensitization of mice to methamphetamine, as a reduction in piccolo expression by chronic, intraventricular infusion of an antisense oligonucleotide increased the methamphetamine-induced behavioral sensitization [2]. Long-term potentiation in the hippocampal CA1 region was reduced in cultured brain slices from the piccolo-reduced expression mice and these mice also showed impaired spatial learning [3]. Moreover, clinical studies suggested that a single nucleotide polymorphism in the piccolo C2A domain might be a causal risk factor for major depression [4,5]. Piccolo may interact with various components of the active zone by its C2A domain and thereby regulate the psychiatric behavior. In this study, we generated a transgenic mouse overexpressing the piccolo C2A domain, as a hindrance to the endogenous piccolo and examined depression-like behavior in these mice.

Materials and methods

Animals and environments

Five mice were housed to a cage under a standard 12-h light/dark cycle (lights on 9:00 a.m.) at a constant temperature (23 ± 1°C) with free access to food and water throughout the experiments. They were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight, 12-week-old male mice were used for the behavioral tests.

Materials

The cytomegalovirus promoter-myc piccolo C2A expression plasmid (amino acids 4704-5610) was constructed as described earlier [6]. The following compounds were purchased from commercial sources: total RNA extraction kit (QIAGEN, Tokyo, Japan), reverse transcriptase and reagents for real-time reverse transcription polymerase chain reaction (Invitrogen, Carlsbad California, USA), and fluvoxamine (Sigma-Aldrich, Japan).

Production of piccolo C2A domain transgenic mice

Transgenic mice ubiquitously expressing the Myc-tagged C2A domain (myc-C2A) of the piccolo was produced by Unitech (Chiba, Japan). In brief, the transgene cassette including the cytomegalovirus promoter followed by the myc-C2A domain sequence was obtained from the cytomegalovirus promoter-myc piccolo C2A expression plasmid. The transgene cassette was microinjected into the fertilized eggs from C57BL/6J females mated with males.

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Reverse transcription polymerase chain reaction

The level of piccolo C2A mRNA was determined by the real-time reverse transcription polymerase chain reaction using a fast real-time PCR system (Applied Biosystems, Foster City, California, USA). Total RNA was isolated from the whole brain of E15 fetal mice using Trizol (Invitrogen). For reverse transcription, 1 μg of RNA was converted into cDNA using prime script reverse transcription [3]. Total cDNA (1 μl) was amplified in a 25-μl reaction mixture using 0.1 μM each of forward and reverse primers and the Power SYBR-Green kit (Applied Biosystems). The following mouse piccolo C2A primers were used: 5′-CAGCCAGCAGTCCCCAAA-3′ (forward) and 5′-GGGAAGATACCGTGGCTTCTG-3′ (reverse). For the internal control, the mouse GAPDH primers 5′-C ATGGCCTTCCGTGTTCCTA-3′ (forward), and 5′-AT GCCTGCTTCACCACCTTCT-3′ (reverse).

Southern blotting

Southern blotting analyses of myc-C2A transgenic mice were conducted by Unitech Co. Ltd., to determine the copy number of C2A domain-coding DNAs. The probe for southern blotting was prepared by PCR using the following primers: 5′-ATGACCTTATGGGACTTTCCTACTT-3′ (forward) and 5′-CTGGAAGTAGGTACACCTTCACAA-3′ (reverse). Genomic DNA of myc-C2A transgenic mice was cut by the restriction enzymes (AseI and Acc65I) and detected on the southern blots. Copy number was calculated based on a standard curve consisting of 10 concentrations of cytomegalovirus promoter-myc-piccolo C2A plasmid (1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 copies; data not shown).

Conditioned place preference

The apparatus used for the place-conditioning task consisted of a box with two compartments: one of transparent plexiglas and the other of black plexiglas (both 15 × 15 × 15 cm). The compartments of the box were separated from one another by a sliding door (10 × 15 cm high). The place-conditioning paradigm was performed as described earlier with a minor modification [7,8]. After habituation for 2 days, we used a Scanet SV-20 LD (Melquest, Toyama, Japan) to measure the time that the mice spent in each compartment during a 15-min period with the door open (preconditioning test). The compartment in which the mouse spent most of the time was referred to as its 'preferred side' and the other as the 'nonpreferred side'. The mice were given methamphetamine (0.3 mg/kg, subcutaneously) or saline and placed in one side or the other for 20 min with the sliding door closed. On the next day, they were given saline and placed in the compartment opposite to the methamphetamine-conditioning side for 20 min. These treatments were repeated for three cycles (6 days). In the postconditioning test, the sliding door was opened for 15 min, and we measured the time that the mice spent in each compartment again. Place-conditioning behavior was expressed as [(postvalue)-(prevalue)], of which postvalue and prevalue were the differences in time spent in the drug-conditioning and saline-conditioning compartments in the postconditioning and preconditioning tests, respectively.

Forced swim test

Mice were placed in a transparent plastic cylinder (14.5 cm diameter × 19 cm high), containing water (15 cm deep and 24–25°C) for 6 min. The immobility time was measured with a SCANET MV-10 AQ apparatus (Melquest) during the last 5 min.

Tail suspension test

The mice were suspended by the tail, such that the body dangled in the air, facing downward. The duration of immobility during 6 min was recorded visually. Fluvoxamine (90 mg/kg, intraperitoneally) treatment was done 30 min before the test.

Social interaction test

The social-interaction apparatus was an open-field box made of a gray polycarbonate (30 × 25 × 25 cm high) [9]. After habituation for 2 days, the mice were randomly assigned to an unfamiliar partner from another cage. The pairs of unfamiliar mice were placed in the apparatus for 10 min and the total amount of time spent in active social interaction, such as sniffing, grooming, following, mounting, and crawling over or under the partner, was recorded.

Results

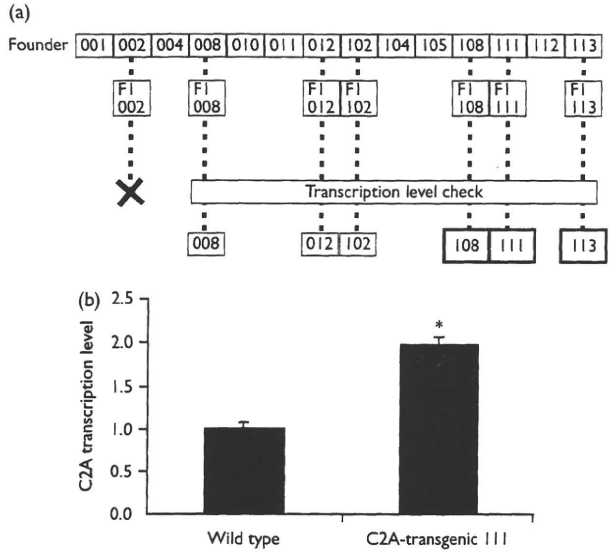
Choice of piccolo C2A domain overexpression transgenic mice

We prepared 14 lines of myc-C2A transgenic mice (Fig. 1a). We chose three lines (lines 108, 111, and 113) showing C2A mRNA expression, and compared its copy level in two different mice each (Table 1). Southern blotting showed that the mice in line 111 had 532 and 456 copies of piccolo C2A domain cDNA (Table 1), whereas the copy numbers in the other two lines were near the wild-type levels. We confirmed by the reverse transcription polymerase chain reaction that piccolo C2A mRNA levels in the fetal brains of line 111 mice were 1.96 ± 0.09 times more than those of the wild-type mice (Fig. 1b). Thus, we decided to use the line 111 transgenic mice for behavioral and biological investigations. There seemed to be no differences in the general behavior or development between myc-C2A transgenic and wild-type mice.

