Recreational drugs, 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA) and diphenylprolinol, inhibit neurite outgrowth in PC12 cells

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ABSTRACT — 3,4-Methylenedioxymethamphetamine (MDMA) is widely abused as a psychoactive recreational drug. It is well known that MDMA induces neurotoxic damage of serotonergic nerve endings. Although drug abuse is increasing among youths, it is unclear whether recreational drugs affect the development of nerve growth. Thus, the present study examined the effect of recreational drugs, such as MDMA, 3,4-methylenedioxyamphetamine (MDA) and diphenylprolinol, a novel recreational drug with a similar chemical structure as that of psychoactive agent pipradrol, on nerve growth factor (NGF)-induced neurite outgrowth. These recreational drugs induced a dose-dependent cell death in PC12 cells. The IC₅₀ values of MDMA, MDA, R-diphenylprolinol and S-diphenylprolinol were 4.11 mM, 2.75 mM, 1.00 mM and 0.77 mM, respectively, at 24 hr. To examine the effects of these recreational drugs on NGF-induced neurite outgrowth, PC12 cells were treated with NGF together with MDMA, MDA, S-diphenylprolinol or R-diphenylprolinol at low toxic concentrations. The recreational drugs significantly suppressed neurite outgrowth of PC12 cells induced by NGF. The results suggest that these psychoactive recreational drugs may inhibit neurite growth and thus be implicated in their elicited neurotoxicity.

Key words: Recreational drug, MDMA, MDA, Diphenylprolinol, Neurite outgrowth, PC12

INTRODUCTION

Many countries have specific regulations to control recreational drugs, but markets that supply novel recreational drugs are growing. Although these newly introduced chemicals show similar clinical effects as their respective analogs, they are not covered under appropriate legislation because of their divergent chemical structures. In addition, novel recreational drugs are often distributed on the street or through the internet making application of legislation difficult.

Tablets called “ecstasy” contain 3,4-methylenedioxyamphetamine (MDMA), but may also contain varied amounts of 3,4-methylenedioxyamphetamine (MDA), methamphetamine, ephedrine, caffeine and ketamine (Makino et al., 2003). Ecstasy is widely abused as a psychoactive recreational drug (Parrott, 2002).

MDMA was first synthesized and patented by the German pharmaceutical company Merck in Darmstadt around 1912, with plans to market an anorectic drug or appetite suppressor (Freudemann et al., 2006); but the drug, however, was never marketed. Apart from some US Army trials in the 1950s, MDMA passed more or less into oblivion until the 1970s when it was evaluated by an American biochemist, Shulgin (Parrott, 2001). Subsequently, MDMA was subjected to limited use as an adjunct in psychotherapy; it was considered beneficial by enhancing the patients’ communicative skills and thus making them more accessible to psychotherapy (Downing, 1986). In addition, MDMA was sporadically used as a recreational drug in non-therapeutic settings (Parrott, 2001). The therapeutic use of MDMA was gradually abolished during the 1970s. The substance was banned in the UK in 1977, in the USA in 1985 and in
Japan in 1989.

It has been demonstrated that acute MDMA administration induces a rapid release of serotonin (5-HT) (Gdelsky and Nash, 1996) indicating a decrease of 5-HT concentration in brain tissues (Colado et al., 1993). MDMA binds serotonin transporter with high affinity and then inhibits reuptake of 5-HT; MDMA also binds noradrenaline and dopamine transporters with lower affinities (Rothman and Baumann, 2002; Liechti et al., 2000). MDMA inhibits the enzyme tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, and produces a degeneration of serotonergic nerve terminals (Ricaurte et al., 1985; Battaglia et al., 1987; Schmidt and Taylor, 1987; O’Shea et al., 1998). In addition, MDMA is a weak inhibitor of both A and B subtypes of monoamine oxidase (Torre et al., 2004; Parrott, 2005). These observations indicate that MDMA affects brain activity by altering neurotransmission, particularly the serotonergic system.

Although use of MDMA by the young generation is widely spread, the effects of the drug on pregnancy and fetal brain are not obvious. It is, therefore, important to clarify the effect of recreational drugs on nerve growth.

