P=0.185), and active (F(5,10)=0.0185, P=0.982) and inactive (F(5,10)=0.200, P=0.822) lever responses.

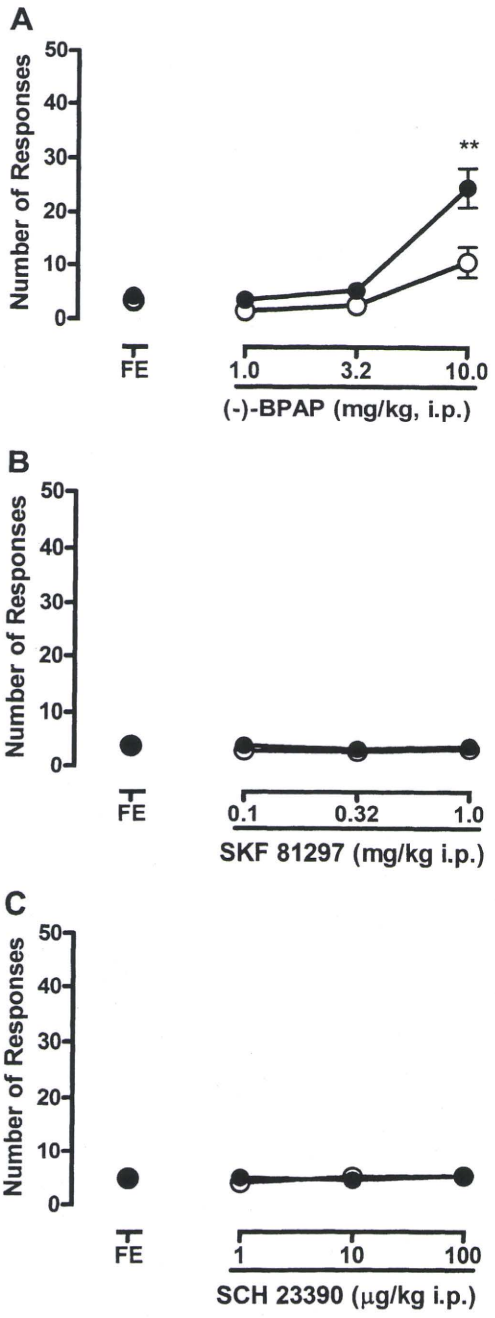

Fig. 9. Priming effects of pre-session treatment with (−)-BPAP, (A) SKF-81297, (B) and SCH-23390, (C) alone under extinction condition in methamphetamine self-administered rats after extinction sessions. (−)-BPAP was administered i.p. 30 min before the sessions, whereas SKF-81297 and SCH-23390 were administered s.c. 15 and 30 min before the session. Closed and open circles indicate responding on active and inactive levers. ** P<0.01 versus responding on active or inactive levers on final extinction (FE) day. (A) The sample sizes of the FE session and (−)-BPAP at the dose of 1.0, 3.2, and 10 mg/kg were 16, six, four, and six, respectively. (B) The sample sizes of the FE session, and SKF-81297 at the dose of 0.1, 0.32, and 1.0 mg/kg were 18, six, six, and six, respectively. (C) The sample sizes of the FE session, and SCH-23390 at the dose of 1.0, 10, and 100 μg/kg were 18, six, six, and six, respectively.

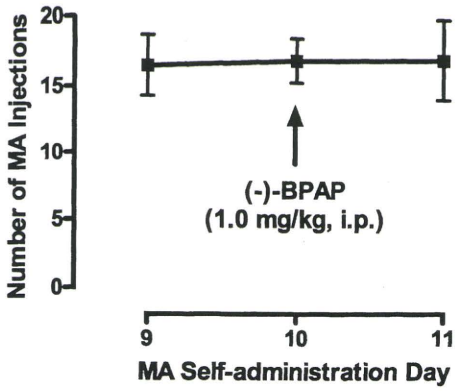

Fig. 10. Effects of pre-session treatment with (−)-BPAP in methamphetamine self-administering rats (n=6). (−)-BPAP was administered i.p. 30 min before the sessions.

Effects of (−)-BPAP and SKF-81297 on food-maintained behavior 5 min after the reinstatement sessions

In contrast to reinstatement of methamphetamine-seeking behavior, no pre-treatment with (−)-BPAP or SKF-81297 across the dose ranges tested had significant effects on food-maintained responses (Table 1). Two-way measures ANOVA indicated non-significant effect of repeated administration of (−)-BPAP on the dose (F(1,23)=2.824, P=0.106), reinstatement factor (F(1,23)=0.192, P=0.665), and the interaction (F(1,23)=0.295, P=0.592). Regarding single administration of (−)-BPAP, two-way measures ANOVA indicated a non-significant effect of (−)-BPAP on the dose (F(2,48)=0.170, P=0.844), reinstatement factor (F(1,48)=0.150, P=0.700), and the interaction (F(2,48)=0.244, P=0.784). In addition, two-way measures ANOVA indicated non-significant effect of SKF-81297 on SKF-81297 dose (F(2,42)=0.162, P=0.851), reinstatement factor (F(1,42)=0.119, P=0.732), and the interaction (F(2,42)=0.0315, P=0.969).

DISCUSSION

Repeated administration of (−)-BPAP during extinction sessions attenuated reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associ-

Table 1. Effect of (−)-BPAP or SKF-81297 on food-maintained behavior 5 min after the reinstatement sessions. Regarding the sample sizes, see Figs. 2, 3 and 7

Treatment	MA-associated cue	MA-priming injection
Repeated (−)-BPAP (mg/kg/day, i.p.)		
0	10.8±1.2	12.0±1.3
1.0	9.5±0.4	9.4±1.2
Single (−)-BPAP (mg/kg, i.p.)		
0	11.4±1.3	11.0±1.6
0.32	10.7±0.9	10.9±1.5
1.0	10.9±1.4	12.4±1.7
SKF-81297 (mg/kg, s.c.)		
0	10.6±0.4	11.1±0.4
0.32	10.9±0.4	10.8±0.4
1.0	10.8±0.5	10.8±0.6

ated cues and methamphetamine-priming injections. This result may suggest a preventive role of (−)-BPAP against the development of relapse to methamphetamine craving. Moreover, even a single pre-session treatment with (−)-BPAP also attenuated the reinstatement induced by methamphetamine-associated cues and methamphetamine-priming injections in a dose-related manner. Surprisingly, neither pre-session treatment with SCH-23390, a dopamine D_1-like receptor antagonist, nor amisulpride, a dopamine D_2-like receptor antagonist, across dose ranges tested appreciably reversed the inhibitory effect of single pre-session treatment with (−)-BPAP. In addition, combined pre-session treatment with SCH-23390 and amisulpride failed to reverse the effect of a single pre-session treatment with (−)-BPAP. This finding suggests that (−)-BPAP blocks the reinstatement of methamphetamine-seeking behavior through mechanisms other than dopamine receptors. On the other hand, pre-session treatment with SKF-81297, a dopamine D_1-like receptor agonist, dose-dependently attenuated the reinstatement of methamphetamine-seeking behavior induced by either methamphetamine-associated cues or methamphetamine-priming injections similar to the result obtained from the single pre-session treatment with (−)-BPAP. In contrast to (−)-BPAP, SCH-23390 dose-dependently reversed the inhibitory effect of SKF-81297. Additionally, SCH-23390 alone failed to reinstate methamphetamine-seeking behavior. Therefore, these results suggest an inhibitory role of dopamine D_1-like receptors to reinstate methamphetamine-seeking behavior in rats. Several studies demonstrated inactivated function of dopamine D_1-like receptors. For example, clinical study demonstrated reduced activity of adenyl cyclase after striatal dopamine D_1-like receptor-stimulation in methamphetamine abusers (Tong et al., 2003). In preclinical study, rats that self-administered methamphetamine exhibited downregulation of dopamine D_1-like receptor protein in the nucleus accumbens during withdrawal (Stefanski et al., 1999). However, inability of SCH-23390 to reinstate methamphetamine-seeking behavior in the present study suggests that blockade of dopamine D_1-like receptors by itself is insufficient to reinstate methamphetamine-seeking behavior.

Decrease in lever responding might result from a general overactivation or suppression of behavioral activity. However, neither repeated nor single pretreatment of (−)-BPAP decreased in responding maintained by food reinforcement. Furthermore, pre-session treatment with SKF-81297 also failed to affect food-maintained responding. Additionally, the half-life of radio-labeled [(−)-BPAP-14C] has been reported to be 5.5 to 5.8 h, which is long enough for (−)-BPAP to continue the action during sessions on food reinforcement (Magyar et al., 2002). Therefore, blocking effects of (−)-BPAP or SKF-81297 on the reinstatement of methamphetamine-seeking behavior do not result from nonspecific behavioral effects.

