Modification of Physical Dependence on Central Depressants by Neuromodulation

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This dissertation is dedicated to my father, mother, grand mother, sister and brother.

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MAJOR DRUGS USED IN THIS STUDY

barbital	*barbiturates*
imipramine	*tricyclic antidepressants* *norepinephrine and serotonin re-uptake inhibitor*
bifemelane	*atypical antidepressants* *norepinephrine and serotonin re-uptake inhibitor*
teniloxazine	*atypical antidepressants* *norepinephrine re-uptake inhibitor*
diazepam	*benzodiazepine full agonist*
FG 7142	*benzodiazepine partial inverse agonist*
buspirone	*serotonin (5-HT_{1A}) agonist*
mianserin	*serotonin (5-HT_{1C}) antagonist*
ketanserin	*serotonin (5-HT_{2}) antagonist*
ondansetron	*serotonin (5-HT_{3}) antagonist*
nordiazepam	*active metabolite of diazepam*
oxazepam	*active metabolite of diazepam*
nifedipine	*L-type Ca^{2+} channel sensitive blocker*
diltiazem	*L-type Ca^{2+} channel sensitive blocker*
flunarizine	*T-type Ca^{2+} channel sensitive blocker*

ABBREVIATIONS OF THE MONOAMINES AND ITS METABOLITES

norepinephrine	*NE*
dopamine	*DA*
serotonin	*5-HT*
5-hydroxyindoleacetaldehyde	*5-HIAAld*
5-hydroxytryptophol	*5-HTOL*
5-hydroxyindoleacetic acid	*5-HIAA*
γ-aminobutylic acid	*GABA*

ABBREVIATIONS OF THE DRUGS

FG 7142	*N-methyl-β-carboline-carboxamide*
Ro 15-4513	*Ethyl-8-azido-5,6-dihydro-5-methyl-6-Oxo-4H-imidazo [1,5α]-[1,4] benzodiazepine-3-carboxylate*
β-CCE	*Ethyl β-carboline-3-carboxylate*
β-CCM	*Methyl β-carboline-3-carboxylate*
DMCM	*Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate*
ZK 93426	*Ethyl 5-isopropoxy-4-methyl-β-carboline-3-carboxylate*

GENERAL INTRODUCTION

Sedative hypnotics, barbiturates and benzodiazepines, are widespread in clinical therapy. Barbiturates have been derived from barbituric acid, a chemical discovered in 1863 by von Baeyer (Henningfield and Ator, 1986a). Forty years later, two German scientists, Fischer and von Mering, used von Baeyer's acid to synthesize a new drug which they called barbital. Before synthesis of barbital, many researchers had been looking for a drug that would combat the effects of anxiety and nervousness (Rall, 1990). Although some drugs succeeded to combat the effects of anxiety and nervousness, these drugs induced the side effects such as narcosis (like morphine and codeine), chronic toxicity (like bromide salts) and user bad breath (like chloral hydrate) (Henningfield and Ator, 1986a). Since barbiturates are tasteless, odorless and do not give the user bad breath, and show the anxiolytic and hypnotic effects at low and high doses, respectively, barbiturates had been widely used as a useful, safe and low-side effect sedative hypnotics from the beginning to middle of 20th century, and several popular old drugs (bromide salts, chloride hydrate, paraldehyde, urethane and sulfonal) have slipped into oblivion (Henningfield and Ator, 1986a; Rall, 1990; Yutrzenka and Patrick, 1992). Moreover, the partial separation of sedative-hypnotic-anesthetic from anticonvulsant properties, was embodied in phenobarbital (Rall, 1990). Unfortunately, the profound complications, such as deaths by overdosages, abuse and physical dependence, associated with barbiturates use became apparent at middle of 20th century (Henningfield and Ator, 1986a; Miller, 1991; Yutrzenka and Patrick, 1992). For instance, during the mid-1950's, 70 % of all admissions at the poison treatment center in Copenhagen, Denmark, were due to barbiturates overdosages (Henningfield and Ator, 1986a). Moreover, from the 1950's, the illicit use (nonmedical use) of barbiturates was epidemic and was becoming an accepted way to get "high" (Henningfield and Ator, 1986c). Additionally, anxiolytics,

anticonvulsants and hypnotics such as barbiturates, meprobamate and trimethadione, which have been used at that time, have been reported to possess the tolerance and physical dependence liability (Rall, 1990). On 1960, chlordiazepoxide was developed by Roche as the first compound of benzodiazepines and then many of benzodiazepines have been synthesized (Miller and Greenblatt, 1992; Rall, 1990). Because toxicity and dependence liability of benzodiazepines are weaker than those of barbiturates, benzodiazepines are now widely used as anxiolytics, anticonvulsants, hypnotics and muscle-relaxants, selecting for individual pharmacological profiles, and have largely replaced barbiturates and other sedative hypnotics (Miller and Greenblatt, 1992; Rall, 1990). Today, barbiturates are scarcely used, except for phenobarbital as an antiepileptic in clinical therapy and pentobarbital as an anesthetic in animal studies (Rall, 1990).

Sedative hypnotics, barbiturates and benzodiazepines, commonly show the anxiolytic, anticonvulsant, muscle-relaxant and hypnotic effects, memory impairment and hypothermia in human and experimental animals (Rall, 1990). Action sites of these drugs have been accepted to be the respective binding sites on $GABA_A$ · benzodiazepine receptor / Cl^- channel complex. $GABA_A$ · benzodiazepine receptor / Cl^- channel complex consists of five subunits of $GABA_A$ receptor, containing Cl^- channel as a core (Doble and Martin, 1992). The subunits of $GABA_A$ receptor are grouped into five sequence classes (α, β, γ, δ and ρ) with most classes containing several variants: α_{1-6}, β_{1-4}, γ_{1-3} (Doble and Martin, 1992; Wafford et al., 1992). It is known that two α subunits and two β subunits must be present in the native $GABA_A$ · benzodiazepine receptor / Cl^- channel complex (Doble and Martin, 1992). The recognition sites for $GABA_A$ agonists is present on the β subunit of the $GABA_A$ receptor, and one α subunit and one β subunit of $GABA_A$ receptor may be required to the positive responses of $GABA_A$ agonists (Casalotti et al., 1986). On the other hand,

the recognition sites for benzodiazepine agonists are present on the α subunit of the $GABA_A$ receptor, and one α subunit, one β subunit and one γ subunit may be required to the positive responses of benzodiazepine agonists (Ymer et al., 1990). Therefore, there are many types of $GABA_A$ · benzodiazepine receptor / Cl^- channel complex consisted with various combination of the subunits of $GABA_A$ receptor. $GABA_A$ agonists, barbiturates and benzodiazepine agonists may be considered to bind respective $GABA_A$ · benzodiazepine receptor / Cl^- channel complex according to the own selectivity to the subunit of $GABA_A$ receptor. For example, in the studies (Luddens and Wisden, 1991) using the recombinant $GABA_A$ · benzodiazepine receptor / Cl^- channel complex consisting $\alpha_x\beta_2\gamma_2$ subunits of $GABA_A$ receptor, benzodiazepine agonists, benzodiazepine antagonists and benzodiazepine inverse agonists show the individual selectivities to α subunits of $GABA_A$ receptor as follows: diazepam and flunitrazepam ($\alpha_1 = \alpha_2 = \alpha_3 = \alpha_5$), zolpidem and alpidem ($\alpha_1 > \alpha_2 = \alpha_3 >> \alpha_5$), flumazenil ($\alpha_1 = \alpha_2 = \alpha_3 = \alpha_5 > \alpha_6$), Ro 15-4513 ($\alpha_1 = \alpha_2 = \alpha_3 = \alpha_5 = \alpha_6$), β-carboline analogues FG 7142, β-CCE and DMCM ($\alpha_1 > \alpha_2 = \alpha_3 > \alpha_5 > \alpha_6$). However, the efficacy of these drugs may be related not only to the selectivity to the α subunits of $GABA_A$ receptor but also to the combination of $\alpha_x\beta_y\gamma_z$ subunits of $GABA_A$ receptor (Ducic et al., 1993). Recently, the relation of pharmacological profile of these drugs in behavioral study to the combination of $\alpha_x\beta_y\gamma_z$ subunits of $GABA_A$ receptor has been beginning to be determined. In these researches, it was suggested that anxolytic and anticonvulsant effects of these drugs may be mediated by $\alpha_1\beta_y\gamma_2$ subunits of $GABA_A$ receptor, sedative and muscle-ralaxant effects of these drugs may be mediated by $\alpha_{2,3,5}\beta_y\gamma_2$ subunits of $GABA_A$ receptor, and hypnotic effect of these drugs may be mediated by $\alpha_6\beta_y\gamma_2$ subunits of $GABA_A$ receptor (Doble and Martin, 1992; Guidotti et al., 1990; Luddens et al., 1990). Since the selectivity of these drugs to respective α subunits of $GABA_A$ receptor disappeared at high doses of these drugs, and the densities of respective $GABA_A$ · benzodiazepine receptor / Cl^- channel complex in

various brain regions are not necessarily to be related to the behavioral pharmacological profile of respective brain regions (Doble and Martin, 1992; Potier et al., 1988), it is still difficult to explain the mechanisms for producing the behavioral characterization of these drugs, today.

Barbiturates and benzodiazepines bind to respective binding sites on $GABA_A$ · benzodiazepine receptor / Cl^- channel complex in the nerve terminals or cell bodies of other neurotransmitter-related neurons, and increase Cl^- influx in the nerve terminals and cell bodies (Akaike, 1989; Harrison et al., 1988). In the nerve terminals of other neurotransmitter-related neurons, the equilibrium potential of Cl^- on cell membrane is high (-30 - -40 mV), since intracellular Cl^- concentration is high (Akaike, 1989). Therefore, the Cl^- which gets into a cell through Cl^- channels, induces the depolarization of cell membrane and blocks the Na^+ channels; as a result, Ca^{2+} influx through the voltage-dependent Ca^{2+} channels on the nerve terminals is indirectly suppressed (Akaike, 1989; Maruyama et al., 1988). In the nerve terminals, the Ca^{2+} which gets into a cell through voltage-dependent Ca^{2+} channels, mediates release of neurotransmitters from nerve terminals (Rogawski and Porter, 1990). By the above mechanisms, which called presynaptic inhibition, barbiturates and benzodiazepines suppress the release of neurotransmitters (Akaike, 1989; Maruyama et al., 1988). On the other hand, in the cell bodies of other neurotransmitter-related neurons, increase in Cl^- influx induces the inhibitory postsynaptic potential and produces the hyperpolarization of cell membrane; as a result, Ca^{2+} influx through the voltage-dependent Ca^{2+} channels on the cell bodies is directly suppressed (Harrison et al., 1988). Therefore barbiturates and benzodiazepines suppress the postsynaptic neuron activities by inhibiting the depolarization of cell membranes of postsynaptic neurons, that is called postsynaptic inhibition (Harrison et al., 1988). As shown by the above findings, presynaptic and

postsynaptic inhibitions by barbiturates and benzodiazepines are due to inhibition of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels. On the other hand, it is considered that the Ca^{2+} which gets into a cell through voltage-dependent Ca^{2+} channels, binds to calmodulin and activates phosphatase. Activated phosphatase induces the dephosphorylation of ATP-sensitive phosphorylation sites in β subunit of $GABA_A$ receptor; as a result, the increased intracellular Ca^{2+} may suppress GABA response which is induced by barbiturates and benzodiazepines (Akaike et al., 1989; Yakushiji et al., 1987). Therefore, intracellular Ca^{2+} may play a very important role on the appearance of central depressing effects of barbiturates and benzodiazepines. Barbiturates and benzodiazepines suppress the release of neurotransmitters from nerve terminals, and suppress the cell body firing rates of several neurons (Rogawski and Porter, 1990). It is believed that central depressing effects of barbiturates and benzodiazepines are due to this suppression of several neuron activities in the brain. Anxiolytic effects of these drugs may be derived from due to the suppression of the activities of ascending serotonergic neurons from raphe neucleus to amygdala (Blackburn, 1992; Costall and Naylor, 1991; Costall et al., 1989b). Moreover, memory impairment of these drugs may be derived from suppression of cholinergic neuron activities in hippocampus (Nabeshima et al., 1990; Tohyama et al., 1991). However, anticonvulsant, muscle-relaxant and sedative effects of these drugs can not yet be completely characterized by specific neurotransmitters and brain regions. Sedative hypnotics, especially benzodiazepines, are widely used in clinical therapy. Therefore, there is a possibility that these sedative hypnotics may be used concurrently with other drugs which affect central nervous system. In fact, barbiturates and benzodiazepines are used concurrently with other anticonvulsants (e.g. phenytoin and carbamazepine) in epilepsy therapy (Delgado-Escueta and Enrile-Bacsal, 1983). Moreover, benzodiazepines are used concurrently with antidepressants (tricyclic and tetracyclic antidepressants) (Nutt and Glue, 1991) or antipsychotic

drugs (e.g. haloperidol) (Tamminga and Gerlach, 1987) in anxiety and depression or schizophrenia therapy, respectively. These drugs combined with barbiturates or benzodiazepines have their own effects mediated by neurotransmitters directly or indirectly (through ion channels). Therefore, it is possible that the combined drugs affect the efficacy and dependence liability of these sedative hypnotics. However, there are few reports that these drug interactions and their mechanisms have been determined.

Sedative hypnotics, barbiturates and benzodiazepines are known to possess the same physical dependence liability. Physical dependence of these drugs are categorized in the same class of substance dependence, barbiturates type, by the World Health Organization (Kramer and Cameron, 1975). As a generalization, the withdrawal signs seen after cessation of a drug tends to be opposite to the signs produced by acute administration of that drug (Nutt et al., 1991). Therefore, withdrawal signs of sedative hypnotics are characterized by apprehension, high-excitability, loss of appetite, piloerection (hair standing on end), muscle rigidity, impaired motor activity, retching, vomiting, weight loss, tremor, convulsion, delirium, hallucinations and hyperthermia (Henningfield and Ator, 1986b). The development of physical dependence on these drugs is related to the frequency, duration and dosage of the treatment (Suzuki, 1990; Woods et al., 1992). The withdrawal signs of sedative hypnotics with more frequency or greater intensity appear following administration of higher doses or doses with greater effects, longer duration of treatment, or continuous rather than intermittent drug administration (Woods et al., 1992). Barbiturates and benzodiazepines are classified to 3 groups for their half life as follows; short-acting (barbiturates: 4 hr or less, benzodiazepines: 6 hr or less), intermediate-acting (barbiturates: 4-6 hr, benzodiazepines: 6-20 hr) and long-acting (barbiturates: 6 or more hr, benzodiazepines: 20 or more hr) (Henningfield and

Ator, 1986a; Rall, 1990). Long-acting barbiturates and benzodiazepines are easy to develop the physical dependence rather than short-acting barbiturates and benzodiazepines, because the brain concentration of long-acting drugs is maintained for a longer time by single injection, but short-acting drugs are required several injections to maintain the brain concentration for the same time (Henningfield and Ator, 1986a; Hollister, 1985). On the other hand, the intensities of physical dependence on short-acting barbiturates and benzodiazepines are stronger than those on long-acting barbiturates and benzodiazepines, because severities of withdrawal signs of barbiturates and benzodiazepines correlate to the disappearance rate of these drugs from the brain after withdrawal (Henningfield and Ator, 1986a; Hollister, 1985).

Physical dependence on these drugs have been evaluated so far in various animal species and by various experimental methods (Suzuki, 1990; Woods et al., 1992). These studies have been started with dogs in 1931. Seevers and Tatum (1931) reported that several withdrawal signs (including convulsions) were observed in dogs after the chronic intragastric injection of barbital. Moreover, Fraser and Isbell (1954) achieved to develop the physical dependence on several barbiturates (secobarbital, pentobarbital and barbital) in dogs. In those days, the evaluation of physical dependence on barbiturates were also examined using the monkeys, baboons and cats, but not rodents; therefore, a lot of money was required for the evaluation. In 1960's, the evaluations using the rodents, rats and mice, were feasible. Crossland and Leonard (1963) and Essig (1966) succeeded to develop barbital physical dependence and to induce the withdrawal convulsion in rats using a drinking method. Kaneto et al. (1973) reported that barbital withdrawal signs were observed in mice after oral treatment with barbital for 10 days. It was also reported by Yoshimura and Yamamoto (1979) that phenobarbital withdrawal signs appeared after oral

treatment with phenobarbital for 10 weeks. However, these procedures require prolonged treatment with barbiturates to develop the physical dependence (Fraser and Isbell, 1954; Seevers and Tatum, 1931; Yoshimura and Yamamoto, 1979), and the intensity of withdrawal signs observed in these procedures is so weak (Kaneto et al., 1973; Yoshimura and Yamamoto, 1979). Importantly, these procedures can not develop physical dependence on benzodiazepines. In 1970's, Tagashira et al. (1978) reported that natural withdrawal signs (including tremor and convulsions) of barbiturates were observed in rats chronically treated with barbiturates using a drug-admixed food method. Using this method, Suzuki et al. (1992a) established animal models of physical dependence on benzodiazepines in rats. This method has successfully induced severe natural withdrawal signs, including tremors and convulsions in rats, but this procedure requires a prolonged and continuous exposure to barbiturates and benzodiazepines (at least 1 month) (Suzuki et al., 1992a; 1992b; Woods et al., 1992). In the cases of opioids such as morphine, evaluations of physical dependence on opioids in rodents are generally determined using the precipitation of opioid withdrawal by opioid antagonists such as naloxone (Suzuki, 1990). However, the studies with antagonists of barbiturates and benzodiazepines have hardly progressed as compared with opioids, partly because competitive antagonists of barbiturates and benzodiazepines have not been available. Recently, competitive antagonists of benzodiazepines, flumazenil (benzodiazepine analogue) and ZK 93426 (β-carboline analogue), were synthesized and began to use to evaluate physical dependence on benzodiazepines (Little et al., 1987). Lukas and Griffiths (1982) reported that treatment with flumazenil was able to precipitate the withdrawal signs of diazepam in baboons after intragastric injection of diazepam for 7 days. Moreover, Loscher et al. (1989) reported that treatment with ZK 93426 was also able to precipitate the withdrawal signs of diazepam in dogs after treatment with diazepam for 7 days. Although the two antagonists precipitate withdrawal signs, the

signs of withdrawal observed are somewhat different (Loscher et al., 1989). Withdrawal by flumazenil produced rigidity in posture and walking with increased muscle tone, tremor, twitches and jerks. In contrast, ZK 93426 induced myoclonic jerks and clonic-tonic seizures but did not alter motility. The investigators noted that the withdrawal signs observed after ZK 93426 were similar to those observed after natural withdrawal, whereas the rigid postures and immobility observed after flumazenil were unique signs which may be due to its partial agonistic efficacy (Woods et al., 1992). Unfortunately, the precipitation of benzodiazepine withdrawal signs using ZK 93426 has been reported only in dogs, but not in rodents (Loscher et al., 1989). Moreover, to get appearance of severe precipitated withdrawal signs (including convulsions) of benzodiazepines by flumazenil, prolonged exposure to benzodiazepines (for about 1 month) is required in rodents (Little et al., 1992; Woods et al., 1992). Recently, it has been shown that chronic treatment with benzodiazepines in mice causes supersensitivity to proconvulsant effect of benzodiazepine partial inverse agonist FG 7142 following termination of the treatment with benzodiazepines (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; Nutt and Costello, 1988). The supersensitivity to FG 7142 lasts for 24 hr to 1 week after the last treatment with benzodiazepines (Little et al., 1988); this period coincides with the period that natural withdrawal signs of benzodiazepines appear (Suzuki et al., 1992a). Since this procedure requires only 1 week, the production of convulsions by FG 7142 at proconvulsant doses after chronic treatment with benzodiazepines in rodents has been accepted as a reliable and useful, although preliminary, index for evaluating benzodiazepine-like physical dependence liability (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; Moreau et al., 1990; Nutt and Costello, 1988; Piot et al., 1990).

The object of the proposed research is the investigation on the involvement of some neuromodulations, especially changes in the activities of noradrenergic and serotonergic neurons and adaptations of some 5-HT receptors and Ca^{2+} channels, in the development of physical dependence on barbiturates and benzodiazepines, which commonly affect $GABA_A$ · benzodiazepine receptor / Cl^- channel complex, and the dependence of which are categorized in the same class of substance dependence "barbiturates type". The detailed aims of the proposed research are:

(1) To determine how monoamine-related neuromodulations are involved in the physical dependence on barbiturates, the effects of different types of antidepressants (monoamine re-uptake inhibitors) on the development of physical dependence on barbital were investigated.

(2) To determine the involvement of adaptation of 5-HT receptors in the physical dependence on benzodiazepines, the effects of the several serotonergic anxiolytics which act through different subtypes of 5-HT receptors on the development of physical dependence on diazepam were investigated.

(3) To determine the involvement of brain Ca^{2+} channels in the physical dependence on barbiturates and benzodiazepines, the effects of several Ca^{2+} channel blockers which block different types of Ca^{2+} channels both on the development of physical dependence on barbital and diazepam, and on the appearance of barbital withdrawal signs were investigated.

CHAPTER 1

EFFECTS OF ANTIDEPRESSANTS ON THE CENTRAL DEPRESSION BY AND THE DEVELOPMENT OF PHYSICAL DEPENDENCE ON BARBITAL IN RATS

Introduction

Barbiturates are widely known to possess physical dependence liability. Physical dependence on barbiturates has been evaluated in various animal species and with various experimental methods (Suzuki, 1990). Biochemical changes associated with barbiturate dependence and after withdrawal from barbiturates have been determined, and the effects of psychotropic drugs on physical dependence on barbiturates have been examined. Several reports have indicated that changes in brain monoamine concentrations and monoamine-related neuron activities may be involved in physical dependence on barbiturates.