Diphenylprolinol is a novel recreational drug with a structure similar to that of pipradrol, a drug initially developed to treat obesity (Gelvin et al., 1955). However, pipradrol was found to possess a stimulant effect on the central nervous system (CNS); it induced incoordinated motor activity and ataxia, followed by tremor and clonic convulsions (Portoghese et al., 1968). Therefore, pipradrol has been removed from the Japanese market and controlled under drug legislation since the 1970s. The psychoactive feature of racemic pipradrol is based on R-pipradrol, which is twice as likely to be active than the racemate. S-pipradrol does not show CNS stimulation (Portoghese et al., 1968). It has recently been reported that diphenylprolinol causes high blood pressure and ischemic chest pain (Lidder et al., 2008). However, the effect of diphenylprolinol on nerve growth is unknown. Thus, we studied the effects of recreational drugs on neurite outgrowth.

A rat pheochromocytoma cell line, PC12 as a model for the neuronal cell, was selected. PC12 is known to differentiate into sympathetic neuron-like cells when exposed to NGF (Greene and Tischler, 1976) and has been widely used for the analysis of neuronal cells (Mérot et al., 2009).

**MATERIALS AND METHODS**

**Cell culture**

PC12 cells were maintained in Dulbecco’s modified eagle medium (DMEM) containing 10% horse serum (HS), 5% fetal bovine serum (FBS), 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 4 mM glutamine, 0.2% sodium hydrogen carbonate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under 95% O₂/5% CO₂.

**MDMA and MDA synthesis**

MDMA was synthesized from 3,4-methylenedioxyphenyl acetone and methylamine by reductive amination. MDA was also synthesized from 3,4-methylenedioxyphenyl acetone and ammonium acetate by reductive amination (Shulgin and Shulgin, 1991) under the control of a narcotics researcher (T. Yoshida) licensed under the narcotics and psychoactive control law. All analytical data were consistent with the assigned structures.

**Cell viability assay**

PC12 cells were plated in poly-L-lysine-coated 96-well plates. After 24 hr, the medium was changed to low serum (2% HS and 1% FBS) DMEM, and PC12 cells were treated with NGF (25 ng/ml; Upstate Biotechnology Inc., Lake Placid, NY, USA) together with various concentrations of recreational drugs for 24 or 48 hr. After incubation, cell viability was assessed by luminescent cell viability assay with Cell Titer-Glo® (Promega, Madison, WI, USA).

**Measurement of neurite outgrowth**

PC12 cells were plated in collagen type IV-coated 24-well plates. At 24 hr after incubation, the medium was changed to low serum DMEM, and PC12 cells were treated with NGF (25 ng/ml) together with 1 mM MDMA, 1 mM MDA, 0.1 mM S-diphenylprolinol or 0.1 mM R-diphenylprolinol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Twenty-four hours after the treatment, neurite outgrowth was assessed using a phase-contrast microscope BZ-8000 (Keyence Corporation, Osaka, Japan). Four fields were randomly chosen in each well. The total number of cells in each field was counted and the percentage of neurite positive cells was determined. When the length of the neurite was greater than the cell body diameter, it was counted as a neurite positive cell (Mérot et al., 2009). The length of the neurite extending from the cell was measured with a BZ-Analyzer (Keyence Corporation, Osaka, Japan). If a neurite positive cell had some neurites, we measured the longest neurite.

**Western blot analysis**

PC12 cells were stimulated by NGF (25 ng/ml) together with MDMA, MDA, S-diphenylprolinol or R-diphenylprolinol. After 5 or 15 min incubation, a culture medi-
um was aspirated and lysed by scraping into SDS-PAGE sample buffer containing 2 mM PMSF. Then the lysates were boiled for 5 min. Protein samples were separated on 10% SDS-PAGE gels, transferred to a polyvinylidene difluoride (PVDF) membrane (Pall Bio Support, Glen Cove, NY, USA), and probed with a monoclonal mouse anti-Phospho-ERK 1/2 antibody (1:2,000 dilution, Cell Signaling, Danvers, MA, USA, product number 9106) or rabbit anti-ERK1/2 antibody (1:1,000 dilution, Cell Signaling, Danvers, MA, USA, product number 9102).

**Statistical analysis**

Statistical analysis was performed with ANOVA followed by Dunnett’s test.