Pre-session treatment with (−)-BPAP at a dose of 1.0 mg/kg selectively affected reinstatement of methamphetamine-seeking behavior, but not methamphetamine self-administration. Radio-labeled [(−)-BPAP-14C] has been reported to be well-absorbed after the i.p. and s.c. treatment and the peak concentration in the rat brain has been reached at 30 to 60 min following s.c. administration (Magyar et al., 2002). Therefore, during the session on reinstatement and self-administration in the present study, the concentration of (−)-BPAP in the brain appears to reach a peak. Considering these findings, our data suggest that methamphetamine's reinforcing effect might be less sensitive to actions of (−)-BPAP compared to the reinstatement of methamphetamine-seeking behavior. Clinical study also demonstrated that the reinforcing effect of psychostimulants is extremely robust and simply unaltered by even substantial medication effects on drug "craving" or its subjective effects. Thus, maintenance on the antidepressant desipramine in volunteers with a history of cocaine abuse resulted in a 40% decrease in ratings of "I want cocaine," yet had no effect on the amount of cocaine self-administered (Fischman et al., 1990). Therefore, (−)-BPAP may be effective as an anti-relapse therapeutic; however, (−)-BPAP may not work sufficiently as anti-methamphetamine abuse medication.

Pre-session treatment with (−)-BPAP alone at the dose 1.0 and 3.2 mg/kg did not reinstate methamphetamine-seeking behavior, whereas (−)-BPAP at only the highest dose (10.0 mg/kg) demonstrated moderate reinstatement. A tenfold higher dose was needed to reinstate methamphetamine-seeking behavior compared with the dose at which pre-session treatment with (−)-BPAP (1.0 mg/kg) attenuated the reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associated cues and methamphetamine-priming injections. On the other hand, agonist/substitution therapies for opiate abuse with methadone and tobacco addiction with various formulations of nicotine have been reported to be effective (Henningfield, 1995; Kreek, 1996). In addition to the beneficial results, self-administration of methadone (Altshuler et al., 1975; Werner et al., 1976; Oei et al., 1980; Martin et al., 2007) and nicotine (Le Foll and Goldberg, 2005) in humans or experimental animals has been reported. Considering the positive and negative reports on methadone and nicotine, the potential ability of (−)-BPAP at the high dose to reinstate methamphetamine-seeking behavior in clinical situations may not discredit its clinical application as an anti-relapse agent for methamphetamine abusers.

(−)-BPAP has been reported to be an inhibitor of monoamine uptake in HEK cell (IC_{50} values: [^{3}H] dopamine, [^{3}H] noradrenaline, and [^{3}H] serotonin; 42, 52, and 640 nM, respectively) (Shimazu et al., 2003b), suggesting possible involvement of the relatively higher affinity for noradrenaline transporters in inhibitory effects of (−)-BPAP on the reinstatement of methamphetamine-seeking behavior. Moreover, methamphetamine has an at least twofold higher affinity for noradrenaline transporters than dopamine transporters but negligible affinity for serotonin transporters (Rothman and Baumann, 2003). However, dissimilar to methamphetamine (Yoneda et al., 2001) and tyramine (Shimazu et al., 2003b), (−)-BPAP alone does not release catecholamines. In addition, (−)-BPAP blocked tyramine-induced noradrenaline and dopamine

release from rat brain synaptosomes (Shimazu et al., 2003b), rather than potentiating the release. If binding of (−)-BPAP at noradrenaline transporters contributes to an inhibitory effect of (−)-BPAP on the reinstatement of methamphetamine-seeking behavior, selective noradrenaline uptake inhibitors would mimic the effect. So far, no studies in rats have reported pre-session treatment effect of selective noradrenaline uptake inhibitors on reinstatement of drug-seeking behavior, whereas a few studies in squirrel monkeys and humans have reported such effects. In squirrel monkeys, pre-session treatment with selective noradrenaline uptake inhibitors nisoxetine and talsupram both produced leftward shift of dose-effect curve of reinstatement of cocaine-seeking behavior induced by cocaine-priming injections; however, neither of the two selective noradrenaline uptake inhibitors affected the dose-effect curve of reinstatement of cocaine-seeking behavior induced by priming injections of GBR 12909, a selective dopamine uptake inhibitor (Platt et al., 2007). Furthermore, priming injections of nisoxetine and talsupram alone reinstated cocaine-seeking behavior (Platt et al., 2007). On the contrary, one clinical study has found positive results of the selective noradrenaline uptake inhibitor reboxetine to maintain cocaine abstinence (Szerman et al., 2005). Although the findings of clinical and preclinical studies seem to be inconsistent, the clinical evidence may support possible involvement of monoamine uptake inhibition in blocking effect of (−)-BPAP on the reinstatement of methamphetamine-seeking behavior, especially via noradrenaline transporters.

Alternatively, (−)-BPAP has been reported to be a highly potent enhancer (0.1 μg/kg s.c.; Yoneda et al., 2001) of electrically-stimulated monoamine release (Miklya and Knoll, 2003), whereas standard monoamine uptake inhibitors do not share this effect (Miklya and Knoll, 2003). Therefore, these findings suggest (−)-BPAP as an atypical monoamine uptake inhibitor. Meanwhile, previous studies reported "atypical" dopamine uptake inhibitors, including benztropine analogues with pharmacological profiles unlike that of cocaine (Newman et al., 1995; Katz et al., 1999; Beuming et al., 2008; Loland et al., 2008). Among benztropine analogues, several *N*-substituted benztropine analogues exhibited reduced cocaine-like effects (Katz et al., 2004), antagonized cocaine-stimulated activity (Desai et al., 2005), and failed to substitute for cocaine in rats trained to self-administration cocaine (Hiranita et al., 2009). One of the possible targets underlying the "atypical" property of *N*-substituted benztropines analogues appear to be the σ1 receptor (σ1-R), because (1) *N*-substituted benztropine analogues have a high affinity for this protein with the nanomolar order of Ki values (Katz et al., 2004), (2) rimcazole, a σ1-R antagonist with a high affinity for the dopamine transporters (Cao et al., 2003), shows reduced cocaine-like behavioral effects (Katz et al., 2003) and (3) these two analogues show different molecular interactions at the dopamine transporters from cocaine (Loland et al., 2008). Interestingly, (−)-BPAP has been reported to be a ligand at σ1-R (Hamabe et al., 2000). Furthermore, *in vitro* study demonstrated that enhancement of cellular survival activity on cortical neurons by incubation with (−)BPAP was blocked by pre-incubation with *N*-[2-(3, 4-dichlorophenyl) ethyl]-4-methylpiperazine (BD 1063), a σ1-R antagonist (Hamabe et al., 2000). A behavioral study also demonstrated that (1) methamphetamine self-administration upregulated σ1-R mRNA and protein levels in several limbic regions (Stefanski et al., 2004). Meanwhile, we have reported that donepezil, an inhibitor of choline esterase, dramatically attenuated the reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associated cues and methamphetamine-priming injections (Hiranita et al., 2006). Interestingly, donepezil has been reported to have very high affinity for σ1-Rs (IC_{50} value; 14.6 nM) (Kato et al., 1999). Although the involvement of σ1-Rs in the reinstatement of methamphetamine-seeking behavior has not been well understood, the σ1-Rs may be considered as the possible target underlying blocking effect of (−)-BPAP on the reinstatement of methamphetamine-seeking behavior.

In the present study, pre-session treatment with SCH-23390 dose-dependently reversed the blocking effect of SKF-81297 on the reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associated cues and methamphetamine priming injections. In contrast, at even the tenfold higher dose, pre-session treatment with SCH-23390 appreciably failed to reverse the effect of the single pre-session treatment with (−)-BPAP on the reinstatement of methamphetamine-seeking behavior. The role of dopamine D_1-like receptors on reinstatement of drug-seeking behavior seems complicated. Systemic administration of the agonist (SKF-81297) and antagonist (SCH-23390) both have been reported to attenuate cocaine-seeking behavior induced by cocaine-priming injections (15 mg/kg i.p.) or cocaine-associated cues in rats (Alleweireldt et al., 2002, 2003). On the contrary, systemic administration of SCH-23390 (up to 10 μg/kg) has been reported to fail to attenuate reinstatement of cocaine-seeking behavior induced by priming injections of cocaine (5.0, 10 and 20 mg/kg i.p.) or a selective dopamine uptake inhibitor, WIN 35,428 (Schenk and Gittings, 2003). The mechanisms underlying the inhibitory effect of both the dopamine D_1-like receptor agonist and antagonist on the reinstatement of cocaine-seeking behavior and inconsistent results of SCH-23390 on the primed-cocaine-induced reinstatement are unknown. However, considering the inhibitory effect of SCH-23390 on reinstatement of cocaine-seeking behavior in the studies by Alleweireldt et al. (2002, 2003), inability of SCH-23390 to reverse the attenuating effect of pre-session treatment with (−)-BPAP on the reinstatement of methamphetamine-seeking behavior in the present study might result from the possible inhibitory action of SCH-23390 on reinstatement of drug-seeking behavior.