Morgan et al. (1977, 1978) suggested that brain DA concentrations increase after barbital withdrawal, and that α-methyl-*p*-tyrosine suppresses barbital withdrawal convulsions. Tagashira et al. (1983a) reported that barbital withdrawal convulsions are suppressed by disulfiram, which decreases brain NE concentrations. Moreover, they found that 5-HT turnover increases after barbital withdrawal, and that this increase in 5-HT turnover and barbital withdrawal convulsions are suppressed by nitrazepam (Tagashira et al., 1982a). Therefore, it is believed that the concentrations and/or turnovers of 5-HT, NE and DA increase after barbiturate withdrawal, and that the increases in brain monoamine concentrations and monoamine-related neuron activities may be directly involved in the appearance of barbiturate withdrawal signs.

On the other hand, co-administration of α-methyl-*p*-tyrosine or reserpine during barbital treatment potentiates the development of physical dependence on barbital (Tagashira et al., 1983b). While, co-administration of methamphetamine or *p*-chlorophenylalanine suppresses the development of physical dependence on barbital (Tagashira et al., 1983b). Therefore, the changes in brain monoamine concentrations and/or monoamine-related neuron activities may affect not only the appearance of barbiturate withdrawal signs, but also the development of physical dependence on barbiturates.

Tricyclic antidepressants, such as imipramine and desipramine, inhibit the re-uptake of NE and 5-HT into the terminals of noradrenergic and serotonergic neurons, and increase 5-HT and NE concentrations in the synaptic lacuna (Baldessarini, 1990). However, chronic treatment with tricyclic antidepressants down-regulates 5-HT_{1A}, 5-HT_2, α_2 and β receptors (Blier and de Montigny, 1980; Cohen et al., 1982; Fuxe et al., 1983; Heninger and Charney, 1987; Wolfe et al., 1978). On the other hand, new psychotropic drugs, bifemelane and teniloxazine, are potent antidepressants (Anami et al., 1985; Tobe et al., 1981), and possess antianoxic (Izumi and Yasuda, 1985; Tobe et al., 1983), antiischemic (Egawa et al., 1984) and memory retrieval (Anami et al., 1985; 1987; Tobe et al., 1985) actions. Moreover, bifemelane inhibits the re-uptake of 5-HT and NE into the nerve terminals of respective neurons, and affects brain 5-HT and NE concentrations (Egawa et al., 1983; Ohizumi et al., 1982). While, teniloxazine only inhibits the re-uptake of NE (Anami et al., 1986).

Tricyclic antidepressants have been known to potentiate the barbital-induced central depression, for example, barbital-induced hypnosis (Bahattacharya, 1978; Baldessarini, 1990; Liu et al., 1975). This potentiation of barbital-induced hypnosis

by tricyclic antidepressants may be due to the inhibition of monoamine re-uptake (Bahattacharya, 1978; Liu et al., 1975). In general, it is considered that increase in magnitude of barbiturate-induced central depression by combination of a dependence liability-free drug during the barbiturate treatments, induces the potentiation of the development of physical dependence on barbiturates (Tagashira et al., 1981). Therefore, in the present study, the effects of imipramine, bifemelane and teniloxazine on barbital-induced hypnosis and on the development of physical dependence on barbital were examined. Moreover, the effects of imipramine and bifemelane or teniloxazine on the barbital-induced hypnosis and on the development of physical dependence on barbital were compared, and the involvement of monoamine re-uptake inhibition in the development of physical dependence on barbital was determined.

EXPERIMENT 1-1: Effects of antidepressants on the central depression by barbital in rats

Materials and Methods

Animals

Male Sprague-Dawley rats (Tokyo Animal Laboratories Inc., Tokyo, Japan) that weighed 170-290 g at the beginning of the experiment, were used. Animals were housed individually under a 12 hr light-dark cycle (lights on 8:30 to 20:30) with free access to food and tap water. The room temperature and the relative humidity were maintained at 22 $\pm$ 1 °C and 55 $\pm$ 5 %, respectively.

Barbital-induced Hypnosis

Hypnosis induced by barbital (200 mg/kg, i.p.) was measured in rats. Rats were pretreated with saline (1 ml/kg, i.p.), imipramine (20 mg/kg, i.p.), bifemelane (40 mg/kg, i.p.) or teniloxazine (4 mg/kg, i.p.) 30 min before the treatment with barbital. The onset time of barbital hypnosis was considered to be the time between the injection of barbital and loss of the righting reflex, whereas sleep time was defined as the time between the loss and regaining of the righting reflex.

Drugs

Barbital sodium (Tokyo Chemical Ind., Tokyo, Japan), imipramine hydrochloride (Sigma Chemical Co., St. Louis, USA), bifemelane hydrochloride (Mitsubishi Chemical Ind. Ltd., Yokohama, Japan) or teniloxazine maleate (Yoshitomi Pharmaceutical Ind. Ltd., Fukuoka, Japan) were dissolved in saline.

Statistical Analysis

Analyses for onset time and sleep time of barbital-induced hypnosis were performed by Student's t-test.

Results

Table 1-1 shows effects of several antidepressants on barbital-induced hypnosis. Pretreatment with imipramine or bifemelane significantly prolonged barbital-induced sleep time ($p<0.01$ and $p<0.001$, respectively). Moreover, pretreatment with bifemelane also significantly shortened onset time of barbital-induced hypnosis ($p<0.01$). On the other hand, pretreatment with teniloxazine only significantly prolonged onset time of barbital-induced hypnosis ($p<0.05$), but did not affect the sleep time of barbital-induced hypnosis.

Table 1-1
Effects of several antidepressants on barbital-induced hypnosis in rats

Pretreatment	*Onset time (min)*	*Sleep time (min)*
Saline (1 ml/kg, i.p.)	*47.5 ± 3.9*	*209.6 ± 16.3*
Imipramine (20 mg/kg, i.p.)	*68.3 ± 16.4*	*420.4 ± 60.4* **
Saline (1 ml/kg, i.p.)	*70.2 ± 7.8*	*243.6 ± 23.8*
Bifemelane (40 mg/kg, i.p.)	*29.1 ± 7.1* **	*699.1 ± 66.9* ***
Saline (1 ml/kg, i.p.)	*47.5 ± 3.9*	*209.6 ± 16.3*
Teniloxazine (4 mg/kg, i.p.)	*89.7 ± 15.6* *	*195.0 ± 23.0*

*Rats were pretreated with imipramine, bifemelane or teniloxazine 30 min prior to barbital injection (200 mg/kg, i.p.). Each group consisted of 6 animals. *p<0.05, **p<0.01, ***p<0.001 vs. respective saline-pretreated group.*

EXPERIMENT 1-2: Effect of a tricyclic antidepressants, imipramine, on the development of physical dependence on barbital in rats

Materials and Methods

Animals

Male Sprague-Dawley rats (Tokyo Animal Laboratories Inc., Tokyo, Japan) that weighed 210-290 g at the beginning of the experiment, were used. Animals were housed individually under the conditions as described in EXPERIMENT 1-1.

Development of Physical Dependence

To prepare the drug-admixed food, barbital and imipramine were mixed with a normal powdered food (CA-1, Japan Clea, Tokyo, Japan) in a mortar (Suzuki et al., 1990, 1992b). Each rat was fed either the barbital alone- or barbital+imipramine-admixed food for 28 days and could drink tap water *ad libitum*. The concentration of barbital in the food was gradually increased during the treatment (Table 1-2); the schedule of the barbital treatment was according to the method of Suzuki et al. (1992b) with minor modifications. The concentration of imipramine in the food was fixed at 0.25 mg/g of food. Body weight and food consumption were measured every day at 16:00. Daily barbital intake was calculated as follows:

$$\text{Barbital intake (mg/kg/day)} = \frac{\text{Food intake (g/day)} \times \text{Drug concentration (mg/g of food)}}{\text{Body weight (kg)}}$$

Barbital Withdrawal

Withdrawal was achieved by substituting normal food for drug-admixed food at 18:00 on the last day of the treatment. Body weight was measured and withdrawal

signs were observed after termination of the drug treatment. Changes in body weight after withdrawal were calculated as percent changes from the body weight at the beginning of withdrawal. To quantify the intensity of physical dependence on barbital, a rating score for withdrawal signs, according to the method of Suzuki et al. (1992b) with minor modifications, was used (Table 1–3). Withdrawal scores are the sum of the rating scores of individual animals, and the withdrawal scores for 204 hr after the withdrawal are shown as total withdrawal scores.

Drugs

Barbital and imipramine hydrochloride were purchased from Wako Pure Chemical Ind. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, USA), respectively.

Statistical Analysis

Analyses for the changes in body weight and withdrawal scores were performed by two factor (groups x times) repeated measures analysis of variance (ANOVA). All other analyses were carried out using Student's t–test.

Table 1-2
Progressively increasing dosage schedule for development of physical dependence on barbital in rats

Barbital concentration (mg/g of food)	*Duration (days)*
0.5 and 1.0	*4*
1.0 and 2.0	*6*
2.0 and 4.0	*6*
4.0 and 6.0	*6*
6.0	*6*

Table 1-3
Score chart for barbital withdrawal signs

Characteristic signs		*Score*
Weight loss	*5-10 %*	*1*
	10-15 %	*2*
	15 % <	*3*
Piloerection		*2*
Vocalization		*2*
Irritability		*2*
Aggression		*2*
Teeth-chattering		*2*
Diarrhea		*2*
Muscle rigidity		*2*
Straub's tail		*2*
Ear-twitch		*2*
Lacrimation		*3*
Nose-bleed		*3*
Fascicular-twitch		*3*
Jerk		*3*
Tremor		*3*
Handling-elicited convulsion		*3*
Spontaneous convulsion		*4*
Death		*4*

Results

There was no significant difference in daily barbital intake during barbital treatment between the barbital alone and barbital+imipramine groups. The mean barbital intakes at the final barbital concentration (6 mg/g of food) was 318.6 $\pm$ 7.3 mg/kg/day for the barbital alone group and 316.3 $\pm$ 6.9 mg/kg/day for the barbital+ imipramine group.

After termination of the barbital treatment, several withdrawal signs were observed. These signs included body weight loss, piloerection, vocalization, irritability, aggression, muscle rigidity, Straub's tail, ear-twitching, teeth-chattering, fascicular-twitch, lacrimation, nose-bleed, jerk, tremor, handling-elicited convulsions, spontaneous convulsions and death. Total withdrawal scores after barbital withdrawal in the barbital alone and barbital+imipramine groups were 26.8 $\pm$ 1.9 and 31.8 $\pm$ 2.1, respectively (Fig. 1-1). The total withdrawal scores were significantly potentiated by co-administration of imipramine ($p<0.05$). Figure 1-2 shows the time course changes in withdrawal scores after termination of the barbital treatment. Withdrawal scores were also significantly potentiated by co-administration of imipramine ($F[1,133]=23.604$, $p<0.01$). As shown in Fig. 1-3, body weight decreased after termination of the barbital treatment. The maximum weight loss was 7.20 $\pm$ 1.04 % at 72 hr after withdrawal in the barbital alone group and 10.40 $\pm$ 1.81 % at 72 hr after withdrawal in the barbital+imipramine group. Weight loss after the withdrawal was significantly increased by co-administration of imipramine ($F[1,133]=47.861$, $p<0.01$).

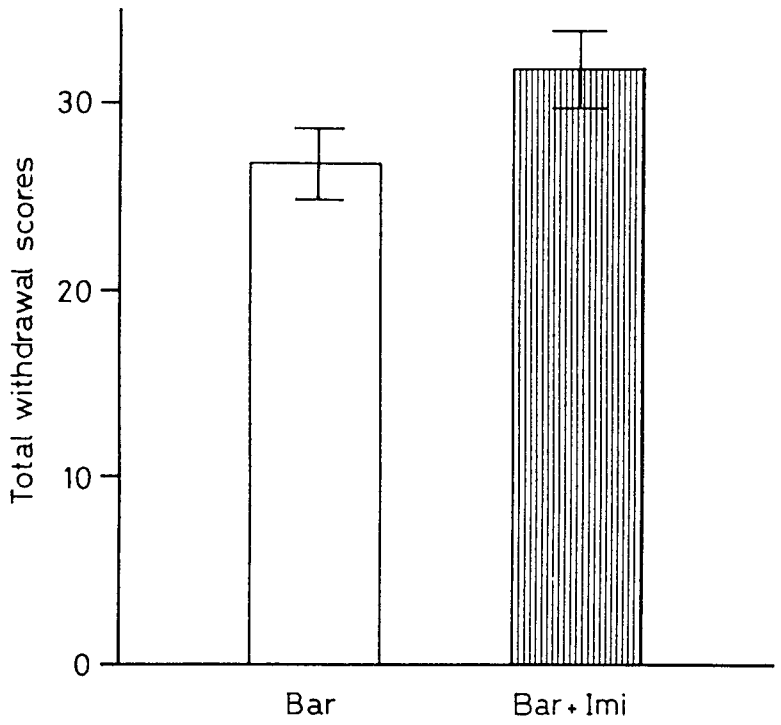


Fig. 1-1
*Total withdrawal scores for 204 hr after withdrawal from treatment with barbital (Bar) alone or barbital+imipramine (Imi; 0.25 mg/g of food). Each column represents the mean ± SEM of 5-6 observations. *p<0.05 vs. barbital alone group.*

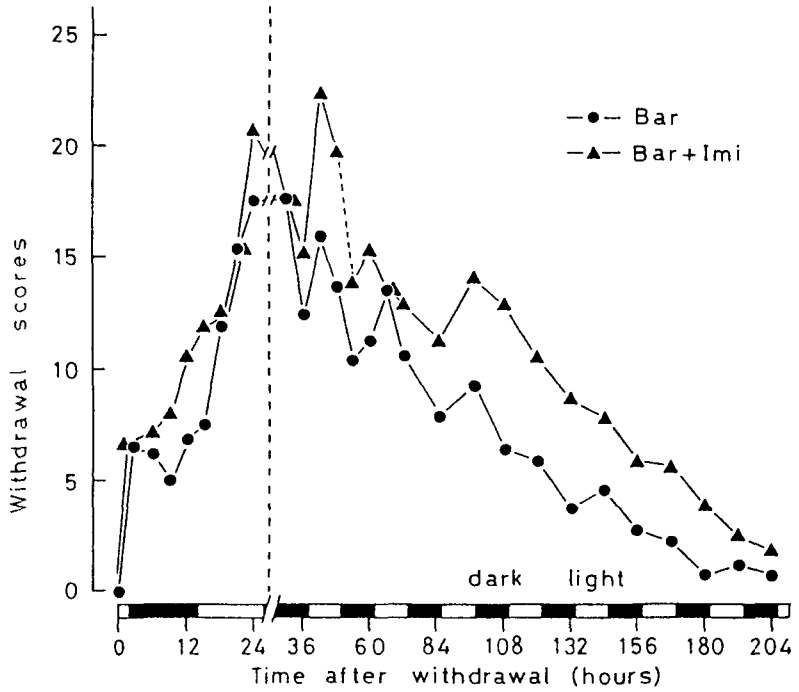


Fig. 1-2
Time course changes in withdrawal scores after withdrawal from treatment with barbital (Bar) alone or barbital+imipramine (Imi; 0.25 mg/g of food). Each point represents the mean of 5-6 observations. Withdrawal scores were significantly potentiated by co-administration of imipramine ($F[1,133]= 23.604$, $p<0.01$).

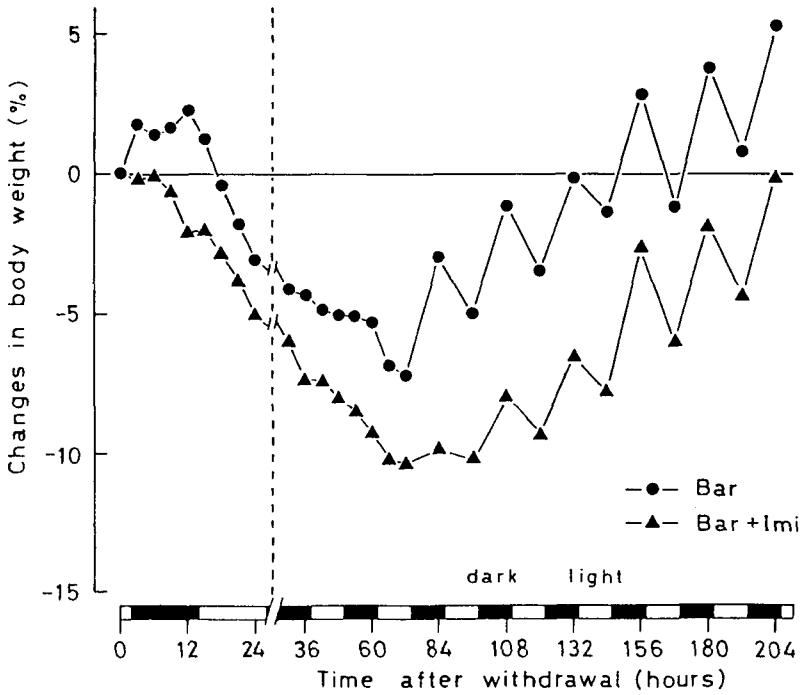


Fig. 1-3
Time course changes in body weight loss (%) after withdrawal from treatment with barbital (Bar) alone or barbital+imipramine (Imi; 0.25 mg/g of food). Each point represents the mean of 5-6 observations. Body weight loss was significantly potentiated by co-administration of imipramine ($F[1,133]= 47.861$, $p<0.01$).

EXPERIMENT 1-3: Effects of new antidepressants, bifemelane and teniloxazine, on the development of physical dependence on barbital in rats

Materials and Methods

Animals

Male Sprague-Dawley rats (Tokyo Animal Laboratories Inc., Tokyo, Japan) that weighed 170-200 g at the beginning of the experiment, were used. Animals were housed individually under the conditions as described in EXPERIMENT 1-1.

Development of Physical Dependence

As described in EXPERIMENT 1-2, barbital alone-, barbital+bifemelane- or barbital+teniloxazine-admixed food was prepared. Each rat was fed any of drugs-admixed foods for 28 days and could drink tap water *ad libitum*. The concentration of barbital in the food was gradually increased during the treatment according to EXPERIMENT 1-2. The concentrations of bifemelane and teniloxazine in the food were fixed at 1.0 and 0.5 mg/g of food, respectively. Body weight and food consumption were measured everyday at 16:00. Daily barbital intake was calculated according to EXPERIMENT 1-2.

Barbital Withdrawal

Withdrawal was conducted according to EXPERIMENT 1-2. Body weight was measured and withdrawal signs were observed after termination of the drug treatment. Changes in body weight and withdrawal scores after withdrawal were calculated according to EXPERIMENT 1-2. The withdrawal scores for 156 hr after the withdrawal are shown as total withdrawal scores.

Drugs

Barbital was purchased from Wako Pure Chemical Ind. (Tokyo, Japan), and bifemelane hydrochloride and teniloxazine maleate were supplied from Mitsubishi Chemical Ind. Ltd. (Yokohama, Japan) and Yoshitomi Pharmaceutical Ind. Ltd. (Fukuoka, Japan), respectively.

Statistical Analysis

Analysis of the incidence of withdrawal signs was performed using the chi-square (2 x 2) test. Analyses for the changes in body weight and withdrawal scores were performed by two factor (groups x times) repeated measures analysis of variance (ANOVA). All other analyses were carried out using Student's t-test.

Results

There was no significant difference in daily barbital intake during barbital treatment between the barbital alone- and barbital+bifemelane or barbital+ teniloxazine group. The mean barbital intakes at the final barbital concentration (6 mg/g of food) in the barbital alone, barbital+bifemelane and barbital+teniloxazine groups were 298.9 $\pm$ 13.6, 344.5 $\pm$ 10.5 and 330.2 $\pm$ 14.4 mg/kg/day, respectively.

After termination of the barbital treatment, several withdrawal signs were observed (Table 1-4). The incidence of all withdrawal signs were not affected by co-administration of bifemelane or teniloxazine. Figure 1-4 shows the time course changes in withdrawal scores after termination of the barbital treatment. The maximum withdrawal scores in the barbital alone, barbital+bifemelane and barbital+ teniloxazine groups were 20.2 $\pm$ 3.0 (at 42 hr after withdrawal), 20.0 $\pm$ 3.7 (at 39 hr after withdrawal) and 19.2 $\pm$ 2.5 (at 36 hr after withdrawal), respectively. Co-administration of bifemelane or teniloxazine did not affect the withdrawal scores. The total withdrawal scores after barbital withdrawal in the barbital alone, barbital+ bifemelane and barbital+teniloxazine groups were 30.2 $\pm$ 1.7, 30.2 $\pm$ 2.2 and 27.8 $\pm$ 2.2, respectively (Table 1-4). There was no significant difference in the total withdrawal scores between barbital alone and barbital+bifemelane or barbital+teniloxazine groups. Body weight of animals in all groups slightly increased immediately after the withdrawal, and then decreased abruptly. The maximum weight losses in the barbital alone, barbital+bifemelane and barbital+teniloxazine groups were 7.95 $\pm$ 1.49 % (at 54 hr after withdrawal), 8.47 $\pm$ 1.54 % (at 45 hr after withdrawal) and 8.20 $\pm$ 1.28 % (at 45 hr after withdrawal), respectively; then body weight gradually increased and regained to the levels of the beginning of withdrawal at 108 hr after withdrawal. Co-administration of bifemelane or teniloxazine did not affect the weight loss after

withdrawal.