Role of piccolo in methamphetamine-induced conditioned place preference

The effect of myc-C2A overexpression on methamphetamine-induced conditioned place preference was examined in mice that learned the association of an environment with drug exposure. The experimental schedule is shown in Fig. 2a. Methamphetamine (0.3 mg/kg, subcutaneously) induced place preference in both transgenic and wild-type

Fig. 1



Transgenic mice. (a) Germ line of piccolo C2A transgenic mice. The copy numbers of inserted myc-C2A cDNA were determined by southern hybridization in lines 108, 111, and 113. (b) The levels of C2A mRNA in transgenic mice were compared with those of wild-type mice. Values indicate the mean ± standard error of the mean ($n=6$). *$P<0.005$ versus wild-type mice (Student's *t*-test).

Table 1 Copy numbers of myc-C2A cDNA

Mouse ID (line, mouse no.)	Copy number
C2A108 #1	2
C2A108 #2	2
C2A111 #1	532
C2A111 #2	415
C2A113 #1	6
C2A113 #2	5

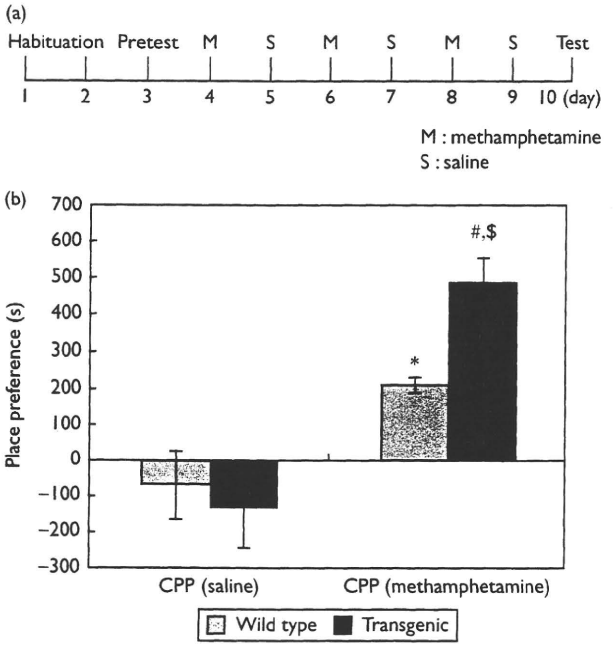
The copy numbers of inserted myc-C2A cDNA were determined by southern hybridization in the three lines of transgenic mice showing C2A mRNA expression.

mice. In myc-C2A mice, methamphetamine-induced conditioned place preference was significantly greater than in the wild-type mice (Fig. 2b).

The myc-C2A transgenic mice showed depression-like behavior

We investigated depression-like behavior in myc-C2A transgenic mice using the forced swim and tail suspension tests. These tests are usually used for evaluating the antidepressant effects of new therapeutic tools [10,11]. In both the tests, the myc-C2A transgenic mice remained immobile significantly longer than the wild-type mice (forced swim: wild type, 66.0 ± 12.8 s; myc-C2A, 119.5 ± 16.1 s; Fig. 3a; tail suspension: wild type, 388.4 ± 34.8 s; myc-C2A transgenic, 485.9 ± 17.0 s; Fig. 3b), suggesting that overexpression of myc-C2A induced depression-like

Fig. 2



myc-C2A transgenic mice exhibited increased methamphetamine-sensitization. (a) Experimental schedule of the conditioned place preference (CPP) test. The experiment was performed from 13:00 to 17:00 for 10 continuous days. Conditioning was performed during six successive days. (b) On day 10, the postconditioning test was conducted. Values indicate the mean ± standard error of the mean ($n=6$). *$P<0.05$ versus saline-treated, wild-type mice, $^{\#}P<0.005$ versus saline-treated myc-C2A transgenic mice, $^{\$}P<0.01$ versus methamphetamine-treated, wild-type mice.

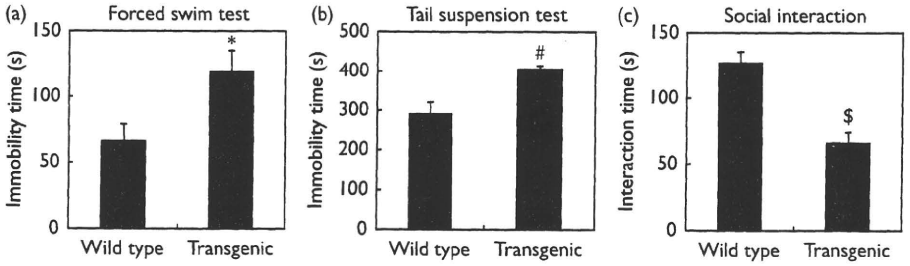
behavior in these mice. Interestingly, the myc-C2A transgenic mice also showed less social interaction with unfamiliar mice than the wild-type mice did (wild type, 128.2 ± 9.1 s; myc-C2A, 66.6 ± 9.1 s; Fig. 3c).

In contrast, the myc-C2A mice showed no significant phenotypes in either the Y-maze test or the novel object recognition test (data not shown), suggesting that myc-C2A overexpression had no effect on short-term memory and recognition memory.

Discussion

The piccolo C2A domain is important for the homodimer formation of piccolo or its interaction with synaptotagmin-1 [12]. Recently, a clinical study suggested that the single nucleotide polymorphism, rs2522833, in the piccolo C2A domain was a causal risk factor for major depression [4,5]. This polymorphism codes for a non-synonymous amino acid change (Ala4814Ser) in piccolo near its C2A calcium-binding domain. To evaluate the role of piccolo in mental disorders, we made transgenic mice overexpressing the C2A domain of piccolo. Overexpression of myc-C2A, which does not include any of

Fig. 3



Behavioral tests. Comparisons of immobility times in the forced swim (a) and tail suspension (b) tests in the myc-C2A transgenic mice and wild-type mice. (c) Comparison of social interaction times of the wild type and transgenic mice in the presence of novel partners. Values indicate the mean ± standard error of the mean ($n=12$). *$P<0.05$ versus wild-type mice, #$P<0.005$ versus wild-type mice, $$P<0.005$ versus wild-type mice.

the other piccolo domains, may inhibit the role of the endogenous piccolo protein as a dominant negative form. Leal-Ortiz *et al.* [13] showed that piccolo influences the presynaptic function by negatively regulating synaptic vesicle exocytosis. Mechanistically, this regulation seems to be calmodulin kinase II dependent and mediated through the modulation of synapsin1a dynamics. Previously, we showed that the reduction of piccolo expression by an antisense oligonucleotide increased dopamine levels in the brain [2] and increased the preference induced by methamphetamine treatment. In this study, the overexpressed myc-C2A domain also increased the preference induced by the methamphetamine treatment (Fig. 2), confirming that reduction of piccolo increases the methamphetamine preference.