On the other hand, pre-session treatment with amisulpride up to 10 mg/kg appreciably failed to reverse the effect of the single pre-session treatment with (−)-BPAP. In Chinese hamster ovary cells expressed with human dopamine D_2 and D_3 receptors, amisulpride has been reported to have very high selectivity (Ki values; 21 and 2.9 nM) (Schoemaker et al., 1997). The effective dose of amisulpride for 50% occupancy for dopamine $D_{2/3}$ recep-

tors in rat brain has been reported to be 4.68 mg/kg (s.c.) (Natesan et al., 2008). Furthermore, pretreatment with 10 mg/kg of amisulpride has been reported to reverse amphetamine (1.0 or 2.0 mg)-stimulated locomotor activity to the vehicle level (Perrault et al., 1997; Natesan et al., 2008). In addition, administration of a higher dose of amisulpride alone (20 mg/kg i.p.) has been reported to suppress 10% sucrose feeding in rats (Schneider et al., 1986). Therefore, it is unlikely that the dose of amisulpride tested (10 mg/kg) is insufficient to work as a dopamine D_2-like receptor antagonist without impairment of non-specific operant behavior in the present study. Alternatively, systemic administration of dopamine D_2-like (eticlopride (Schenk and Gittings, 2003), raclopride (Cervo et al., 2003) and haloperidol (Gal and Gyertyan, 2006)) and selective dopamine D_3 receptor antagonists (SB-277011-A (Vorel et al., 2002; Gilbert et al., 2005; Gal and Gyertyan, 2006; Cervo et al., 2007) and NGB 2904 (Gilbert et al., 2005; Xi et al., 2006; Xi and Gardner, 2007)) were found consistently to attenuate reinstatement of cocaine-seeking behavior induced by cocaine-priming injections or cocaine-associated cues in rats. Therefore, lack of amisulpride effect to reverse the attenuating effect of pre-session treatment with (−)-BPAP on the reinstatement of methamphetamine-seeking behavior in the present study might be also masked due to the possible inhibitory action of amisulpride on reinstatement of drug-seeking behavior.

CONCLUSION

In summary, activation of dopamine D_1-like receptors resulted in attenuation of the reinstatement of methamphetamine-seeking behavior in rats. Although the attenuating effect of pre-session treatment with (−)-BPAP may be unrelated to dopamine D_1-like receptors, our results suggest a specific blocking effect of pre-session treatment with (−)-BPAP without affecting the reinforcing effect of methamphetamine. Extending this conclusion to the treatment of drug dependence, (−)-BPAP and dopamine D_1-like receptor agonists may be useful as anti-relapse agents in methamphetamine dependence.

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III. 精神薬理学

12. エンドカンナビノイドと依存

大麻（*Cannabis sativa*）は，最も古い「幻覚生薬/快楽植物」の一つであり，多幸感や気分の高揚（報酬効果/強化効果）をもたらし，その精神作用を発現する主要な活性成分がΔ^9-テトラヒドロカンナビノール（Δ^9-tetrahydrocannabinol：THC）である．一方，脳内にカンナビノイドCB受容体，その内在性リガンド（エンドカンナビノイド〈endocannabinoid〉）としてのアナンダミド（anandamide）および2-AG（2-arachidonoylglycerol）ならびにそれらの合成・代謝に関与する酵素系を包含してのカンナビノイド・システムも明らかにされている．このカンナビノイドCB受容体は，グルタミン酸，GABA，ACh（アセチルコリン）等の神経シナプス前膜に存在し，神経シナプス後膜から遊離される内在性カンナビノイドを介して各種伝達物質の遊離を抑制することが知られている．これまで摂食・摂水や依存性薬物の報酬効果の発現には，内在性オピオイドや腹側被蓋野を起始核とし前頭前野や中脳辺縁系に走行するDA（ドパミン）神経を中心とする脳内報酬系が重要な役割を演じていることが知られている．近年，脳内カンナビノイド・システムも脳内報酬系の一翼を担い，諸種報酬効果の発現に関与していることが明らかにされつつある．

カンナビノイドの報酬効果（カンナビノイド自己投与行動）

大麻の活性成分Δ^9-テトラヒドロカンナビノール（THC）の反復投与後の自然退薬では著明な退薬症状の発現はないが，CB_1受容体拮抗薬の投与によって誘発される．オペラント実験装置を用いての薬物自己投与実験では，安定したTHC自己投与行動が形成されないので，その依存性はほとんどないとされていた．しかし，Goldbergらは，コカイン自己投与行動の履歴をもつサルを用い，THCの自己投与訓練を再開するとTHC自己投与行動が成立し，THCにも強化効果があることを明らかにした．彼らはさらに，薬物履歴のないサルを用いての実験でもコカイン履歴のサルの場合の2倍用量でTHC自己投与行動を成立させている．さらに，エンドカンナビノイドのアナンダミドやその合成活性物質メタアナンダミドの自己投与行動がラットで成立することも明らかにされている．合成カンナビノイドCB_1受容体アゴニストWIN-55,212-2は，それ自体でマウスでの静脈内自己投与行動が成立することも近年報告されている．

ラットにおけるカンナビノイドの自己投与行動の成立に関してのこれまでの不一致は，ラットの種差にあるとの指摘がある．すなわち，Long Evans系およびLister系ラットではカンナビノイド自己投与行動が成立し，Sprague-Dawley系ラットでは成立していない．一方，オピオイドκ受容体ノックアウト（KO）マウスではカンナビノイド自己投与行動の習得が促進される．このことを念頭におくと，カンナビノイド自己投与行動が認められがたいのは，オピオイドκ受容体の活性化に基づく不快効果のために報酬効果がマスクされている可能性が指摘されている．いずれにしても，大麻/カンナビノイドに依存性はないとするのは危険であり，覚醒剤やコカインに比べて「弱いが依存の危険性はある」ととらえるべきである．

依存性薬物の報酬効果とその渇望の再燃におけるエンドカンナビノイドの関与

モルヒネの自己投与行動はCB_1受容体KO

マウスでは成立せず，さらにヘロイン自己投与行動はカンナビノイド CB_1 受容体拮抗薬 SR141716A（リモナバン）により抑制される．これらのことは，オピオイドの報酬効果の発現には CB_1 受容体の活性化が介在していることを示唆している．また逆に，THC 自己投与行動もオピオイド受容体拮抗薬ナルトレキソン（naltrexone）で抑制されることから，カンナビノイドの報酬効果発現にもオピオイド受容体がかかわっていることがわかる．

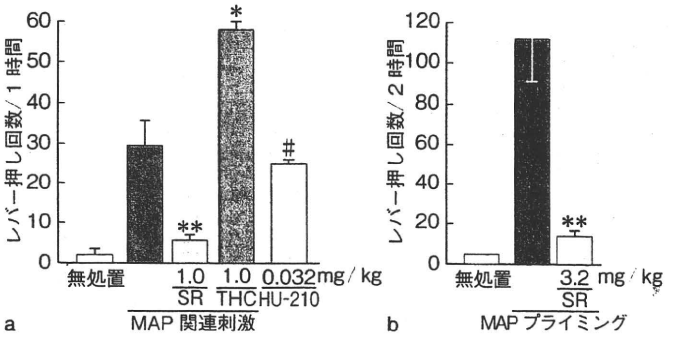

図 1　覚醒剤メタンフェタミン自己投与ラットにおけるメタンフェタミン探索行動（渇望）の発現に対するカンナビノイド CB_1 受容体アゴニスト/アンタゴニストの作用

$^*p < 0.01$　$^{**}p < 0.001$ vs 薬物関連刺激または MAP プライミング単独群，$^{\#}p < 0.001$ vs 無処置群，SR：SR141716A（リモナバン），なお，実験時間は，a：MAP 関連刺激による探索行動実験では 1 時間，および b：MAP プライミングによる探索行動実験では 2 時間．

（Anggadiredja K, et al. *Neuropsychopharmacology* 2004[1]）

薬物自己投与行動が成立した後に，薬物から生理食塩水注入に切り替えると，レバー押し行動は減弱する（消去過程）．このとき，薬物摂取と関連した刺激（たとえば，音・光；薬物関連刺激）または中枢興奮薬の少量投与（薬物プライミング投与）によりレバー押し行動が出現し，ヒトでの渇望の再燃に基づく薬物探索行動を彷彿させる行動モデルと考えられている．これら 2 つの刺激により誘発されるコカイン，ヘロインおよびメタンフェタミン探索行動は，CB_1 受容体アンタゴニスト SR141716A によって抑制され，そのアゴニスト HU-210 で誘発される[1]（**図 1**）．このように，エンドカンナビノイドは薬物摂取行動のみならず探索行動の発現にも関与していることが示唆されている．

薬物の報酬効果/薬物摂取行動ならびに薬物探索行動の発現メカニズムにエンドカンナビノイド・システムが関与していることは明らかであるが，特に後者の場合，依存性薬物や誘発要因によってそのメカニズムが異なる可能性が指摘されている．一方，エンドカンナビノイドは食欲の制御をつかさどる視床下部で食欲増進的に関与し，さらに食欲を抑制する神経ホルモンであるレプチン（leptin）と拮抗的調節をしている．摂食などの自然報酬と薬物報酬におけるカンナビノイド・システムの関与に相違があるのか否かは興味のあるところであるが，この点はまだ不明な点が多い．さらに，エンドカンナビノイドは依存性薬物の退薬症状（不快・不安感など）の発現，諸種精神疾患の成因およびその再燃・再発の機構にも関与している可能性も近年指摘されている．今後，エンドカンナビノイドの異常と諸種精神疾患の成因に関するブレークスルーを期待したい．