Table 1-4
Behavioral withdrawal signs for 156 hr after the termination of treatment with barbital alone, barbital+bifemelane or barbital+teniloxazine in rats

Withdrawal signs (scores)	Positive animals / Total number of animals		
	Barbital	Barbital+ Bifemelane	Barbital+ Teniloxazine
Weight loss			
5-10 % (1)	2/6	3/6	2/6
10-15 % (2)	3/6	3/6	3/6
15 % < (3)	0/6	0/6	0/6
Piloerection (2)	6/6	6/6	6/6
Vocalization (2)	6/6	6/6	5/6
Irritability (2)	6/6	5/6	5/6
Aggression (2)	2/6	1/6	3/6
Diarrhea (2)	0/6	0/6	0/6
Teeth-chattering (2)	4/6	4/6	4/6
Muscle rigidity (2)	6/6	6/6	6/6
Straub's tail (2)	6/6	6/6	6/6
Ear-twitch (2)	6/6	6/6	6/6
Lacrimation (3)	0/6	0/6	1/6
Nose-bleed (3)	6/6	6/6	5/6
Fascicular-twitch (3)	6/6	6/6	6/6
Jerk (3)	6/6	6/6	6/6
Tremor (3)	5/6	5/6	4/6
Convulsion			
Handling-elicited (3)	5/6	5/6	2/6
Spontaneous (4)	0/6	1/6	1/6
Death (4)	0/6	0/6	0/6
Total withdrawal scores	30.2 $\pm$ 1.7	30.2 $\pm$ 2.2	27.8 $\pm$ 2.2

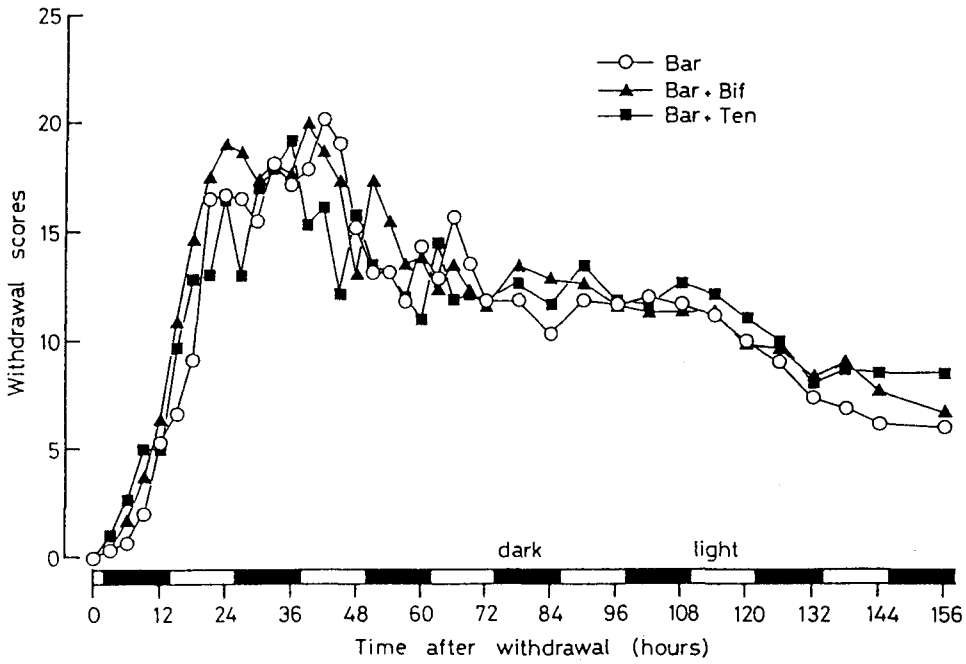


Fig. 1-4
Time course changes in withdrawal scores after withdrawal from treatment with barbital (Bar) alone, barbital+bifemelane (Bif; 1.0 mg/g of food) or barbital+teniloxazine (Ten; 0.5 mg/g of food). Each point represents the mean of 6 observations.

Discussion

The mechanism of barbiturate-induced hypnosis has not yet been clarified. However, several reports have suggested that barbiturate-induced hypnosis is affected by changes in the brain 5-HT concentrations. Barbiturates are known to inhibit the metabolism of 5-HIAAld (a metabolite of 5-HT) to 5-HTOL, as a result, the concentrations of 5-HT increase (Fukumori et al., 1979: Tabakoff and Erwin, 1970). Pretreatment with 5-HT potentiates barbiturate-induced hypnosis, while pretreatment with *p*-chlorophenylalanine, a 5-HT-synthesis inhibitor, suppresses it (Jouvet, 1969). Moreover, tricyclic antidepressants, imipramine and desipramine, which possess 5-HT and NE re-uptake inhibiting actions, potentiate the barbiturate-induced hypnosis (Baldessarini, 1990; Bhattacharya, 1978; Liu et al., 1975), and this potentiation of barbiturate-induced hypnosis by the tricyclic antidepressants was suppressed by co-treatment with methysergide, a 5-HT antagonist and *p*-chlorophenylalanine (Bhattacharya, 1978). Based on these findings, it has been considered that barbiturate-induced hypnosis is potentiated by an increase in the 5-HT concentrations, but is suppressed by a decrease in the 5-HT concentrations. In the present study, pretreatment with imipramine and bifemelane, but not teniloxazine, potentiated barbital-induced hypnosis. Imipramine and bifemelane inhibit the re-uptakes of 5-HT and NE into the nerve terminals of noradrenergic and serotonergic neurons, which increases 5-HT and NE concentrations in the synaptic lacuna of cortex and hippocampus (Baldessarini, 1990; Egawa et al., 1983). While teniloxazine only inhibits the re-uptake of NE (Anami et al., 1986). Therefore, the potentiating effects of imipramine and bifemelane on barbital-induced hypnosis may be due to an increase in 5-HT concentrations induced by its 5-HT re-uptake inhibition. In the present study, teniloxazine slightly prolonged onset time of barbital-induced hypnosis. Since teniloxazine possesses 5-HT receptor antagonistic action

(Anami et al., 1987), this effect of teniloxazine observed may have resulted from 5-HT receptor blockade.

In general, the severity of physical dependence on barbiturates is related to the magnitude of central depression during the treatment. For example, Tagashira et al. (1981) reported that increase in the magnitude of central depression by combination of a dependence liability-free drug such as chlorpromazine during the phenobarbital treatment, induces the potentiation of the development of physical dependence on phenobarbital. However, in the present study, pretreatment with both imipramine and bifemelane potentiated the central depressing effect of barbital, but the development of physical dependence on barbital was potentiated only by co-administration of imipramine. The potentiating effects of both imipramine and bifemelane on the barbital-induced central depression may be due to their re-uptake inhibition of 5-HT. Therefore, increase of magnitude of central depression by chronic drug combination during barbital treatment, may not relate to the development of physical dependence on barbital. The differences in the effects of imipramine and bifemelane on the development of physical dependence on barbital may be due to the differences of both drugs in other pharmacological profiles.

Physical dependence on barbiturates may involve changes in brain monoamine concentrations and in the activities of monoamine-related neurons. Barbiturates suppress NE and DA release from nerve terminals (Rogawski and Porter, 1990), and chronic treatment with barbiturates decreases DA and NE concentrations in the brain (Morgan et al., 1977; 1978; Tagashira et al., 1983a). After withdrawal from barbiturates, the release of NE and DA from nerve terminals increases, and barbiturate withdrawal signs are suppressed by decreases in NE and DA concentrations (Morgan et al., 1977; 1978; Tagashira et al., 1983a). Moreover, the

development of physical dependence on barbiturates is potentiated by further reduction of brain NE and DA concentrations induced by co-administration of α-methyl-*p*-tyrosine or reserpine (Tagashira et al., 1983b), but is suppressed by a recovery from the decrease in brain NE and DA concentrations induced by co-administration of methamphetamine (Tagashira et al., 1983b). Therefore, the development of physical dependence on barbital is potentiated and suppressed by further inhibition and enhancement of noradrenergic neuron activity, respectively.

Barbiturates suppress 5-HT release (Rogawski and Porter, 1990), but increase 5-HT and 5-HIAAld concentrations in synaptic lacuna by inhibiting NADPH-dependent aldehyde reductase (Fukumori et al., 1979: Tabakoff and Erwin, 1970). Satoh et al. (1979) reported that NADPH-dependent aldehyde reductase activity is increased by chronic treatment with barbiturates. Although 5-HT and 5-HIAA concentrations in the brain do not change after chronic treatment with barbital (Tagashira et al., 1982a), 5-HT release may increase, since 5-HT turnover increases due to the increase in NADPH-dependent aldehyde reductase activity. Moreover, 5-HT and 5-HIAA concentrations in the brain increase after withdrawal from barbiturates, and barbital withdrawal convulsions are suppressed by reduction of the increases in 5-HT and 5-HIAA concentrations (Tagashira et al., 1982a). Tagashira et al. (1983b) reported that co-administration of *p*-chlorophenylalanine suppresses the development of physical dependence on barbital. Since *p*-chlorophenylalanine decreases 5-HT metabolites and suppresses the activity of serotonergic neurons (Koe and Weissman, 1966), the suppressive effect of *p*-chlorophenylalanine on the development of physical dependence on barbital may result from suppression of the potentiated serotonergic neuron activity by chronic treatment with barbital. The development of physical dependence on barbital is potentiated and suppressed by further enhancement and inhibition of serotonergic

neuron activity, respectively.

In the present study, co-administration of imipramine, but not bifemelane or teniloxazine, potentiated the development of physical dependence on barbital. Since imipramine and bifemelane are both 5-HT and NE re-uptake inhibitors, monoamine re-uptake inhibition of these drugs may not be directly involved in the development of physical dependence on barbital. Imipramine inhibits the re-uptake of 5-HT and NE into nerve terminals (Baldessarini, 1990), and decreases 5-HT and NE turnovers (Egawa et al., 1983). Moreover, chronic treatment with imipramine down-regulates 5-HT_{1A} (presynaptic autoreceptor), 5-HT_2, α_2 and β receptors (Blier and de Montigny, 1980; Cohen et al., 1982; Fuxe et al., 1983; Heninger and Charney, 1987; Wolfe et al., 1978). Functional changes in these receptors suppress the activity of noradrenergic neurons, but potentiate the activity of serotonergic neurons and increase the release of 5-HT from nerve terminals (Blier and de Montigny, 1980; Cohen et al., 1982; Fuxe et al., 1983; Heninger and Charney, 1987; Wolfe et al., 1978). On the other hand, teniloxazine inhibits the re-uptake of NE only, and decreases NE turnover, but increases 5-HT turnover (Anami et al., 1986). Moreover, bifemelane inhibits the re-uptake of 5-HT and NE, and increases NE turnover, and does not affect 5-HT turnover (Egawa et al., 1983). Chronic treatment with bifemelane decreases NE and 5-HT turnovers, but does not regulate β receptor (Egashira et al., 1989). Therefore, the differences in manipulation to serotonergic and noradrenergic neuron activities between imipramine and bifemelane or teniloxazine seem to be involved in differences in the effects of these drugs on the development of physical dependence on barbital. If the neurochemical changes induced by chronic treatment with the combined drugs are similar to the changes induced by chronic treatment with barbital, these drugs may potentiate the development of physical dependence on barbital. Furthermore, if chronic treatment with the combined drugs

produces neurochemical changes contrary to those induced by chronic treatment with barbital, these drugs may suppress the development of physical dependence on barbital. Chronic treatment with both barbital and imipramine produce the suppression of noradrenergic neuron activities and increase 5-HT release. While, chronic treatment with bifemelane produces the suppression of noradrenergic and serotonergic neuron activities. From these findings, the potentiating effect of imipramine on the development of physical dependence on barbital may have resulted from the further enhancement of 5-HT release and the further suppression of the activities of noradrenergic neurons by chronic treatment with imipramine during the barbital treatment.

In conclusion, these results suggest that imipramine and bifemelane, but not teniloxazine, potentiate barbital-induced central depression, and that co-administration of imipramine, but not bifemelane and teniloxazine, potentiates the development of physical dependence on barbital. The increase in magnitude of central depression by chronic drug combination during barbital treatment, may not relate to the development of physical dependence on barbital. If the neurochemical changes induced by chronic treatment with the combined drugs are similar to those induced by chronic treatment with barbital, then these drugs may potentiate the development of physical dependence on barbital. Therefore, the development of physical dependence on barbital may involve neurochemical changes in serotonergic and noradrenergic neuron activities; the further suppression of noradrenergic neuron activities and the further potentiation of serotonergic neuron activities that are induced by chronic treatment with both barbital and monoamine re-uptake inhibitors may potentiate the development of physical dependence on barbital.

CHAPTER 2

EFFECTS OF SEROTONERGIC ANXIOLYTICS ON THE DEVELOPMENT OF PHYSICAL DEPENDENCE ON DIAZEPAM IN MICE AND RATS

Introduction

Treatment with benzodiazepines is the major clinical therapy for anxiety (Katz et al., 1991; Lader, 1991; Woods et al., 1992) and chronic treatment with benzodiazepines is required in many clinical applications. As a result, tolerance to and physical dependence on benzodiazepines develop, and withdrawal signs of benzodiazepines, such as excess anxiety and convulsions, appear after termination of benzodiazepine treatment (Hallstrom and Lader, 1981; Katz et al., 1991; Lader, 1991; Woods et al., 1992). Because of these problems, anxiolytic agents that lack physical dependence liability are required. Several reports have suggested that the central serotonergic system is involved in the induction of anxiety and in the anxiolytic effect of benzodiazepines (Collines et al., 1979; Costall and Naylor, 1991; Costall et al., 1989b; Engel et al., 1984; Jones, 1990; Kahn et al., 1988; Pei et al., 1989; Treit, 1991; Tye et al., 1979). Moreover, it has been reported that some serotonergic agents, such as 5-HT_{1A} agonists and 5-HT_3 antagonists, are effective anxiolytics (Colpaert et al., 1985; Costall and Naylor, 1991; Costall et al., 1989b; 1990b; Dourish et al., 1986; Engel et al., 1984; Gardner, 1986; Higgins et al., 1991; 1992; Jones et al., 1988; Kennett, 1992; Sprouse, 1991; Traber and Glaser, 1987; Treit, 1991). Since the anxiolytic effects of serotonergic agents seem to be weaker than those of benzodiazepines (Jann, 1988; Treit, 1991), there is a possibility that these drugs may be used concurrently with benzodiazepines, or may replace benzodiazepines in some cases in clinical therapy. Especially, 5-HT_3 antagonists

have been expected as a useful and dependence liability-free anxiolytics, since 5-HT_{1A} agonists have anxiolytic effects at low doses, but elicit anxiety at high doses (File and Andrews, 1991; Moser et al., 1990). Although the effects of these serotonergic agents on benzodiazepine withdrawal signs, especially withdrawal anxiety, have been previously reported (Costall and Naylor, 1991; Costall et al., 1989a; 1990a; File and Andrews, 1991; Goudie and Leathley, 1990; 1991; Woods et al., 1992), there is little information available regarding the effects of these drugs on the development of physical dependence on benzodiazepines.

Physical dependence on benzodiazepines has been evaluated in various animal species and with various experimental methods (Chan et al., 1989; Goudie and Leathley, 1990; 1991; Katz et al., 1991; Miller et al., 1990; Suzuki et al., 1992a; Woods et al., 1992). Natural and benzodiazepine antagonist-precipitated withdrawal signs are observed in rats chronically treated with benzodiazepines (Suzuki et al., 1992a; Woods et al., 1992). However, appearance of precipitated withdrawal signs (including convulsions) of benzodiazepines requires prolonged exposure to benzodiazepines (for about 1 month) (Little et al., 1992; Woods et al., 1992). On the other hand, a reliable demonstration and quantitative characterization of benzodiazepine withdrawal signs are feasible only by examining natural withdrawal from benzodiazepines. The drug-admixed food method has successfully induced severe natural withdrawal signs, including tremors and convulsions, in rats, but this procedure also requires a prolonged and continuous exposure to benzodiazepines (at least 1 month) (Suzuki et al. 1992a; Woods et al., 1992). Recently, it has been shown that chronic treatment with benzodiazepines in mice causes hypersensitivity to the proconvulsant effect of the benzodiazepine partial inverse agonist FG 7142 following termination of the treatment (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; Nutt and Costello, 1988). Since this procedure requires only 1 week, the

production of convulsions by FG 7142 at proconvulsant doses after chronic treatment with benzodiazepines in rodents has been therefore accepted as a reliable and useful, but preliminary index for evaluating benzodiazepine-like physical dependence liability (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; Moreau et al., 1990; Nutt and Costello, 1988; Piot et al., 1990). This procedure only evaluates FG 7142-induced convulsion. In the present study, the effects of some serotonergic anxiolytics on the development of physical dependence on diazepam in mice were indirectly examined using FG 7142. In addition, the effect of ondansetron on the development of physical dependence on diazepam in rats was directly examined by natural withdrawal using the drug-admixed food method.

EXPERIMENT 2-1: Effects of serotonergic anxiolytics on the development of physical dependence on diazepam in mice.

Materials and Methods

Animals

Male ICR mice (Charles River Japan Inc., Atsugi, Japan) that weighed 25–30 g at the beginning of the experiment, were used. Animals were housed in groups of 5 under the conditions as described in EXPERIMENT 1-1.

FG 7142-induced Convulsions

Mice were treated with vehicle (10 ml/kg, i.p.)+saline (10 ml/kg, i.p.), diazepam (16 mg/kg, i.p.)+saline, diazepam+buspirone (5-HT_{1A} agonist; 0.3, 1 and 3 mg/kg, i.p.), diazepam+mianserin (5-HT_{1C} antagonist; 5, 10 and 20 mg/kg, i.p.), diazepam+ketanserin (5-HT_2 antagonist; 0.1, 0.3 and 1 mg/kg, i.p.), diazepam+ondansetron (5-HT_3 antagonist; 0.003, 0.01 and 0.03 mg/kg, i.p.), vehicle+buspirone (3 mg/kg, i.p.) or vehicle+ondansetron (0.03 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment with drugs, mice were treated with FG 7142 (8, 10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p.), and then clonic-tonic convulsions induced by FG 7142 were observed for 30 min after the injection.

Plasma Concentrations of Diazepam and Its Metabolites

Plasma concentrations of diazepam and its metabolites, nordiazepam and oxazepam, were measured after the treatment with diazepam (16 mg/kg, i.p.)+saline (10 ml/kg, i.p.), diazepam+buspirone (3 mg/kg, i.p.) or diazepam+ondansetron (0.03 mg/kg, i.p.). After the treatment, blood was drawn from the femoral artery, and centrifuged at 3000 rpm for 10 min to separate plasma samples. The plasma

concentrations of diazepam and its metabolites were analyzed by high-performance liquid chromatography according to the method of McNicholas et al. (1985) with minor modifications. Diazepam, nordiazepam and oxazepam were used as standards.

Drugs

Diazepam (Profarma Co., Italy) and FG 7142 (Research Biochemicals Inc., MA, USA) were suspended in vehicle consisting of 9 % Tween 80 (Kishida Chemical Co., Osaka, Japan) in saline. Buspirone hydrochloride (Sigma Chemical Co., St. Louis, USA), mianserin hydrochloride (supplied by Zeria Pharmaceutical Co., Saitama, Japan), ketanserin (Sigma Chemical Co., St. Louis, USA) and ondansetron (synthesized at Nisshin Flour Milling Co., Saitama, Japan) were dissolved in saline. Nordiazepam and oxazepam were synthesized at Nisshin Flour Milling Co. (Saitama, Japan).

Statistical Analysis

ED_{50} values and their 95 % confidence limits of FG 7142 for the clonic-tonic convulsions were calculated by the method of Litchfield and Wilcoxon (1949). Statistical analysis for the comparison between ED_{50} values was carried out by analyzing the potency ratio. This analysis was performed according to a statistical program (Program 47 of the Pharmacologic Calculations System) (Tallarida and Murray, 1987).

Results

Chronic treatment with diazepam did not cause any mortality or deterioration in the condition of the animals. No convulsions were observed in chronically vehicle+saline-treated mice which just received FG 7142. On the other hand, mice chronically treated with diazepam showed clonic-tonic convulsions following administration of FG 7142 in a dose-dependent manner (Fig. 2-1).

The incidence of the clonic-tonic convulsions induced by FG 7142 40 mg/kg after the chronic treatment with diazepam in combination with serotonergic anxiolytics is shown in Fig. 2-2. The incidence of the clonic-tonic convulsions was 54.05 % for the diazepam+saline group. While, the incidences of the clonic-tonic convulsions for diazepam+buspirone 3 mg/kg and diazepam+ondansetron 0.03 mg/kg groups were 72.73 and 80.00 %, respectively (Fig. 2-2A, D). The incidence of the clonic-tonic convulsions tended to be potentiated by co-administration of buspirone or ondansetron. However, the incidence of the clonic-tonic convulsions was not affected by co-administration of mianserin or ketanserin (Fig. 2-2B, C). Dose-response line of FG 7142 for clonic-tonic convulsions was shifted toward left by co-administration of buspirone 3 mg/kg or ondansetron 0.03 mg/kg (Fig. 2-3). ED_{50} values of FG 7142 for clonic-tonic convulsions were significantly decreased by co-administration of buspirone 3 mg/kg or ondansetron 0.03 mg/kg (Table 2-1, $p<0.05$). On the other hand, no convulsions were observed in mice chronically treated with vehicle+buspirone 3 mg/kg or vehicle+ondansetron 0.03 mg/kg, when mice had just received FG 7142.