Furthermore, myc-C2A transgenic mice exhibited significantly increased periods of immobility in both the forced swim and tail suspension tests compared with the wild-type mice (Fig. 3a and b), suggesting that myc-C2A transgenic mice may show depression-like behavior. Piccolo showed increased expression in brain, is localized to the presynaptic active zone, and is suggested to be involved in synaptic vesicle clustering [14]. Piccolo dysfunction may lead, not only to a reduced dopamine uptake, but also to the modified vesicle transport in the presynapse, and to depression-like behavior. We showed that overexpression of the myc-C2A domain induced depression-like behavior and methamphetamine induced conditioned place preference. These results confirm that the C2A domain of piccolo plays an important role in the psychiatric behavior.

Conclusion

Our results indicate that piccolo plays important roles in reducing psychiatric disorders and in drug dependency. Furthermore, they confirm the interaction of piccolo and depression symptoms, which was suggested in the clinical studies.

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薬物依存におけるピッコロの役割

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Identification of Piccolo as a regulator of behavioral plasticity and dopamine transporter internalization

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Summary

Dopamine transporter (DAT) internalization is a mechanism underlying the decreased dopamine reuptake caused by addictive drugs like methamphetamine (METH). We found that Piccolo, a presynaptic scaffolding protein, was overexpressed in the nucleus accumbens (NAc) of the mice repeatedly administrated with METH. Piccolo downexpression by antisense technique augmented METH-induced behavioral sensitization, conditioned reward and synaptic dopamine accumulation in NAc. Expression of Piccolo C2A domain attenuated METH-induced inhibition of dopamine uptake in PC12 cells expressing human DAT. Consistent with this, it slowed down the accelerated DAT internalization induced by METH, thus maintaining the presentation of plasmalemmal DAT. In immunostaining and structural modeling Piccolo C2A domain displays an unusual feature of sequestering membrane phosphatidylinositol 4,5-bisphosphate, which may underlie its role in modulating DAT internalization. Together, our

※教育委員会からの推薦論文：現在のアルコールを含む依存性薬物の基礎的および臨床的研究のトピックス

results indicate that Piccolo upregulation induced by METH represents a homeostatic response in the NAc to excessive dopaminergic transmission. Piccolo C2A domain may act as a cytoskeletal regulator for plasmalemmal DAT internalization, which may underlie its contributions in behavioral plasticity.

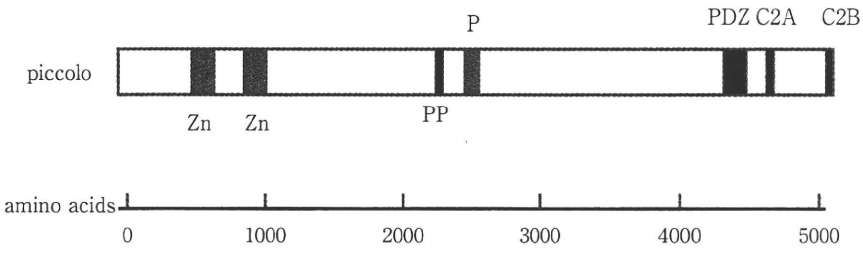
Key words: Piccolo, dopamine transporter, methamphetamine, behavioral plasticity, C2A domain
ピッコロ，薬物依存，ドパミン，ドパミントランスポータ，覚せい剤

薬物が依存性を形成するメカニズムについては解明されていないことが多い．興味深いことに依存性を持つ薬物の中には，精神興奮作用を持つものと鎮静作用を持つものが併せて存在する．両者の薬物依存形成過程の差異と共通性について全ては明らかになっていない．ここでは，覚せい剤の依存と関係する分子として我々が研究を進めているPiccoloについて紹介をする．

依存性薬物の中でも，メタンフェタミンの依存性の形成過程を明確にし，依存抑制薬の開発をすることが，当面の目標である．加えて，一旦，メタンフェタミン摂取を中止した後の渇望感から生じる再燃の機構を明らかにすることは，さらに重要な課題である．薬物依存が日本より深刻な社会問題となっているアメリカでは，多くの研究者によって薬物依存や再燃についての研究が分子生物学的，行動薬理学的および遺伝疫学的にパワフルに実施されている．アメリカで使用頻度の高い覚せい剤はコカインであることから，コカインの研究が主となり，いくつかのコカイン依存治療薬も報告されている．しかしながら，薬物依存治療薬が市販されるには至っていない．一方で，我が国において，深刻な問題となっているのはメタンフェタミンである．残念なことに，国内では依存性薬物についての研究はアメリカほど大規模に行われていず，治療薬の開発やリスクファクター解明に繋がる研究も不十分な部分が否定できない．即ち，メタンフェタミンの依存形成を阻害するような遺伝子や治療薬の開発は，日本国内でなさねばならぬところであるが，立ち遅れているのが現状である．腫瘍壊死因子，組織型プラスミノーゲンアクティベータ，グリア細胞株由来神経栄養因子などの分子が，メタンフェタミンの依存に関与していることを我々のグループでは報告している．これら一連の報告から，薬物依存形成や再燃のメカニズムは単純でなく，未知の分子が関与している可能性が考えうる．

我々はこのような現状を打破するためにcDNAサブトラクション法を用いて，メタンフェタミンの依存に関与する分子の検索を行った．メタンフェタミンまたは生理食塩水を10日間連続投与したマウス側坐核をそれぞれ取り出し，その組織のmRNAをもとに市販のキットを用いてcDNAサブトラクションを行った．メタンフェタミンを連続投与した脳で発現が著しく増加していた遺伝子のシークエンスを行い，タンパク質に翻訳される可能性のあるものについて詳細に照合したところ，Piccoloという名の分子を見出した．

PiccoloはPDZ，C2AやC2Bのドメインから成る400KDを超える巨大タンパク質である．Aczoninとの相同性が高く，共通してN末端にglutamine-richな配列を持ち，C末端にPDZドメインを持っていることも共通している（図1）．C2Aドメインは，Ca^{2+}結合部位を持ち，結合の有無で立体構造が変化すると考えられている．ピッコロはシナプス前膜の細胞膜マトリックスに存在し，active zoneと呼ばれる部位に存在する他の分子と結合することも分かっているが，詳細な生理機能は分かっていない．Piccoloと同様にシナプス小胞体に存在するタンパク質



Zn; zinc finger, PP; polyproline,

図1　Piccolo遺伝子の配列

には，ELKS，Liprin 1 α，RIM，RIMBPなどが知られているが，それらの相互関係は分かっていないものの，共通のアミノ酸配列があるため，それらの機能の網羅的な解明が期待される．

Piccoloは膵臓において，インスリン分泌に関係することも報告されている．Piccoloは細胞膜の内側に接触しており，ATP依存性カリウムチャンネルや電位依存性のカルシウムチャンネルの開閉によって，インスリンの細胞外放出を調整することが証明された．このように小胞体内に存在するタンパク質が相互作用していることを論理的に証明される例は多くなく，価値あるデータである．

Piccoloの機能については，上述したような点を中心に研究がなされているが，脳ではmRNAが検出されていることが分かっているのみで，その機能に踏み込んだ検討はなされていなかった．