（山本経之）

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4 脳内報酬系における内因性／外因性カンナビノイドの作用

（山本経之）

はじめに

大麻（*Cannabis sativa*）は知覚効果（subjective）としての多幸感や陶酔感，また空間認知障害や感覚異常等の強い精神作用を誘発する．近年，この大麻の乱用は，我が国でも急速に広がりを見せ，大きな社会問題となっている．大麻の主要活性成分はΔ^9-テトラヒドロカンナビノール（Δ^9-THC）を筆頭に60種以上存在し，カンナビノイドと総称されている．1980年代末に，カンナビノイドに特異的に結合するカンナビノイドCB受容体とその内在性リガンドとして不飽和脂肪酸である*N*-アラキドニルエタノールアミド（アナンダマイド）や2-アラキドニルグリセロール（2-AG）が相次いで発見された．さらにカンナビノイドCB受容体は，グルタミン酸（Glu），GABA，ACh等の神経シナプス前膜に存在し，神経シナプス後膜から遊離さるカンナビノイド（脳内“大麻様物質”）を介して各種伝達物質の遊離を抑制する役割を演じている事も明らかにされている．内因性カンナビノイドの発見は，過っての脳内オピオイドの発見と同様に，多彩な脳機能の解明に新たな糸口を与える事が示唆されている．本項では，脳内カンナビノイド・システムの役割を脳内報酬系に関わる中枢作用を中心に，薬物依存，食欲および痛覚について概説したい．

1 カンナビノイド受容体と内因性カンナビノイド

1988年，Devaneと Howlett等はΔ^9-THCの誘導体［^{3}H］-CP55,940を用いて，ラットの脳シナプトソームにカンナビノイドに対する特異的な受容体が存在することを初めて明らかにした（1）．Masuda等はウシのサブスタンスK受容体遺伝子の一部をプローブとしたラットの大脳皮質cDNAライブラリーのスクリーニングからカンナビノイドCB_1受容体を偶然発見し，クローニングした（2）．このCB_1受容体（ヒト472個，ラット473個のアミノ酸配列）は中枢神経と一部の末梢神経に存在する（3）．その脳内分布は，海馬，大脳皮質，線条体，黒質，前脳基底部，嗅脳および小脳に多い．カンナビノイド受容体にはサブタイプがあり，もう1つはヒト白血病細胞（HL-60）のcDNAライブラリーからクローニングされたCB_2受容体（ヒト・ラット共に360個のアミノ酸）であり主に末梢神経に存在する．CB_1受容体とCB_2受容体とのアミノ酸配列の相同性は，48％とされている．これらの両受容体は7回膜貫通型ドメインを持つ典型的なGTP結合タンパク質共役型の受容体で，アデニル酸シクラーゼを阻害し，MAPK（mitogen-activated protein kinase）を活性化させる．さらに，CB_1受容体は電位依存性カルシウムチャネル（N型またはO型）を抑制し，

電位依存性カリウムチャネルを活性化させる．

その内在リガンドとして，Devane 等（4）によってブタ脳からアラキドン酸誘導体の 1 種であるアナンダマイドが，また Sugiura 等（5）および Mechoulam 等（6）によってそれぞれラット脳およびイヌ小腸から 2-AG が相次いで発見された．さらに 2002 年には，カンナビノイド受容体アンタゴニストの作用を持つとされるビロダミンも発見されている．これらの内因性カンナビノイドの産生はシナプス後膜でなされ，強い脱分極（細胞内 Ca^{2+} 濃度の上昇；$Ca^{2+} > 1\mu M$）または G_q タンパク質活性化後の PLCbeta の活性化の 2 つの経路により行われる（7）．またその遊離は，担体輸送系（carrier-mediated transport）を介して行われる．その後速やかに酵素的に不活性化されるのでシナプス前膜でのカンナビノイド CB_1 受容体の生理的作用は，制限される事になる．脂肪酸アミド加水分解酵素（FAAH）はアナンダマイドのような内因性 signaling lipid である脂肪酸ファミリーを加水分解する．この酵素は基質特異性に欠ける面があり，脂肪酸エタノールアミドのみならず，2-AG のような脂肪酸エステルにも加水分解を起こす．しかし，最近の報告では 2-AG は FAAH よりも主に脳内 monoglyceride lipase により分解される事が分っている（8）．

薬物報酬効果の発現に関与する腹側被蓋野から側坐核へ投射するドパミン（DA）神経系を中心にカンナビノイド受容体の局在を図 **1** に示した（9）．腹側被蓋野におけるカンナビノイド CB_1 受容体は，グルタミン酸ならびに GABA 神経系のシナプス前膜に局在している．それに対し，側坐核へ入力する DA 神経系には，CB_1 受容体は存在していない．内因性カンナビノイドはシナプス後膜で産生され，逆行的に放出される．このカンナビノイ

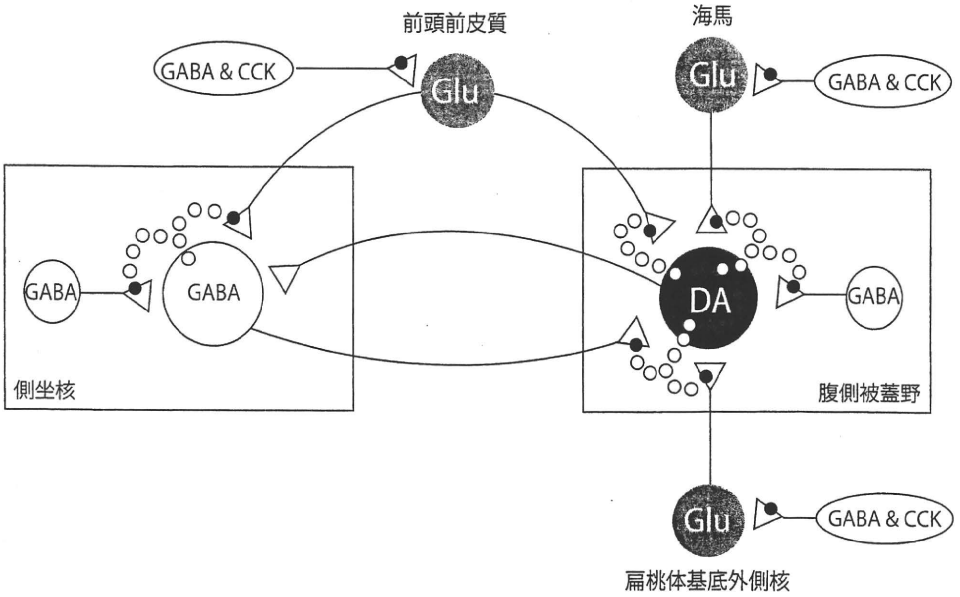

図 1　薬物報酬効果の発現に関与する腹側被蓋野および側坐核を中心とするカンナビノイド受容体の局在性（文献 9. Maldonard らの図より改変）
●：シナプス前膜に存在するカンナビノイド CB 受容体
○：シナプス後膜で生産され逆行性に遊離される内因性カンナビノイド

ドは，腹側被蓋野のDA神経に入力する抑制性GABA神経と興奮性グルタミン酸神経の2つの神経系を抑制的に制御している．薬物報酬効果の発現には，情動や記憶が重要な役割を演じている．情動／記憶に関わる扁桃体基底外側核および海馬から投射するグルタミン酸神経も，GABA／コレシストキニン神経の支配を受け，そのシナプス前膜のCB_1受容体の活性化を介してGABA／コレシストキニン遊離が抑制される．一方，側坐核の内因性カンナビノイドは，前頭前皮質から投射するグルタミン酸神経の終末部に存在するカンナビノイドCB_1受容体に対しても逆行性モデュレーターとして働く．これにより興奮性伝達物質であるグルタミン酸の遊離は抑制される．その結果，側坐核のGABA神経が抑制され，腹側被蓋野のDA神経系は間接的に活性化される（9）．この様に，内因性カンナビノイドは，シナプス前膜において逆行性の抑制性モデュレーターとして働き，興奮性神経伝達物質グルタミン酸や抑制性神経伝達物質GABA等の遊離応答を抑制的に制御する役割を演じている．