Plasma concentrations of diazepam and its metabolites, nordiazepam and oxazepam after the treatment with diazepam were not affected by co-administration

of buspirone 3 mg/kg or ondansetron 0.03 mg/kg (Fig. 2–4)

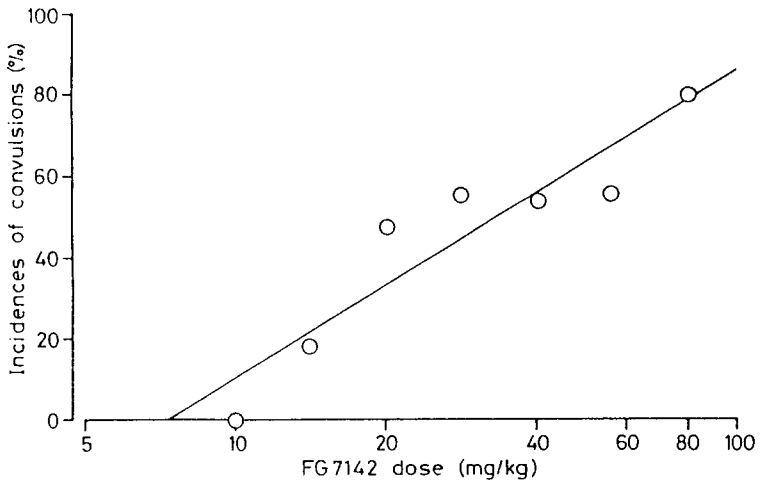
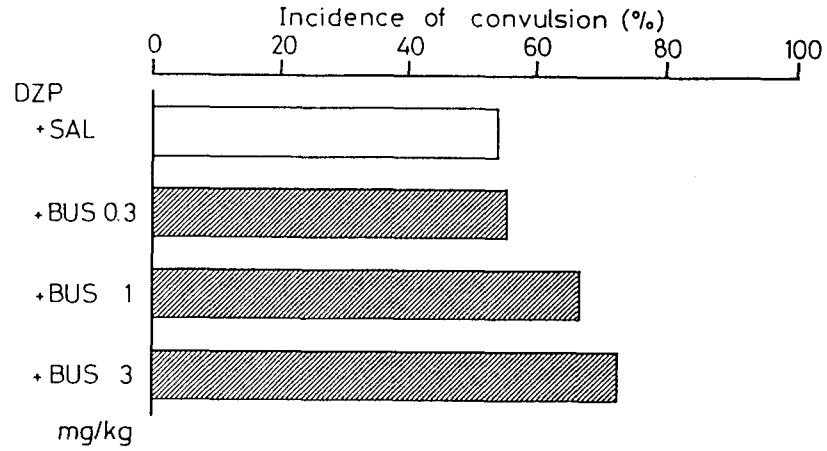
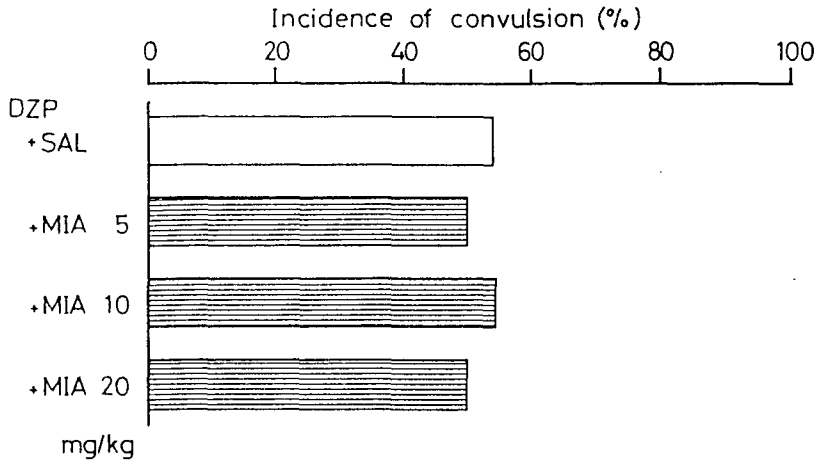


Fig. 2-1
Dose-response line of FG 7142 for clonic-tonic convulsions after the chronic treatment with diazepam. Mice were treated with diazepam (16 mg/kg, i.p.)+ saline (10 ml/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p.), and clonic-tonic convulsions were observed for 30 min. Each point represents the incidence of clonic-tonic convulsions calculated from 9-37 observations. ED_{50} value of FG 7142 for clonic-tonic convulsions was 34.16 (25.69-45.43) mg/kg.

<A>


Incidence of convulsion (%)
0
20
40
60
80
100
DZP
+SAL
+BUS 0.3
+BUS 1
+BUS 3
mg/kg

<B>


Incidence of convulsion (%)
0
20
40
60
80
100
DZP
+SAL
+MIA 5
+MIA 10
+MIA 20
mg/kg

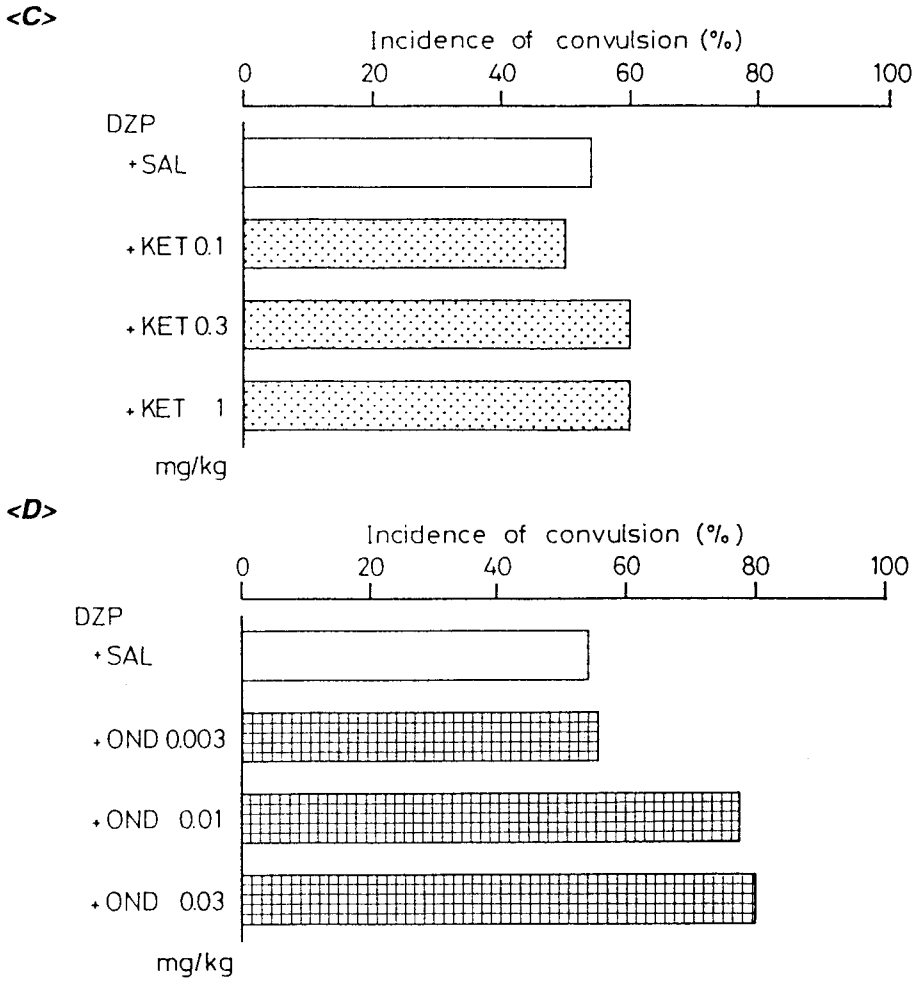


Fig. 2-2
Effects of chronic co-administration of serotonergic anxiolytics with diazepam (DZP) on FG 7142-induced clonic-tonic convulsions. Mice were treated with diazepam (16 mg/kg, i.p.)+saline (SAL; 10 ml/kg, i.p.), <A> diazepam+buspirone (BUS; 0.3, 1 and 3 mg/kg, i.p.), <B> diazepam+ mianserin (MIA; 5, 10 and 20 mg/kg, i.p.), <C> diazepam+ketanserin (KET; 0.1, 0.3 and 1 mg/kg, i.p.) or <D> diazepam+ondansetron (OND; 0.003, 0.01 and 0.03 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with FG 7142 (40 mg/kg, i.p.), and clonic-tonic convulsions were observed for 30 min. Each column represents the incidence of clonic-tonic convulsions calculated from 9-37 observations.

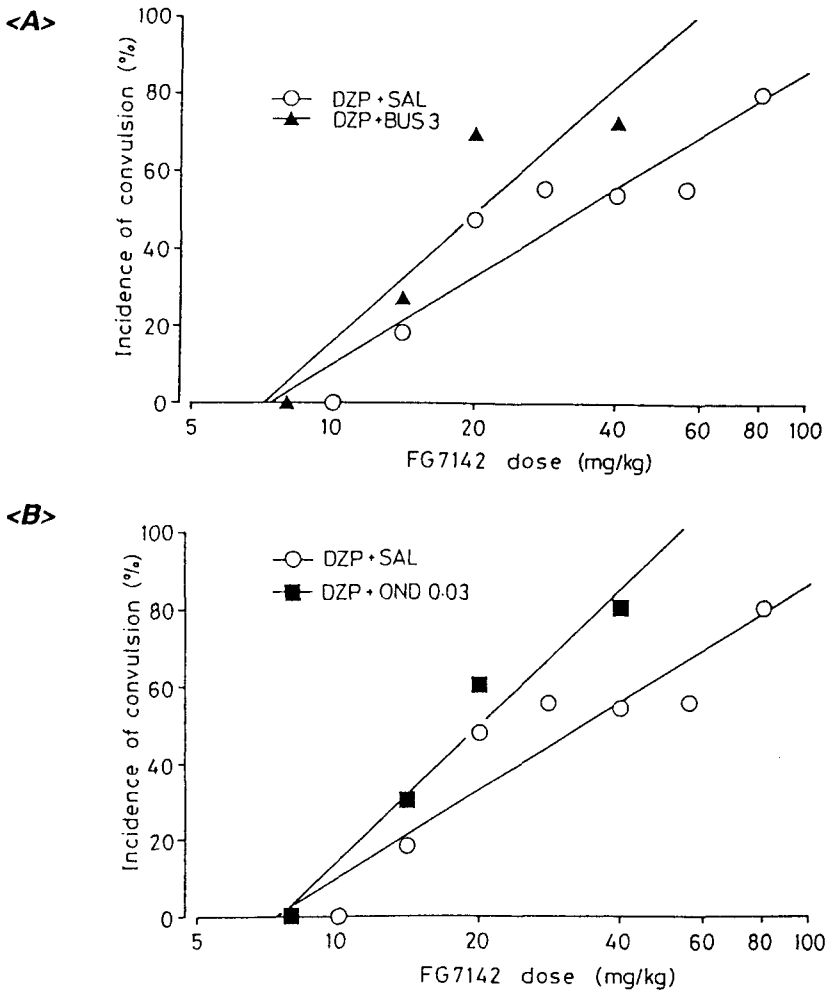


Fig. 2-3
Dose-response lines of FG 7142 for clonic-tonic convulsions after the chronic treatment with <A> diazepam (DZP)+buspirone (BUS) or <B> diazepam+ondansetron (OND). Mice were treated with diazepam (16 mg/kg, i.p.)+saline (SAL; 10 ml/kg, i.p.), diazepam+buspirone (3 mg/kg, i.p.) or diazepam+ondansetron (0.03 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with several doses of FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p. in diazepam+saline group, and 8, 14, 20 and 40 mg/kg, i.p. in diazepam+buspirone or diazepam+ondansetron groups), and clonic-tonic convulsions were observed for 30 min. Each point represents the incidence of clonic-tonic convulsions calculated from 9-37 observations. ED_{50} value of FG 7142 for clonic-tonic convulsions in diazepam+saline, diazepam+buspirone and diazepam+ondansetron groups were 34.16 (25.69-45.43), 20.85 (14.78-29.41) and 20.68 (14.04-30.46) mg/kg, respectively.

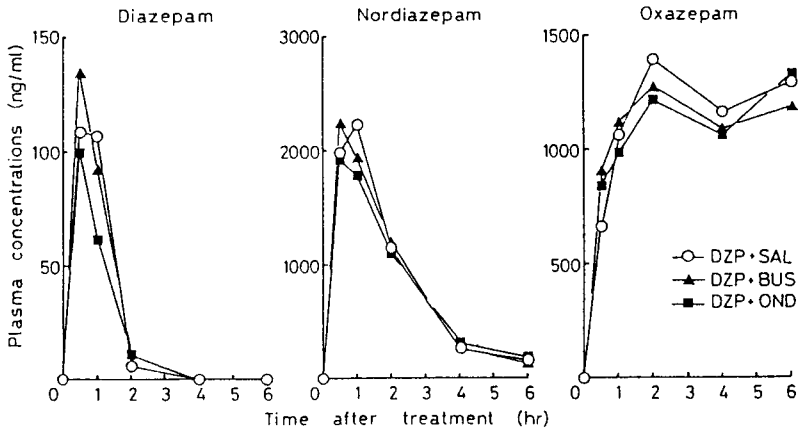


Fig. 2-4
Effects of co-administration of buspirone (BUS) or ondansetron (OND) with diazepam (DZP) on plasma concentrations of diazepam and its metabolites, nordiazepam and oxazepam. Mice were treated with diazepam (16 mg/kg, i.p.)+saline (SAL; 10 ml/kg, i.p.), diazepam+buspirone (3 mg/kg, i.p.) or diazepam+ondansetron (0.03 mg/kg, i.p.). Blood samples were drawn from the femoral artery at 0.5, 1, 2, 4 and 6 hr after treatment. Each point represents the mean of 5-6 observations.

Table 2-1
Effects of chronic co-administration of serotonergic anxiolytics with diazepam on convulsive threshold of FG7142

Treatment	*ED_{50} of FG 7142 (95 % CL) mg/kg, i.p.*	*Potency ratio (95 % CL)*
Diazepam 16 mg/kg, i.p.		
+ saline	*34.16*	
10 ml/kg, i.p.	*(25.69-45.43)*	
+ buspirone	*20.85*	*1.64 **
3 mg/kg, i.p.	*(14.78-29.41)*	*(1.05-2.56)*
+ ondansetron	*20.68*	*1.65 **
0.03 mg/kg, i.p.	*(14.04-30.46)*	*(1.02-2.67)*

*Mice were treated with diazepam (16 mg/kg, i.p.)+saline, diazepam+ buspirone (3 mg/kg, i.p.) or diazepam+ondansetron (0.03 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with several doses of FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p. in the diazepam+saline group, and 8, 14, 20 and 40 mg/kg, i.p. in diazepam+ buspirone or diazepam+ondansetron groups), and clonic-tonic convulsions were observed for 30 min. ED_{50} values and their 95 % confidence limits (CL) were calculated from the incidence of clonic-tonic convulsions. *$p<0.05$ vs. diazepam+saline group.*

EXPERIMENT 2-2: Effect of a serotonergic anxiolytics, ondansetron, on the development of physical dependence on diazepam in rats

Materials and Methods

Animals

Male Fischer 344 rats (Charles River Japan Inc., Atsugi, Japan) that weighed 130–150 g at the beginning of the experiment, were used. Animals were housed individually under the conditions as described in EXPERIMENT 1–1.

Drug Treatment

As described in EXPERIMENT 1–2, diazepam alone–, diazepam+ondansetron– and ondansetron alone–admixed food was prepared. Each rat was fed either the diazepam alone– or diazepam+ondansetron–admixed food for 26 days, or the ondansetron alone–admixed food for 33 days, and could drink tap water *ad libitum*. The concentration of diazepam in the food was gradually increased during the treatment (Table 2–2); the schedule of the diazepam treatment was according to the method of Suzuki et al. (1992a) with minor modifications. The concentration of ondansetron in diazepam–admixed food was fixed at 0.1, 0.2 or 0.4 mg/g of food. On the other hand, the concentration of ondansetron in food which contained ondansetron alone was gradually increased during the treatment (Table 2–2). Body weight and food consumption were measured everyday at 16:00. Daily drug intake was calculated according to EXPERIMENT 1–2.

Abrupt Withdrawal

Abrupt withdrawal was conducted according to EXPERIMENT 1–2. Withdrawal signs were observed after termination of the drug treatment. To quantify the intensity

of physical dependence on diazepam, a rating score for withdrawal signs, according to the method of Suzuki et al. (1992a) with minor modifications, was used (Table 2-3). Withdrawal scores were calculated according to EXPERIMENT 1-2. When rats died, their withdrawal scores in all points after death were considered to be 40.

Drugs

Diazepam was purchased from Profarma Co. (Italy) and ondansetron was synthesized at Nisshin Flour Milling Co. (Saitama, Japan).

Statistical Analysis

Analysis of the incidence of withdrawal signs was performed using the chi-square (2 x 2) test. Analysis for the changes in withdrawal scores was performed by two factor (groups x times) repeated measures analysis of variance (ANOVA). All other analyses were carried out using the Student's t-test.

Table 2-2
Progressively increasing dosage schedule for chronic treatment with diazepam or ondansetron in rats

Diazepam concentration (mg/g of food)	*Duration (days)*
1 and 2	*3*
2 and 4	*3*
4 and 6	*4*
6	*4*
8	*3*
10	*3*
12	*6*
Ondansetron concentration (mg/g of food)	*Duration (days)*
0.05	*3*
0.1	*3*
0.2	*3*
0.4	*3*
0.8	*3*
1.6	*3*
3.2	*5*
6.4	*10*

Results

There was no significant difference in daily diazepam intake during diazepam treatment between the diazepam alone- and the diazepam+ondansetron (0.1 or 0.2 mg/g of food) groups. However, diazepam intake was significantly increased by co-administration of ondansetron 0.4 mg/g of food ($p<0.01$). The mean diazepam intakes in the diazepam alone and diazepam+ondansetron (0.1, 0.2 or 0.4 mg/g of food) groups at the final diazepam concentration (12 mg/g of food) were 780.7 $\pm$ 13.7, 846.2 $\pm$ 17.7, 794.8 $\pm$ 31.3 and 861.1 $\pm$ 14.3 mg/kg/day, respectively.

After the abrupt withdrawal from diazepam, several withdrawal signs were observed (Table 2-3). The incidence of withdrawal signs tended to be potentiated by co-administration of ondansetron. In fact, the appearance of jerks, tremors and convulsions were significantly potentiated ($p<0.05$). Moreover, the withdrawal scores after abrupt withdrawal were significantly potentiated by co-administration of ondansetron (0.1 mg/g of food: $F[1,140]=33.409$, $p<0.01$, 0.2 mg/g of food: $F[1,126]=33.352$, $p<0.01$, 0.4 mg/g of food: $F[1,154]=25.930$, $p<0.01$) (Fig. 2-5). The most potentiation of withdrawal signs was observed in the ondansetron 0.2 mg/g of food co-administered group (vs. 0.1 mg/g of food: $F[1,126]=8.821$, $p<0.01$, vs. 0.4 mg/g of food: $F[1,140]=4.060$, $p<0.05$).

In the rats that were treated with ondansetron alone, body weight gradually increased until the ondansetron concentration reached 6.4 mg/g of food, at which weight gain was suppressed. Therefore, this concentration of ondansetron (6.4 mg/g of food) was regarded as the final concentration of ondansetron treatment. The mean ondansetron intake at the final ondansetron concentration was 421.6 $\pm$ 8.4 mg/kg/day. After the abrupt withdrawal from ondansetron, any marked withdrawal signs were not

observed.

Table 2-3
Behavioral withdrawal signs for 48 hr after the abrupt withdrawal from diazepam alone or diazepam+ondansetron in rats

Withdrawal signs (scores)	Positive animals / Total number of animals			
	Diazepam alone	Diazepam+ondansetron 0.1 mg/g	0.2 mg/g	0.4 mg/g
Weight loss				
5-10 % (1)	2/6	1/6	1/5	2/7
10-15 % (2)	4/6	5/6	4/5	5/7
15 % < (3)	0/6	0/6	0/5	0/7
Piloerection (2)	6/6	6/6	6/6	7/7
Vocalization (2)	3/6	1/6	2/5	4/7
Irritability (2)	6/6	6/6	5/5	6/7
Aggression (2)	0/6	0/6	0/5	0/7
Diarrhea (2)	2/6	1/6	0/5	0/7
Teeth-chattering (2)	3/6	5/6	4/5	6/7
Muscle rigidity (2)	6/6	6/6	5/5	7/7
Straub tail (2)	4/6	4/6	5/5	7/7
Ear-twitch (2)	6/6	6/6	5/5	7/7
Lacrimation (3)	1/6	0/6	3/6	0/7
Nose-bleed (3)	4/6	4/6	3/5	4/7
Dysuria (3)	1/6	3/6	3/6	1/7
Hematuria (3)	0/6	0/6	2/6	1/7
Fascicular-twitch (3)	4/6	6/6	5/5	7/7
Jerk (3)	2/6	6/6 *	4/5	6/7
Tremor (3)	1/6	4/6	4/5 *	6/7 *
Convulsion (4)	1/6	2/6	4/5 *	4/7
Death (4)	0/6	0/6	2/6	1/7

*$p<0.05$ vs. diazepam-alone group.

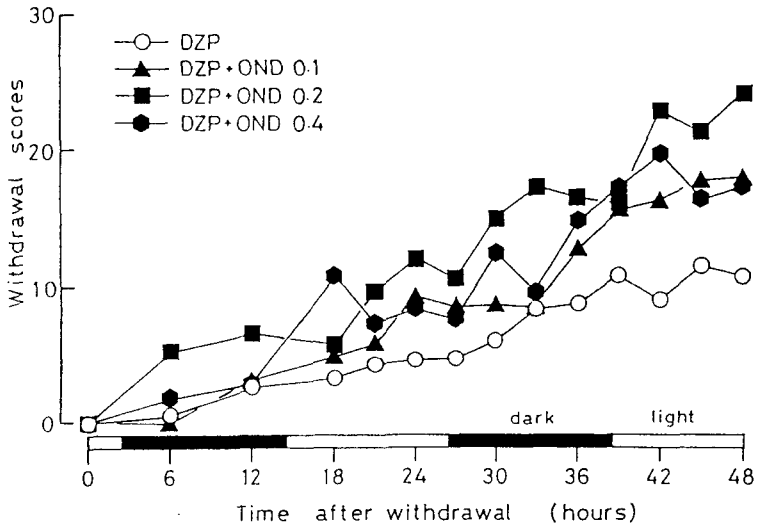


Fig. 2-5
Time course changes in withdrawal scores after the abrupt withdrawal from the treatment with diazepam (DZP) alone or diazepam+ondansetron (OND; 0.1, 0.2 or 0.4 mg/g of food). Each point represents the mean of 5-7 observations. Withdrawal scores were significantly potentiated by co-administration of ondansetron (0.1 mg/g of food: $F[1,140]=33.409$, $p<0.01$, 0.2 mg/g of food: $F[1,126]=33.352$, $p<0.01$, 0.4 mg/g of food: $F[1,154]=25.930$, $p<0.01$).

