Neuroscience 163 (2009) 731–734

RAPID REPORT

ROLE OF α_2/δ SUBUNIT IN THE DEVELOPMENT OF MORPHINE-INDUCED REWARDING EFFECT AND BEHAVIORAL SENSITIZATION

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Abstract—Previous data demonstrate that L-type voltage-gated calcium channel (VGCC) blockers, which bind to α_1 subunits of VGCC to suppress Ca^{2+} entry into cells, inhibit the development of psychological dependence on drugs of abuse, suggesting the upregulation of L-type VGCC in the development of psychological dependence. However, there are few available data on changes of the auxiliary subunit α_2/δ modifying L-type VGCC under such conditions. We therefore investigated here the role of α_2/δ subunits of VGCCs in the brain of mouse after repeated treatment with morphine. The treatment with morphine increased α_2/δ subunit expression in the frontal cortex and the limbic forebrain of mice showing rewarding effect and sensitization to hyperlocomotion by morphine. The morphine-induced behavioral sensitization and place preference were also suppressed by gabapentin, which binds to an exofacial epitope of the α_2/δ auxiliary subunits of VGCCs. These findings indicate that the upregulation of α_2/δ subunit as well as α_1 subunits of VGCC in the frontal cortex and the limbic forebrain plays a critical role in development of morphine-induced rewarding effect and behavioral sensitization following neuronal plasticity.

Key words: gabapentin, morphine, α_2/δ subunit of voltage-gated calcium channels, rewarding effect, behavioral sensitization, neuronal plasticity.

Psychological dependence on opioids produces dynamic behavioral responses as the result of molecular and/or cellular changes in the brain following chronic morphine (MRP) treatment. Various studies have provided the data to support a substantial role of mesolimbic dopaminergic transmission, which originates from the ventral tegmental area and projects to the nucleus accumbens, in rewarding effect of opioids (Narita et al., 2003). MRP indirectly activates dopaminergic neurons localized in the ventral tegmental area as a consequence of its inhibiting actions on non-dopaminergic neurons, presumably GABAergic neurons, to increase dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988).

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) play an essential role in regulating various functions such as learning and memory (Berridge et al., 2000). Voltage-gated calcium channel (VGCC) controls selective entry of Ca^{2+} ion in response to changes of membrane potential and forms heteromeric complexes consisting of α_1, α_2, β, δ and γ subunits. VGCCs contribute to some forms of synaptic plasticity, including long-term potentiation (Kapur et al., 1998) and long-term depression (Wickens and Abraham, 1991). Recent reports have demonstrated that α_2/δ subunit is widely distributed in the brain (Taylor and Garrido, 2008), and gabapentin (GBP) binds with high affinity to an exofacial epitope of the α_2/δ subunit to suppress α_1 subunits (Gee et al., 1996; Marais et al., 2001). However, there are few available data on the role of α_2/δ subunit in the development of MRP psychological dependence. In the present study, we investigated whether α_2/δ subunit in the frontal cortex and limbic forebrain could be involved in the development of MRP-induced rewarding effect and sensitization to hyperlocomotion.

MATERIALS AND METHODS

Animals

Male ddY mice (Japan SLC, Inc., Hamamatsu, Japan) were housed in a room maintained at 22±1 °C, 55±0.5% with a 12-h light/dark cycle (light on 8:00 AM to 8:00 PM). Food and water were available *ad libitum*. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Kawasaki Medical School, as adopted by the Committee on Animal Research of Kawasaki Medical School. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments.

Place conditioning

The place conditioning procedure is used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in animals (Narita et al., 2005). The conditioning place preference schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). For the preconditioning and postconditioning tests the time spent in each compartment during 900 s session was recorded (Recorder System BS-CPP-MS; BrainSienceIdea Co., Ltd. Osaka, Japan). Conditioning sessions (three sessions for MRP: three sessions for saline (SAL)) were started on the next day after preconditioning test and conducted once daily for 6 days. Immediately after s.c. injection of MRP (5 mg/kg), these animals were placed in the opposite compart-

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Abbreviations: GBP, gabapentin; mGluR5, metabotropic glutamate receptor 5; MRP, morphine; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SAL, saline; VGCC, voltage-gated calcium channel; Vps34, vacuolar protein sorting 34.

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doi:10.1016/j.neuroscience.2009.07.017

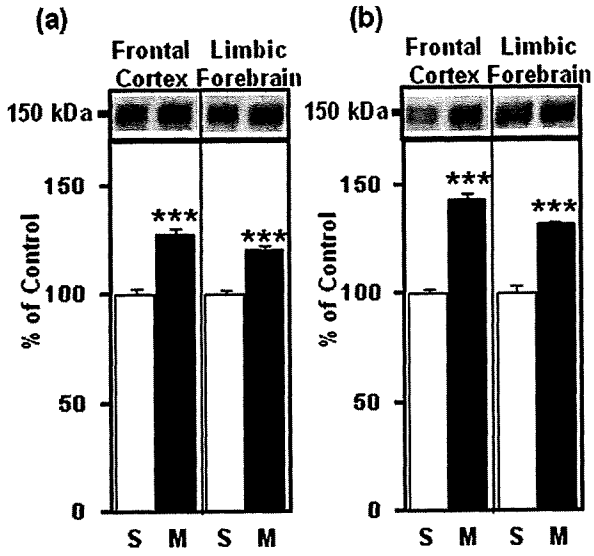

Fig. 1. Changes in the protein levels of the α_2/δ subunit in membrane fractions of the mouse frontal cortex and limbic forebrain obtained from MRP-induced sensitization to hyperlocomotion (a) and rewarding effect (b) in mice. The membrane fraction was prepared 24 h after of the last MRP treatment for behavioral sensitization to hyperlocomotion and after the postconditioning test for rewarding effect. Each column represents the mean±SEM of four samples. *** $P<0.001$ vs. SAL group (Bonferroni's test). S, saline; M, morphine.

ment in which they had spent the most time during the preconditioning test, for 1 h. On alternate days, these animals received SAL and were placed in another compartment for 1 h. GBP was administered i.c.v. 30 min before the treatment with MRP (5 mg/kg s.c.) or SAL. The preference for drug-paired place was shown as the mean difference between the times spent during the postconditioning and preconditioning tests.

Locomotor assay

The locomotor activity of mice was measured by an animal movement analyzing system (Actimo-100 system, Shintechno Ltd., Fukuoka, Japan). Total activity counts in each 10 min segment were automatically recorded for 30 min prior to and for 180 min after MRP (10 mg/kg s.c.) administration. In order to induce behavioral sensitization to MRP-induced hyperlocomotion, mice were given five intermittent treatments with MRP (10 mg/kg s.c.) every 96 h. Their activities were counted after every injection to confirm the development of MRP-induced sensitization. To investigate implication of α_2/δ subunit in the locomotor activity and development of sensitization to hyperlocomotion by MRP, mice were pretreated with vehicle or GBP 30 min prior to MRP treatment. GBP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in SAL.

Western blotting

Immediately after the postconditioning test for conditioned place preference and after 24 h of the last MRP treatment for behavioral sensitization to hyperlocomotion, the frontal cortex and the limbic forebrain (containing the nucleus accumbens) were quickly removed from the decapitated mice and homogenized in ice cold lysis buffer. The homogenate was centrifuged at 1000×g for 10 min and the supernatant was centrifuged at 100,000×g for 60 min at 4 °C. Proteins were separated by 5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes in Tris–glycine buffer. For immunoblot detection, the membrane was incubated with primary antibody diluted 1:1000 (α_2/δ; Alomone Laboratories Ltd., Jerusalem, Israel) overnight at 4 °C and horseradish peroxidase–conjugated goat anti-rabbit IgG diluted 1:5000 for 2 h at room temperature. Finally, blots were detected with chemiluminescence.

Statistical analysis

Each data point was expressed as the mean±SEM. The statistical significance was assessed by the methods described in each figure legend following the application of the one-way or two-way ANOVA.

RESULTS

As shown in Fig. 1, the immunoreactivity for α_2/δ subunit significantly increased in the MRP-sensitized (a) and -conditioned (b) mice compared to those in mice treated repeatedly with SAL in both the frontal cortex and limbic forebrain.

To investigate the role of α_2/δ subunit in the hyperlocomotion by MRP, GBP was treated before a single administration of MRP (10 mg/kg s.c.) or with intermittent administration of MRP. GBP (3, 10 and 30 nmol/mouse, i.c.v.) had no effects on the locomotion induced by a single MRP administration (Fig. 2a). On the other hand, the intermittent administration of MRP (10 mg/kg s.c.) enhanced the locomotion in a frequency of administration-dependent manner, that is, the MRP-treated mice in 4th and 5th challenges showed marked enhancement of hyperlocomotion as compared to those in the 1st challenge, and this enhanced locomotion was significantly suppressed by GBP (30 nmol/mouse, i.c.v.).

The effect of α_2/δ subunit on the MRP-induced rewarding effect was examined by place preference paradigm

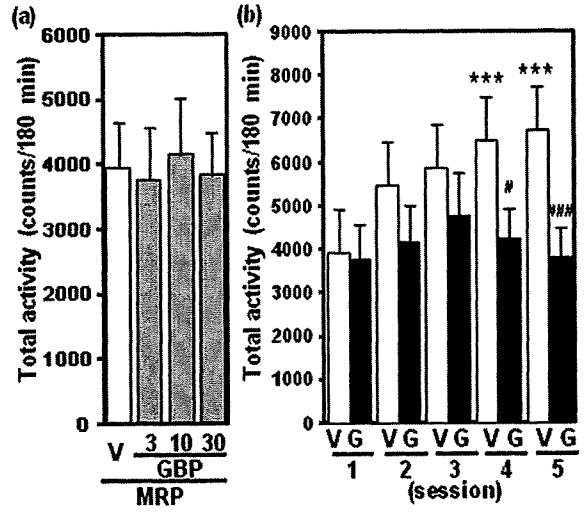

Fig. 2. (a) Total activity of the MRP-induced hyperlocomotion in mice pretreated with V or GBP. Each point or column represents the mean locomotor activity counts±SEM of 10 mice. (b) Groups of mice were given five treatments of MRP every 96 h and pretreated with V or GBP. Total activity was counted for 180 min after each treatment. The data represent the mean±SEM of 10 mice. *** $P<0.001$ vs. the 1st drug administration (Dunnett's test), # $P<0.05$, ### $P<0.001$ vs. V-MRP group (Bonferroni's test). V, vehicle; G, gabapentin.