我々は，cDNAサブトラクション法を用いてメタンフェタミンの連続投与によって増加している分子としてpiccoloの断片を見出した．Piccoloは，他のタンパクと共通した配列を持つことから，本当にpiccoloの発現が増加しているのか否かが明確でなく，ピッコロの配列の全領域をカバーするように10組のプライマーのデザインの行い，RT-PCR法で検討した．設計した全ての組み合わせの配列で，メタンフェタミンを連続投与したマウス側坐核における該当配列のmRNAが増加していることが分かった．またメタンフェタミンを1回だけ投与した場合および連続投与しても側坐核以外の部位では，発現量の変化が観察されなかった．メタンフェタミンを連続投与した場合のみ，かつ，側坐核でのみpiccoloの発現量が増加していることは，薬物依存との関連を考えた時に意味あることである．

我々の通常の研究手法では，次にpiccoloの遺伝子欠損マウスを用いた実験を行うところであるが，上述したようにpiccoloは巨大分子であることから全長を欠損させるのは難しい．そこでアンチセンスヌクレオチド（以下ASとする）を脳に注入することで，生理機能を検討することにした．ASは，Alzet社製のミニ浸透圧ポンプで脳室に2週間持続投与を行い，注入中に行動実験を行った．メタンフェタミンを投与した直後1時間の行動量の測定を3日間おこなった．メタンフェタミン投与後の行動量は経日的に増加するが，AS持続注入されているマウスでは，その増加の程度が有意に高かった．引き続き，場所嗜好性反応試験を行ったところ，メタンフェタミンへの嗜好性がAS投与群は増強していた．さらに，プローブを側坐核に挿入してin vivoマイクロダイアリシスを行いドパミン遊離量の測定を行った．メタンフェタミン投与後にドパミ

ンの遊離量が4倍程度増加するが，Piccolo-ASを注入したマウスでは増加の程度が有意に上昇した．このようにASによってpiccoloの発現量を抑制すると，メタンフェタミンによる精神障害がより強く観察されたことから，piccoloはメタンフェタミンが生体に与える作用を減弱すると考えられる．特に，in vivoマイクロダイアリシスでの実験結果から，メタンフェタミン投与によって引き起こされるドパミンの遊離量の増加への影響が本質ではないかと考えられた．

さらにpiccoloがメタンフェタミン投与の影響を抑制するメカニズムについて検討した．マウス脳神経細胞を培養して，piccoloとチロシンハイドロキシラーゼ，または，ドパミントランスポータとそれぞれ2重免疫組織染色をしたところ，piccoloはドパミントランスポータと同一細胞で発現していて，チロシンハイドロキシラーゼ陽性細胞とは異なることが観察された．メタンフェタミンが脳に存在すると，①シナプス小胞からのドパミンの排出量が増加すること，②ドパミントランスポータの内在化が起こり，不活性化されて，シナプス間隙での遊離量が増加する，ことが分かっている．Piccoloは，①か②のどちらか，または両方を抑制する作用があると考えられるが，ドパミントランスポータ陽性細胞に発現していることから，②を抑制している可能性が高いと考えられた．

そこで，PC12細胞にドパミントランスポータ遺伝子を導入して安定発現させて使用した．ピッコロ遺伝子全長を導入することは難しいため，ドメインごとに導入したところ，C2Aドメインを強制発現した時，メタンフェタミン添加によるドパミントランスポータの内在化を抑制した．膵臓におけるインスリン分泌には，piccoloのPDZドメインが重要であることと比べ対照的である．C2Aドメインは，カルシウムと結合することから，ドパミントランスポータとの関係を解明しようと色々実験を行ったが，明確な結果を得ることができなかった．

MOEモデルを用いてシュミレーションを行ったところ，phosphatidylinositol4,5-bisphosphateとpiccoloが結合したときが一番安定であることが分かった。

今後は，ドパミントランスポータとCa+との関係についての解明を行っていきたいと考えている．

本総説に記述した内容は，富山大学大学院医学薬学研究部　薬物治療学研究室，名古屋大学大学院医学系研究科　医療薬学講座で行われた．実験に携わってくださった方々にお礼を申し上げます．

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Opinion

Cell PRESS

Neuropathic and chronic pain stimuli downregulate central μ -opioid and dopaminergic transmission

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Although morphine and other μ-opioid agonists are the main analgesics for severe pain, these compounds have potential for abuse and/or addiction. This has complicated the use of μ-agonists in the treatment of chronic pain. However, clinical studies show that when μ-agonist analgesics are appropriately used to control pain, actual abuse or addiction does not usually occur, although some risk factors that increase vulnerability need to be considered, including genetic variation. We review recent findings on molecular adaptations in sustained pain models, and propose how these adaptations (including sustained release of the endogenous μ-agonist β-endorphin) can result in decreased abuse potential of μ-agonists in chronic pain states. We also review data on particular gene polymorphisms (e.g. in the μ-receptor gene) that could also influence the relative abuse potential of μ-agonists in clinical pain populations.

Introduction

Morphine and other μ-opioid agonists (μ-agonists) are frequently used for the treatment of cancer pain and moderate to severe non-cancer pain, as well as post-surgical or traumatic pain [1–3]. Longer-acting opioid medications and formulations such as methadone, buprenorphine and sustained-release oxycodone also have utility in the treatment of neuropathic pain [3–5]. However, μ-agonists also have a constellation of side-effects (e.g. acute respiratory depression, chronic constipation and pruritus), in addition to abuse or addiction potential. Furthermore, chronic administration of μ-agonists results in tolerance and dependence.

There has been a substantial increase in the non-medical use of prescription μ-opioids, possibly because of their widespread availability compared with illicit compounds such as heroin. However, abuse or addiction does not usually occur when μ-agonists are used to treat substantial somatic pain [1,6,7]. Patients do show withdrawal signs when there is abrupt cessation of chronic μ-agonist administration. However, this physical dependence *per se* is not sufficient for a diagnosis of abuse or addiction [7,8]. The relative infrequency of developing an addictive disorder *de novo* in this setting lends support to the safe use of μ-agonists for the treatment of severe acute pain, as well as cancer and non-cancer chronic pain [1,6,7].

Chronic pain, including neuropathic pain, often has a negative effect on quality of life, can function as a stressor and increases the incidence of anxiety and depression. The endogenous opioid system has been strongly implicated in nociception, anxiety and stress-responsive hypothalamic–pituitary–adrenal (HPA) axis modulation. Alterations of the expression of genes involved in stress responsiveness have been reported after chronic intermittent exposure to μ-agonists, cocaine, other stimulants and alcohol in animals and in clinical settings [9].

Glossary

Drug abuse: abuse has been defined by various scientific, national and international policy and clinical groups. Among commonly used diagnostic criteria, the Diagnostic and Statistical Manual IV (DSM-IV) focuses on non-medical use of a particular substance resulting in maladaptive patterns of behavior and leading to clinically significant impairment or distress. It is often considered that reward properties of the drug can drive the initial trajectory of drug abuse from early experimentation to regular usage.
Drug addiction: often described as a clinical disorder with greater severity than abuse and including some cardinal signs such as escalation of drug exposure, compulsive drug use, presence of dependence and withdrawal, and clinical and social consequences. Of note, the specific clinical and neurobiological characteristics of addiction are related to the particular substance that is used (e.g. μ-agonists vs. dopaminergic psychostimulants).
Drug dependence: altered physiological state that develops with persistent drug exposure (e.g. of a μ-agonist). This is observed *in vivo* and clinically as the emergence of a pharmacologically characteristic withdrawal syndrome after sudden drug discontinuation.
Neuropathic pain: pain caused by nerve or neuron injury or its related molecular adaptations.
Nociceptive pain: pain caused by ongoing noxious slimuli, such as heat, cold and chemicals, or acute injury.
Place preference paradigm: an experimental paradigm used to investigate the conditioned reward effects of a drug (e.g. a μ-agonist) by repeated pairing with a novel environment. After repeated pairings, an experimental animal can exhibit drug-induced place preference. In other words, it will spend more time in an environment previously paired with a dose of a rewarding drug than in the complementary environment previously paired with vehicle.
Reward: stimulus (e.g. certain drugs) that can be perceived as pleasant or euphoric. This can result in self-administration of the drug by experimental animals or humans.
Reinforcer: event (in this case, administration of a particular drug) that when paired with a particular behavior, will increase the probability of reoccurrence of that behavior. Reinforcing effects of many drugs abused by humans (including μ-agonists) can thus be studied in self-administration paradigms whereby a subject emits a behavior (e.g. a lever press) that results in drug delivery.