Discussion

Co-administration of buspirone or ondansetron with diazepam potentiated the development of physical dependence on diazepam in evaluations using both experimental methods: FG 7142 injection method and drug-admixed food method. Especially, in evaluation using the drug-admixed food method, appearance rates of severe withdrawal signs of diazepam, jerks, tremors and convulsions, after withdrawal from diazepam were potentiated by co-administration of ondansetron. Therefore, the potentiation of supersensitivity observed to a benzodiazepine partial inverse agonist by co-administration of buspirone or ondansetron with diazepam may reflect enhancement of the appearance rates of jerks, tremors and convulsions after withdrawal.

In evaluation using the drug-admixed food method, diazepam intake during the treatment was increased only by co-administration of ondansetron 0.4 mg/g of food. In general, an increase in diazepam intake during treatment potentiates the development of physical dependence on diazepam. However, although diazepam intake was not increased by co-administration of ondansetron 0.1 and 0.2 mg/g of food, co-administration of ondansetron potentiated the development of physical dependence on diazepam at all of the doses of ondansetron used. Moreover, the development of physical dependence on diazepam was most potentiated in the ondansetron 0.2 mg/g of food co-administered group. These results suggest that the potentiation by ondansetron does not result from an increase in diazepam intake.

Co-administration of buspirone or ondansetron with diazepam potentiated the development of physical dependence on diazepam. To determine whether or not the enhancing effects of buspirone and ondansetron on the development of physical

dependence on diazepam result from the pharmacokinetic interactions between these drugs, plasma concentrations of diazepam and its metabolites were measured after the treatment. However, plasma concentrations of diazepam and its metabolites were not affected by co-administration of buspirone or ondansetron. These results suggest that the potentiation of development of physical dependence on diazepam by co-administration of buspirone or ondansetron is not ascribable to the pharmacokinetic interactions between diazepam and buspirone or ondansetron.

It is well known that anxiety is elicited by the overstimulation of ascending serotonergic neurons from the raphe nucleus (Blackburn, 1992; Collines et al., 1979; Costall and Naylor, 1991; Engel et al., 1984; Jones, 1990; Kahn et al., 1988; Treit, 1991; Tye et al., 1979). Benzodiazepines suppress the cell body firing rate of these serotonergic neurons in the dorsal raphe nucleus, and reduce the release of 5-HT from the nerve terminals of these serotonergic neurons in the amygdala, hippocumpus and cortex (Blackburn, 1992; Costall and Naylor 1991; Costall et al., 1989b; Laurent et al., 1983; Pei et al., 1989; Thiebot et al., 1980; Treit 1991). As a result, benzodiazepines appear to produce anxiolytic effects. On the other hand, chronic treatment with benzodiazepines results in physical dependence and causes withdrawal signs, including excess anxiety and convulsions (Costall and Naylor 1991; Treit 1991; Woods et al. 1992). These signs of withdrawal from benzodiazepines have been suggested to result from increases in neurotransmitter release. In fact, following the termination of chronic treatment with benzodiazepines, ascending serotonergic neurons are overstimulated. As a result, the release of 5-HT from the nerve terminals of these neurons is increased (Hitchcott et al. 1990; Wagner et al. 1985). Low doses of buspirone have been shown to bind to 5-HT_{1A} autoreceptors and then to decrease the cell body firing rate in the dorsal raphe nucleus and the release of 5-HT in the amygdala (Blackburn, 1992; Dourish et al., 1986; Engel et al.,

1984; Higgins et al., 1992; Jann, 1988; Sprouse, 1991; Traber and Glaser, 1987; Treit, 1991). However, high doses of buspirone have been shown to bind to postsynapse 5-HT_{1A} receptor in the amygdala and hippocumpus, and to potentiate these neuron activities (File and Andrews, 1991; Moser et al., 1990). On the other hand, ondansetron has been shown to suppress the cell body firing rate in the dorsal raphe nucleus and to block 5-HT_3 receptors in ascending serotonergic neurons (Costall and Naylor 1991; Costall et al., 1989a; 1989b; 1990a; 1990b; Higgins et al., 1991; Jones et al., 1988). Ondansetron and low doses of buspirone also suppress the signs of withdrawal from benzodiazepines, withdrawal anxiety and body weight loss (Costall and Naylor 1991; Costall et al., 1989a; 1990a; 1990b; File and Andrews, 1991; Goudie and Leathley 1990; 1991; Woods et al., 1992). Since suppression of general anxiety and benzodiazepine withdrawal anxiety occurs following microinjection of ondansetron and buspirone into the dorsal raphe nucleus and amygdala, but not into the median raphe nucleus, nucleus accumbens or caudate-putamen (Costall and Naylor 1991; Costall et al. 1989b; 1990a; 1990b; Higgins et al., 1991; 1992), the suppression of benzodiazepine withdrawal signs by ondansetron and low doses of buspirone may be due to the suppression of overstimulation of ascending serotonergic neurons, especially those from the dorsal raphe nucleus to the amygdala (Costall and Naylor 1991; Costall et al., 1989a; 1990a; 1990b; File and Andrews, 1991; Goudie and Leathley 1990; 1991; Woods et al., 1992). Therefore, overstimulation of these serotonergic neurons may be involved in the appearance of benzodiazepine withdrawal signs. The present results suggest that co-administration of buspirone or ondansetron, but not mianserin or ketanserin, potentiates the development of physical dependence on diazepam. Therefore, the potentiating effects of buspirone and ondansetron on the development of physical dependence on diazepam may eventually result from enhancement of the overstimulation of ascending serotonergic neurons following the termination of benzodiazepine

treatment. Moreover, it has been found that, in the rodent brain, a high density of 5-HT_{1A} and 5-HT_3 receptors is present in the dorsal raphe necleus and amygdala, but the densities of 5-HT_{1C} and 5-HT_2 receptors in these regions are low in comparision with those in other regions (Hoyer et al., 1992). Therefore, 5-HT_{1A} and 5-HT_3 receptors rather than 5-HT_{1C} and 5-HT_2 receptors may be involved in the overstimulation of these serotonergic neurons and in the development of diazepam physical dependence.

In the present evaluation using the drug-admixed food method, the development of physical dependence on diazepam was potentiated the most by co-administration of ondansetron 0.2 mg/g of food, and the potentiation by ondansetron exhibits in the bell-shaped dose-response fashion. It is known that high dose of ondansetron does not possess anxiolytic effect (Jones, 1990; Jones et al., 1988). Moreover, Goudie and Leathley (1990) reported that high dose of ondansetron did not suppress the benzodiazepine withdrawal signs. Since suppressing effect on diazepam withdrawal signs and anxiolytic effect of ondansetron result from suppression of ascending serotonergic neurons, suppression of serotonergic neuron activities by ondansetron may exhibit in a bell-shaped dose-response curve. As a result, ondansetron suppress the development of physical dependence on diazepam in a bell-shaped dose-response fashion.

Benzodiazepines bind to benzodiazepine binding sites in the $GABA_A$ · benzodiazepine receptor / Cl^- channel complex, and increase Cl^- influx in the brain (Allan et al., 1992a; Miller et al., 1988; Woods et al., 1992). Consequently, benzodiazepines show central depressing effects. Chronic treatment with benzodiazepines causes the down-regulation of benzodiazepine binding sites and subsensitivity to benzodiazepine-elicited Cl^- influx in the brain (Allan et al., 1992a;

Miller et al., 1988; Woods et al., 1992). On the other hand, after the termination of chronic treatment with benzodiazepines, a supersensitivity to benzodiazepine partial inverse agonists, β-carboline analogues (FG 7142, β-CCE, β-CCM, etc.), has been observed in behavioral (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; Nutt and Costello, 1988; Woods et al., 1992) and electrophysiological (Little et al., 1992) studies. Moreover, the decrease in the Cl^- influx in the brain by FG 7142 is potentiated following the termination of chronic treatment with benzodiazepines (Allan et al., 1992a). These changes in the Cl^- influx in the brain following the chronic treatment with benzodiazepines may be directly involved in the development of physical dependence on benzodiazepines and the appearance of benzodiazepine withdrawal signs. In the present study, chronic treatment with buspirone or ondansetron alone did not affect the sensitivity to the proconvulsant effect of FG 7142. These results show that buspirone and ondansetron do not possess a benzodiazepine-like physical dependence liability. It is known that neither buspirone nor ondansetron has affinity to benzodiazepine binding sites (Blackburn, 1992). Therefore, it is possible that chronic co-administration of buspirone or ondansetron indirectly potentiates the decrease in Cl^- influx after the termination of benzodiazepine treatment, and/or directly enhances the overstimulation of ascending serotonergic neurons following benzodiazepine withdrawal. As a result, chronic co-administration of buspirone or ondansetron with diazepam may potentiate the development of physical dependence on diazepam.

Ondansetron has been shown to suppress anxiety, including that induced by withdrawal from benzodiazepines (Costall and Naylor 1991; Costall et al., 1989a; 1989b; 1990a; 1990b; Higgins et al., 1991; Jones et al., 1988). While, buspirone also suppresses it at only low doses, but potentiates at high doses (Blackburn, 1992; Dourish et al., 1986; Engel et al., 1984; File and Andrews, 1991; Higgins et al., 1992;

Jann, 1988; Moser et al., 1990; Sprouse, 1991; Traber and Glaser, 1987; Treit, 1991). Therefore, the valuability of buspirone in clinical therapy seems to be lower than that of ondansetron. The present results and several reports (Eison, 1986; Jann, 1988, Katz et al., 1991; Lader, 1991) suggest that buspirone and ondansetron do not possess a benzodiazepine-like dependence liability, but the present study revealed that they may potentiate the development of physical dependence on benzodiazepines. Based on these findings, since co-administration of buspirone or ondansetron with benzodiazepines potentiates the development of physical dependence on benzodiazepines, buspirone and ondansetron should be avoided in benzodiazepine users, even if there is a possibility of potentiating the anxiolytic effects of the benzodiazepines.

In conclusion, the present results suggest that co-administration of buspirone or ondansetron with diazepam potentiates the development of physical dependence on diazepam, and that regulation of serotonergic neurons through 5-HT_{1A} and 5-HT_3 receptors may be involved in the development of this physical dependence.

CHAPTER 3

EFFECTS OF Ca^{2+} CHANNEL BLOCKERS ON PHYSICAL DEPENDENCE ON BARBITAL AND DIAZEPAM IN RATS AND MICE

Introduction

Ca^{2+} channel blockers are widely used as vasodilators and antiarrhythmic agents. Specific binding sites for these drugs have been found in the periphery and the brain (Gould et al., 1985), and recently effects of these drugs on the central nervous system have been reported (Pucilowski, 1992; Rogawski and Porter, 1990; Shibuya and Watanabe, 1992). Several studies suggested that Ca^{2+} channel blockers possess some central depressing effects such as anticonvulsant (Czuczwar et al., 1990a; 1990b; De Sarro et al., 1988; Desmedt et al., 1976; Dolin et al., 1988), ataxic (Johnston et al., 1986) and anxiolytic effects (Akaike et al., 1991).

On the other hand, acute treatments with barbiturates (Blaustein and Ector, 1975; Elrod and Leslie, 1980; Friedman et al., 1979), ethanol (Farrar et al., 1989; Friedman et al., 1980; Harris and Hood, 1980; Leslie et al., 1983; Stokes and Harris, 1982) and benzodiazepines (Leslie et al., 1980; 1986; Taft and DeLorenzo, 1984) decrease Ca^{2+} influx or Ca^{2+} uptake, suggesting that central depressing effects of these drugs may result from reduction of central Ca^{2+} concentration (Friedman et al., 1979; 1980; Leslie et al., 1980; 1986). It is known that Ca^{2+} channel blockers potentiate the anesthetic effects of ethanol, pentobarbital and midazolam (Dolin and Little, 1986; Dolin et al., 1991), the anticonvulsant effect of phenobarbital (Czuczwar et al., 1990a; 1990b), the hypothermia induced by ethanol and diazepam (Draski et al., 1985; Isaacson et al., 1985), and the motor incoordination by ethanol, midazolam

and clonazepam (Dolin et al., 1991; Isaacson et al., 1985). Thus, Ca^{2+} channel blockers seem to potentiate the central depressing effects of central depressants that affect $GABA_A$ · benzodiazepine receptor / Cl^- channel complex.

Chronic administration of barbiturates, ethanol and benzodiazepines develops physical dependence and tolerance (Suzuki, 1990; Suzuki et al., 1992a; 1992b; Woods et al., 1992). Administration of Ca^{2+} channel blockers, especially dihydropyridines, during chronic ethanol treatment prevents development of tolerance to and physical dependence on ethanol (Dolin and Little, 1989; Whittington et al., 1991; Wu et al., 1987). Furthermore, Ca^{2+} channel blockers suppress ethanol withdrawal signs, when these drugs were administered systemically to ethanol–dependent rats at the termination of ethanol treatment (Little et al., 1986; Littleton et al., 1990). Therefore, it is possible that Ca^{2+} channel blockers may be used to suppress withdrawal signs of barbiturates or benzodiazepines, and may be used concurrently with barbiturates or benzodiazepines to potentiate the central depressing effects of these drugs, such as the anticonvulsant or anxiolytic effects, and to reduce the physical dependence and tolerance liability by decreasing the required dosage of these drugs. However, the effects of Ca^{2+} channel blockers on physical dependence on barbiturates and benzodiazepines, especially on the development of physical dependence on these central depressants, have been scarcely investigated.

In the present study, the effects of three Ca^{2+} channel blockers, flunarizine, nifedipine and diltiazem on the development of physical dependence on barbital and diazepam, and on the barbital withdrawal signs were studied. It is well known that nifedipine and diltiazem are L–type Ca^{2+} channel sensitive blockers, while flunarizine is a T–type Ca^{2+} channel sensitive blocker (Akaike et al., 1989; Louvel et al., 1986;

Pucilowski, 1992; Rogawski and Porter, 1990; Shibuya and Watanabe, 1992; Takahashi and Akaike, 1991; Tygat et al., 1988; Wang et al., 1990). Moreover, flunarizine and nifedipine possess a property of high penetration into the brain (Shibuya and Watanabe, 1992), but diltiazem possesses a property of poor penetration (Pani et al., 1990; Shibuya and Watanabe, 1992). Therefore, the role of two types of Ca^{2+} channels in physical dependence on barbital and diazepam were discussed.

EXPERIMENT 3-1: Effects of Ca^{2+} channel blockers on the appearance of barbital withdrawal signs in rats

Materials and Methods

Animals

Male Sprague-Dawley rats (Tokyo Animal Laboratories Inc., Tokyo, Japan) that weighed 180-230 g at the beginning of the experiment were used. Animals were housed individually under the conditions as described in EXPERIMENT 1-1.

Development of Physical Dependence

As described in EXPERIMENT 1-2, barbital-admixed food was prepared. Each rat was fed the barbital-admixed food for 28 days, and could drink tap water *ad libitum*. The concentration of barbital in the food was gradually increased during the treatment according to EXPERIMENT 1-2. Body weight and food consumption were measured everyday at 16:00. Daily barbital intake was calculated according to EXPERIMENT 1-2.

Barbital Withdrawal

Withdrawal was conducted according to EXPERIMENT 1-2. Body weight was measured and withdrawal signs were observed after termination of the drug treatment. Changes in body weight after withdrawal were calculated according to EXPERIMENT 1-2. To quantify the intensity of physical dependence on barbital, a rating score for withdrawal signs, according to EXPERIMENT 1-2 with minor modifications, was used (Table 3-1). Withdrawal scores after the withdrawal were calculated according to EXPERIMENT 1-2. The withdrawal scores for 42 hr (from 18 hr to 60 hr) after the withdrawal are shown as total withdrawal scores.

Substitution of Calcium Channel Blockers

After the withdrawal from barbital, rats were treated with saline or diltiazem (20 and 40 mg/kg, i.p.) from 17 hr to 57 hr after the withdrawal at intervals of 4 hr, or vehicle or flunarizine (20, 40 and 80 mg/kg, i.p.) from 17 hr to 53 hr after the withdrawal at intervals of 6 hr.

Drugs

Barbital was purchased from Wako Pure Chemical Ind. (Tokyo, Japan). Diltiazem hydrochloride (Sigma Chemical Co., St. Louis, USA) was dissolved in saline, and flunarizine dihydrochloride (Sigma Chemical Co., St. Louis, USA) was dissolved in vehicle consisting of 9 % Tween 80 (Kishida Chemical Co., Osaka, Japan) in saline.

Statistical Analysis

Analysis for the incidence of withdrawal signs was performed using the chi–square (2 x 2) test. Analysis for the changes in body weight and withdrawal scores were performed by two factor (groups x times) repeated measures analysis of variance (ANOVA). All other analyses were carried out using the Student's t–test.

Results

The barbital intake was gradually increased during the treatment. The mean barbital intake at the final barbital concentration (6 mg/g of food) was 389.3 $\pm$ 6.9 mg/kg/day.

After termination of the barbital treatment, several withdrawal signs were observed (Table 3-1). The incidence of withdrawal signs during the substitution (from 18 hr to 60 hr after withdrawal) was affected by substitution of diltiazem or flunarizine. Flunarizine significantly suppressed the appearance of hematuria ($p<0.05$), tremor ($p<0.05$), handling-elicited convulsions ($p<0.05$) and spontaneous convulsions ($p<0.01$) as compared with vehicle treatment. On the other hand, diltiazem significantly suppressed handling-elicited convulsions ($p<0.05$) as compared with saline treatment, but did not affect other withdrawal signs.

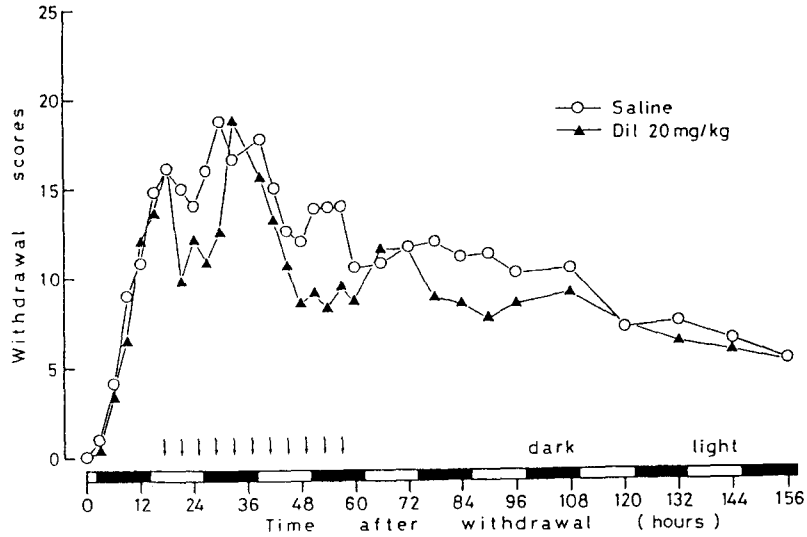
Figure 3-1A and 3-2A show the time course changes in withdrawal scores after the termination of barbital treatment. Substitution of flunarizine or diltiazem significantly suppressed withdrawal scores during the substitution (flunarizine 20 mg/kg: $F[1,150]=34.239$, $p<0.01$, flunarizine 40 mg/kg: $F[1,150]=39.673$, $p<0.01$, diltiazem 20 mg/kg: $F[1,150]=19.502$, $p<0.01$). While diltiazem partially suppressed the withdrawal scores during the substitution (Fig. 3-1A), flunarizine constantly suppressed the withdrawal scores (Fig. 3-2A). Figures 3-1B and 3-2B show the areas under the curve (AUC: scores x hr) of withdrawal scores during the substitution. The AUC of withdrawal scores during the substitution of flunarizine (20 mg/kg: 543 $\pm$ 32, $p<0.05$, 40 mg/kg: 511 $\pm$ 39, $p<0.05$) were significantly lower than that during the substitution of vehicle (775 $\pm$ 89) (Fig. 3-2B). However, there was no significant difference in the AUC of withdrawal scores during the substitution between

saline group (722 $\pm$ 22) and diltiazem 20 mg/kg group (593 $\pm$ 63) (Fig. 3-1B). On the other hand, total withdrawal scores during the substitution of flunarizine (20 and 40 mg/kg) were significantly lower than that during the substitution of vehicle ($p<0.01$) (Table 3-1). However, there was no significant difference in the total withdrawal scores during the substitution between saline group and diltiazem group (Table 3-1).

Figure 3-3 shows the time course changes in body weight loss after the barbital withdrawal. During the substitutions, weight loss was significantly reduced by flunarizine (20 mg/kg: $F[1,150]=28.179$, $p<0.01$, 40 mg/kg: $F[1,150]=20.168$, $p<0.01$), but not by diltiazem.

More than half of the barbital withdrawn rats died in diltiazem 40 mg/kg or flunarizine 80 mg/kg substitution group with showing intraperitoneal hemorrhage, but naive rats did not. As a result, these dosage of diltiazem or flunarizine may be toxic dosage for barbital withdrawn rats.