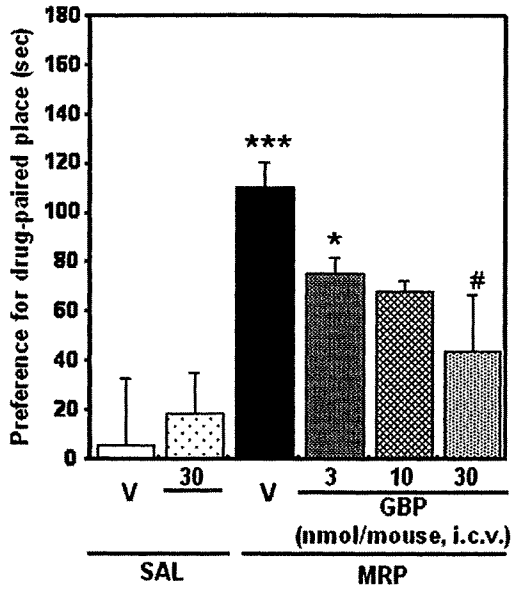

Fig. 3. Place preference of the MRP-induced rewarding effect in mice pretreated with V or GBP. Groups of mice were conditioned with SAL or MRP three times every other day for 6 days according to the conditioning schedule descried in the Experimental Procedures. Each column represents the mean±SEM of 10 mice. * $P<0.05$, *** $P<0.001$ vs. SAL-conditioned group (Bonferroni's test), # $P<0.05$ vs. V pretreatment group (Bonferroni's test). V, vehicle.

using MRP-conditioned mice with or without GBP. Chronic administration of MRP (5 mg/kg s.c.) produced significant place preference in mice, which was dose-dependently inhibited by GBP (3, 10 and 30 nmol/mouse, i.c.v.) (Fig. 3).

DISCUSSION

VGCCs play an important role in long-term synaptic plasticity (Deisseroth et al., 2003) and Ca^{2+} influx in psychostimulant-induced behavioral and neurochemical changes (Karler et al., 1991; Suzuki et al., 1992). In the latter investigations the data that the nifedipine suppresses the development of conditioned place preference and behavioral sensitization induced by psychostimulants such as cocaine and amphetamine are reported (Karler et al., 1991; Suzuki et al., 1992). We report here the role of α_2/δ subunit in MRP-induced rewarding effect and sensitization to hyperlocomotion with the data showing the increase in α_2/δ subunit protein in the frontal cortex and limbic forebrain.

In the present behavioral study, no significant changes in MRP-induced hyperlocomotion were observed by the i.c.v. pretreatment with GBP. However, the development of behavioral sensitization to MRP was abolished by the cotreatment with GBP. Accumulating evidence suggests that the behavioral sensitization phenomenon induced by MRP should be accompanied by long-lasting neuronal plasticity (Narita et al., 2002, 2003, 2005), which may involve structural modifications in the CNS. In particular, Ca^{2+} dependent-signaling activity such as Ca^{2+}/calmodulin-mediated kinase II and protein kinase C (PKC), is stimulated by a rise of intracellular Ca^{2+} concentration through VGCCs (Anderson et al., 2008) and participates in drug addiction (Narita et al., 2002, 2004). Taken together, these findings including those in this study suggest that the enhancement of intracellular Ca^{2+} concentration due to the upregulation of α_2/δ subunit induced by MRP may lead to neuronal plasticity of CNS, resulting in the development of rewarding effect and sensitization to hyperlocomotion on MRP.

A recent study has reported that phosphatidylinositol 3-kinase (PI3K) promotes VGCC trafficking to the plasma membrane (Viard et al., 2004). On the other hand, our previous study revealed that PKCγ directly regulated the activation of PI3K class III vacuolar protein sorting 34 (Vps34) (Shibasaki et al., 2009). The known functions of Vps34 were the regulation of vesicular trafficking in the endosomal/lysosomal system (Lindmo and Stenmark, 2006). Our preliminary data also implicate that upregulation of the VGCC α1c subunit via facilitating trafficking induced by Vps34 occurs during the development of rewarding effect on MRP (data not shown). In addition, previous reports have demonstrated that upregulation of $G_{\alpha q/11}$ protein and PKC is important for the development of behavioral sensitization to MRP (Narita et al., 2002) and that $G_{\alpha q}$-coupled metabotropic glutamate receptor 5 (mGluR5) participates in the development of MRP-induced rewarding effect in mice (Aoki et al., 2004). When these findings are taken together, it is supposed that the upregulation of α_2/δ subunit in the frontal cortex and the limbic forebrain may be regulated by $G_{\alpha q}$-coupled mGluR5 through PKCγ-Vps34 on MRP psychological dependence and cause a change in functional and structural plasticity in the following Ca^{2+} dynamic state.

Previous investigations reported that MRP physical dependence was inhibited by L-type VGCC blockers such as verapamil and diltiazem (Baeyens et al., 1987; Caro et al., 1988). N-type VGCC is also reported to participate in the development of MRP physical dependence (Meng et al., 2008). In addition, chronic treatment with ethanol and nicotine induces not only physical dependence in animals but also upregulation of α_1 and α_2/δ subunits of VGCC in the cerebral cortex (Hayashida et al., 2005; Katsura et al., 2005). These data therefore suggest that the α_2/δ subunit of VGCC may play important roles in the development of physical dependence on MRP as well as ethanol and nicotine, though these problems remain to be elucidated at present.

In this study it seems that the magnitude of α_2/δ subunit upregulation after the CPP experiment is more than that after the hyperlocomotion experiment, although the dose and period of MRP treatment in the hyperlocomotion experiment are much more than those in the CPP experiment. Although both methods of measuring CPP and behavioral sensitization are the same as those for checking the development of psychological dependence, these methods observe different expression of signs in animals, which therefore causes the difference of drug administration schedules. We consider that the doses of MRP used in this study are appropriate to investigate effects of GBP on MRP-induced CPP and behavioral sensitization. It is noted

that the expression of the α_2/δ subunit is in the frontal cortex and the nucleus accumbens to which dopaminergic mesolimbic neurons project and that MRP is supposed to indirectly act on these dopaminergic neurons via inhibiting non-dopaminergic neurons, presumably GABAergic neurons terminated on the soma of dopaminergic neurons in the ventral tegmental area. Therefore, the magnitude of α_2/δ subunit upregulation may not be dependent on dose and duration of MRP treatment. However, it is difficult to explain how such a difference in the magnitude of α_2/δ subunit upregulation occurs and the mechanism of the difference remains to be elucidated.

CONCLUSION

In conclusion, the present study indicates that repeated treatment with MRP increases α_2/δ subunit expression in the frontal cortex and limbic forebrain. The MRP-induced behavioral sensitization and place preference were suppressed by GBP, an inhibitor for the α_2/δ subunit. These findings indicate that the upregulation of the α_2/δ subunit in the frontal cortex and limbic forebrain plays a critical role of the development to MRP-induced rewarding effect and behavioral sensitization.

Acknowledgments—This work was supported in part by Grants-in-Aid for Science and Culture of Japan, and the Research Project Grants from Kawasaki Medical University.

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(Accepted 8 July 2009)
(Available online 25 July 2009)

SYNAPSE 64:440–444 (2010)

Upregulation of L-Type Ca_v1 Channels in the Development of Psychological Dependence

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KEY WORDS drugs of abuse; L-type voltage-dependent calcium channels; neuronal plasticity

ABSTRACT Although L-type voltage-dependent Ca^{2+} channels regulate activity-dependent processes including synaptic plasticity and synapse formation, there are few data on the changes of Ca_v1 channel expression in psychological dependence. This study investigated the role of L-type Ca_v1 channel expression in the brain of mouse that was psychologically dependent on methamphetamine (2 mg/kg, subcutaneous injection [s.c.]), cocaine (10 mg/kg, s.c.), and morphine (5 mg/kg, s.c.) with the conditioned place preference paradigm. Intracerebroventricular administration of nifedipine (3, 10, and 30 nmol/mouse) dose-dependently reduced the development of methamphetamine-, cocaine-, and morphine-induced rewarding effect. Under such conditions, protein levels of both $Ca_v1.2$ and $Ca_v1.3$ in the frontal cortex and the limbic forebrain were significantly increased on methamphetamine-, cocaine-, and morphine-induced psychologically dependent mice. These findings suggest that the upregulation of $Ca_v1.2$ and $Ca_v1.3$ participated in the development of psychological dependence. **Synapse 64:440–444, 2010.** ©2010 Wiley-Liss, Inc.