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Neuropathic pain is characterized by burning pain, hyperalgesia (an exaggerated pain in response to painful stimuli) and allodynia (pain evoked by normally innocuous stimuli), and can result from long-term functional alterations of primary afferent neurons and/or spinal dorsal horn neurons (containing diverse receptors, protein kinases and neuropeptides) after nerve injury of different etiologies [10].

In this review, we focus on recent experimental studies detailing molecular and neurobiological adaptations that occur as a result of chronic (e.g. neuropathic) pain stimuli [11–16]. Based on these and prior studies, we propose that these adaptations result in decreased abuse potential of μ-agonists used as analgesics in clinical pain conditions. We present data showing that genetic variation (e.g. in the μ-receptor gene *OPRM1*) can further influence the abuse potential of chronic μ-agonist exposure [17,18].

Role of μ-opioid receptor function in drug reward and in neuropathic pain

The mesolimbic dopaminergic system, projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is a crucial mediator of the reinforcing effects of μ-agonists [19–21] (Box 1). Positron emission tomography studies in humans have mapped μ-opioid receptor distribution in brain and have detected substantial populations in areas involved in pain response (e.g. insular cortex and thalamus) and in reward-related areas (e.g. cingulate cortex, mesolimbic system including NAc) [22]. Intriguingly, release of dopamine in the NAc after morphine treatment is markedly suppressed by sciatic nerve ligation [23], a model of neuropathic pain. μ-Receptor binding sites have also been observed in the pons and medulla regions, critical hindbrain sites that regulate μ-agonist-induced antinociception. We therefore assessed changes in the ability of morphine to activate G proteins in the lower midbrain, including the VTA, limbic forebrain, including the NAc, and pons and medulla regions of sham-operated and sciatic nerve-ligated mice by measuring binding of guanosine-5′-*o*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) to membranes [23]. Morphine-induced [^{35}S]GTPγS binding was decreased in the midbrain, including the VTA, but not in limbic forebrain or pons and medulla of sciatic-nerve-ligated mice [23]. This finding suggests that neuropathic pain induced by sciatic nerve ligation leads to a reduction in μ-opioid receptor function in the midbrain, including the VTA, resulting in inhibition of the reward effect of morphine. This selective reduction in μ-opioid signaling in midbrain, including the VTA, is consistent with a decrease in the reward effects of μ-agonists (and thus their abuse potential) in neuropathic pain states, with relative preservation of analgesic effects.

Molecular adaptations in μ-opioid receptor function due to chronic pain

One mechanism for the aforementioned reduction in μ-opioid receptor signaling in VTA in chronic pain states could be a sustained increase in release of the μ-opioid neuropeptide β-endorphin. Sustained exposure to β-endorphin could result in receptor phosphorylation and uncoupling of receptors from effector systems, and thus desensitization. Of note, β-endorphin tends to cause greater desensitization than exogenous ligands such as morphine [24]. A serine/threonine kinase, G protein receptor kinase 2 (GRK2), promotes μ-agonist-induced phosphorylation [25]. The level of membrane-bound GRK2 in the midbrain, including the VTA, but not in the pons and medulla, was increased in nerve-ligated mice relative to controls [23]. This increase in GRK2 in the midbrain might therefore reduce μ-opioid receptor function during sciatic nerve ligation, leading to decreased morphine-induced place preference (i.e. an apparent decrease in morphine-induced reward effects) [23].

Box 1. Opioid reward and the mesolimbic dopamine system

μ-Agonists have marked effects on mood and motivation. They can produce euphoria in humans and function as positive reinforcers (i.e. they maintain drug-seeking behaviors). These reinforcing effects can become the primary stimuli that motivate behavior, with subsequent compulsive drug-seeking behavior or addiction [50,51]. The mesolimbic dopaminergic system (from the VTA of midbrain, projecting to the NAc) is a critical circuit for this effect [54]. Furthermore, μ-agonists increase dopamine release and dopamine metabolites in mesolimbic terminal fields [21,31,55]. Using the conditioned place preference paradigm, intra-VTA administration of morphine produces a reward effect [21,56]. Morphine-induced place preference is blocked by either dopamine antagonists or neurochemical destruction of the NAc [57]. Dynorphins (endogenous κ-agonists) act on κ-receptors in the NAc, dose-dependently decrease dopamine release [35] and can block the reward effects of μ-agonists (Figure 2).

Changes in β-endorphin levels in chronic pain: impact on abuse potential of exogenous μ-agonists

As alluded to above, the endogenous neuropeptide μ-agonist β-endorphin is released within some brain regions, including the mesolimbic pathway, during pain states [26,27]. Sciatic nerve ligation in rats resulted in suppression of place preference induced by intra-VTA injection of DAMGO (a μ-selective agonist), and intra-VTA injection of a specific antibody to β-endorphin reversed this effect [28]. Furthermore, sciatic nerve ligation also caused suppression of place preference induced by systemic morphine and a parallel decrease in DAMGO-stimulated binding of [^{35}S]GTPγS in the VTA. These phenomena were also abolished in β-endorphin knockout mice [28]. In addition, nerve ligation resulted in inhibition of systemic morphine-induced dopamine release in the NAc, which is consistent with reduced abuse potential of the μ-agonist in this condition; this effect was also abolished in β-endorphin knockout mice [28]. Taken together with data on spinal PKC activation (below), these findings suggest that selective and sustained activation of mesolimbic β-endorphin might be an important proximal mechanism for suppression of the reward effects of exogenous μ-agonists in pain states (Figure 1).