2　報酬効果／強化効果に対する脳内カンナビノイド・システム

依存性薬物は報酬としての“快”の知覚効果（subjective）を有し，食餌・水等の“自然報酬”と区別され，“薬物報酬”と呼ばれている．これらの報酬効果は内因性オピオイドや腹側被蓋野を起始核とし，前頭前野や中脳辺縁系に走行するDA神経を中心とする脳内報酬系によって制御されている事が知られている．大麻およびその主要活性成分Δ^9-THCも他の依存性薬物と同様に，多幸感や陶酔感を起こす事から内因性カンナビノイドと脳内報酬系との関連性が指摘されている（10-15）．ヒトで報酬効果／強化効果を発現し依存を起こす薬物は，一般的に実験動物でオペラント装置を用いた薬物自己投与行動を成立させると考えられている．大麻の活性成分Δ^9-THCは反復投与後に自然退薬しても，著明な退薬症状の発現は起きない．しかし，CB_1受容体アンタゴニストの投与によって退薬症状は，誘発される．一方，1970年初頭に始まったΔ^9-THC自己投与行動に関する研究では，安定したΔ^9-THC自己投与行動が認められないので，その依存性はほとんど無いものと結論付けられていたが，2000年初頭からのGoldbergら（16-18）を中心とする研究によって修正されつつある．コカイン自己投与行動の履歴をもつサルを用い，Δ^9-THCの自己投与訓練を新たに始めるとΔ^9-THC自己投与行動が成立する．この事は，Δ^9-THCにも強化効果がある事を示唆している．さらに，薬物履歴のないサルを用いての実験でも，コカイン履歴のサルの場合の2倍用量でTHC自己投与行動が成立する事も明らかにされている．また合成カンナビノイドCB_1受容体アゴニストWIN 55,212-2（i.v.；19），HU-210（i.v.；20）およびCP 55,940（intra.vent.；21）もマウスやラットでの自己投与行動が成立する．一方，内因性カンナビノイドのアナンダミドやその合成活性物質メタアナンダミドの自己投与行動もサルで成立する事が明らかにされている（10）．これまでのカンナビノイドに関する薬物自己投与行動の成立の可否を**表1**に示した（18）．2000年以前に進められた大部分の研究はΔ^9-THCが中心でその自己投与行動の成立が認められていないが，2000年代以降の

表 1　薬物自己投与実験法（オペラント装置使用）を用いてのカンナビノイドの報酬効果／強化効果の発現

薬物種	自己投与用量 (mg/kg/injection)	ルート	動物種	結果	文献
外因性カンナビノイド					
Δ^9-THC	25-100	i.v.	monkeys	negative	Pickens, et al. 1973
	100-400	i.v.	monkeys	negative（2/6 例成立）	Deneau, et al. 1971
	25-300	i.v.	monkeys	negative	Harris, et al. 1974
	カンナビノイド樹脂懸濁液 30-330 μg THC/ml 含有	oral	rats	negative	Leite, et al. 1974
	ハシシュ水溶性懸濁液 . 1.25-12.5 μg THC/ml 含有	oral	rats	negative	Corcoran, et al. 1974
	3-300	i.v.	monkeys	negative	Carney, et al. 1977
	7.5-300	i.v.	rats	negative	van Ree, et al. 1978
	6.25-50	i.v.	rats	positive（食餌制限下）	Takahashi, et al. 1979, 1980
	17-100	i.v.	monkeys	negative	Mansbach, et al. 1994
	2-8	i.v.	monkeys	positive（cocaine 自己投与サル；特に 4 μg）	Tanda, et al. 2000
	1-16	i.v.	monkeys	positive（特に 4 μg）	Justinova, et al. 2003
WIN 55,212-2	10-500	i.v.	mice	positive ※	Martellotta, et al. 1998
	100	i.v.	mice	positive ※（CB1+/+） negative ※（CB1−/−）	Ledent, et al. 1999
	10-100	i.v.	mice	positive ※	Navarro, et al. 2001
	6.25-50	i.v.	rats	positive（特に 12.5 μg）	Fattore, et al. 2001 Spano, et al. 2004
CP 55,940	0.3-3	i.v.	monkeys	negative	Mansbach, et al. 1994
	0.1-1.6	i. vent	rats	positive	Braida, et al. 2001
HU-210	5	i.v.	mice	positive ※	Navarro, et al. 2001
メタアナンダミド	2.5-80	i.v.	monkeys	positive（特に 40 μg）	Justinova, et al. 2005
内因性カンナビノイド					
アナンダミド	2.5-160	i.v.	monkeys	positive（特に 40 μg）	Justinova, et al. 2005

※ single sel0f administration session: 1 試行 60 分間にわたって nose-poke response（前面ホールへの鼻の突っ込み行動）に基づいて薬物が注入される実験系.（文献 10. Justinova Z ら，に加筆・修正）

研究報告では多くのカンナビノイドで自己投与行動が成立している．この不一致は，以下の事が一因と考えられている．まず第 1 に，ラットの種差にあるとの指摘がある．即ち，Long Evans 系および Lister 系 ラットではカンナビノイド自己投与行動が成立し，Sprague-Dawley 系ラットでは成立していない（15）．一方，オピオイド κ 受容体 KO マウスではカンナビノイド WIN 55,212-2 自己投与行動の習得が促進される．Mendizabal V ら（22）はこの点に着目し，カンナビノイド自己投与行動が認められ難いのは，オピオイド κ 受容体の活性化に基づく不快効果の為に報酬効果がマスクまたは減弱されている可能性を指摘している．いずれにしても，大麻／カンナビノイドに依存性はないとするのは薬理学的に誤りであり，覚せい剤やコカインに比べて“弱いが依存の危険性はある”と捉えるべきである．

3 モルヒネの報酬効果ならびに退薬症候に対する脳内カンナビノイド・システムの関与

モルヒネの自己投与行動はCB_1受容体ノックアウト（KO）マウスでは成立せず（23），さらにヘロイン自己投与行動はCB_1受容体アンタゴニスト リモナバン（SR141716A）により抑制される．これらの事は，オピオイドの報酬効果の発現にCB_1受容体の活性化が介在している事を示唆している．また逆に，THC自己投与行動もオピオイド受容体アンタゴニスト ナルトレキソンで抑制される事（24）から，カンナビノイドの報酬効果発現にもオピオイド受容体が関わっている事が分かる．

一方，依存性薬物の長期使用後の退薬時に，精神的苦痛を避ける為に薬物再摂取への渇望（craving）が誘引される．その為，薬物を入手しようとするが，その行動を薬物探索行動（drug seeking behavior）と呼び，“渇望”の指標としている．薬物探索行動の動因には，①興奮性薬物の少量使用（薬物プライミング投与），②薬物摂取時を連想させる環境刺激（薬物関連刺激）および③ストレスが知られている．覚せい剤メタンフェタミン（MAP）自己投与行動が成立したラットを用い，MAPから生理食塩液注入に切り替える（消去過程）とレバー押し行動は減弱する．この時，MAPの少量投与または薬物関連刺激（音・光）を与えるとレバー押し行動が出現し，ヒトでの薬物探索行動を彷彿させる類似の行動と考えられている．我々はこれまで，リモナバンを投与すると，これら２つの刺激により誘発される薬物探索行動はいずれも有意に抑制される事を明らかにした（25）．

また，ヘロイン探索行動の発現も，リモナバンにより抑制される．De Vriesらも，コカイン探索行動がリモナバンにより抑制され，CB_1受容体アゴニストHU-210それ自体で誘発される事を明らかにした（26）．これらの知見は，薬物探索行動の発現には脳内カンナビノイド・システムの活性化が必須である事を示唆している．一方，MAP探索行動は，消去過程でのΔ^9-THC投与により抑制される（25）．辺縁系における2-AG量は，コカイン反復投与後の退薬時に減少する事も明らかにされている（27）．これらの知見から，薬物退薬時での脳内カンナビノイド・システムの不活性な状態が薬物探索行動の“準備状態”を形成している可能性が示唆される．

これとは別に，モルヒネ反復投与マウスにCB_1受容体アンタゴニスト リモナバンの投与によってモルヒネ退薬症状が出現する事，またナロキソンによるモルヒネ退薬症候が2-AGの脳室内投与により抑制される事（28）を，我々は明らかにした．この事は，モルヒネ退薬症候発現時にはカンナビノイド・システムの機能低下が起こっている可能性を示唆すると同時に，脳内報酬系におけるオピオイドとカンナビノイドの相互関与が推察される．さらに我々は，アスピリンやジクロフェナクといったCOX阻害薬がTHCによるレバー押し行動（餌強化）の抑制作用に拮抗し（29），THCの退薬症候を誘発する事，またプロスタグランジンPGE_2がリモナバンによる退薬症候を抑制する事を報告した（30）．これらの知見はカンナビノイド・システムとアラキドン酸カスケードとの関連性を示すものであり，THCの退薬症候発現にはアラキドン酸カスケードの不活性化が関与している可能性