INTRODUCTION

Voltage-dependent Ca^{2+} channels (VDCCs) are a large family of integral membrane proteins that control the selective flow of Ca^{2+} ion down their electrochemical gradient in response to changes in membrane potential. VDCCs are classified into distinct subtypes (L, N, P/Q, R, and T) based on their pharmacological and biophysical properties (Tsien et al., 1995). L-type VDCCs (α1C subunit: $Ca_v1.2$, α1D subunit: $Ca_v1.3$) are heterometric complexes and show electrophysiological and pharmacological diversity (Catterall, 2000; Wang et al., 2004). $Ca_v1.2$ and $Ca_v1.3$ are dominant calcium channel-forming subunits of L-type VDCCs, are expressed in many types of neurons, and are located in the soma, proximal dendrites, and postsynaptic regions of neurons (Hell et al., 1993). In the nervous system, Ca_v1 VDCCs conduct L-type Ca^{2+} currents that dually regulate membrane excitability and intracellular signal transduction. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) play an essential role in regulating fertilization, proliferation, development, learning and memory, contraction, and secretion (Berridge et al., 2000). In addition, these VDCCs contribute to some forms of synaptic plasticity, including long-term potentiation (Kapur et al., 1998) and long-term depression (Wickens and Abraham, 1991). Through electrical and signaling functions, Ca_v1 channels regulate activity-dependent processes including synaptic plasticity and synapse formation, which are required for the normal development and function of the nervous system. However, only little data are available on the role of L-type VDCCs in the development of psychological dependence due to drugs of abuse.

Accumulating evidence suggests that repeated administration of psychostimulants induces long-lasting neuronal plasticity in the brain (Nestler and Aghajanian, 1997). Animal models for drug dependence with psychostimulants and opioid show dynamic behavioral response as a result of molecular and/or cellular changes in the brain following chronic treatment with abused drugs. Various studies have

Contract grant sponsors: Ministry of Education, Culture, Sports, Science, and Technology of Japan, Kawasaki Medical University, Japan.

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Received 15 May 2009; Accepted 21 August 2009

DOI 10.1002/syn.20745

Published online in Wiley InterScience (www.interscience.wiley.com).

provided the data that support substantial roles of mesolimbic dopaminergic transmission, which originates from the ventral tegmental area and projects to the nucleus accumbens and frontal cortex, in rewarding effect (Bals-Kubik et al., 1993; Narita et al., 2003; Vezina and Stewart, 1984). Drugs of abuse, such as psychostimulants and opiates, are generally considered to exert their locomotor and rewarding effects through an increased dopaminergic transmission in the nucleus accumbens. Dopamine neurotransmission in the nucleus accumbens is essential for the processing of behaviorally relevant stimuli and the attribution of motivational valence to related events (Robinson and Berridge, 2003; Schultz, 2002). The critical involvement of dopamine transmission in drug addiction and reward has been recognized for many years (Koob, 1992; Wise and Rompre, 1989). This study investigated the role of L-type VDCC expression changes in the brain of mice that were psychologically dependent on methamphetamine, cocaine, and morphine, with the conditioned place preference paradigm.

MATERIALS AND METHODS

In this study, we used male ddY mice (Japan SLC, Hamamatsu, Japan). Animals were housed in a room (22°C ± 1°C, humidity 55% ± 0.5%) with a 12-h light/dark cycle (light on from 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum. The place-conditioning procedure was used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in animals (Narita et al., 2005). The conditioning place preference schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). The preconditioning and postconditioning tests were recorded as the time spent in each compartment during a 900-s session (BS-CPP-MS; BrainSienceIdea, Osaka, Japan). In the combination study, nifedipine (Wako Pure Chemical Industries, Osaka, Japan) was administered intracerebroventricularly 30 min before subcutaneous treatment with methamphetamine (Dainippon Pharmaceutical, Tokyo, Japan), cocaine (Shionogi, Osaka, Japan), and morphine (morphine hydrochloride; Sankyo, Tokyo, Japan). The preference for drug-paired place was shown as the mean difference between times spent during the postconditioning and preconditioning tests. Immediately after 24 h of the last conditioning phase, the animals were decapitated, and the frontal cortex and the limbic forebrain (containing the nucleus accumbens) were quickly dissected and homogenized in the ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, 10 mM NaF, 0.5% Triton X-100, with a protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]). The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant was further centrifuged at 100,000*g* for 60 min at 4°C. Proteins in the pellet were separated using 5% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes in Tris-glycine buffer (25 mM Tris and 192 mM glycine). For immunoblot detection, the membrane was incubated with primary antibodies (Alomon Labs, Jerusalem, Israel) for $Ca_v1.2$ and $Ca_v1.3$ diluted 1:1000 overnight at 4°C and horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5000 for 2 h at room temperature. Finally, blots were detected with chemiluminescence.

The data were expressed as the mean ± SEM. The statistical significance was assessed by the methods described in each figure legend following the application of one-way ANOVA.

RESULTS

To investigate the direct involvement of L-type VDCCs as $Ca_v1.2$ and $Ca_v1.3$ in the methamphetamine-, cocaine-, and morphine-induced rewarding effect, mice were conditioned with methamphetamine, cocaine, and morphine in the presence or absence of a L-type VDCC antagonist nifedipine using place preference paradigm. The intracerebroventricular administration of nifedipine caused a dose-dependent inhibition of methamphetamine-, cocaine-, and morphine-induced rewarding effect (Figs. 1A–1C).

Under such conditions when the mice showed methamphetamine-, cocaine-, and morphine-induced rewarding effect, we examined how the protein levels of $Ca_v1.2$ and $Ca_v1.3$ in the frontal cortex and the limbic forebrain altered. As shown in Figure 2, both $Ca_v1.2$ and $Ca_v1.3$ significantly increased in the frontal cortex and the limbic forebrain of mice showed the rewarding effect.

In addition to previous reports suggesting that upregulation of L-type VDCC function is an important factor in the development of physical dependence by drugs of abuse such as ethanol, morphine, and nicotine (Little, 1991; Walter and Messing, 1999), it was reported that the rewarding effects of methamphetamine and cocaine were reduced by nifedipine (Suzuki et al., 1992). Similarly, this study also demonstrated that the administration of nifedipine caused a dose-dependent inhibition of methamphetamine-, cocaine-, and morphine-induced place preference. A noticeable result demonstrated in this study is that $Ca_v1.2$ and $Ca_v1.3$ protein levels also increased in the frontal cortex and the limbic forebrain including the nucleus accumbens of the methamphetamine-, cocaine-, and morphine-conditioned mice, which is considered to explain well the inhibitory effect of nifedipine on abuse drug-induced place preference. Although the upregulation of Cav1 protein in the animal brain has been reported in both physical (Katsura et al., 2005; Shibasaki et al., 2007; Walter

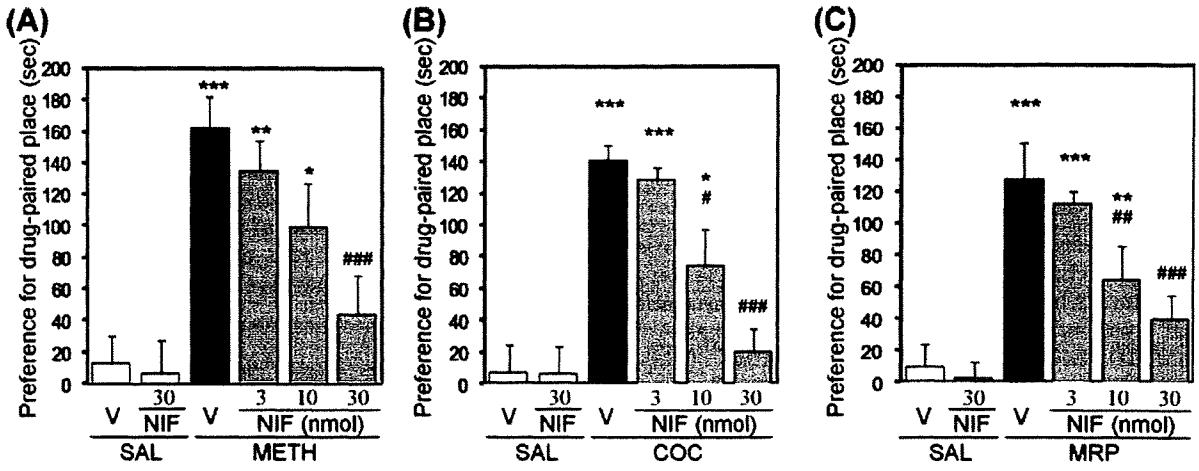

Fig. 1. Effect of a L-type VDCC antagonist nifedipine on (A) methamphetamine-, (B) cocaine-, and (C) morphine-induced place preference in mice. Nifedipine (NIF; 3, 10, and 30 nmol/mouse) or vehicle (V) was intracerebroventricularly administered 30 min before subcutaneous treatment with saline (SAL), methamphetamine (METH; 1 mg/kg), cocaine (COC; 10 mg/kg), and morphine (MRP; 5 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline-conditioned mice (Bonferroni's test). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. vehicle pretreatment (Bonferroni's test). The data represent the mean ± SEM obtained from eight animals.