ERK and dopamine neurons in the VTA in neuropathic pain states

Extracellular signal-regulated kinase (ERK) mediates cellular responses to a wide variety of signals. Chronic administration of morphine increases ERK activity in the VTA, and ERK activation in this region is associated with a morphine-induced increase in the activity of tyrosine

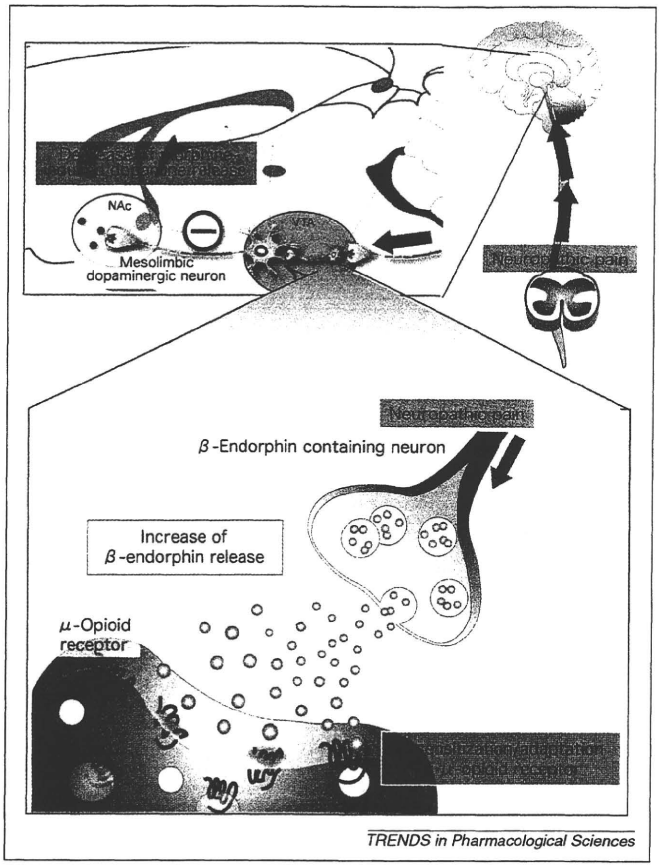

Figure 1. Model of the mechanism of suppression of μ-agonist-induced reward in neuropathic pain. Peripheral nerve injury can cause sustained activation of the endogenous β-endorphinergic system in the brain. β-Endorphin released by chronic nociceptive stimuli can continuously activate μ-opioid receptors in the VTA, thus leading to downregulation of μ-opioid receptor function and resulting in a decrease in dopamine release in the NAc. This phenomenon could explain the mechanism that underlies the suppression of μ-opioid reward under neuropathic pain-like states observed in animal models [28].

hydroxylase (TH) [29], the rate-limiting enzyme in dopamine biosynthesis. We therefore investigated whether ERK could be critical to the reward effects of morphine and whether neuropathic pain could affect ERK in the mouse lower midbrain, including the VTA [15]. Levels of phosphorylated-ERK (p-ERK) in these regions were decreased after sciatic nerve ligation, without changes in basal levels of ERK protein. Furthermore, a double immunolabeling experiment with antibodies against TH and p-ERK demonstrated that almost all of the p-ERK immunoreactivity was localized within TH-positive neurons in the VTA of sham-operated mice. After sciatic nerve ligation, a marked decrease in p-ERK immunoreactivity was detected in the VTA [15]. TH can be phosphorylated at specific serine residues by various protein kinases. The only protein kinase reported to phosphorylate TH at Ser31 *in vitro* are ERKs [30]. Sciatic nerve ligation caused a reduction in p-TH (ser31)-immunoreactivity in NAc projection neurons in the VTA [28]. Taken together, these findings suggest that sustained downregulation of ERK activity in the VTA in neuropathic pain might decrease TH activity and result in decreased dopaminergic tone and potential dysphoria. It can also be hypothesized that this decreases the responsiveness of the mesolimbic system to exogenous μ-agonists during neuropathic pain states.

Upregulation of the κ-opioid–dynorphin system limits drug reward in pain states

κ-Agonists, including the endogenous neuropeptide dynorphin A(1–17), cause a decrease in dopamine dialyzates in terminal fields of the nigrostriatal and mesolimbic systems, and can also block the reward effects of drugs of abuse, including μ-agonists and psychostimulants [21,31–35] (Figure 2). Repeated administration of μ-agonists upregulates expression of the κ-opioid receptor (KOR) and prodynorphin (pDYN) mRNA in brain [36], and this upregulation might decrease the reward effects and abuse potential of chronic μ-agonists in clinical settings. Furthermore, pain stimuli themselves (in the formalin model) decreased reward effects of systemic morphine [12], and this effect was sensitive to κ-receptor antagonism and to dynorphin antibodies in the NAc.

It is also known that both *KOR* and *PDYN* polymorphisms can affect vulnerability to addictive diseases in humans [37,38]. Taken together, these findings suggest that upregulated κ-receptor or dynorphin function, due to chronic pain itself or to chronic μ-agonist therapy, can decrease the abuse potential of μ-agonist analgesics. In addition, genetic variations in *KOR* and *PDYN* could influence the relative impact of these adaptations in clinical pain settings [37,38] (Figure 3).

Role of PKC in neuropathic pain

The protein kinase C (PKC) family of enzymes plays an important role in signal transduction in several physiological processes. PKCγ (the major PKC isoform within mammalian spinal cord) immunoreactivity is clearly increased in the dorsal horn of rat spinal cord after peripheral nerve injury [39]. Furthermore, pain behaviors are decreased after sciatic nerve ligation in mice lacking the PKCγ gene [40,41]. This observation suggests that activated PKC in the spinal cord might result in central sensitization to nociceptive transmission, leading to the development of neuropathic pain.

Interestingly, intrathecal administration of the specific PKC activator phorbol 12,13-dibutyrate (PDBu) induces spontaneous nociceptive pain-like behavior and long-lasting thermal hyperalgesia associated with enhancement of neuronal activity in brain regions related to pain perception [42,43]. In addition, we recently found that intrathecal PDBu-induced activation is observed in brain regions putatively involved with both sensory-discriminative and affective-motivational components of pain (e.g. somatosensory cortex, lateral thalamus, cingulate cortex and medial thalamus) [44] (Box 2). Intrathecal PDBu also induced neuronal activation in the mesolimbic pathway (VTA and NAc) and these effects were abolished in PKCγ-gene knockout mice [44]. We also found that systemic morphine-induced place preference was reduced by intrathecal pretreatment with PDBu [43]. Of interest, this effect of intrathecal PDBu was eliminated in β-endorphin knockout mice, suggesting that spinal PKC activation leads to sustained mesolimbic β-endorphin release, which in turn results in decreased reward by exogenous μ-agonists as detailed above [45]. More broadly, these findings lead to the proposition that activation of spinal PKCγ after sus-