<A>



<B>

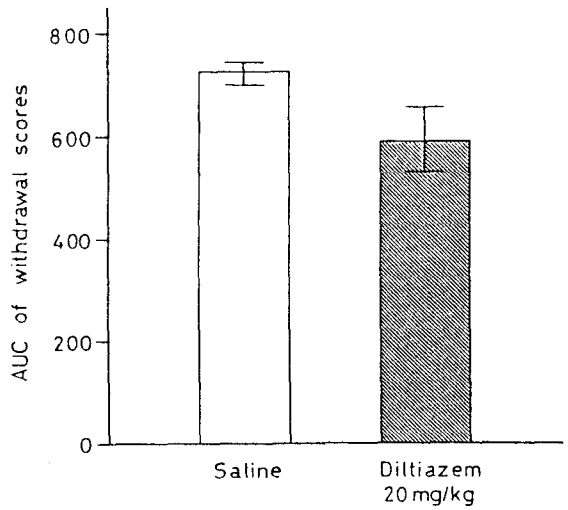


Fig. 3-1
Effect of substitution of saline or diltiazem (Dil) on withdrawal scores after withdrawal from barbital treatment. <A> Time course changes in withdrawal scores after the withdrawal. Each point represents the mean of 6 observations. Each arrow represents the treatment with saline (1 ml/kg, i.p.) or diltiazem (20 mg/kg, i.p.). Withdrawal scores during the substitution were significantly suppressed by substitution of diltiazem 20 mg/kg ($F[1,150]= 19.502$, $p<0.01$). <B> Area under the curve of withdrawal scores during the substitution. Each column represents the mean $\pm$ SEM of 6 observations.

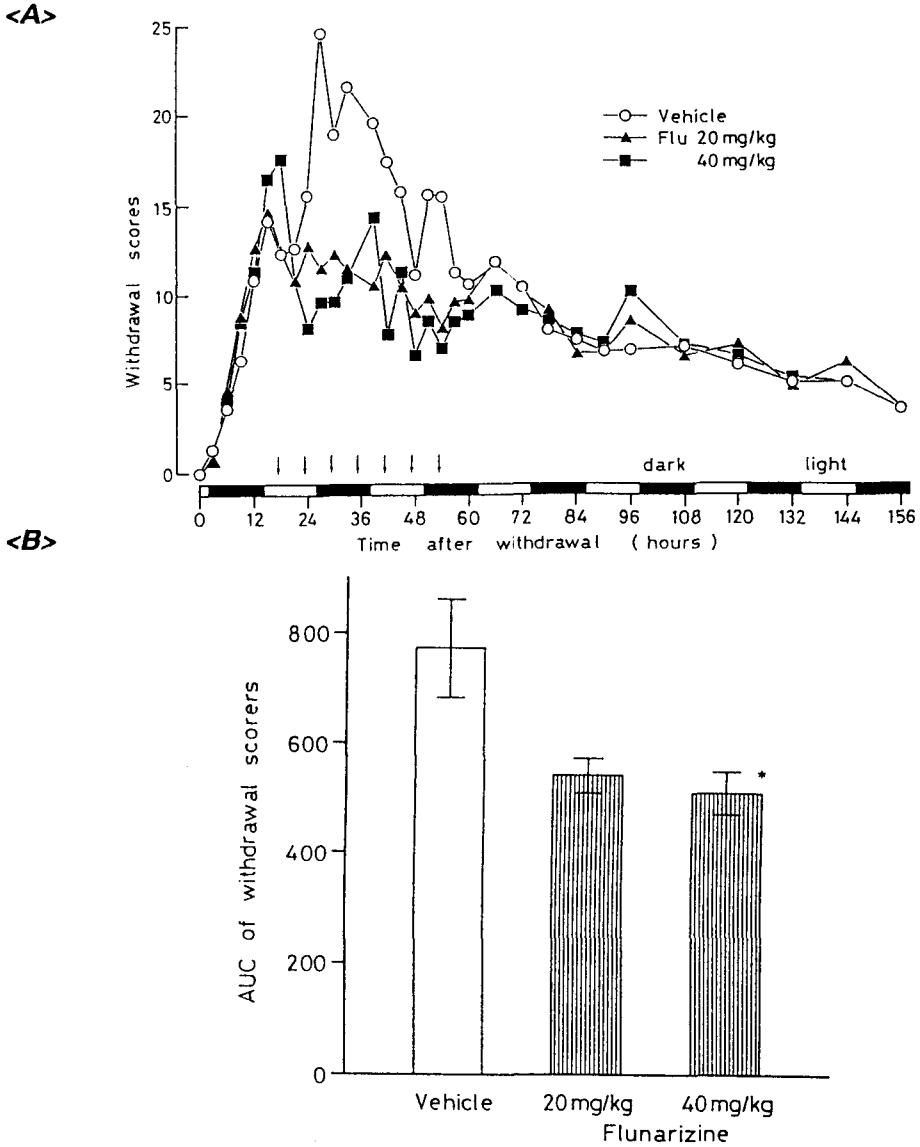


Fig. 3-2

*Effect of substitution of vehicle or flunarizine (Flu) on withdrawal scores after withdrawal from barbital treatment. <A> Time course changes in withdrawal scores after the withdrawal. Each point represents the mean of 6 observations. Each arrow represents the treatment with vehicle (10 ml/kg, i.p.), or flunarizine (20 or 40 mg/kg, i.p.). Withdrawal scores during the substitution were significantly suppressed by substitution of flunarizine (20 mg/kg: $F[1,150]=34.239$, $p<0.01$, 40 mg/kg: $F[1,150]=39.673$, $p<0.01$). <B> Area under the curve of withdrawal scores during the substitution. Each column represents the mean $\pm$ SEM of 6 observations. *$p<0.05$ vs. vehicle sbstitution group.*

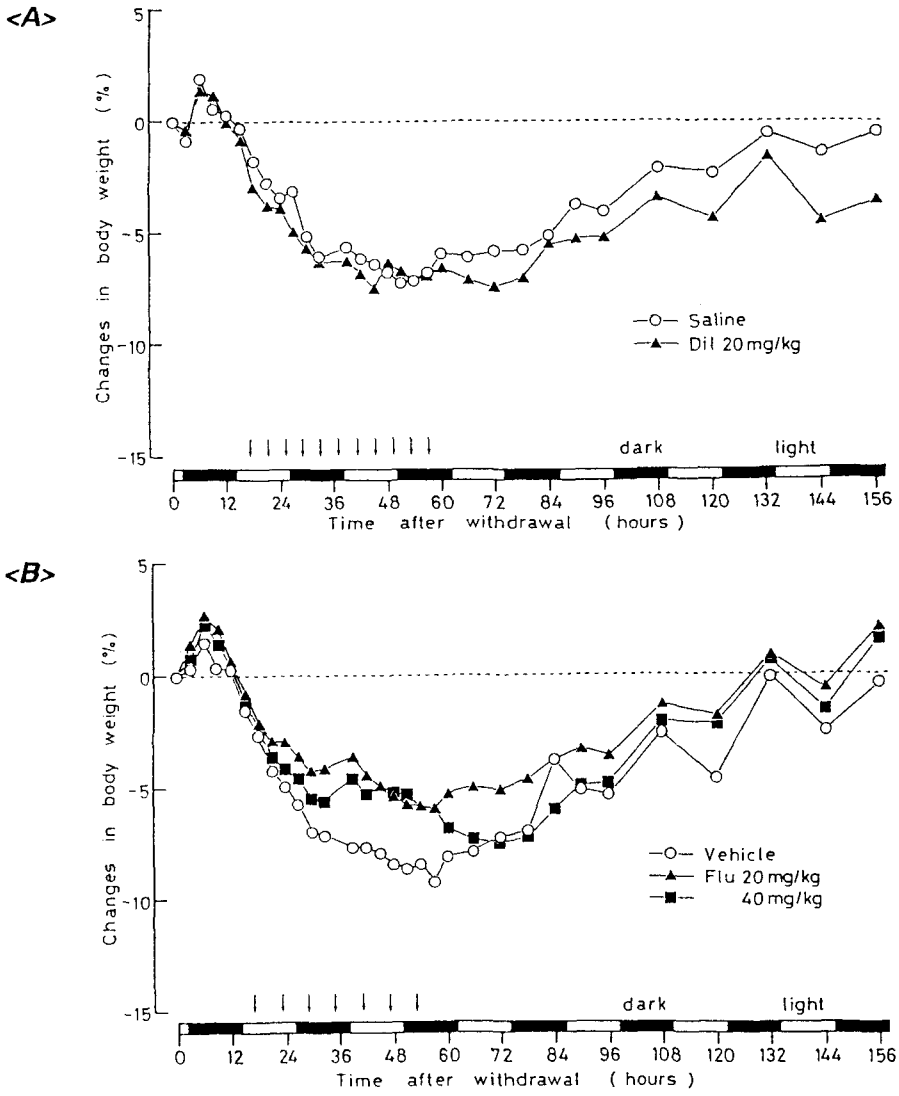


Fig. 3-3

Time course changes in body weight loss (%) after withdrawal from barbital treatment. Each point represents the mean of 6 observations. <A> Effect of substitution of saline or diltiazem (Dil). Each arrow represents the treatment with saline (1 ml/kg, i.p.) or diltiazem (20 mg/kg, i.p.). <B> Effect of substitution of vehicle or flunarizine (Flu). Each arrow represents the treatment with vehicle (1 ml/kg, i.p.), or flunarizine (20 or 40 mg/kg, i.p.). Body weight loss during the substitution was significantly reduced by flunarizine (20 mg/kg: $F[1,150]=28.179$, $p<0.01$, 40 mg/kg: $F[1,150]=20.168$, $p<0.01$).

Table 3-1
Behavioral withdrawal signs during the substitution of diltiazem and flunarizine (from 18 hr to 60 hr) after the termination of barbital treatment in rats

Withdrawal signs (scores)	Positive animals / Total number of animals				
	Saline	Diltiazem 20 mg/kg	Vehicle	Flunarizine 20 mg/kg	Flunarizine 40 mg/kg
Weight loss					
5–10 % (1)	3/6	3/6	1/6	4/6	6/6
10–15 % (2)	2/6	3/6	3/6	1/6	0/6
15 % < (3)	0/6	0/6	1/6	0/6	0/6
Piloerection (2)	6/6	6/6	6/6	6/6	6/6
Vocalization (2)	6/6	6/6	5/6	6/6	6/6
Irritability (2)	6/6	6/6	6/6	6/6	6/6
Aggression (2)	3/6	2/6	1/6	0/6	0/6
Diarrhea (2)	0/6	2/6	1/6	0/6	0/6
Teeth-chattering (2)	5/6	3/6	4/6	3/6	2/6
Muscle rigidity (2)	6/6	6/6	6/6	6/6	6/6
Straub tail (2)	6/6	6/6	6/6	6/6	6/6
Ear-twitch (2)	6/6	6/6	6/6	6/6	6/6
Lacrimation (3)	1/6	0/6	0/6	0/6	0/6
Nose-bleed (3)	2/6	4/6	5/6	4/6	2/6
Dysuria (3)	1/6	4/6	5/6	2/6	2/6
Hematuria (3)	0/6	2/6	4/6	1/6	0/6 *
Fascicular-twitch (3)	6/6	6/6	6/6	6/6	6/6
Jerk (3)	6/6	6/6	6/6	4/6	6/6
Tremor (3)	6/6	5/6	6/6	3/6 *	5/6
Convulsion					
Handling-elicited (3)	4/6	0/6 #	5/6	2/6	1/6 *
Spontaneous (4)	1/6	2/6	5/6	0/6 **	3/6
Death (4)	0/6	0/6	0/6	0/6	0/6
Total withdrawal scores	29.5 ± 1.5	30.7 ± 1.5	37.2 ± 2.6	25.0 ** ± 2.5	26.7 ** ± 1.6

#$p<0.05$ vs. saline substitution group.
*$p<0.05$, **$p<0.01$ vs. vehicle substitution group.

EXPERIMENT 3-2: Effects of Ca^{2+} channel blockers on the development of physical dependence on barbital in rats

Materials and Methods

Animals

Male Sprague-Dawley rats (Tokyo Animal Laboratories Inc., Tokyo, Japan) that weighed 180-230 g at the beginning of the experiment were used. Animals were housed individually under the conditions as described in EXPERIMENT 1-1.

Development of Physical Dependence

As described in EXPERIMENT 1-2, barbital alone-, barbital+diltiazem- and barbital+flunarizine-admixed foods were prepared. Each rat was fed any of drugs-admixed foods for 28 days and could drink tap water *ad libitum.* The concentration of barbital in the food was gradually increased during the treatment according to EXPERIMENT 1-2. The concentrations of diltiazem and flunarizine in the food were fixed at 0.75 and 1.5 mg/g of food, respectively. Body weight and food consumption were measured everyday at 16:00. Daily barbital intake was calculated according to EXPERIMENT 1-2.

Motor Incoordination

Motor incoordination during the drug treatments, was measured for 3 min using rotarod performance apparatus (9 cm in diameter, 7.5 rpm; Natsume Seisakusho Co., Tokyo, Japan). Each rat was trained to run on a rotarod until it could remain there for 3 min without falling, before the drug treatments. The rotarod performance test was carried out every other day.

Barbital Withdrawal

Withdrawal was conducted according to EXPERIMENT 1-2. Body weight was measured and withdrawal signs were observed after the withdrawal from barbital treatment. Changes in body weight and withdrawal scores after withdrawal were calculated according to EXPERIMENT 3-1.

Plasma Concentrations of Barbital after the Withdrawal

Plasma concentrations of barbital were measured 4 times at 8 hr intervals after the withdrawal. Rats were drawn blood samples of 400 μl from a tail caudal vein using hematocrit tubes (100 μl, Drummond Scientific Co., Pa., USA). Blood samples of 400 μl were centrifuged at 5,000 rpm for 10 min and then plasma samples of 100 μl were separated. The plasma concentrations of barbital were analyzed by high-performance liquid chromatography according to the method of Kabra et al. (1977) with minor modifications.

Drugs

Barbital was purchased from Wako Pure Chemical Ind. (Tokyo, Japan), and diltiazem hydrochloride and flunarizine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, USA).

Statistical Analysis

Analysis for the changes in motor incoordination, body weight and withdrawal scores was performed by two factor (groups x times) repeated measures analysis of variance (ANOVA). All other analyses were carried out using the Student's t-test.

Results

There was no significant difference in daily barbital intake during barbital treatment between the barbital alone and barbital+diltiazem or barbital+flunarizine groups. The mean barbital intakes in the barbital alone, barbital+diltiazem and barbital+flunarizine groups at the final barbital concentration (6 mg/g of food) were 389.3 $\pm$ 6.9, 374.2 $\pm$ 13.6 and 374.6 $\pm$ 18.7 mg/kg/day, respectively. During the treatment, motor incoordination gradually increased in a barbital concentration-dependent manner (Fig. 3-4). Barbital-induced motor incoordination during the treatment was significantly potentiated by co-administration of flunarizine ($F[1,240]= 19.634$, $p<0.01$), but not by co-administration of diltiazem.

After termination of the barbital treatment, several withdrawal signs were observed, as described in EXPERIMENT 3-1. Figure 3-5A shows the time course changes in withdrawal scores after the termination of barbital treatment. Withdrawal scores were significantly suppressed by co-administration of flunarizine ($F[1,168]= 20.425$, $p<0.01$), but not by co-administration of diltiazem. Moreover, the AUCs of withdrawal scores were 1,520 $\pm$ 87 for barbital alone, 1,535 $\pm$ 140 for barbital+diltiazem and 1,126 $\pm$ 94 for barbital+flunarizine groups (Fig. 3-5B). The AUC of withdrawal scores in barbital+flunarizine group was significantly lower than that in barbital alone group ($p<0.01$).

As shown in Fig. 3-6, body weight decreased after termination of the barbital treatment. The maximum weight loss was 9.49 $\pm$ 1.20 % at 51 hr after the withdrawal in the barbital alone group, 8.55 $\pm$ 1.53 % at 66 hr after the withdrawal in the barbital+ diltiazem group and 7.23 $\pm$ 2.45 % at 45 hr after the withdrawal in the barbital+ flunarizine group. Weight loss after the withdrawal was significantly reduced by co-

administration of flunarizine ($F[1,168]=20.074$, $p<0.01$), but not by co-administration of diltiazem.

Plasma concentrations of barbital after withdrawal are shown in Fig. 3-7. There were no significant differences in plasma barbital concentrations among groups.

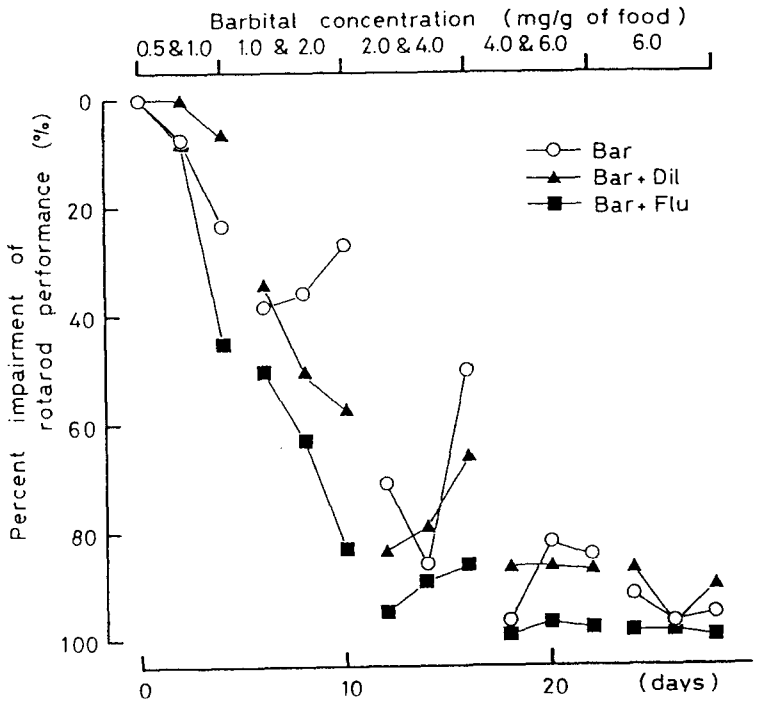


Fig. 3-4
Motor incoordination (%) during the treatment with barbital (Bar) alone, barbital+diltiazem (Dil; 0.75 mg/g of food) or barbital+flunarizine (Flu; 1.5 mg/g of food). Each point represents the mean of 8 observations. Motor incoordination during the treatment was significantly potentiated by co-administration of flunarizine ($F[1,240]=19.634$, $p<0.01$).

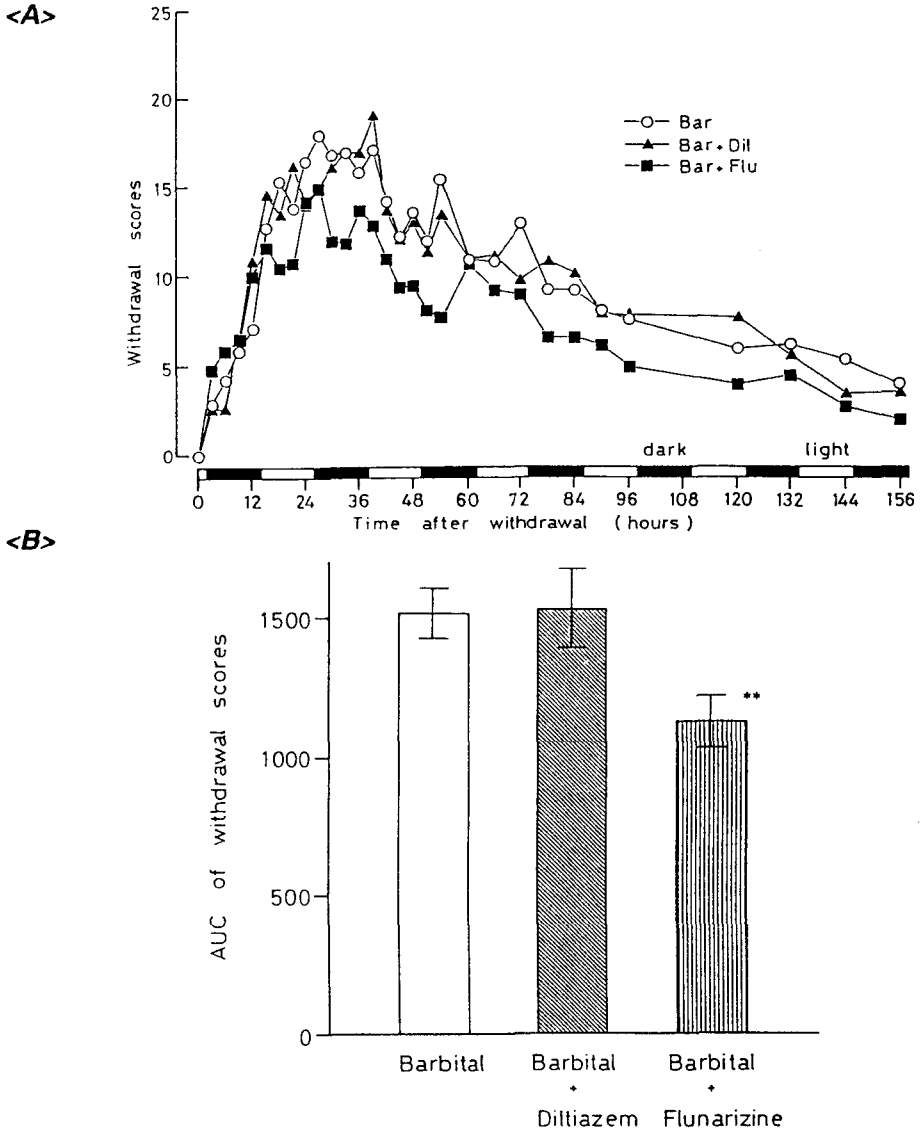


Fig. 3-5
*Withdrawal scores after the withdrawal from treatment with barbital alone, barbital+diltiazem (Dil; 0.75 mg/g of food) or barbital+flunarizine (Flu; 1.5 mg/g of food). <A> Time course changes in withdrawal scores after the withdrawal. Each point represents the mean of 8 observations. Withdrawal scores were significantly suppressed by co-administration of flunarizine ($F[1,168]=20.425$, $p<0.01$). <B> Area under the curve of withdrawal scores after the withdrawal. Each column represents the mean $\pm$ SEM of 8 observations. *$p<0.05$ vs. barbital alone group.*

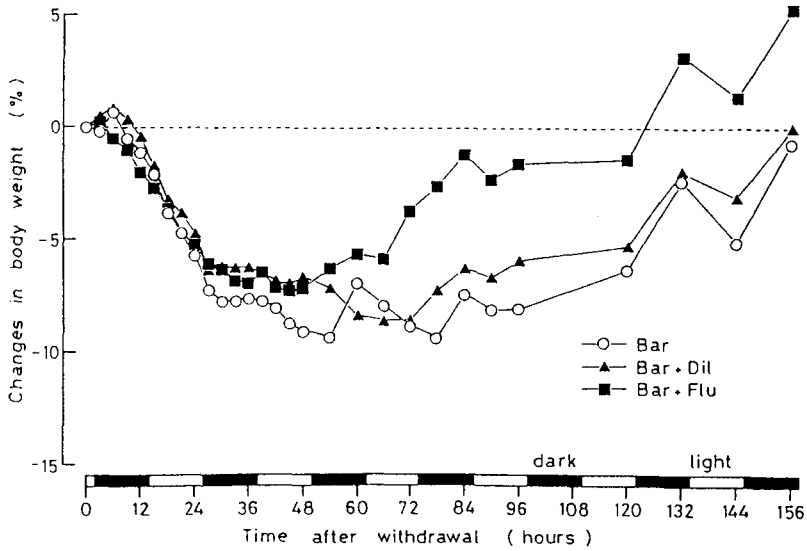


Fig. 3-6
Time course change in body weight loss (%) after the withdrawal from treatment with barbital alone, barbital+diltiazem (Dil; 0.75 mg/g of food) or barbital+flunarizine (Flu; 1.5 mg/g of food). Each point represents the mean of 8 observations. Body weight loss was significantly reduced by co-administration of flunarizine ($F[1,168]=20.074$, $p<0.01$).