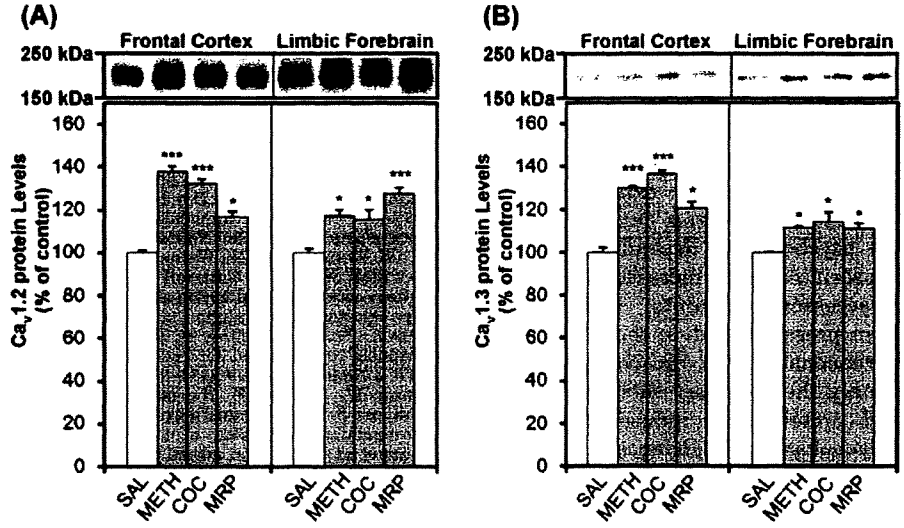

Fig. 2. Changes in the protein levels of $Ca_v1.2$ (A) and $Ca_v1.3$ (B) in the frontal cortex and the limbic forebrain during the conditioned place preference to methamphetamine, cocaine, and morphine. The column represents the mean ± SEM of four animals. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. saline-conditioned group (Bonferroni's test). SAL, saline; METH, methamphetamine; COC, cocaine; MRP, morphine.

and Messing, 1999) and psychological (as shown here) dependences, the differences in the properties of Cav1 protein upregulation under these conditions and its pathophysiological significance remain to be elucidated.

In addition, previous reports have demonstrated that the upregulation of $G\alpha_{q/11}$ protein and protein kinase C (PKC) is important for the development of behavioral sensitization to morphine (Narita et al., 2002) and that $G\alpha_q$-coupled metabotropic glutamate receptor 5 participates in the development of morphine-induced rewarding effect in mice (Aoki et al., 2004). Our recent study revealed that PKCγ directly regulates the activation of Vps34, a kinase classified as PI 3-kinase class III (Shibasaki et al., 2009a). One of the well-known functions of Vps34 is to regulate vesicular trafficking in the endosomal/lysosomal system (Lindmo and Stenmark, 2006). Taken together with these findings it is suggested that the upregulation of $Ca_v1.2$ and $Ca_v1.3$ shown in this study may be

regulated by $G\alpha_q$-coupled receptor cascade through PKCγ-Vps34 during the development of methamphetamine-, cocaine-, and morphine-induced psychological dependence.

On the other hand, the facilitated response of L-type channel $Ca_v1.2$ observed in the brain of animals with psychological dependence may be produced by other mechanisms. As for L-type channel $Ca_v1.2$, various mechanisms for its functional facilitation have been characterized, that is, phosphorylation of the channels by PKA (Catterall, 2000) and association with calmodulin-dependent protein kinase II (CaMKII) (Lee et al., 2006). The activation of dopamine receptors in the nucleus accumbens increases their coupling to guanine nucleotide-binding regulatory protein to act on cAMP-dependent PKA, and the PKA and CaMKII protein levels increase in mice dependent on morphine (Narita et al., 2004; Nestler and Aghajanian, 1997). Therefore, the facilitating Ca^{2+} response observed in the brain of animals with psychological dependence may also be produced via such pathways to modify $Ca_v1.2$ channel functions.

Moreover, VDCCs are known to form heteromeric complexes consisting of $\alpha1$, $\alpha2$, δ, β, and γ subunits and the accessory subunits ($\alpha2$, δ, β, and γ subunits) serve as modulators or regulators for a1 subunit. Therefore, the possibility that the expression and/or functions of these accessory subunits may also be induced during the development of psychological dependence and, in turn, affect the expression of $\alpha1$ subunits has been supposed. Indeed, our recent study revealed that the psychological dependence induced by morphine is associated with increased expression of one of the accessory subunits, $\alpha2/\delta$ subunit (Shibasaki et al., in press). Based on these data, it is considered to be reasonable that the changes in the expression of not only the accessory subunits but also the Cav1 channel proteins are induced under the conditions with psychological dependence.

Calcium influx through L-type calcium channels plays an important role in psychostimulant-induced behavioral and neuronal plasticity as described earlier. On the other hand, it is reported that Ca_v1 activation by membrane depolarization promotes ryanodine receptor (RYR)-mediated Ca^{2+} release from the endoplasmic reticulum (Kim et al., 2007; Mouton et al., 2001). There is considerable evidence indicating that calcium-stimulated second messengers contribute to the induction of long-term potentiation (Baudry and Lynch, 2001; Gnegy, 2000; Lisman et al., 2002). These studies and this study suggest that the upregulation of Ca_v1 channels cause the activity of RYRs and the alteration of calcium signaling on neuronal function, although such functional relationship between Ca_v1 channels and RYRs in psychological dependence due to drugs of abuse remains to be elucidated.

CONCLUSION

In conclusion, this study indicates that the methamphetamine-, cocaine-, and morphine-induced place preference was suppressed by a L-type VDCC antagonist nifedipine. The repeated in vivo treatment with methamphetamine, cocaine, and morphine increases the protein levels of $Ca_v1.2$ and $Ca_v1.3$ in the frontal cortex and limbic forebrain of mouse. These findings provide evidence for a role of the upregulation of $Ca_v1.2$ and $Ca_v1.3$ in the development of psychological dependence.

ACKNOWLEDGMENTS

The authors thanks Noriko Ohtsuki and Junko Katayama for their excellent technical assistance.

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SYNAPSE 00:000–000 (2010)

Methamphetamine-Induced Up-Regulation of α_2/δ Subunit of Voltage-Gated Calcium Channels Is Regulated by DA Receptors

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KEY WORDS methamphetamine; α_2/δ subunit of voltage-gated calcium channels; rewarding effect; DA receptors

ABSTRACT This study was carried out to determine the roles of dopamine D1 and D2 receptors on the up-regulation of α_2/δ subunit of voltage-gated Ca^{2+} channels (VGCCs) induced by methamphetamine (METH). In the conditioned place preference paradigm, METH-induced place preference suppressed with gabapentin, an antagonist for α_2/δ subunit. Under these conditions, the increase in α_2/δ subunit expression was found in the frontal cortex and limbic forebrain. In addition, the METH-induced place preference was significantly attenuated by dopamine D1 and D2 receptor antagonists, SCH23390 and sulpiride, respectively. The expression of α_2/δ subunit protein and its mRNA was significantly enhanced in the METH-treated cortical neurons. These increases in protein and mRNA of α_2/δ subunit were completely abolished by SCH23390 and sulpiride with simultaneous exposure to METH. These findings indicate that up-regulation of α_2/δ subunit is regulated through the activation of dopamine D1 and D2 receptors during METH treatment. **Synapse 00:000–000, 2010.**

INTRODUCTION

Drug addiction has linked to functional changes in neural circuits involving in motivation that can lead to drug dependence, craving, and relapse (Koob et al., 1998). Among these neural circuits, the mesolimbic dopaminergic projection to the nucleus accumbens or frontal cortex is considered to mediate dominantly the rewarding effects of many stimuli including drugs of abuse (Wise and Hoffman, 1992).

Voltage-gated Ca^{2+} channels (VGCCs) are heteromeric complexes composed of α_1, α_2, δ, β, and γ subunits and α_1 subunit forms a pore through which Ca^{2+} enters into cells (Davies et al., 2007). Recent reports have revealed that membrane-anchored α_2/δ subunit of which dominant portion is extracellular is widely distributed in the brain (Taylor and Garrido, 2008), and our previous data show that chronic in vivo administration of morphine increases α_2/δ subunit protein in the nucleus accumbens and frontal cortex of mice showing rewarding effect of morphine (Shibasaki et al., 2009a). Although these data raise the possibility that α_2/δ subunit contributes to the development of rewarding effect induced by methamphetamine (METH), little is known about the mechanisms of functional changes in α_2/δ subunit in the development of rewarding effects of METH. On the other hand, mesolimbic dopamine neurons play a critical role in developing rewarding effect of drugs of abuse, which suggests that dopamine released may regulate α_2/δ subunit expression. Taken together these data, the present study was therefore carried out to examine whether dopamine receptors regulate the METH-induced changes in α_2/δ subunit expression.

MATERIALS AND METHODS

Animal

In this study, we used male ddY strain mice (Japan SLC, Inc., Hamamatsu, Japan). Animals were housed in a room maintained at 22 ± 1°C and 55 ± 0.5% of humidity with 12 h light/dark cycle (light on

Contract grant sponsor: Grants-in-Aid for Science and Culture of Japan; Contract grant sponsor: Kawasaki Medical University; Contract grant number: 20-606

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Received 1 July 2009; Accepted 16 February 2010

DOI 10.1002/syn.20797

Published online 17 March 2010 in Wiley InterScience (www.interscience.wiley.com).

8:00 A.M. to 8:00 P.M.). Food and water were available ad libitum.

All experiments presented in this article were approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the "Guide for Care and Use of Laboratory Animals" of Kawasaki Medical School that is based on the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Place conditioning

The place-conditioning procedure is used to evaluate rewarding effects of METH in animals. The conditioning place preference schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). The time spent in each of two compartments during a 900-s session was recorded (BS-CPP-MS; Brain SienceIdea Co., Ltd. Osaka, Japan) in the preconditioning and postconditioning tests. In the combination study, SCH23390 and sulpiride was administered intraperitoneally (i.p.), and gabapentin (GBP) was intracerebroventricularly (i.c.v.) injected 30 min before subcutaneous administration of METH. The preference for drug-paired place was shown as mean difference between the duration spent during the postconditioning and preconditioning tests. GBP, SCH23390, and sulpiride were dissolved in saline.

Primary culture of cerebral cortical neurons

Isolation and primary culture of cerebral cortical neurons were carried out according to the method described previously (Ohkuma et al., 1986) with a minor modification. The neurons were used for the experiments described blow on the 13th day of the culture. More than 95% of cultured cells were identified as the neurons by immunohistochemical analysis (Ohkuma et al., 1986).