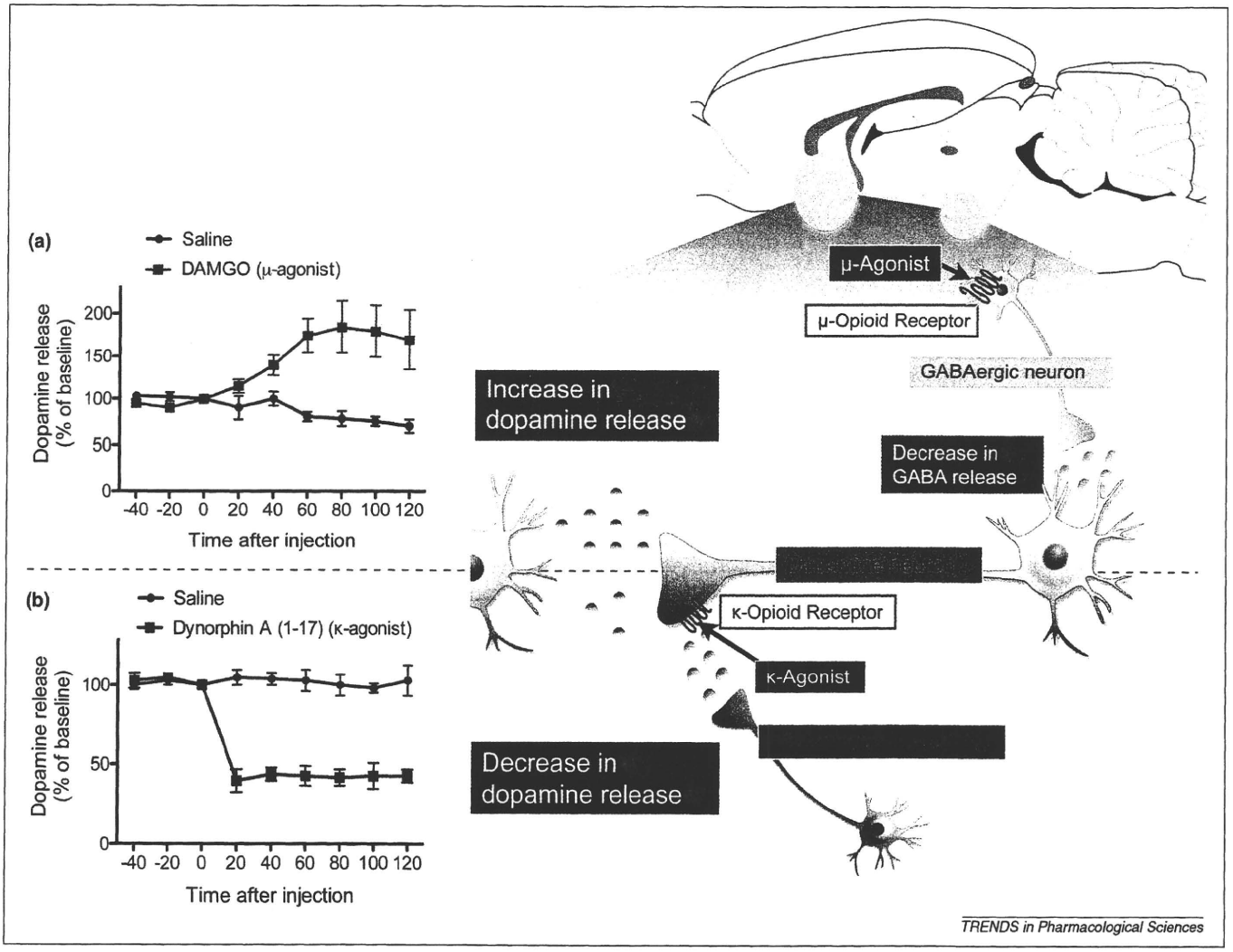

Figure 2. Mechanism of modulation of dopamine release by μ- and κ-opioid systems. **(a)** μ-Agonists inhibit γ-aminobutyric acid (GABA)ergic neurons that normally inhibit dopaminergic neurons in the VTA. This 'disinhibition' leads to an increase in the release of dopamine in the NAc. Inset: DAMGO (0.1 nmol) administered intra-VTA in rats (Narita *et al.*, unpublished observations). **(b)** Dynorphins act through κ-opioid receptors in the NAc as a countermodulatory system. Acute dynorphins (or exogenous κ-agonists) tend to decrease the release of dopamine [35] and the reward effects of μ-agonists [12]. Inset: dynorphin (4.4 nmol) administered intra-striatally in mice; replotted from [35].

tained ascending pain transmission is an important factor in the development of neuropathic pain-like states. Furthermore, by affecting mesolimbic function, this process can lead to changes in emotional and affective function in pain states.

Impact of *OPRM1* genetic variation on clinical analgesia and addiction to μ-agonists

Of relevance to both patient-specific and population-wide patterns of adaptation to chronic pain and μ-agonist administration, a major functional SNP (known as

Box 2. Ascending pain pathways and interactions of pain and reward functions

The axons of second-order spinal nociceptive neurons ascend via the spinothalamic tract and terminate in two different parts of the thalamus. The lateral nociceptive system terminates in the ventrobasal complex, which in turn projects to primary and secondary somatosensory cortices. This system is involved in the sensory-discriminative aspects of responses to noxious stimuli [58]. The medial component of the spinothalamic tract terminates in the medial and intralaminar thalamic nuclei, which project in turn to limbic regions including the anterior cingulate and anterior insular cortices and amygdala. This system is involved in the affective-motivational aspects of pain processing, namely the conscious perception of pain affect, memory and motor outputs associated with prediction and avoidance of noxious stimulation also termed nocifensive behaviors [59,60]. The actions of μ-opioid receptors predominate in areas with the highest densities of such receptors; responses to chronic pain states and surgical treatment are associated with changes in opioid receptor binding in the medial system [61]. The limbic system, including the VTA, is involved in coding the reward properties of appetitive stimuli, and plays a role in selecting between reward and pain resolution outcomes. These dual functions, particularly of the VTA and anterior cingulate cortex, predispose parts of the limbic system to modulation of what at first seem to be diametrically opposed functions: pain and reward. Thus, modulation of dopaminergic functions by chronic pain such as neuropathic pain is a natural outcome of pain and reward processing in the medial pain system and is modulated by opioid compounds.