を示唆している．

4　自然報酬と脳内カンナビノイド・システムの関与

強化子としての食物や水は，自然報酬（natural reward）と呼ばれ，先に述べた薬物報酬とは分けて考えられている．摂食行動，摂水行動および性行動等の本能行動の発現も，腹側被蓋野を起点とし視床下部外側野を通る内側前脳束を中心とする脳内報酬系が重要な役割を演じている．特に食欲は，脳の視床下部腹内側核（満腹中枢）と外側核（摂食中枢）での拮抗的調節でコントロールされている．一方，視床下部外側野のニューロンから分泌されるオレキシンは摂食／食欲促進物質として，また側坐核から腹側淡蒼球に向かう伝達物質 GABA や神経ホルモンであるレプチンは摂食／食欲抑制物質として働いている．また Δ^9-THC やアナンダマイド等の CB_1 受容体アゴニストは，げっ歯類の餌の摂食量を増強させる（31）．さらに，脳内カンナビノイド濃度は絶食によって増加し，逆に摂食によって減少する事も分かっている．CB_1 受容体アゴニストのラットでの摂食亢進作用は，中脳辺縁系および視床下部への微量注入により誘発される事も明らかにされている．これらの事から，視床下部を中心とする食欲のコントロールには，内因性カンナビノイドが促進的に関与している事が分かる．これらのカンナビノイドアゴニストによる摂食亢進作用はリモナバンにより拮抗され，正常摂食量をも減少させる．また CB_1 受容体 KO マウスでも餌の摂食量が減少している．また餌ペレット強化のオペラント行動も選択的な CB_1 受容体アンタゴニストにより抑制されるが，AM630 のような選択的 CB_2 受容体アンタゴニストでは抑制されない．一連の CB_1 受容体アンタゴニストには自発運動の低下や記憶障害を起こす作用がない事から，このオペラント行動の抑制作用も食欲減退作用に基づくとされている．一方，これまでの CB_1 受容体アンタゴニストはアンタゴニスト作用と共に，大部分がアゴニスト作用を有している（inverse agonist）．従ってこの食欲減退作用が CB_1 受容体の拮抗作用そのものに基因するのか否かは明らかではなかった．しかし，最近，アゴニストならびに inverse agonist 作用を持たない CB_1 受容体の“silent antagonist”として O-2050 が合成された．この O-2050 は絶食をかけない条件下で，リモナバンと同様にラットの餌摂取量を著しく抑制する（32）．従って，CB_1 受容体アンタゴニストの食欲減退作用は CB_1 受容体の拮抗作用そのものに基づいている事が分かる．

一方，食欲を抑制する神経ホルモンとして知られているレプチンとの関係も指摘されている（33）．レプチンは視床下部でのカンナビノイド濃度を減少させ，逆にレプチン欠損ラットでは視床下部でのカンナビノイド濃度は増加している．このことは，食欲が内因性カンナビノイドとレプチンとの相互関与によって調節されている事を示している．Van Gaal らは，リモナバンの 1 年間にわたる臨床試験データから肥満の治療薬としての可能性を指摘している（34）．現在，このリモナバンはフランスの Sanofi 社から“抗肥満薬”（“anti-obesity drug”）として acomplia の商品名で，2006 年から英国およびドイツで販売されている．逆に，カンナビノイド CB_1 受容体アゴニストは臨床的にも食欲を亢進させ，体重増加

を起こす事からエイズや癌等の消耗性疾患症候群の患者に対する延命効果が期待されている．

以上，内因性カンナビノイドは食欲の制御を司る視床下部で食欲増進的に関与し，さらに食欲を抑制する神経ホルモン レプチンと拮抗的関与をしている．摂食等の自然報酬と薬物報酬におけるカンナビノイド・システムの関与に相違があるのか否かは興味のあるところであるが，この点はまだ不明な点が多い．

5 疼痛と脳内カンナビノイド・システム

カラゲニン誘発炎症反応（浮腫）および熱刺激による疼痛反応（hot plate test および tail-flick test）は，Δ^9-THC，合成カンナビノイド HU-210 や CP 55,940 および内因性カンナビノイド アナンダミドの脊髄内または脳室内投与により，鎮痛および痛覚過敏の緩和作用が認められる（35）．さらにカンナビノイド受容体アゴニストは視床の側部後方と中央下部領域，A5 領域，扁桃体，腹側中脳水道周囲灰白質（PAG）および延髄吻側腹側部への微量注入によっても鎮痛作用を発現し，これらの脳部位はカンナビノイドによる鎮痛効果発現の作用点と考えられている．Freund's adjuvant 注入で誘発されるラットの allodynia（非侵害性の触・熱刺激で発痛する）も，カンナビノイド受容体アゴニスト WIN 55,212-2 の脊髄内投与により，鎮痛作用発現に必要とする用量よりも低用量で抑制される（36）．このようにラットの神経因性疼痛モデルを用いての研究において，カンナビノイドの末梢投与や脊髄内投与によって熱刺激による疼痛反応のみならず，冷刺激や触刺激によるアロディニアも抑制される事が明らかにされている．興味ある事には，CB_1 受容体 KO マウスでは，Δ^9-THC の hot-plate test での鎮痛作用は認められないが，アナンダマイドの鎮痛作用は出現する点である（37）．このようにアナンダミドの鎮痛作用の発現には，少なくとも CB_1 受容体以外にも作用点があるようである．アナンダミドは FAAH によって失活する．この FAAH 欠損マウスは脳内アナンダミド量が正常マウスのそれに比べて 15 倍も高く，侵害刺激に対する痛覚閾値も高い事が報告されている（38）．

一方，末梢性の侵害刺激によって，腹側中脳水道周囲灰白質から，アナンダミドが放出される．また，Seigling らは，ラットの末梢神経損傷後にその末梢神経と対側の視床において，CB_1 受容体のアップレギュレーションが起こっている事を報告した（39）．この事は，慢性疼痛の動物モデルにおけるカンナビノイドの疼痛緩和作用の発現が CB_1 受容体のアップレギュレーションに基づく可能性を示唆している．

さらにカラゲニンによって誘発される痛覚過敏はアナンダミドの末梢投与により抑制され，ホルマリン誘発疼痛反応もアナンダミドや CB_2 受容体の選択的アゴニスト パルミチルエタノールアミドによっても抑制される．カンナビノイドによるこの疼痛抑制作用は，CB_1 受容体および CB_2 受容体アンタゴニストで拮抗される（40）．さらに Malan 等は CB_2 受容体の選択的アゴニスト AM1241 の末梢投与により，CB_1 受容体アゴニストと同様に熱刺激に基づく疼痛反応（tail-flick 法）が減弱される事を明らかにし，カンナビノイドの鎮

痛作用が CB_2 受容体活性化を介しても起こる事を指摘している（41）．また最近，Beltramらも，慢性疼痛における痛みの軽減には CB_2 受容体の末梢神経系のみならず，中枢神経系（脊髄でのミクログリアの不活性化）での関与を明らかにしている（42）．

唐辛子の辛味成分であるカプサイシンはその化学構造式の中にバニリル基を有しバニロイドと呼ばれ，バニロイド受容体の興奮を介して疼痛を起こす事が知られている（43）．このバニロイド受容体のリガンドはカンナビノイドCB受容体やアナンダミド・トランスポーターのリガンドと比べると高級不飽和炭素鎖（20個）やバニロイド構造を有している等の化学構造上の類似点を持ち，受容体／トランスポーター相互に親和性を有している事が分っている．痛覚に対しては相反する作用を示すバニロイド受容体と各種侵害刺激によって活性化されるカンナビノイドCB受容体とバニロイド受容体，またカンナビノイドCB受容体サブタイプの CB_1 受容体と CB_2 受容体が“痛み”の制御に関して如何なるクロストークがなされているか明らかではないが，内因性オピオイドのスコープから見え難かった痛覚過敏や慢性疼痛のメカニズムの解明に新たな糸口を与えてくれるものと期待される．

おわりに：今後の展望

植物としての大麻の活性成分の薬理作用の解明から始まったカンナビノイド研究は，脳内にカンナビノイド受容体とその内因性カンナビノイドの存在が明らかにされてから，生体におけるカンナビノイドの機能的役割を解明する研究へと方向舵を変えている．大麻乱用に基づく精神障害の臨床知見（44, 45）からも，脳内の内因性カンナビノイドの病的な増加または減少は精神機能の異常をきたす可能性が強く推測される．まだまだ不明な点が多いが，統合失調症やアルツハイマー病の患者脳でのカンナビノイド受容体・内因性カンナビノイドの異常性はすでに散見される（46, 47）．

本項で取り上げた脳内報酬系における内因性カンナビノイドの機能的役割に関する研究によって，①物質やプロセス依存症に留まらず意欲減退，情動障害および精神障害等の病因解明，また②過食症／拒食症だけでなく長寿と深く関わる肥満を含む生活習慣病の病因解明，さらには③緩和医療の中でも喫緊の課題である痛覚過敏や慢性疼痛の病因解明に向け内因性カンナビノイドの視点からの新たなブレーク・スルーを期待したい．

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Piccolo knockdown-induced impairments of spatial learning and long-term potentiation in the hippocampal CA1 region

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ABSTRACT

Neurotransmitter release is regulated at a specific site in nerve terminals called the "active zone", which is composed of various cytomatrix proteins such as Piccolo (also known as Aczonin) and Bassoon. These proteins share regions of high sequence similarity and have very high molecular weights (>400 kDa). Since Piccolo knockout mice have not yet been established, the role of Piccolo in the neuronal system remains unclear. In this study, we investigated the effects of Piccolo antisense oligonucleotide injected into the ventricle on hippocampal long-term potentiation (LTP) and learning and memory assessed with the novel object recognition test and the Morris water maze test. There was no significant difference in cognitive memory between Piccolo antisense-treated and vehicle- or sense-treated mice; however, spatial learning in Piccolo antisense-treated mice was impaired but not in sense- or vehicle-treated mice. Next, we investigated LTP formation in these groups in area CA1 and dentate gyrus of the same hippocampal slices. The magnitude of LTP in Piccolo antisense-treated mice was significantly lower than in sense- or vehicle-treated mice, with no change in basal level. Moreover, the level of high K^+-induced glutamate release in the antisense-treated mice was significantly lower than in sense-treated mice. Taken together, these results indicate that Piccolo plays a pivotal role in synaptic plasticity in area CA1 and in hippocampus-dependent learning in mice, and that the extracellular levels of glutamate in the hippocampus under stimulated conditions are controlled by Piccolo.