In this study, the effects of METH on α_2/δ-1 subunit expression were examined by two different patterns of METH exposure. One pattern is to exposé the neurons continuously to 1 μM METH for 24 h and then the neurons were used for the following experiments. Another pattern of METH exposure is an intermittent exposure. Namely, as mice were intermittently treated with METH in examining CPP, the neurons were exposed to METH (1 μM) for 1 h and were cultured for following 23 h in the absence of METH. Such exposure of the neurons to METH was carried out for three days and then protein for analyzing α_2/δ subunit expression was extracted. In both experiments with different pattern of METH exposure, SCH23390 and sulpiride were added in the culture medium 10 min before the addition of METH.

The concentration and the duration of METH exposure of the neurons were determined as described below. In the preliminary experiments (data not shown), 30 mM KCl-evoked [$^{45}Ca^{2+}$] influx into the neurons attained its plateau 1 h after the initiation of 1 μM METH. When examining the influx with various concentrations of METH after three days of its exposure, its maximal influx was observed at 1 μM of METH. So, in the case of continuous exposure of METH, we exposed the neurons to METH (1 μM) for three days. On the other hand, in the case of the intermittent exposure, the neurons were exposed to METH as described above, because 40 ~ 60 min were required to show maximal locomotor activity in mice administered METH and maximal CPP were also found 40 ~ 60 min after METH administration.

Immunofluorescence

On the 13th day in vitro, α_2/δ-1 subunit and dopamine receptors in the neurons were identified by immunofluorescence using rabbit anti-α_2/δ-1 subunit (1:1000), mouse anti-dopamine receptor 1 (DA_1-R, 1:100 in phosphate buffer saline (pH 7.4: PBS), mouse antidopamine receptor 2 (DA_2-R, 1:100). These antibodies were diluted in phosphate buffer saline (pH 7.4: PBS) containing 10% normal donkey serum as described in parenthesis. After the neurons were fixed by the method previously reported (Ohkuma et al., 1986), they were incubated with the antibodies for α_2/δ-1 subunit and dopamine receptor subtypes overnight at 4°C followed by the incubation with Alexa 488-conjugated donkey antirabbit antibody (1:1000) for anti-α_2/δ-1 and Alexa 594-conjugated donkey antimouse antibody (1:1000) for anti- DA_1-R or anti-DA_2-R for 8 h at room temperature. Fluorescence immunolabeling was detected using a Leica TCS SP2 confocal laser scanning microscope.

Western blotting

After 24 h of the last conditioning phase, mice were decapitated for quick removing of the brains, and both the frontal cortex and limbic forebrain including nucleus accumbens were dissected on an ice-cold metal plate. The tissue was homogenized in 10 volumes of ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.5 mM EDTA, 10 mM NaF, 0.5% Triton X-100 with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA) using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 1000*g* for 10 min at 4°C and the resultant supernatant was further centrifuged at 100,000*g* for 60 min at 4°C. The pellets were then resuspended and centrifuged again at 100,000*g* for 60 min at 4°C. The resulting pellets were retained as membrane fractions for the subsequent analysis.

In the case of the cerebral cortical neurons, the neurons were scraped off and homogenized in ice-cold lysis buffer. The homogenate from the neurons was

manipulated as similar to that from the brain to prepare samples for Western blot analysis.

Proteins were separated by 5% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes in Tris-glycine buffer. For immunoblot detection, the membrane was incubated with primary antibody for α_2/δ-1 subunit diluted 1:1000 overnight at 4°C and horseradish peroxidase-conjugated goat antirabbit IgG diluted 1:5000 for 2 h at room temperature. Finally, samples were detected with chemiluminescence.

Real-time reverse transcription-PCR

Total RNA was prepared from the neurons using TRIzol reagents (Invitrogen Co., Tokyo, Japan) according to the standard protocol for RNA extraction. The amount of total RNA was quantified by measuring OD_{260} using a Nonodrop spectrophotometer (Wilimington, USA). Total RNA (200 ng) was reacted with Prime Script reverse transcriptase (Takara, Kyoto, Japan). Quantitative PCR was performed with One SYBR® PrimeScript ®RT-PCR kit II (Takara, Kyoto, Japan) using 7500 real-time PCR system (Applied Biosystems, Foster city, USA) according to a protocol supplied by the manufacturer, and the data were analyzed by 7500 system SDS Software 1.3.1 (Applied Biosystems) using the standard curve method. The sequences of the primers for α_2/δ-1 subunit were as followed; forward: 5′- CAAAGAGGCCGGAGAAAA-3′ and reverse: 5′-AAAGTAGGGCGCAGTGAAAA-3′.

Statistical analysis

All data are presented as the mean ± SEM. The statistical significance was assessed by the method described in each figure legend after the application of one-way ANOVA.

Drugs

Methamphetmaine hydrochloride (METH) was obtained from Dainippon Pharmaceutical Co., Tokyo, Japan. Gabapentin (GBP), SCH23390 and sulpiride were purchased from Sigma-Aldrich (St. Louis, USA). Antibody for α_2/δ-1 subunit was the product of Alomon Labs Ltd. (Jerusalem, Israel). Mouse anti-dopamine receptor 1 and mouse anti-dopamine receptor 2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Alexa 488-conjugated donkey antirabbit antibody for anti-α_2/δ-1 and Alexa 594-conjugated donkey antimouse antibodies for anti- DA_1-R or anti- DA_2-R were the products of Invitrogen, Co. (Tokyo, Japan). Other chemicals used were locally available and of analytical grade.

RESULTS

As shown in Figure 1, METH that induces rewarding effect in mice significantly increased the level of α_2/δ subunit in the frontal cortex and limbic forebrain, both of which are involved in the development of METH psychological dependence.

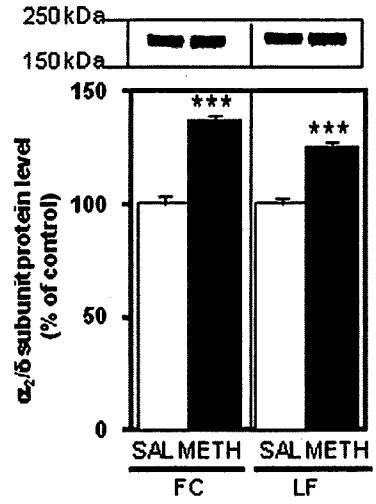

Fig. 1. Immunoreactivity of α_2/δ subunit in the frontal cortex and limbic forebrain of mice by repeated treatment with methamphetamine (METH). Mice were subcutaneously administered saline or METH (1 mg/kg; three times, every other day) for six days according to the conditioning schedule descried in the text. Twenty-four hours after the last conditioning, the membrane fractions were prepared to measure α_2/δ subunit. Representative Western blots of α_2/δ subunit protein in the frontal cortex (FC) and limbic forebrain (LF) following the METH-conditioning were presented in the upper portion of each column. Ordinate: percentage of the respective value of the control (saline-conditioned). Each column represents the mean ± SEM of four mice. ***$P < 0.001$ vs. vehicle plus saline-conditioned group (Bonferroni's test). SAL, saline; METH, methamphetamine.

To examine the role of α_2/δ subunit in the development of rewarding effect of METH, we investigated the effects GBP on rewarding effect of METH in mice using the conditioned place preference paradigm. METH (1 mg/kg, s.c., every two days, totally four times injections) exhibited a significant place preference for the drug-associated place and GBP dose-dependently suppressed this METH-induced place preference (Fig. 2A). Furthermore, the METH-induced place preference was dose-dependently suppressed by intraperitoneal administration of a dopamine D1 receptor antagonist SCH23390 and a dopamine D2 receptor antagonist sulpiride prior to METH administration (Fig. 2B). On the basis of the data in Figure 2B, the doses of SCH23390 and sulpiride were determined as 0.1 mg/kg and 40 mg/kg, respectively, in the following experiments shown in Figures 3A and 3B.

According to the results described above, we attempted to confirm a regulatory role of dopamine receptors on α_2/δ subunit expression using the primary cultures of cerebral cortical neurons exposed to METH (1 μM) for 24 h. Both SCH23390 and sulpiride did not show any affects on the expression of α_2/δ subunit protein in the absence of METH, while the sustained exposure to METH (1 μM) for 24 h significantly increased α_2/δ subunit protein (Fig. 3A). On

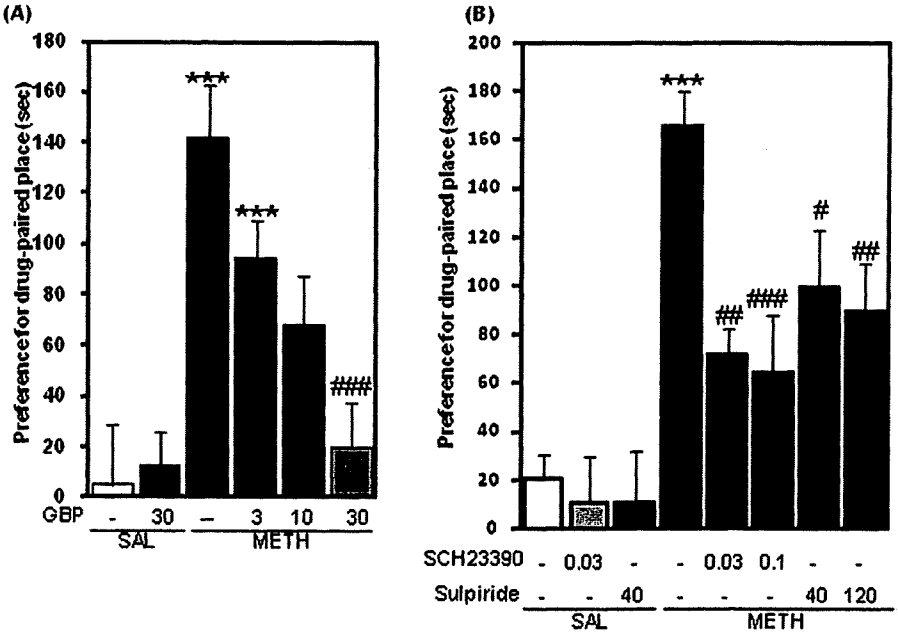

Fig. 2. Effect of gabapentin (GBP), SCH23390, and sulpiride on the METH-induced place preference in mice. Each column represents the mean conditioning scores ±SEM of eight mice. Mice were treated with, (A) saline (SAL) and gabapentin (GBP; 3, 10, and 30 nmol/mouse, i.c.v.), (B) SCH23390 (0.03 and 0.1 mg/kg, i.p.) and sulpiride (40 and 120 mg/kg, i.p.) before the administration of saline or METH (1 mg/kg, s.c.). GBP, SCH23390, and sulpiride were dissolved in saline. $^{***}P < 0.001$ vs. SAL-SAL group (Bonferroni's test), $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. SAL-METH group (Bonferroni's test).