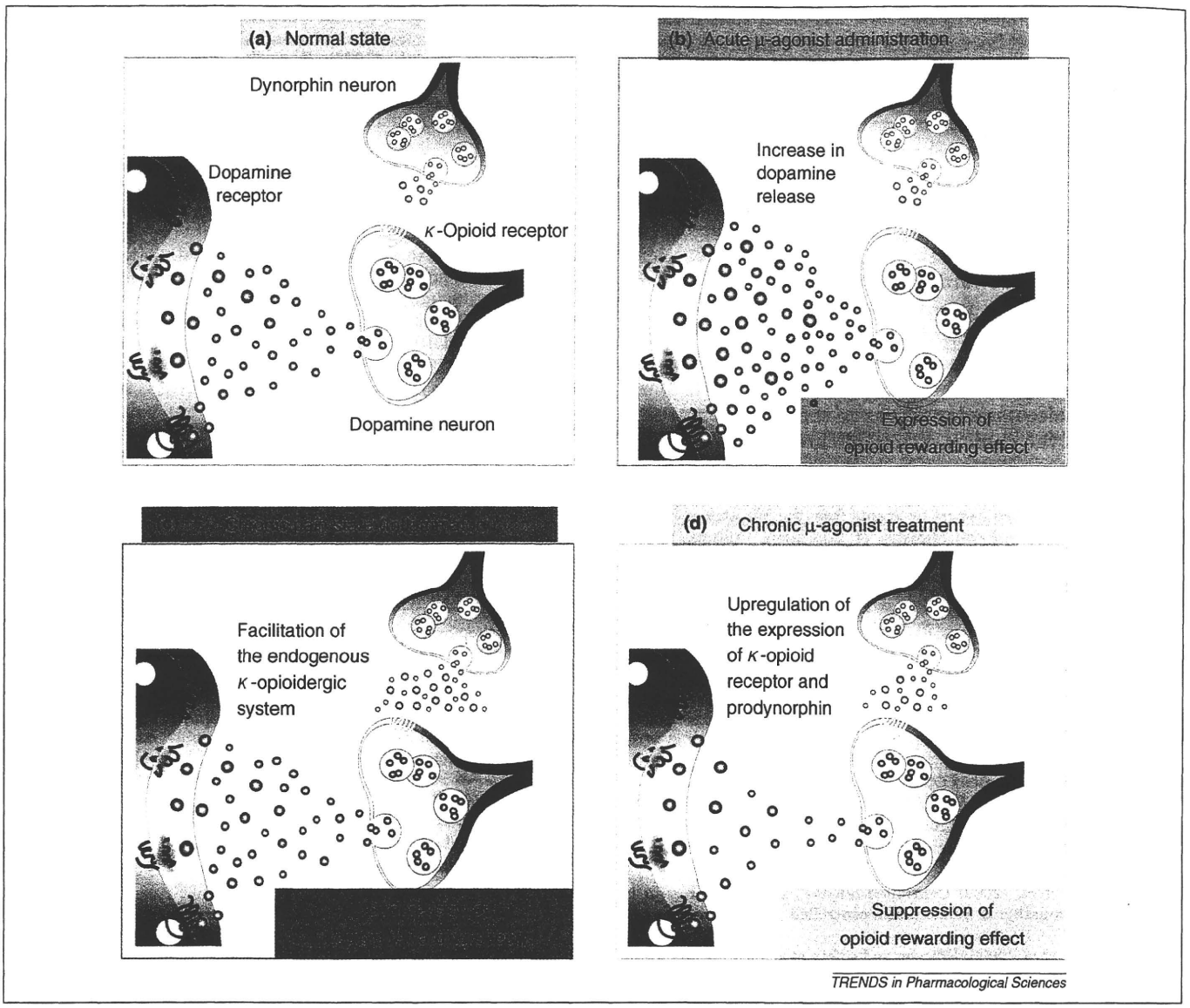

Figure 3. Adaptation of the κ-opioid–dynorphin system limits drug reward in pain states and during chronic μ-agonist administration. **(a)** Normal state: endogenous dynorphin regulates dopamine release as part of a negative feedback system. **(b)** Acute exogenous μ-agonist administration: morphine produces an increase in dopamine release in the NAc, resulting in expression of a reward effect. **(c)** Chronic pain state (inflammatory pain) + μ-agonist administration: the opioid-induced reward effect is suppressed under an inflammatory pain-like state owing to inhibition of dopamine release at dopaminergic terminals caused by facilitation of the endogenous κ-opioid system within the NAc. **(d)** Chronic μ-agonist treatment: repeated administration of a μ-agonist upregulates expression of KOR and pDYN in brain, which in turn might cause inhibition of dopamine release at dopaminergic terminals. In addition, it might cause a decrease in the reward effects of an exogenous μ-agonist.

A118G) in the μ-receptor gene *OPRM1* causes an increase in affinity and potency of β-endorphin [46]. This SNP also leads to lower mRNA and protein expression of μ-opioid receptors *in vitro* [47,48]. As previously discussed, enhanced β-endorphin function can result in decreased abuse potential of exogenous μ-agonists in chronic pain. The A118G SNP is differentially distributed in various ethnic groups, from <2% in African populations to ~50% in Asian populations [37], which is of relevance to population-wide studies of its clinical impact. Consistent with a direct role of this SNP in this area, we found that the A118G SNP imparted substantial attributable risk of developing heroin addiction in a modestly admixed Swedish population [49].

We have long hypothesized that stress responsiveness underlies vulnerability to addiction, adaptation to chronic opioids and neuroendocrine patterns of opioid withdrawal (resulting in prominent activation of the HPA axis) [9,50–52]. Such adaptations might affect vulnerability in the setting of μ-agonist exposure through illicit use or chronic analgesia therapy, and might also be influenced by genetic variation (for recent reviews see [17,37,50,51]). As a direct example, the A118G *OPRM1* SNP imparts greater activation of the HPA stress axis after naloxone challenge in European Americans [53]. Overall, these findings suggest that *OPRM1* SNPs can affect the risk of developing abuse or addiction after chronic μ-agonist exposure in certain patient populations [17,18].

Conclusion

Chronic use of μ-agonists in neuropathic pain has been the subject of several clinical reviews, and its effectiveness with respect to the balance of therapeutic and undesirable

effects (including abuse potential) has been controversial [7]. Work reviewed here reveals that adaptation of specific spinal and supraspinal molecular systems (e.g. PKC and ERK) occurs in neuropathic pain models and that this results in a decrease in the reward effect of exogenous μ-agonists. We further show that functional plasticity occurs in mesolimbic μ- and κ-opioid receptor or neuropeptide systems (involving β-endorphin and dynorphins) as a consequence of pain itself and of repeated exogenous μ-agonist administration. We hypothesize that this plasticity also decreases the reward effects of exogenous μ-agonists, and thus their abuse potential, in chronic pain states. We also propose that variations in specific opioid receptor and neuropeptide genes (e.g. *OPRM1*, *KOR* and *PDYN*) and HPA axis genes can further affect the impact of the aforementioned molecular adaptations on the abuse potential of μ-agonists in the clinical setting of chronic pain.

Acknowledgments

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Addiction Biology

PRECLINICAL STUDY: BRIEF REPORT

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Epigenetic modulation at the CCR2 gene correlates with the maintenance of behavioral sensitization to methamphetamine

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ABSTRACT

The intermittent administration of methamphetamine produces behavioral sensitization to methamphetamine. In the limbic forebrain, mainly including the nucleus accumbens, of mice that had been intermittently treated with methamphetamine, we found a significant increase in mRNA of a chemokine, CCR2. This increase was accompanied by a significant increase in histone H3 lysine 4 (H3K4) trimethylation at its promoter. Interestingly, the maintenance of sensitization to methamphetamine-induced hyperlocomotion was significantly decreased in CCR2 knockout mice. These findings suggest that increased CCR2 associated with epigenetic modification after the intermittent administration of methamphetamine may be associated with the maintenance of sensitization to methamphetamine-induced hyperlocomotion.

Keywords CCR2, drug abuse, epigenetics, histone modification, methamphetamine, sensitization.

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Methamphetamine is a strongly addictive psychostimulant that dramatically affects the central nervous system (CNS) and is highly abused worldwide. In rodents, it has been shown consistently that repeated exposure to psychostimulants results in a progressive and enduring enhancement of the motor stimulant effect elicited by a subsequent drug challenge, which is called behavioral sensitization. Many studies have suggested that the mesolimbic dopaminergic system, which projects from the ventral tegmental area to the nucleus accumbens, is critical for the initiation of methamphetamine-induced hyperlocomotion (Vanderschuren & Kalivas 2000).

A growing body of evidence suggests that the behavioral sensitization induced by psychostimulants may be accompanied by long-lasting neural plasticity (Robinson & Kolb 1999). The neuronal plasticity has been believed to require diverse alterations in gene expression. Although some of the candidate genes that are involved in behavioral sensitization to psychostimulants have been identified (Ujike *et al.* 2002; Sokolov, Polesskaya & Uhl 2003), an important step toward unraveling the complex machinery of psychostimulant-induced behavioral sensitization is a multiplex analysis for both gene expression profiling and epigenetic modifications, which exert lasting control over gene expression without altering the genetic code.

Recent evidence has suggested that epigenetic mechanisms contribute to drug-induced transcriptional and behavioral changes (Renthal & Nestler 2008). Such epigenetic modulation is mainly controlled by histone modification. Histones are modified at many sites. Previously published reports have indicated that the increased acetylation of histone H3 or methylation of H3 at K4 (lysine 4) highly predicts gene activation, while increased methylation of H3 at K9 or K27 (lysine 9 or 27) is predictives of gene repression. The triggering of signaling cascades in target neurons leads to more long-lasting effects, including changes in gene expression via the control of transcription and thereby, chromatin remodeling.