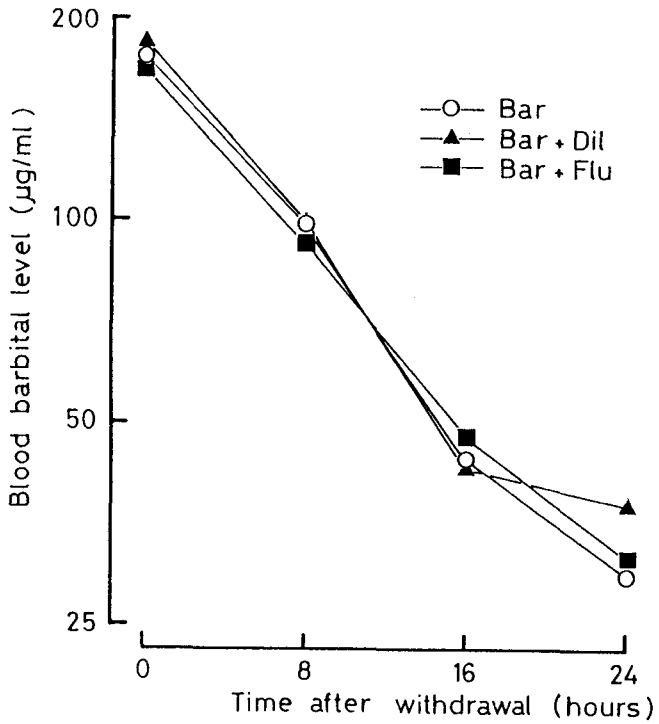


Fig. 3-7
Time course change in plasma concentration of barbital after the withdrawal from treatment with barbital alone, barbital+diltiazem (Dil; 0.75 mg/g of food) or barbital+flunarizine (Flu; 1.5 mg/g of food). Blood samples were drawn from the femoral artery at 0, 8, 16 and 24 hr after the withdrawal. Each point represents the mean of 5-6 observations.

EXPERIMENT 3-3: Effects of Ca^{2+} channel blockers on the development of physical dependence on diazepam in mice

Materials and Methods

Animals

Male ICR mice (Charles River Japan Inc., Atsugi, Japan) that weighed 25–30 g at the beginning of the experiment, were used. Animals were housed in groups of 5 under the conditions as described in EXPERIMENT 1-1.

FG 7142-induced Convulsions

Mice were treated with vehicle (10 ml/kg, i.p.)+vehicle, diazepam (16 mg/kg, i.p.)+vehicle, diazepam+nifedipine (10, 20 and 40 mg/kg, i.p.), diazepam+diltiazem (5, 10 and 20 mg/kg, i.p.) or diazepam+flunarizine (10, 20 and 40 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment with drugs, mice were treated with FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p.), and then clonic-tonic convulsions induced by FG 7142 were observed for 30 min after the injection.

Drugs

Diazepam (Profarma Co., Italy), FG 7142 (Research Biochemicals Inc., MA, USA) and nifedipine (Sigma Chemical Co., St. Louis, USA) were suspended, and flunarizine dihydrochloride (Sigma Chemical Co., St. Louis, USA) was dissolved in vehicle consisting of 9 % Tween 80 (Kishida Chemical Co., Osaka, Japan) in saline. Diltiazem hydrochloride (Sigma Chemical Co., St. Louis, USA) was dissolved in saline.

Statistical Analysis

ED_{50} values and their 95 % confidence limits of FG 7142 for clonic-tonic convulsions were calculated by the method of Litchfield and Wilcoxon (1949). Statistical analysis for the comparison between ED_{50} values was carried out by analyzing the potency ratio. This analysis was performed according to a statistical program (Program 47 of the Pharmacologic Calculations System) (Tallarida and Murray, 1987).

Results

Chronic treatment with diazepam did not cause any mortality or deterioration in the condition of the animals. No convulsions were observed in chronically vehicle+vehicle-treated mice which just received FG 7142. On the other hand, mice chronically treated with diazepam showed dose-dependent clonic-tonic convulsions following administration of FG 7142 (Fig. 2-1).

The incidence of the clonic-tonic convulsions induced by FG 7142 40 mg/kg after the chronic treatment with diazepam in combination with Ca^{2+} channel blockers is shown in Fig. 3-8. The incidence of the clonic-tonic convulsions in the group that had been treated with diazepam+vehicle was 54.05 %. In contrast, the incidences of clonic-tonic convulsions in the diazepam+flunarizine 40 mg/kg and diazepam+nifedipine 40 mg/kg groups were 41.17 and 43.75 %, respectively (Fig. 3-8A, B). The incidence of clonic-tonic convulsions tended to be suppressed by co-administration of either flunarizine or nifedipine. On the other hand, the incidence of clonic-tonic convulsions was not affected by co-administration of diltiazem (Fig. 3-8C). Figure 3-9 shows dose-response lines of FG 7142 for clonic-tonic convulsions. Dose-response line of FG 7142 was shiffted toward the right by co-administration of flunarizine 40 mg/kg, but not by co-administration of nifedipine 40 mg/kg. ED_{50} values of FG 7142 for clonic-tonic convulsions in the diazepam+flunarizine 40 mg/kg and diazepam+nifedipine 40 mg/kg groups were 59.37 (39.82-88.51) and 45.28 (22.47-91.25) mg/kg, respectively. The potency ratio of the ED_{50} values of FG 7142 for the diazepam+vehicle and diazepam+flunarizine 40 mg/kg groups was 1.74 (1.06-2.84), which indicates that FG 7142-induced clonic-tonic convulsion was significantly suppressed by the co-administration of flunarizine (Table 3-2, $p<0.05$). However, the potency ratio of the ED_{50} values of FG 7142 for the diazepam+vehicle

and diazepam+nifedipine 40 mg/kg groups was 1.33 (0.62–2.82), suggesting that the co-administration of nifedipine did not affect FG 7142-induced clonic-tonic convulsion (Table 3–2).

Table 3-2
Effects of chronic co-administration of Ca^{2+} channel blockers with diazepam on convulsive threshold of FG 7142

Treatment	*ED_{50} of FG 7142 (95 % CL) mg/kg, i.p.*	*Potency ratio (95 % CL)*
Diazepam 16 mg/kg, i.p.		
+ vehicle	*34.16*	
10 ml/kg, i.p.	*(25.69-45.43)*	
+ flunarizine	*59.37*	*1.74 **
40 mg/kg, i.p.	*(39.82-88.51)*	*(1.06-2.84)*
+ nifedipine	*45.28*	*1.33*
40 mg/kg, i.p.	*(22.47-91.25)*	*(0.62-2.82)*

*Mice were treated with either diazepam (16 mg/kg, i.p.)+vehicle (10 ml/kg, i.p.), diazepam+flunarizine (40 mg/kg, i.p.) or diazepam+nifedipine (40 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with several doses of FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p. in the diazepam+vehicle group, and 14, 20, 40, 56 and 80 mg/kg, i.p. in diazepam+flunarizine or diazepam+nifedipine groups), and clonic-tonic convulsions were observed for 30 min. ED_{50} values and their 95 % confidence limits (CL) were calculated from the incidence of clonic-tonic convulsions. *$p<0.05$ vs. the diazepam+vehicle group.*

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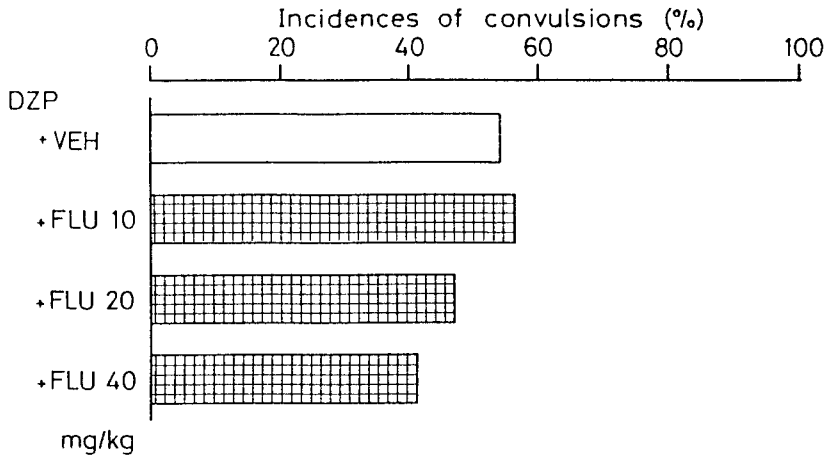
Incidences of convulsions (%)
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40
60
80
100
DZP
+VEH
+FLU 10
+FLU 20
+FLU 40
mg/kg

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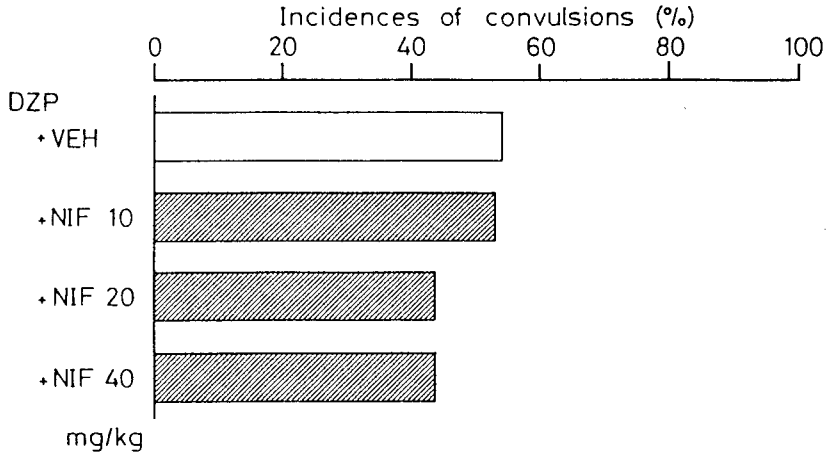
Incidences of convulsions (%)
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20
40
60
80
100
DZP
+VEH
+NIF 10
+NIF 20
+NIF 40
mg/kg

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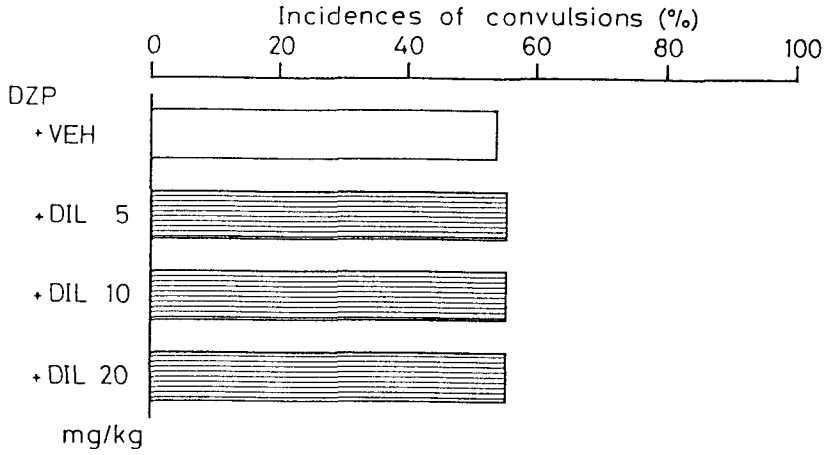

Fig. 3-8
Effects of chronic co-administration of Ca^{2+} channel blockers with diazepam (DZP) on FG 7142-induced clonic-tonic convulsions. Mice were treated with diazepam (16 mg/kg, i.p.)+vehicle (10 ml/kg, i.p.), <A> diazepam+flunarizine (FLU; 10, 20 and 40 mg/kg, i.p.), <B> diazepam+nifedipine (NIF; 10, 20 and 40 mg/kg, i.p.) or <C> diazepam+diltiazem (DIL; 5, 10 and 20 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with FG 7142 (40 mg/kg, i.p.), and clonic-tonic convulsions were observed for 30 min. Each column represents the incidence of clonic-tonic convulsions calculated from 9-37 observations.

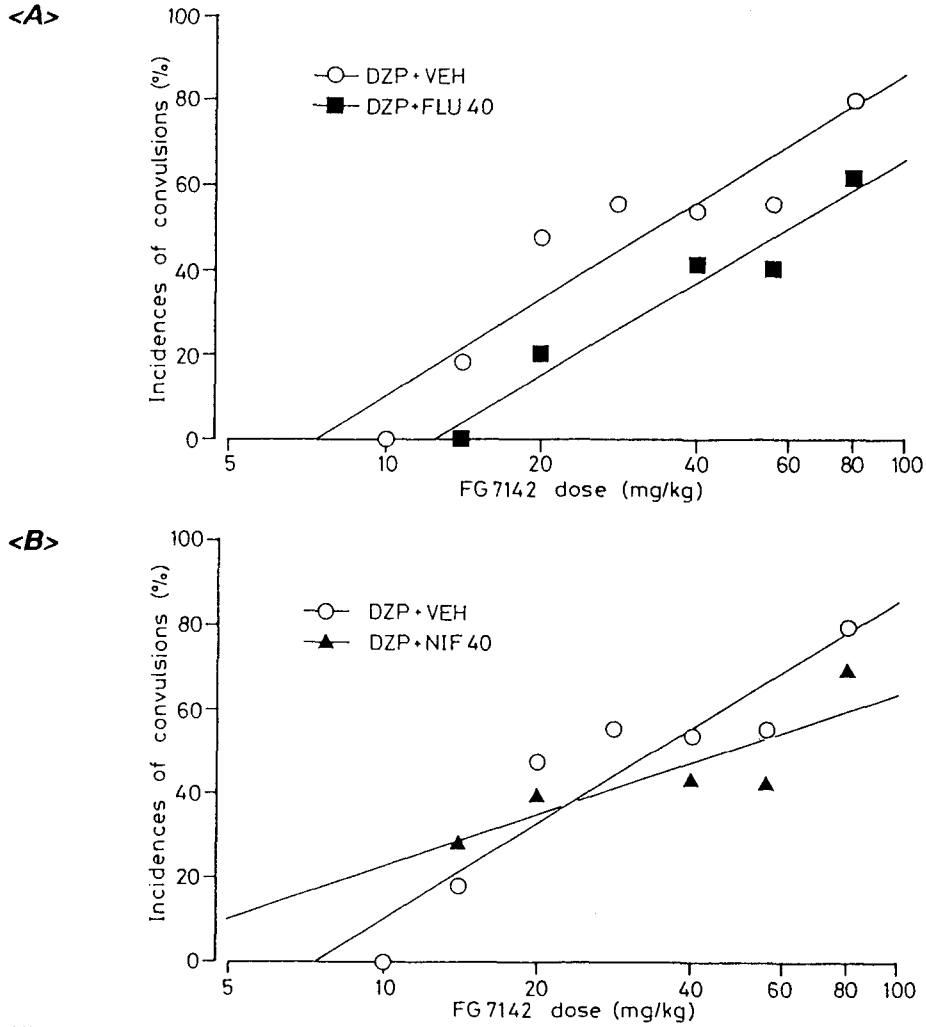

Fig. 3-9

Dose-response lines of FG 7142 for clonic-tonic convulsions after the chronic treatment with <A> diazepam (DZP)+flunarizine (FLU) or <B> diazepam+nifedipine (NIF). Mice were treated with diazepam (16 mg/kg, i.p.)+vehicle (10 ml/kg, i.p.), diazepam+flunarizine (40 mg/kg, i.p.) or diazepam+nifedipine (40 mg/kg, i.p.), once a day for 7 days. Twenty-four hr after the last treatment, mice were treated with several doses of FG 7142 (10, 14, 20, 28, 40, 56 and 80 mg/kg, i.p. in diazepam+vehicle group, and 14, 20, 40, 56 and 80 mg/kg, i.p. in diazepam+flunarizine or diazepam+nifedipine groups), and clonic-tonic convulsions were observed for 30 min. Each point represents the incidence of clonic-tonic convulsions calculated from 9-37 observations. ED_{50} value of FG 7142 for clonic-tonic convulsions in diazepam+vehicle, diazepam+flunarizine and diazepam+nifedipine groups were 34.16 (25.69-45.43), 59.37 (39.82-88.51) and 45.28 (22.47-91.25) mg/kg, respectively.

Discussion

Co-administration of flunarizine with barbital significantly potentiated barbital-induced motor incoordination, and suppressed development of physical dependence on and appearance of withdrawal signs of barbital. While, diltiazem did not affect the barbital-induced motor incoordination and the development of physical dependence on barbital. Moreover, the suppression of barbital withdrawal signs by diltiazem was partial and very weak. In addition, co-administration of flunarizine, but not of either nifedipine or diltiazem, suppressed the development of physical dependence on diazepam. It is well known that diltiazem penetrates poorly into the brain, as compared to either flunarizine or nifedipine (Pani et al., 1990; Shibuya and Watanabe, 1992). Therefore, L-type Ca^{2+} channels in the periphery may not be involved in the development of physical dependence on and appearance of withdrawal signs of these central depressants.

Benzodiazepines (Leslie et al., 1980; 1986; Taft and DeLorenzo, 1984), barbiturates (Blaustein and Ector, 1975; Elrod and Leslie, 1980; Friedman et al., 1979) and ethanol (Farrar et al., 1989; Friedman et al., 1980; Harris and Hood, 1980; Leslie et al., 1983; Stokes and Harris, 1982) decrease Ca^{2+} influx or uptake into brain synaptosomes, which suggests that the central depressing effects of these drugs may be related to the reduction of Ca^{2+} concentrations in brain synaptosomes (Elrod and Leslie, 1980; Friedman et al., 1979; 1980; Leslie et al., 1980; 1986). Ca^{2+} channel blockers are known to potentiate the central depressing effects of these drugs (Czuczwar et al., 1990a; 1990b; Dolin and Little, 1986; Dolin et al., 1991; Draski et al., 1985; Isaacson et al., 1985). In fact, in the present study, co-administration of flunarizine potentiated the central depressing effect (motor incoordination) of barbital. It is believed that these potentiation by Ca^{2+} channel

blockers may result from further reduction of Ca^{2+} concentrations in brain synaptosomes (Czuczwar et al., 1990a; 1990b; Dolin and Little, 1986; Dolin et al., 1991; Draski et al., 1985; Isaacson et al., 1985).

In general, it is known that the severity of physical dependence on sedative hypnotics such as barbiturates, ethanol and benzodiazepines, relates to the magnitude of central depression during treatment (Suzuki, 1990; Tagashira et al., 1978; 1979). For example, Tagashira et al. (1981) reported that increase in magnitude of central depression by combination of a dependence liability-free drugs (such as chlorpromazine) during the phenobarbital treatment, induced the potentiation of the development of physical dependence on phenobarbital. On the contrary, in the present study, although co-administration of flunarizine potentiated the barbital-induced motor incoordination, it suppressed the development of physical dependence on barbital and diazepam. The potentiating effect of chlorpromazine on central depression by and the development of physical dependence on phenobarbital, may result from the inhibition of monoamine re-uptakes and changes in activities of monoamine-related neurons by chlorpromazine (Tagashira et al., 1981). Furthermore, it has been suggested that the potentiating effects of Ca^{2+} channel blockers on the central depression of these sedative hypnotics may be ascribable to further reduction of central Ca^{2+} concentration (Czuczwar et al., 1990a; 1990b; Dolin and Little, 1986; Dolin et al., 1991; Draski et al., 1985; Isaacson et al., 1985). On the other hand, it is known that tolerance to and physical dependence on sedative hypnotics may well relate to changes in regulation of central Ca^{2+} channels (Dolin and Little, 1989; Elrod and Leslie, 1980; Farrar et al., 1989; Friedman et al., 1980; Harris and Hood, 1980; Leslie et al., 1980; 1983; Little et al., 1986; Littleton et al., 1990; Wu et al., 1987). Therefore, the suppressing effect of flunarizine on the development of physical dependence on barbital and diazepam

may result from changes in regulation of central Ca^{2+} channel rather than from changes in central Ca^{2+} concentration.