the other hand, the increase in α_2/δ subunit protein in the METH-treated neurons was clearly abolished by simultaneous treatment with SCH23390 and sulpiride (Fig. 3A). Similarly, both SCH23390 and sulpiride suppressed METH-induced enhancement of α_2/δ subunit mRNA although they show no affects mRNA expression in the absence of METH (Fig. 3B).

In addition to the effects of continuous exposure, we also examined the effects of intermittent exposure to METH because the neurons in the brains of mice used for examining METH-induced CPP were considered to be intermittently exposed to METH. The intermittent exposure to METH also increased α_2/δ subunit protein expression and both dopamine antagonists also significantly suppressed this METH-induced increase of the expression (Fig. 3C), which was similar to those observed after the continuous exposure to METH as described above.

Figure 4 shows immunofluorescent double staining for both α_2/δ-1 subunit and dopamine receptor 1 (DA_1-R) or dopamine receptor 2 (DA_2-R) and reveals that α_2/δ-1 subunit is colocated either DA_1-R or DA_2-R in the cultured neurons used here (Figs. 4C and 4F).

DISCUSSION

The present study demonstrates that α_2/δ subunit of VGCCs is up-regulated in the brains of animals psychologically dependent on METH. Similarly, we have previously reported the increase of this subunit protein in both the frontal cortex and limbic forebrain of mice showing rewarding effect of morphine (Shibasaki et al., 2009a). In addition, GBP significantly suppressed the METH-induced rewarding effect as presented here. GBP binds with high affinity to α_2/δ subunit to suppress its function and has been used as an anticonvulsant (Gee et al., 1996; Marais et al., 2001). It is therefore reasonable to conclude that the up-regulation of α_2/δ subunit is an essential and common neurochemical event in the brain of animals psychologically dependent on drugs of abuse.

As shown in this study, the place preference produced by repeated METH injection was significantly suppressed by GBP, indicating that the up-regulation of α_2/δ subunit participates in the development of METH-induced rewarding effect. In addition, the blockade of dopamine D1 and D2 receptors by their respective antagonists, SCH23390 and sulpiride, attenuated the expression of rewarding effect induced by METH. Similar inhibitory roles of both dopamine D1 and D2 receptors in the development and expression of rewarding effect of METH were previously reported (Hamamura et al., 1991; Kuribara and Uchihashi, 1993). These data suggest that the expression of α_2/δ subunit of VGCCs involved in rewarding effect of METH may be regulated by dopamine receptors.

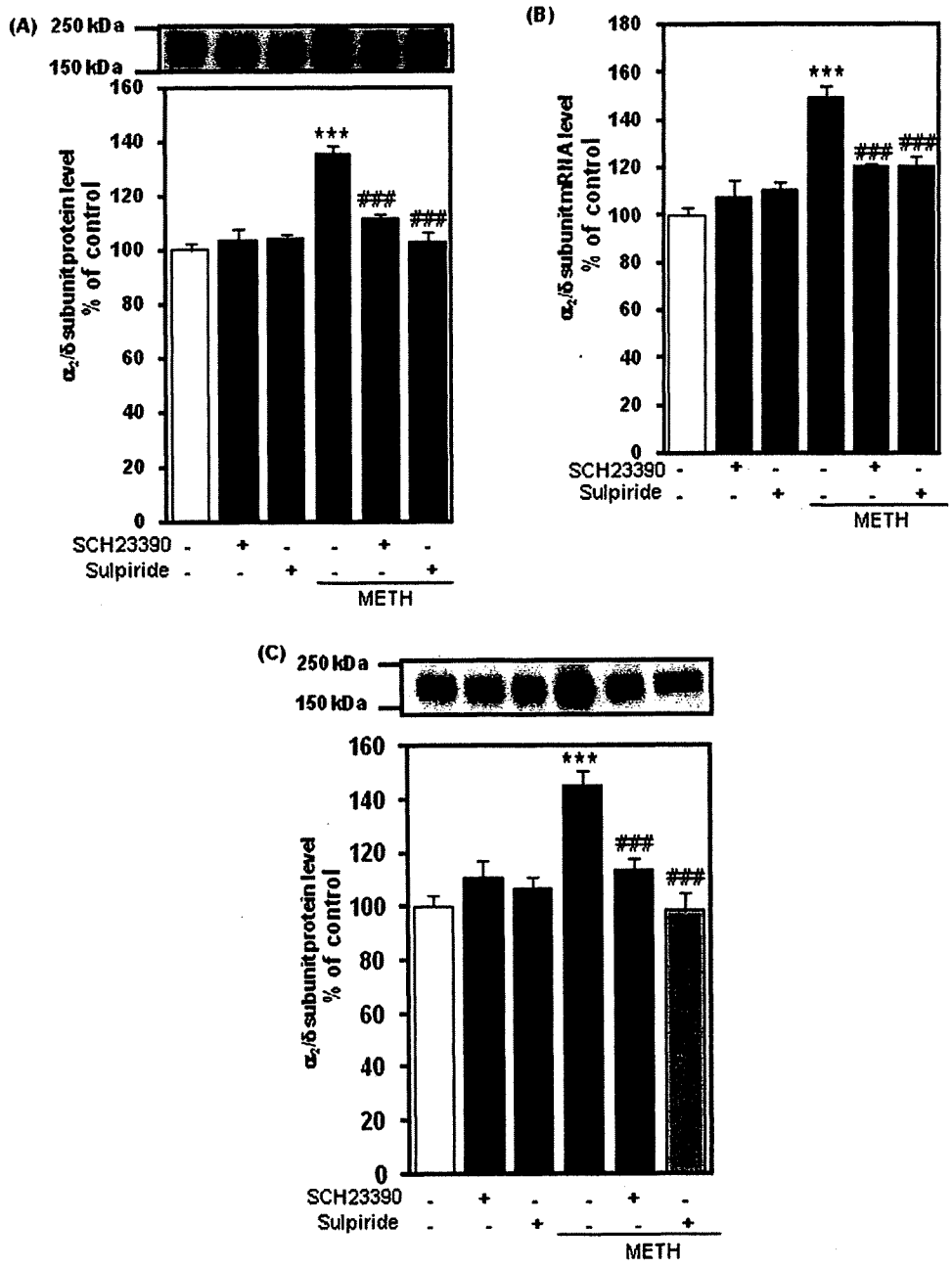

Fig. 3. Effects of SCH23390 and sulpiride on expression of α_2/δ subunit protein and its mRNA in the cortical neurons after (A and B) continuous and (C) intermittent exposure to METH. (A) Protein and (B) mRNA of α_2/δ subunit in the neurons treated with 1 μM SCH23390 and 1 μM sulpiride 10 min before the treatment with 1 μM METH for 24 h. (C) α_2/δ subunit in the neurons after intermittent exposure to METH. The neurons were exposed to METH for 1 h followed by exposure to the culture medium without METH for following 23 h every day. The neurons were treated with such exposure schedule to METH as described above for three days and thereafter subjected to the extraction of protein. DA receptor antagonists were exposed 10 min before METH exposure. The data were obtained from 4 separate experiments. $^{***}P < 0.001$ vs. control, $^{\#\#\#}P < 0.001$ vs. METH-treated neurons (Bonferroni's test).