1. Introduction

Higher brain functions including learning and memory are a result of accurate signal transduction by neuronal networks that are controlled in time and space. Impairment of the capacity of these networks is often associated with neurological disease and major insults to the brain, resulting in deterioration of cognitive function and quality of life. Learning and memory are complex sets of processes involving the acquisition, consolidation and retrieval of information, and have their fundamental basis in synaptic plasticity (Hou et al., 2004). In contrast to the extensive evidence for roles of post-synaptic proteins and post-synaptically expressed forms of synaptic plasticity in learning and memory, relatively little attention has been given to the influence of presynaptic proteins and presynaptic plasticity in cognitive function (Powell et al., 2004).

The synapse is a primitive unit in complex neuronal networks composed of large neuronal cells. In recent years, significant progress has been made in understanding the molecular assembly of the post-synaptic density of the synapse, whereas little is yet known about the components of the presynaptic active zone. Presynaptic buttons are highly specialized cellular compartments that have evolved for the rapid, efficient and regulated release of neurotransmitters. The active zone beneath the presynaptic membrane is the principal site for Ca^{2+}-dependent exo-cytosis of neurotransmitters. Synaptic vesicles dock to the active zone and fuse with the plasma membrane, resulting in exo-cytosis of neurotransmitters (Matteoli et al., 2004). To date, five protein families whose members are highly enriched at active zones, Munc13s, Rab3a-interacting molecules (RIMs), Piccolo, Bassoon, and the liprins-α, have been characterized (Schoch and Gundelfinger, 2006).

Piccolo is a protein of the cytoskeletal matrix associated with the active zone (CAZ) of both excitatory and inhibitory synapses (Dick et al., 2001) and a scaffolding protein involved in endo- and

Abbreviations: LTP, long-term potentiation; PPF, paired-pulse facilitation; ACSF, artificial cerebrospinal fluid; CAZ, cytoskeletal matrix associated with the active zone.

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exo-cytosis of synaptic vesicles (Shibasaki et al., 2004; Fenster et al., 2003). Piccolo and Bassoon are found presynaptically at glutamatergic ribbon synapses and at conventional GABAergic and glycinergic synapses (Dick et al., 2001). They share regions of high sequence similarity, including two double zinc finger motifs in the N-terminal region and three coiled-coil domains in the central part of the molecule (Fenster et al., 2000). Piccolo additionally harbors a post-synaptic density-25/Discs large/zona occludens (PDZ) and one or two C2 domains near the C terminus (Wang et al., 1997). Studies of Bassoon knockout mice have shown that Bassoon is essential for the formation of normal glutamatergic neurons (Altrock et al., 2003). On the other hand, the roles of Piccolo in the neuronal system are not well understood.

Recent studies have implicated that Piccolo plays a role in particular mental disorders associated with memory dysfunction (Fenster and Garner, 2002; Weidenhofer et al., 2005). Our group has found previously that Piccolo is overexpressed in the nucleus accumbens of mice treated with methamphetamine, and that Piccolo down-expression by an antisense technique augmented methamphetamine-induced behavioral sensitization, conditioned reward and synaptic dopamine accumulation in the nucleus accumbens (Cen et al., 2008), suggesting that abnormalities of Piccolo are associated with drug dependence, a mental disorder. Further, we have reported that methamphetamine impairs spatial working memory (Nagai et al., 2007). Taken together, Piccolo may play a role in learning and memory. Therefore, demonstration of the involvement of Piccolo in memory and learning and synaptic plasticity might help to elucidate the mechanisms of memory dysfunction caused by these mental disorders.

In the present study, we used a Piccolo antisense oligonucleotide to investigate whether Piccolo plays a significant role in learning and memory, one of the higher brain functions. Here we report that intact Piccolo is required for hippocampus-dependent learning and for hippocampal synaptic plasticity, and appears to act by modulating extracellular levels of glutamate in the hippocampus.

2. Materials and methods

2.1. Animals

Male 8–10-week-old C57BL/6J (Japan SLC Inc., Hamamatsu, Japan) mice were used for the Western blotting, behavioral and electrophysiological studies. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity), with a 12-h-light/12-h-dark cycle (lights on at 8:00 a.m.). Food and tap water were available ad libitum. All procedures used in this study were performed in accordance with the guidelines established by the College of Pharmacy, Nihon University for the care and use of laboratory animals.

2.2. Microinjection of oligonucleotide into the ventricle

Antisense phosphorothioate-substituted oligonucleotide against mouse Piccolo (the region corresponding to bases 2452–2466) and sense oligonucleotide (5′-AACGTAGTCACGTAG-3′) were designed and manufactured as described by previous authors (Fujimoto et al., 2002). Oligonucleotides were dissolved in vehicle (NaCl, 147 mM; KCl, 3 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1 mM). C57BL/6J mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. An infusion cannula was stereotaxically implanted hemilaterally into the ventricle (−0.5 mm anteroposterior, +1 mm mediolateral from bregma, and −2 mm dorsoventral from the skull). An Alzet osmotic mini-pump (model 1002; ALZA Pharmaceuticals, Palo Alto, CA, USA), filled with the desired oligonucleotide (0.6 nmol/6 μL/day for 7 days) or vehicle, was embedded under the animal's skin on the back and was connected to the Alzet infusion cannula (BRAIN INFUSION KIT 3; ALZA Pharmaceuticals). The infusion rate of the mini-pump was 0.25 μL/h.

2.3. Behavioral analyses

2.3.1. Novel object recognition test

The novel object recognition test was performed by using C57BL/6J mice at 8–10 weeks of age as described previously (Hammond et al., 2004; Kamei et al., 2005). Mice were individually habituated to an open box (30 cm × 30 cm × 35 cm high) for 3 days. During the training session, two novel objects were placed into the open field and the animal was allowed to explore for 10 min. The time spent for exploring each object was recorded. During the retention sessions, at 2 h and 24 h after the training session, the animal was placed back into the same box in which one of the familiar objects used during training had been replaced by a novel object, and allowed to explore freely for 10 min. The preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure recognition memory.

2.3.2. Morris water maze test

The Morris water maze test was carried out as described previously (Miyamoto et al., 2005; Sakata et al., 2005). The test utilized a pool (diameter 120 cm, height 30 cm) made from gray plastic in which the water temperature was maintained at 21–23 °C. Swimming paths were analyzed by a computer system with a video tracking system purchased from Muromachi Kikai Co. Ltd. (Tokyo, Japan). Three starting positions were used pseudorandomly, and each mouse was subjected to the hidden platform test with three trials per day for 7 days (days 1–7) and the visible test (day 8). In the hidden platform test, the platform (7 cm in diameter) was submerged 1 cm below the water surface. After reaching the platform, the mouse was allowed to remain on it for 30 s. If the mouse did not find the platform within 60 s, the trial was terminated and the animal was placed on the platform for 30 s. After the hidden platform test for 7 days, mice were subjected to the probe test on day 8 in which the animal swam for 60 s in the pool without the platform. We measured the time spent in each quadrant of the pool as a measure of spatial memory. One hour after the probe test, to measure swimming ability or motivation, mice were subjected to the visible test in which the platform was marked with a flag that protruded 12 cm above the water surface to be highly visible, but in a new location.

2.3.3. Western blot analysis

Western blotting for Piccolo was carried out as described by Dick et al. (2001). Mouse hippocampus was homogenized in buffer (10 mM HEPES [pH 7.4], 320 mM sucrose) containing a protease inhibitor cocktail. The homogenate was precipitated with trichloroacetic acid, and the resulting pellet was washed with acetone and dissolved in buffer (106 mM Tris HCl, 141 mM Tris base, 2% sodium dodecyl sulfate [SDS], 0.51 mM EDTA). For denaturing gel electrophoresis, 20 μg of proteins per lane were separated on Tris acetate-buffered (500 mM, pH 7.0) 4% polyacrylamide gels and electroblotted onto PVDF membranes (Millipore, Eschborn, Germany). Piccolo and β-actin immunoreactivities were detected on the blots with rabbit anti-Piccolo (1:1000; Synaptic Systems, Albany, OR, USA) and mouse anti-β-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA) as primary antibody and secondary antibodies coupled to horseradish peroxidase, followed by visualization with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.4. Electrophysiology

2.4.1. Preparation of hippocampal slices

A C57BL/6J mouse 8–10 weeks of age was killed by decapitation and the whole brain was removed carefully. The brain was immediately soaked for 5 min in ice-cold oxygenated artificial cerebrospinal fluid (ACSF; NaCl, 124 mM; $NaHCO_3$, 26 mM; glucose, 10 mM; KCl, 5 mM; NaH_2PO_4, 1.24 mM; $CaCl_2$, 2.4 mM; and $MgSO_4$, 1.3 mM). Appropriate portions of the brain were trimmed and placed on the ice-cold stage of a Vibratome. The stage was immediately filled with both oxygenated and frozen ACSF. The thickness of each tissue slice was 400 μm. Each slice was gently taken off the blade with a paintbrush, trimmed to area CA1 and dentate gyrus (DG), and immediately soaked in the oxygenated ACSF for 1 h at room temperature as previously reported (Akaishi et al., 2004). Then a slice containing area CA1 and DG was placed on the center of a Multi Electrode Dish (MED) (MED-P515A; Panasonic, Osaka, Japan) probe. The slice was positioned to cover the 8 × 8 array. After positioning the slice, the MED probe was immediately placed in a box. The slice was preincubated in 95% O_2–5% CO_2-saturated ACSF for at least 1 h at room temperature and perfused with the same ACSF.