![Graphs showing cell survival for MDMA, MDA, S-diphenylprolinol, and R-diphenylprolinol](attachment:graphs.png)

**RESULTS AND DISCUSSION**

**Cytotoxic effects of recreational drugs**

Firstly, we examined the cytotoxic effects of recreational drugs on PC12 cells. PC12 cells were exposed to NGF (25 ng/ml) together with 0.5–8 mM of MDMA or MDA, or 0.01–2 mM of S-diphenylprolinol or R-diphenylprolinol for 24 and 48 hr. All the recreational drugs induced a dose-dependent cell death in PC12 cells (Fig. 1). The IC$_{50}$ values of MDMA, MDA, R-diphenylprolinol and S-diphenylprolinol were 4.11 mM, 2.75 mM, 1.00 mM and 0.77 mM, respectively, at 24 hr after incubation. S-Diphenylprolinol and R-diphenylprolinol were more toxic than MDMA and MDA. Therefore, it is possible to conclude that diphenylprolinol would have a greater effect on the nerve cell than MDMA and MDA. We also checked cyto-

![Graph showing cell survival for S-diphenylprolinol and R-diphenylprolinol](attachment:graphs2.png)

**Fig. 1.** Cytotoxic effects of recreational drugs on PC12 cells. PC12 cells were treated with NGF (25 ng/ml) together with MDMA, MDA, S-diphenylprolinol or R-diphenylprolinol at the concentrations indicated. 24 hr (solid column) or 48 hr (open column) after treatment, cell viability was determined by luminescent cell viability assay. Values are represented as percentage of fluorescence intensity compared to the control cells cultured in the absence of a recreation drug. Values represent the mean ± S.E.M. (n = 3). Statistical analysis was performed with ANOVA followed by Dunnett’s test. *P < 0.05, **P < 0.01 compared with the control (only NGF treated cells).
toxic effects of recreational drugs in PC12 cells without NGF. The obtained results were similar to those observed with NGF (data not shown).

**Effects of recreational drugs on NGF-induced neurite outgrowth**

We next examined the effects of these recreational drugs on NGF-induced neurite outgrowth of PC12 cells. PC12 cells were treated with NGF together with 1 mM MDMA, 1 mM MDA, 0.1 mM R-diphenylprolinol or 0.1 mM S-diphenylprolinol for 24 hr. The rates of cell survival were 93.23%, 85.14%, 88.16% and 93.81%, respectively, 48 hr after treatment.

Twenty-four hours after NGF treatment, most cells (73%) became neurite positive cells (Figs. 2 and 3a). However, the addition of MDMA, MDA, S-diphenylpro-
linol or R-diphenylprolinol significantly decreased the number of NGF-induced neurite positive cells by 37%, 38%, 37% and 30%, respectively (Fig. 3a). The average neurite length was 57 µm in NGF-treated PC12 cells (Fig. 3b). MDMA, MDA, S-diphenylprolinol and R-diphenylprolinol significantly decreased the length of the neurite by 30, 35, 41 and 35 µm, respectively (Fig. 3b).

NGF induces ERK activation in PC12 cells and leads to cell differentiation manifested as neurite outgrowth (Marshall, 1995). To further evaluate the effect of recreational drugs on neuronal differentiation of PC12 cells, the expression of phospho-ERK protein as a marker of activation was assessed by Western blot analysis. NGF significantly induced the level of phospho-ERK protein; however, recreational drugs seemed to have no effect on NGF-evoked ERK activation (Fig. 4).

These results indicate that the recreational drugs decreased the rate of neurite positive cells and the length of the neurite at a low cytotoxic dose in PC12 cells. Although it is reported that ERK has an important role in NGF-induced neurite outgrowth, the present study indicates that recreational drugs have no effect on NGF-induced ERK activation.

Therefore, it is suggested that the MAP kinase pathway is not the target of recreational drugs. It has been reported that Rho kinase (ROCK) is associated with neurite outgrowth (Altun-Gultekin et al., 1998), so it is possible that recreational drugs might induce ROCK activation. Further studies are required to clarify the mechanism of inhibitory effect of these drugs on neurite outgrowth.

From the results, these recreational drugs might effect neuronal development in a drug-taking teenager and in the fetus of a mother who is an abuser. Illicit drugs, such as cocaine, heroin and amphetamines, have been shown to impair fetal growth (Wagner et al., 1998). Infants who are born from mothers who use recreational drugs have lower birth weights and head circumferences in addition to an increased risk of altered neurodevelopment (Wagner et al., 1998; O’Donnell et al., 2009). It is possible that MDMA, MDA and diphenylprolinol might also have the same effects. It is, therefore, of interest to clarify the effect of these novel recreational drugs on nerve development.

In conclusion, the present study has shown that MDMA, MDA and diphenylprolinol produce inhibitory effects on NGF-induced neurite outgrowth suggesting
that these recreational drugs may cause impaired neuronal development.

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