We attempted to confirm the above mentioned hypothesis on the regulatory roles of dopamine receptors on α_2/δ subunit expression in METH psychological dependence using the primary cultures of cerebral cortical neurons by examining colocalization of α_2/δ subunit and dopamine receptor subtypes. The results showing the colocalization of the subunit and dopamine D1 or D2 receptors in the same neurons as dem-

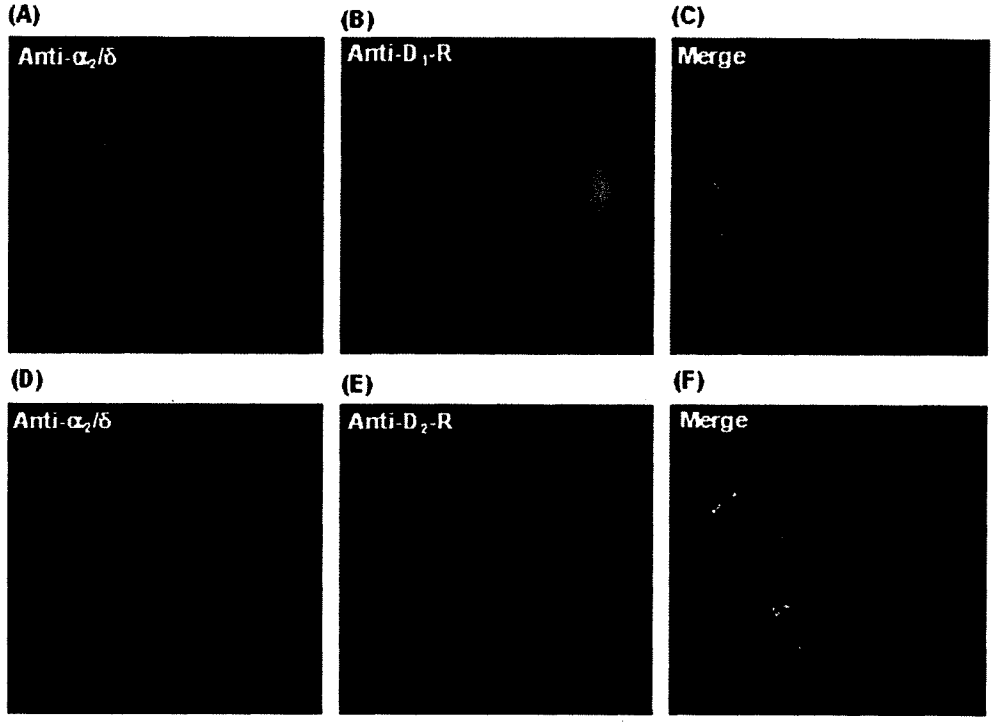

Fig. 4. Immunostaining of α_2/δ subunit and DA receptor subtypes in the cultured cortical neurons. A: α_2/δ subunit was shown as green. B: Anti-DA receptor 1 (DA_1-R) was shown as red. C: Merged (A) and (B). Colocalization of α_2/δ subunit and DA_1-R staining. D: α_2/δ subunit was shown as green. E: Anti-DA receptor 2 (DA_2-R) was shown as red. F: Merged (D) and (E). Colocalization of α_2/δ subunit and DA_2-R staining. Scale bars, 20 μm.

onstrated by the immunohistochemical analysis as well as the suppressive effects of dopamine receptor antagonists on METH-induced increase of α_2/δ subunit, suggested that the up-regulation of α_2/δ subunit is mediated through the activation of dopamine D1 and D2 receptors during METH treatment.

Although the mechanisms of regulatory effects of dopamine receptors on α_2/δ subunit expression are not clear at present, two possibilities are supposed. First, dopamine receptors are generally grouped into two subfamilies, D1-like and D2-like receptors (Missale et al., 1998). Opposing influences of dopamine D1 and D2 receptor activation on cAMP-dependent signaling have been reported in many studies (Stoof and Kebabian, 1981), with dopamine D1 receptors acting through stimulatory Gαs protein, and dopamine D2 receptors acting through inhibitory Gαi/o protein. Despite these opposing actions on cellular signaling via protein kinase A (PKA), both dopamine D1 and D2 receptors can mediate reinforcing signals of abuse (Hiroi and White, 1991). A recent investigation demonstrates interesting data that $G_\beta\gamma$ provides a possible mechanism to explain reforcing signals of abuse by these receptors. That is, Gαs- and Gαi/o-coupled receptors can act cooperatively to activate PKA (Sunahara et al., 1996; Watts and Neve, 1997) and in turn, PKA signaling in the nucleus accumbens involves in expression of animal behaviors (Nestler and Aghajanian, 1997; Self et al., 1998; Sutton et al., 2000). Therefore, it is likely that dopamine receptors may regulate α_2/δ subunit expression via alteration of PKA signaling to induce rewarding effect of METH as presented here.

The second possibility is described below. Vacuolar protein sorting 34 (Vps34), the sole Class III enzyme in phosphoinositide (PI) 3-kinase family phosphorylating 3′ hydroxyl position of the phosphatidylinositol ring and selectively catalyzing phosphatidylinositol to produce only PI(3)P (Volinia et al., 1995), regulates vesicular trafficking in the endosomal/lysosomal system and in the recruitment of proteins with PI(3)P-binding domains to intracellular membranes (Lindmo and Stenmark, 2006). Our recent study also reveals that Vps34 is translocated to Golgi and early endosomes by activation of PKCγ and produces the PI(3)P for regulation of membrane trafficking (Shibasaki et al., 2009b). In addition, repeated administration of METH produces several long-lasting changes in brain function such as phospholylated PKCγ up-regulation in the nucleus accumbens (Narita et al., 2004, 2005), and PKC has also modulatory roles in calcium channel trafficking via insertion of channels into plasma

membrane (Zhang et al., 2008). Such accumulating evidence not only indicates that the functional relationship between PKCγ and Vps34 is important for trafficking of intracellular proteins to plasma membrane but also suggests that the up-regulation of α_2/δ subunit induced by METH may be mediated through facilitation of α_2/δ subunit trafficking to plasma membrane by activation of PKCγ-Vps34 during METH treatment, although exact mechanisms of α_2/δ subunit up-regulation are remain to be elucidated.

In conclusion, this data demonstrated that METH increases α_2/δ subunit in the frontal cortex and limbic forebrain of mice showing rewarding effect of METH. Under the conditions METH-induced rewarding effect was inhibited by GBP as well as both of D1 and D2 dopamine receptor antagonists, SCH23390 and sulpiride, respectively. The sustained exposure of the cortical neurons to METH produces significant increase in α_2/δ subunit protein and its mRNA, which was completely suppressed by both dopamine D1 and D2 receptor antagonists. These results indicate that the METH-induced up-regulation of α_2/δ subunit, that can lead to the expression of psychological dependence, is regulated by both dopamine D1 and D2 receptors.

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VI. 薬物依存の基礎と臨床

2. ベンゾジアゼピン依存

a. ベンゾジアゼピン依存の基礎

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はじめに

ベンゾジアゼピン系薬物（ベンゾジアゼピン）は1961年にクロルジアゼポキシドが登場して以来、精神・神経疾患の治療に用いられており、現用の鎮静・催眠薬、抗不安薬の90%以上は本薬物である。きた。1970年初頭、ベンゾジアゼピンがGABA（γ-アミノ酪酸）受容体におけるGABAの効果を促進すること薬理学的に証明され、次いでベンゾジアゼピンは $GABA_A$ 受容体に直接結合し、GABAによる抑制性シナプス伝達を促進することが明らかにされた。

ベンゾジアゼピン結合部位はGABA結合部位と異なり、GABA結合をアロステリックに修飾する。ベンゾジアゼピン結合部位は $GABA_A$ 受容体を構成するサブユニットのうち、αサブユニットとβサブユニットが隣接する部分に形成され、γサブユニットはベンゾジアゼピンの作用発現には必要であることが明らかにされている。ベンゾジアゼピンは、$GABA_A$ 受容体に内蔵されるクロライド（Cl^-）チャネルの開口頻度を増加させ、しかもバルビツール酸誘導体と異なり、開口時間を延長せず、高用量負荷によってもアゴニスト活性を示さない。したがって、バルビツール酸誘導体に比して安全性が高いと考えられ、抗不安薬、睡眠薬、抗てんかん薬、筋弛緩薬として現在頻用されているが、長期にわたる服用により常用量でも精神および身体依存が形成されることがある。

ベンゾジアゼピンは抑制性依存性薬物に分類されるが、その精神依存性ならびに身体依存性はオピオイドやバルビツール酸誘導体に比べ弱いとされている。この依存性の弱さは、オピオイドなどはそれぞれに対応する受容体などを直接活性化するのに対し、ベンゾジアゼピンの作用がアロステリックであることに起因すると考えられるが、依存形成機序を解明するためのモデル動物作製ならびにこれらのモデル動物から得られたデータの評価は非常に難しいとされてい

る。このため、詳細なベンゾジアゼピン依存の形成機序の解明は十分な進展が未だみられないのが現状である。

本稿ではベンゾジアゼピン依存に関し、$GABA_A$受容体を中心としたベンゾジアゼピンの作用機序と薬理学的役割、およびベンゾジアゼピン依存形成機序や退薬症候発現機序について、基礎研究による知見および臨床的データなどを基盤にして概説したい。

GABA受容体の分類と分子構造

従来よりGABAは微生物や植物に見出されるアミノ酸の1つとして知られていたが、1950年に至り正常な哺乳動物の中枢神経系に存在する物質として同定された。中枢神経系と網膜を除く哺乳動物組織には、ごく微量のGABAが存在するのみである。GABAの作用はこれに対応する受容体であるGABA受容体への結合により発揮される。脊椎動物において、GABA受容体は中枢神経系のほとんどの神経細胞に広く存在し、細胞における局在部位は主に神経細胞膜である。

GABA受容体には$GABA_A$、$GABA_B$、$GABA_C$受容体の3種類のサブタイプが存在し、イオンチャネル内臓型の$GABA_A$および$GABA_C$受容体と、代謝型の$GABA_B$受容体という2つのグループに分けられる。$GABA_A$受容体はイオンチャネルを内蔵するタイプの受容体であり、その活性化はクロライドイオン（Cl^-）の神経細胞内への流入を増加させ、神経細胞膜に過分極を誘発させて神経興奮を抑制させる。$GABA_A$受容体はGABA結合部位の競合的アンタゴニストであるビククリンによって遮断される。$GABA_B$受容体は細胞膜を7回貫通する三量体G蛋白質共役型受容体であり、cAMP生成を抑制して神経細胞機能を抑制する。$GABA_B$受容体はビククリン非感受性で、競合的アゴニストであるバクロフェンによって活性化され、また競合的アンタゴニストであるファクロフェンにより抑制される。いずれの受容体