2.4.2. Measurement of LTP in hippocampal area CA1 and dentate gyrus with the MED system

The hippocampal slices were gently placed on the center of a 0.1% polyethyleneimine-coated MED equipped with a multi-channel extracellular recording system (Alpha MED Sciences Co. Ltd., Tokyo, Japan) (Oka et al., 1999). The device has an array of 64 planar microelectrodes with interelectrode spacing of 150 μm (MED-P515AP; Alpha MED Sciences Co. Ltd.). Electrophysiological experiments were performed in an incubator with perfusion with ACSF at room temperature. ACSF was oxygenated with a 95% O_2 and 5% CO_2 gas mixture before introduction into the MED probe. Evoked field potentials at all 64 sites were recorded with the MED64 system (Alpha MED Sciences Co. Ltd.). Stimulations for recordings were performed with biphasic constant current pulses (0.2 ms, 0.033 Hz) through one of the 64 electrodes. A stimulation electrode was selected in the stratum radiatum to stimulate the Schaffer collateral fibers projecting to area CA1 and the lateral perforant fibers projecting to the DG. Field-excited potentials caused by stimulation were recorded from 64 points around area CA1 and the DG. Control fEPSPs were recorded for 20 min before the conditioning stimulation. LTP

was induced by high-frequency stimulation (HFS), tetanic stimulation (1 s, 100 Hz). After tetanic stimulation, fEPSPs were recorded in area CA1 and DG every 20 s for more than 60 min. LTP occurred and lasted for at least 60 min.

The effect of Piccolo antisense on paired-pulse facilitation (PPF) was examined before LTP was induced. PPF was measured using inter-pulse intervals of 40 ms, 80 ms and 200 ms. As PPF is mediated by presynaptic mechanisms, a change in PPF induced by a specific treatment indicates that a presynaptic mechanism of action of that treatment.

2.4.3. Glutamate release analysis

Glutamate released from mouse hippocampal slices was measured with a glutamic acid/glutamate oxidase assay kit as described by the previous authors (Fujimoto et al., 2004; Yamamoto et al., 2000). C57BL/6J mice were decapitated and the brain was removed. The brain was placed in ice-cold oxygenated preparation buffer (NaCl, 124 mM; $NaHCO_3$, 26 mM; glucose, 10 mM; KCl, 5 mM; NaH_2PO_4, 1.24 mM; $CaCl_2$, 2.4 mM; and $MgSO_4$, 1.3 mM), which was gassed with 95% O_2 and 5% CO_2, cut into 400 μm thick slices with a Vibratome, and the hippocampus was dissected out. After preincubation with oxygenated preparation buffer at room temperature for 1 h, 5 slices were placed in each well of a 6-well plate and incubated in 1 mL of oxygenated preparation buffer at room temperature for 5 min as the pre-stimulation phase (S1). After collecting the buffer from S1, the slices were again incubated in 1 mL of fresh oxygenated preparation buffer containing 50 mM KCl at room temperature for 5 min as the post-stimulation phase (S2). Glutamate released in S1 and S2 was analyzed with an Amplex red glutamic acid/glutamate oxidase assay kit (Invitrogen, Eugene, OR, USA).

2.4.4. Statistical analysis

Statistical analysis was performed by using ANOVA and the Bonferroni test when three groups were compared. When two groups were compared, Student's *t*-test was used. Data are expressed as means ± SEM. p values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of Piccolo antisense injection on the expression of Piccolo in the hippocampus and cerebral cortex

To confirm whether the level of Piccolo protein was reduced by the antisense treatment, we analyzed expression of Piccolo in the hippocampus and cerebral cortex on the 7th day after starting antisense administration. Western blot analysis with anti-Piccolo antibody showed a lower level of Piccolo protein in the hippocampus of the antisense-injected group compared with the vehicle- and sense-treated groups (Fig. 1A). In contrast, the levels of Piccolo protein in the cerebral cortex were similar among these groups (Fig. 1B).

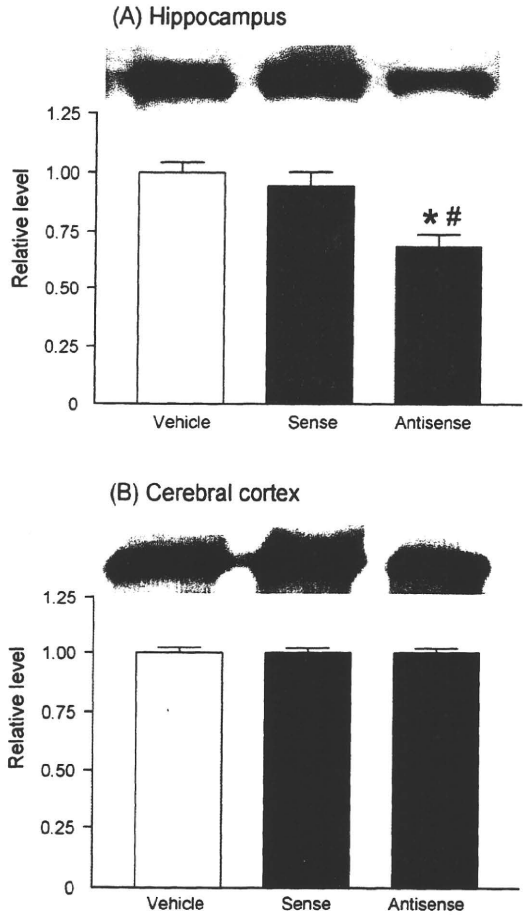

Fig. 1. Effect of Piccolo antisense injection on the expression of Piccolo in the hippocampus (A) and cerebral cortex (B). An osmotic mini-pump was used to deliver a continuous infusion of Piccolo antisense (0.6 nmol/6 μL/day), sense (0.6 nmol/6 μL/day) or vehicle (6 μL/day) into the right ventricle (AP −0.5 mm, ML +1.0 mm from bregma, DV −2.0 mm from the skull). Each value represents the mean ± SEM for 7 experiments. *$p < 0.05$ vs. vehicle-treated mice. #$p < 0.05$ vs. sense-treated mice.

3.2. Effect of Piccolo antisense injection on spatial learning and recognition memory

The Morris water maze is commonly used in mice to measure spatial learning and memory, which requires hippocampal activity. To determine the role of Piccolo in spatial memory formation, we used Piccolo antisense. Mice were assigned to three groups: vehicle, sense and antisense. The hidden platform test in the Morris water maze began on the day after surgery. As shown in Fig. 2A, the escape latencies of vehicle- and sense-injected mice became shorter after hidden platform test for 7 days (ANOVA with repeated measures: day, $F_{(6,90)} = 20.949$: $p < 0.001$). However, a significantly longer escape latency [ANOVA with repeated measures: group, $F_{(2,22)} = 5.528$; $p < 0.05$; day × group, $F_{(12,90)} = 5.513$; $p < 0.05$] was observed in the antisense-treated group compared with the vehicle and sense groups, showing impairment of spatial learning. To examine the effect of Piccolo antisense on the spatial memory, the probe test was carried out 24 h after final hidden platform test. Although vehicle- and sense-injected animals spent significantly longer time in the target quadrant than in opposite quadrants, there was no difference between the times spent in these two quadrants in antisense-treated animals (Fig. 2A). Furthermore, the antisense-treated group showed no performance deficit in the visible platform test, suggesting that the difference in escape latency in hidden platform test between the Piccolo antisense and the vehicle or sense groups was not due to impairment of swimming ability or motivation. Taken together, these results indicate that infusion of Piccolo antisense impaired spatial learning in the Morris water maze.

To examine visual recognition memory in mice treated with Piccolo antisense, we employed a novel object recognition test. We used a 10 min training protocol to assess the enhancement of learning and memory. There was no difference in exploratory preference during training among the vehicle-, sense- and antisense-treated groups, indicating that each group essentially had the same levels of curiosity and/or motivation to explore the two objects. In both 2 h and 24 h retention, all of mice showed significant increase in exploratory preference, but there was no difference in exploratory preference among these groups (Fig. 2B).

3.3. Effect of Piccolo antisense injection on LTP in area CA1 and the DG in the hippocampus

To investigate synaptic activity in the hippocampus of mice treated with antisense, we performed electrophysiological analyses using hippocampal slices. LTP is a form of synaptic plasticity, and LTP in area CA1 and the DG is considered to be one of the cellular mechanisms underlying hippocampus-dependent