Boisse and Okamoto (1978) reported that barbiturate withdrawal signs may be inversely related to residual blood barbiturate concentrations. There is a possibility that co-administration of flunarizine affects the disappearance rate of barbital. Therefore, plasma concentrations of barbital after the withdrawal were mesured. However, there were no differences in plasma concentrations of barbital after the withdrawal between barbital alone and barbital+flunarizine groups. These results suggest that the suppression of development of physical dependence on barbital by flunarizine is not ascribable to a pharmacokinetic interaction.

Chronic treatment with central depressants such as ethanol, barbiturates and benzodiazepines, develops tolerance and physical dependence, and causes an up-regulation of central Ca^{2+} channel and/or an increase in central Ca^{2+} influx in brain synaptosomes (Dolin and Little, 1989; Elrod and Leslie, 1980; Farrar et al., 1989; Friedman et al., 1980; Harris and Hood, 1980; Leslie et al., 1980; 1983; Little et al., 1986; Littleton et al., 1990; Wu et al., 1987). Withdrawal signs of these drugs have been believed to result from increases in transmitter release that are evoked by supersensitivity of the nerve terminals to Ca^{2+} (Chugh et al., 1992; Dolin et al., 1990; Little et al., 1986; Littleton et al., 1990). Ca^{2+} channel blockers may suppress this evoked increase in transmitter release, and thus suppress the signs of withdrawal from these drugs (Chugh et al., 1992; Dolin et al., 1990; Little et al., 1986; Littleton et al., 1990). Moreover, nitrendipine prevents the development of tolerance to and physical dependence on ethanol by preventing the up-regulation of dihydropyridine binding sites in the brain (Dolin and Little, 1989; Whittington et al., 1991). In addition, chronic treatment with some Ca^{2+} channel blockers, e.g. nifedipine, verapamil, etc.

induces a down-regulation of central Ca^{2+} channel (Panza et al., 1985). Based on these findings, it is believed that up-regulation of Ca^{2+} channels in the brain induced by chronic treatment with these central depressants may be involved in the development of tolerance to, physical dependence on, and the appearance of signs of withdrawal from these drugs. In particular, L-type Ca^{2+} channels in the brain may be involved in the tolerance to and physical dependence on ethanol (Dolin and Little, 1989; Whittington et al., 1991). In the present study, co-administration of flunarizine significantly suppressed the development of physical dependence on diazepam and barbital, but co-administration of nifedipine did not affect the development of physical dependence on diazepam. Suzuki et al. (1990) reported that co-administration of nifedipine did not suppress the development of physical dependence on barbital. Moreover, Dolin et al. (1990) reported that co-administration of nitrendipine does not suppress the development of tolerance to and physical dependence on benzodiazepines. These findings and the present results suggest that the developments of tolerance to and physical dependence on benzodiazepines and barbiturates are suppressed by co-administration of a T-type Ca^{2+} channel sensitive blocker, but not of L-type Ca^{2+} channel sensitive blockers, and that the up-regulation of T-type Ca^{2+} channels in the brain, but not of L-type Ca^{2+} channels, may be involved in tolerance to and physical dependence on benzodiazepines and barbiturates.

Benzodiazepines and barbiturates bind to each binding site in the $GABA_A$ · benzodiazepine receptor / Cl^- channel complex, and increase Cl^- influx in the brain (Allan et al., 1992a; 1992b; Woods et al., 1992). Consequently, benzodiazepines and barbiturates show central depressing effects. Chronic treatment with benzodiazepines and barbiturates develops tolerance and causes the subsensitivity to benzodiazepine and barbiturates-elicited Cl^- influx in the brain (Allan et al., 1992a;

1992b; Miller et al., 1988; Woods et al., 1992). On the other hand, after the termination of chronic treatment with benzodiazepines or barbiturates, a supersensitivity to benzodiazepine partial inverse agonists, i.e., β-carboline analogues (FG 7142, β-CCE, β-CCM, etc.), and Cl^- channel negative modulators, e.g. pentylenetetrazol, picrotoxin, has been observed (Lister and Nutt, 1986; Little, 1988; Little et al., 1988; 1992; Nutt and Costello, 1988; Tagashira et al., 1982b; Woods et al., 1992). Moreover, the decrease in the Cl^- influx in the brain by FG 7142 is potentiated following the termination of chronic treatment with benzodiazepines (Allan et al., 1992). These changes in the Cl^- influx in the brain following the chronic treatment with benzodiazepines or barbiturates may be directly involved in the development of physical dependence on and the appearance of withdrawal signs of benzodiazepines and barbiturates. In the present study, co-administration of flunarizine, but not of nifedipine, suppressed the development of physical dependence on diazepam and barbital. Therefore, changes in the sensitivity of T-type Ca^{2+} channels in the brain, rather than L-type Ca^{2+} channels, may be related to the changes in the Cl^- influx in the brain following chronic treatment with benzodiazepines or barbiturates.

In the present study, flunarizine markedly suppressed the barbital withdrawal signs, especially convulsions. Flunarizine selectively blocks T-type Ca^{2+} channel (Akaike et al., 1989; Louvel et al., 1986; Pucilowski, 1992; Rogawski and Porter, 1990; Shibuya and Watanabe, 1992; Takahashi and Akaike, 1991; Tygat et al., 1988; Wang et al., 1990). Phenytoin, an antiepileptic drug and a selective T-type Ca^{2+} channel and Na^+ channel blocker (Coulter et al., 1989; Rogawski and Porter, 1990; Takahashi et al., 1989; Yaari et al., 1987), suppresses withdrawal convulsions induced by barbital (Tagashira et al., 1981). Moreover, Little et al. (1986) and Littleton et al. (1990) reported that flunarizine prevents ethanol withdrawal

convulsions, and Chugh et al. (1992) also reported that cinnarizine, a selective T-type Ca^{2+} channel blocker, prevents diazepam withdrawal signs. Since withdrawal signs of ethanol and diazepam are caused by increases in evoked transmitter release owing to supersensitivity of the nerve terminals to Ca^{2+}, Ca^{2+} channel blockers may prevent the supersensitivity to Ca^{2+}. As a result, Ca^{2+} channel blockers may prevent withdrawal signs of these sedative hypnotics (Chugh et al., 1992; Dolin et al., 1990; Little et al., 1986; Littleton et al., 1990). Therefore, the suppression of barbital withdrawal signs by flunarizine may result from blockade of T-type Ca^{2+} channel.

Flunarizine has been shown to possess anticonvulsant properties in laboratory animals and humans (Astarloa et al., 1989; De Sarro et al., 1988; Desmedt et al., 1976), and this anticonvulsant effect of flunarizine may result from T-type Ca^{2+} channel blockade (Pucilowski, 1992; Rogawski and Porter, 1990; Shibuya and Watanabe, 1992). In the present study, chronic co-administration of flunarizine suppressed the development of physical dependence on barbital and diazepam. These findings suggest that the adequate co-administration of T-type Ca^{2+} channel sensitive blockers and either benzodiazepines or barbiturates may be a useful method for producing a potent antiepileptic action accompanied by decreasing the potential for physical dependence on the benzodiazepines or barbiturates.

In conclusion, the present results suggest that chronic co-administration of flunarizine, but not of either nifedipine or diltiazem, with barbital or diazepam suppresses the development of physical dependence on barbital or diazepam, and substitution of flunarizine, but not diltiazem, suppresses the appearance of barbital withdrawal signs. This effects of flunarizine may be mainly due to suppression of withdrawal convulsions of barbital and diazepam by reducing the Ca^{2+} influx and

preventing up-regulation of Ca^{2+} channel. The differences between flunarizine and either nifedipine or diltiazem may result from differences in the types of Ca^{2+} channel in the brain; unlike ethanol, T-type Ca^{2+} channels in the brain rather than L-type Ca^{2+} channels may be involved in the physical dependence on barbital and diazepam.

Table 3-3
Modification of pysical dependence on central depressants by two types of Ca^{2+} channel blockers

	L-type Ca^{2+} channel sensitive blockers	*T-type Ca^{2+} channel sensitive blockers*
Ethanol		
Physical dependence	*suppression (Whittington et al., 1991)*	———
Withdrawal signs	*suppression (Little et al., 1986)*	*suppression (Little et al., 1986)*
Barbiturates		
Physical dependence	*no effect (Suzuki et al., 1990)*	*suppression (present results)*
Withdrawal signs	*suppression (Brown et al., 1988)*	*suppression (present results)*
Benzodiazepines		
Physical dependence	*no effect (Dolin et al., 1990)*	*suppression (present results)*
Withdrawal signs	*suppression (Dolin et al., 1990)*	*suppression (Chugh et al., 1992)*

GENERAL DISCUSSION

Barbiturates and benzodiazepines both categorized in sedative hypnotics are widely known to commonly work on $GABA_A$ · benzodiazepine receptor / Cl^- channel complex, to increase Cl^- influx (Akaike, 1989; Harrison et al., 1988) and decrease Ca^{2+} influx (Friedman et al., 1979; Leslie et al., 1980); as a result, these drugs suppress several central neuron activities (Akaike, 1989; Harrison et al., 1988). It is considered that central depressing effects of these drugs such as anxiolytic, anticonvulsant, sedative and hypnotic effects and ataxia, may be induced by the suppression of neuron activities (Rogawski and Porter, 1990). As a vital question, chronic treatment with any these drugs produce physical dependence. Physical dependences on barbiturates and benzodiazepines are able to substitute each other (Yanagita and Takahashi, 1973). This phenomenon is called cross physical dependence. Therefore, barbiturates and benzodiazepines are categorized in the same class of substance dependence "barbiturates type" in the WHO classification (Kramer and Cameron, 1975). In this context, barbital and diazepam were chosen in the present study as representatives of barbiturates and benzodiazepines respectively to study "barbiturates type" dependence.

The mechanisms of physical dependence on these drugs have not yet been clear. These drugs have been suggested to show the central depressing effects by suppressing the Ca^{2+} influx and several neuron activities including noradrenergic and serotonergic neuron activities (Akaike, 1989; Friedman et al., 1979; Harrison et al., 1988; Leslie et al., 1980; Rogawski and Porter, 1990). The development of physical dependece on these drugs may therefore relate to changes in the responsiveness of Ca^{2+} channel and the neuron activities. In the present study, the involvements of 1) changes in the activities of noradrenergic and serotonergic neurons, 2) four types of

5-HT receptors and 3) two types of Ca^{2+} channels, in the development of physical dependence on barbiturates and benzodiazepines were thus examined. Barbiturates and benzodiazepines suppress the activities of noradrenergic and serotonergic neurons by increasing Cl^- influx and decreasing Ca^{2+} influx. It is known that chronic treatments with barbiturates and benzodiazepines produce the down-regulation of $GABA_A$ · benzodiazepine receptor / Cl^- channel complex (Allan et al., 1992a; 1992b; Miller et al., 1988; Woods et al., 1992) and the up-regulation of Ca^{2+} channels (Elrod and Leslie, 1980; Leslie et al., 1980). As a result, the suppressing effects of barbiturates and benzodiazepines on the noradrenergic and serotonergic neuron activities become gradually tolerant during chronic treatment with these drugs (Tagashira et al., 1982a; 1983a; Woods et al., 1992). Therefore, the noradrenergic and serotonergic neurons are overstimulated as a rebound phenomenon after the termination of barbiturates or benzodiazepines (Costall and Naylor, 1991; Tagashira et al., 1982a; 1983a; Woods et al., 1992), and withdrawal signs of these drugs are considered to result from the overstimulation of noradrenergic and serotonergic neurons. In the present study, chronic co-administration of imipramine, tricyclic antidepressant, with barbital potentiated the development of physical dependence on barbital. This raises the possibility that chronic co-administration of imipramine with barbital produces the potentiation of the overstimulation of noradrenergic and serotonergic neurons after the termination of barbital. While, acute treatment with imipramine suppresses the activities of noradrenergic and serotonergic neurons (Egawa et al., 1983), the suppressing effect of imipramine on the noradrenergic and serotonergic neuron activities becomes gradually tolerant during chronic treatment with imipramine by down-regulation of α_2 autoreceptor in locus coeruleus (Svensson and Usdin, 1978) and by an increase in 5-HT release in cortex and hippocampus (Heninger and Charney, 1987). As a consequence, serotonergic neuron activities become above the pre-drug level, but noradrenergic neuron activites are still slightly

below the pre-drug level after chronic treatment with imipramine (Heninger and Charney, 1987; Svensson and Usdin, 1978). As above described, barbital suppresses these neuron activities by increasing Cl^- influx and decreasing Ca^{2+} influx, but this suppression becomes gradually tolerant during chronic treatment with barbital by down-regulating $GABA_A$ · benzodiazepine receptor / Cl^- channel complex and up-regulating Ca^{2+} channels (Satoh et al., 1979; Tagashira et al., 1982a; 1983a). Serotonergic neuron activities are also increased by enhancement of NADPH-dependent aldehyde reductase activities which is induced by chronic treatment with barbital (Satoh et al., 1979). As a result, serotonergic neuron activities are totally potentiated, but noradrenergic neuron activites are still slightly suppressed after chronic treatment with barbital. After all, chronic treatment with barbital or imipramine apparently suppresses noradrenergic neuron activities and potentiates serotonergic neuron activities. Therefore, chronic co-administration of imipramine with barbital may potentiate the development of physical dependence on barbital by chronically superimposition of the alteration of noradrenergic and serotonergic neuron activities induced by chronic treatment with barbital. In addition, 5-HT_{1A} agonist, buspirone, and 5-HT_3 antagonist, ondansetron, but not 5-HT_{1C} antagonist, mianserin, and 5-HT_2 antagonist, ketanserin, potentiated the development of physical dependence on diazepam in the present study. It is known that anxiolytic effects of buspirone and ondansetron result from suppression of activities of ascending serotonergic neuron (from dorsal raphe neucleus to amygdala) through 5-HT_{1A} receptors (autoreceptors) and 5-HT_3 receptors, respectively (Blackburn, 1992; Costall and Naylor, 1991; Treit, 1991). After the termination of chronic treatment with tricyclic antidepressants, rebound symptoms (hyper-responsiveness of noradrenergic and serotonergic neurons) are induced by gradual tolerance to the suppression of noradrenergic and serotonergic neuron activities during chronic treatment with tricyclic antidepressants (Nutt and Glue, 1991). Whereas, unlike

tricyclic antidepressants, after the termination of chronic treatment with buspirone or ondansetron, general behavior did not change. Therefore, the suppression of ascending serotonergic neuron activities by buspirone or ondansetron may not become tolerant during chronic treatment with buspirone or ondansetron. While acute diazepam also suppresses the ascending serotonergic neuron activities by increasing Cl^- influx and decreasing Ca^{2+} influx (Costall and Naylor, 1991; Treit, 1991), this suppression become gradually tolerant during chronic treatment with diazepam by down-regulating $GABA_A$ · benzodiazepine receptor / Cl^- channel complex and up-regulating Ca^{2+} channels in dorsal raphe nucleus (Hitchcott et al., 1990; Wagner et al., 1985). Therefore, chronic co-administration of buspirone or ondansetron with diazepam may have potentiated the development of physical dependence on diazepam, owing to the potentiation of these functional changes in $GABA_A$ · benzodiazepine receptor / Cl^- channel complex and Ca^{2+} channels by further suppressing the ascending serotonergic neuron activities through $5\text{-}HT_{1A}$ and $5\text{-}HT_3$ receptors. On the other hand, in the present study, chronic co-administration of T-type Ca^{2+} channel sensitive blocker, flunarizine, with barbital or diazepam suppressed the development of physical dependence on barbital or diazepam, but L-type Ca^{2+} channel sensitive blockers, nifedipine and diltiazem did not. Chronic treatment with Ca^{2+} channel blockers reportedly produces the down-regulation of Ca^{2+} channels (Panza et al., 1985). Therefore, flunarizine may have suppressed the development of physical dependence on barbiturates and benzodiazepines by suppressing the up-regulation of T-type Ca^{2+} channel that was inevitably induced by chronic treatment with barbiturates and benzodiazepines. In these perspectives, it would be claimed that the development of "barbiturates type" physical dependence may be modified by modulating the alterations of $GABA_A$ · benzodiazepine receptor / Cl^- channel complex, Ca^{2+} channel and noradrenergic and serotonergic neuron activities which are induced by chronic treatments with barbiturates and

benzodiazepines.

Barbiturates and benzodiazepines are widely used as anxiolytics, antiepileptics, hypnotics, muscle-relaxants or antidepressants, in clinical therapy. However, as a matter of significance, chronic treatments with barbiturates and benzodiazepines produce physical dependence. Therefore, there is a diverse probability that barbiturates or benzodiazepines may be used concurrently with other drugs to potentiate their central depressing action and to decrease their potential for physical dependence liability. In fact, barbiturates or benzodiazepines have been treated in combination with other antiepileptics (Delgodo-Escueta and Enrile-Bacsal, 1983) and tricyclic antidepressants (Nutt and Glue, 1991) in clinical use. In the present study, a tricyclic antidepressant, imipramine, potentiated the development of physical dependence on barbital. Therefore, it is recommended that tricyclic antidepressants should be avoided to be used concurrently with barbiturates or benzodiazepines. Furthermore, in the present study, 5-HT_{1A} agonist, buspirone, and 5-HT_3 antagonist, ondansetron, potentiated the development of physical dependence on diazepam. Buspirone and ondansetron have recently been expected as safe and useful anxiolytics which possess no physical dependence liability (Costall and Naylor, 1991; Treit, 1991). Buspirone and ondansetron may well be expected to be used together with benzodiazepines clinically, since anxiolytic effects of these drugs are weaker than those of benzodiazepines (Jann, 1988; Treit, 1991). However, it is suggested with a warning from the present results that these combinations potentially have a risk of augmentation of physical dependence in clinical use. On the other hand, in the present study, T-type Ca^{2+} channel sensitive blocker, flunarizine, suppressed the developments of physical dependence on barbital and diazepam and the appearance of barbital withdrawal signs. It is also reported that T-type Ca^{2+} channel sensitive blockers suppress the appearance of withdrawal signs of benzodiazepines (Chugh et

al., 1992). The T-type Ca^{2+} channel sensitive blockers, ethosuximide, flunarizine and cinnarizine share anticonvulsive actions (Desmedt et al., 1976; Rogawski and Porter, 1990). Especially, ethosuximide has been used for therapy of absence status epilepticus (Rogawski and Porter, 1990). Flunarizine has been used to cerebral circulation trouble in clinical therapy. However, flunarizine was recently reported to potentiate anticonvulsive action of antiepileptics in animals and humans (Overweg et al., 1984; Rogawski and Porter, 1990). Therefore, the combination of T-type Ca^{2+} channel sensitive blockers and barbiturates or benzodiazepines may be a useful strategy for producing a potent antiepileptic action and, from the present finding, for decreasing the potential for physical dependence liability on barbiturates or benzodiazepines.

CONCLUSION

Barbiturates and benzodiazepines commonly affect $GABA_A$ · benzodiazepine receptor / Cl^- channel complex and produce the same class of substance dependence "barbiturates type". In the present study, the involvements of changes in the activities of noradrenergic and serotonergic neurons and adaptation of some 5-HT receptors and Ca^{2+} channels, in the development of physical dependence on barbiturates and benzodiazepines were investigated. The conclusion of the present study are shown as follows:

1. The development of physical dependence on barbital was potentiated by chronic co-administration of imipramine, but not bifemelane or teniloxazine. The development of barbiturates physical dependence may be potentiated by further suppression of noradrenergic neuron activities and further potentiation of serotonergic neuron activities during the development of physical dependence.

2. The development of physical dependence on diazepam was potentiated by chronic co-administration of buspirone or ondansetron, but not mianserin or ketanserin. The development of benzodiazepine physical dependence may be potentiated by superimposition of suppression of the ascending serotonergic neuron activities, that are suppressed by chronic treatment with diazepam alone, through $5\text{-}HT_{1A}$ and $5\text{-}HT_3$ receptors.

3. The development of physical dependence on barbiturates and benzodiazepines was suppressed by chronic co-administration of flunarizine, but not nifedipine or diltiazem. The development of physical dependence on barbiturates and benzodiazepines may be suppressed by prevention of the up-regulation of T-

type Ca^{2+} channel which was induced by the development of physical dependence on barbiturates and benzodiazepines. Moreover, the appearance of withdrawal signs of central depressants is suppressed by T-type Ca^{2+} channel blockade.

4. The intensity of physical dependence on barbiturates and benzodiazepines may be related to the several neuromodulation rather than to the changes in the magnitude of central depression by chronic drug combination during the development of physical dependence.

5. Since tricyclic antidepressants, 5-HT_{1A} agonists or 5-HT_3 antagonists potentiate the development of physical dependence on barbiturates and benzodiazepines, these combinations should be avoided. On the other hand, co-administration of T-type Ca^{2+} channel sensitive blockers and barbiturates or benzodiazepines may be a useful method for producing a potent antiepileptic action and decreasing the potential for physical dependence on barbiturates and benzodiazepines.

ACKNOWLEDGEMENTS

This research would never have been materialized without the help of the following people/organizations:

First, I would like to express my gratitude and appreciation to Professor Miwa Misawa (Department of Pharmacology, Hoshi University) and Associate Professor Tsutomu Suzuki (Department of Pharmacology, Hoshi University) for their helpful guidance in my research work and preparing this dissertation.

Further, I wish to thank Dr. Junzo Kamei (Department of Pharmacology, Hoshi University), Dr. Toshio Yoshii, Mr. Hideto Awano, Mr. Hiroshi Motegi and Mr. Hideaki Noguchi for their helpful guidance in my research work.

Also, I wish to thank Ms. Keiko Takakusaki, Ms. Akiko Murai, Ms. Naomi Shirayama, Mr. Makoto Tsuda and Ms. Shiho Iwanuma for their technical assistance in my research work.

Finally, I wish to thank staffs and students of Department of Pharmacology, Hoshi University.

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