もシナプス前部と後部に存在し、それぞれ独立してシナプス情報伝達に関わる。

ベンゾジアゼピンが結合するのは$GABA_A$受容体であり、この受容体は5個のサブユニットにより構成される五量体であり、$GABA_B$受容体に比べ圧倒的に多く存在している。現在知られている範囲ではアミノ酸配列の相違から19種類のサブユニットが存在し、7つの機能的に異なるファミリーに分類される。それらはα、β、γ、δ、ε、π、θ、ρと名付けられ、αサブファミリーに6個（$\alpha_{1\sim6}$）、βサブファミリーに4個（$\beta_{1\sim4}$）、ρサブファミリーに3個（$\rho_{1\sim3}$）、γサブファミリーに2個（$\gamma_{1\sim2}$）、δ、ε、π、θサブファミリーは各々1個ずつのサブユニットが含まれている。これらの組み合わせの相違により多様なサブタイプが形成される。また、$GABA_C$受容体は、ビククリン、バクロフェンの両者に非感受性であり、$GABA_A$受容体同様イオンチャネルを内蔵するタイプの受容体で、その活性化によりCl^-の神経細胞内への流入を増加させ、神経細胞膜に過分極を誘発させる。$GABA_C$受容体は、ρサブユニットの単一構成による受容体であり、網膜にのみ存在するが、その機能は殆ど知られておらず、$GABA_A$受容体の亜型とする考えもある。

ベンゾジアゼピンの作用発現にはGABA受容体のうち$GABA_A$受容体のみが関与しているので、以下に$GABA_A$受容体について詳述する。

$GABA_A$受容体の機能

$GABA_A$受容体サブユニットはおよそ50kDaの大きさで、すべて長いN末端細胞外ドメイン、4つのαヘリックスの膜貫通領域（M1-M4）、M3とM4の間の長い細胞内配列、そして短い細胞外C末端ループからなる。特に、M3-M4領域にはプロテインキナーゼA（PKA）やプロテインキナーゼC（PKC）、プロテインチロシンキナーゼ（PTK）によるリン酸化部位が存在し、リン酸化によりチャネル活性が低下する。また、M2領域両端の塩基性アミノ酸がCl^-チャネル内壁を構成してお

り、また後述するようにGABA結合部位のみならずベンゾジアゼピン結合部位を有しているので、$GABA_A$受容体を$GABA_A$受容体--ベンゾジアゼピン受容体-Cl^-チャネル（Cl^-イオノフォア）複合体と称することも多い。

$GABA_A$受容体がGABAの結合により活性化されると、5個のサブユニットの中心部に形成されるチャネルを通してCl^-の細胞内への流入が増加し、その結果細胞膜には過分極が誘発され、神経細胞興奮が抑制される[1]（図1）。五量体としての$GABA_A$受容体複合体の大きさは、各サブユニットのサイズの5倍のおよその275kDaと見積もられているが、生体内で発現する受容体がどのサブユニットで構成されているのかは分かってはいない。$GABA_A$受容体における GABA 結合部位はαおよびβサブユニットにより形成され、その結合は高親和性である。一方、ベンゾジアゼピンはαおよびγサブユニットとの境界部位に結合する[2]。また、バルビツール酸誘導体や抗痙攣薬であるビククリン、痙攣誘導薬のピクロトキシンは、βサブユニットに結合する。

$GABA_A$受容体サブユニットの病態生理的役割

表2に示すように、$GABA_A$受容体は、mRNA[3,4]や蛋白質[5]の測定により、$GABA_A$受容体は脳内に広く分布しており、しかも各脳部位により、サブユニットの発現が多様であることが明らかになっている。$GABA_A$受容体は非常に多様で部位特異的なサブユニットの発現を示すことから、これは機能する神経細胞の特性に関わるが、このようなサブユニット発現の相違が脳内各部位の神経細胞の機能に大きく関わると考えられるとともに、ベンゾジアゼピンによる薬理作用の特徴に深く関与すると考えられる。

$GABA_A$受容体存在部位のシナプスとの位置関係により$GABA_A$受容体はシナプス

受容体（synaptic receptors）とシナプス外受容体（extrasynaptic receptors）とに分類される（図2）。シナプス受容体はGABA作動性神経細胞の神経終末のGABA放出部位の対極であるシナプス後膜に局在しており、後述するようにα_2やα_3サブユニットを有する$GABA_A$受容体がこれに相当する。これに対して、α_4あるいはα_5サブユニットを有する$GABA_A$受容体はシナプス外受容体であり、シナプス直下以外の樹状突起棘基底部やその周辺部位に存在する。$GABA_A$受容体のGABAに対するEC_{50}値は50μM以下であり、通常シナプス間隙におけるGABA濃度は最も高い時には0.3～3.0mMになる（図2）。したがって、シナプス外$GABA_A$受容体においてもその周辺部には低濃度のGABAが存在し、ベンゾジアゼピンの$GABA_A$受容体増強作用は十分に発揮される。γ_2サブユニットはgephyrinを介して$GABA_A$受容体がシナプスに局在するように調節しており、γ_2サブユニットを欠く受容体はシナプス外受容体として存在することが多い。

βあるいはγ_2サブユニットと受容体を構成するα_1、α_2、α_3およびα_5サブユニットを含む$GABA_A$受容体は脳内では最も普遍的に存在し、ベンゾジアゼピンに対して感受性を示す[1]（表1）。これらの中で$\alpha_1\beta_2\gamma_2$受容体は最も多く、すべての$GABA_A$受容体の40~60%を占めるが、嗅球顆粒細胞層、視床網状核、脊髄運動ニューロンには存在しない[5]。

α_1サブユニットの細胞外ドメインの101番目のヒスチジン残基はベンゾジアゼピン作用上必須である[6]。α_2、α_3およびα_5サブユニットにもヒスチジン残基が存在し、かつベンゾジアゼピン感受性であるが、ヒスチジン残基存在部位に相当する部位にアルギニン残基を有するα_4およびα_6サブユニットはベンゾジアゼピンに対し感受性がみられない。α_1サブユニットはベンゾジアゼピンの薬理作用である鎮静、健忘、抗痙攣作用に重要な役割を果たす[7]が、抗不安作用には関

与しない。α_2サブユニットの点変異を加えた$GABA_A$受容体では、ジアゼパムにより誘発されるβ速波頻度の増加やREM睡眠時のq波の増加が減弱することから、古典的ベンゾジアゼピンによりもたらされるREM睡眠の減少、徐波睡眠の減少、β速波頻度の増加などにはα_2GABA_A受容体が関与していると考えられる[8]。

ジアゼパムのα_2サブユニットへの結合量は極めて低いにも関わらず、抗不安作用を示す。一方、α_3GABA_A受容体欠損マウスではベンゾジアゼピンの抗不安作用が減弱するが、この場合でもベンゾジアゼピンのα_3サブユニットへの結合量が高いことから、抗不安作用の発現の主体はα_2サブユニットと考えられている。α_5サブユニットはベンゾジアゼピンの抗不安効果に影響しないが、動物実験における記憶や学習に関係する可能性が示唆されている。このサブユニットを含む受容体は視床錐体細胞樹状突起軸部近傍やその樹状突起棘基底部に分布しており、シナプス外受容体として存在する。

ベンゾジアゼピンに感受性を示さないα_4およびα_6サブユニットの脳内分密度は低く、前者は視床や歯状回に分布し、後者は主に小脳顆粒細胞層に限定して分布するが、この受容体は小脳内に存在する全$GABA_A$受容体の30～50%を占める。

なお、これらのサブユニットの機能的役割については最近の文献[4]を参照されたい。

ベンゾジアゼピン依存

1．精神依存性

側坐核は報酬をはじめとする情動反応に重要な役割を果たす脳内部位で、メ

タンフェタミン、コカイン、モルヒネ、アルコールによる精神依存では、腹側被蓋野（ventral tegmental area）を起始核とし側坐核へ投射する中脳辺縁ドパミン神経系が重要な役割を果たす[9]。側坐核では$GABA_A$受容体の$\alpha_2>\alpha_5>\alpha_1=\alpha_3$、$\beta_{2/3}$、$\gamma_2$サブユニットの発現が多く認められ、腹側被蓋野では$\alpha_1=\alpha_3$、$\beta_{2/3}$、$\gamma_2$サブユニットの発現が多く認められるが、どのサブユニットが精神依存の形成に関与するのかについては未だ不明である（表２、図３）。

メタンフェタミン、コカイン、モルヒネ、アルコールなどの依存性薬物は、側坐核でのドパミン遊離量の亢進が誘発され、依存性薬物による精神依存形成において共通した反応であると考えられる。一方、ジアゼパムの末梢投与は側坐核におけるドパミン遊離量の低下を生じさせ、この反応は線条体での反応より強い[10]。またミダゾラム急性処置によっても側坐核でのドパミン遊離量の低下が認められるが、ミダゾラムの14日間の慢性処置によっても、対照群と同程度の側坐核におけるドパミン流離量の低下が認められることが報告されている[11]。さらに、フルラゼパムの側坐核への微量注入も、側坐核でのドパミン遊離量の低下をおこし、これはそれぞれベンゾジアゼピンおよび$GABA_A$受容体遮断作用を有するフルマゼニルならびにピクロトキシンにより回復することが報告されている[12]。ベンゾジアゼピン逆作動薬（inverse agonist：作動薬が示すのと反対の生物学的反応を示す薬物）であるFG-7142を腹腔内投与すると、側坐核のshell領域においてドパミン遊離量の増加が引き起こされ、この増加はベンゾジアゼピン拮抗薬Ro 15-1788により消失する[13]。さらに、ジアゼパム、ゾルピデム、オキサゼパムの前処置は、アンフェタミン、メタンフェタミン、コカイン、モルヒネの報酬効果を抑制し[14-16]、クロルジアゼポキシドあるいはクロナザパム