Title: Decrease of D₂ receptor binding but increase in D₂ stimulated G protein activation, dopamine transporter binding and behavioural sensitisation in brains of mice treated with a chronic escalating dose "binge" cocaine administration paradigm

Authors: A. Bailey, A. Metaxas, J.H. Yoo, T. McGee, I. Kitchen.

Faculty of Health and Medical Sciences, AY building, University of Surrey, Guildford, Surrey, GU2 7XH, UK

Corresponding author: Dr Alexis Bailey

Faculty of Health and Medical Sciences

AY building

University of Surrey

Guildford

Surrey

GU2 7XH, UK

Tel: +44 (0)1483 689701, Fax: +44 (0)1483686401; E.mail: a.bailey@surrey.ac.uk

Number of figures: 6

Number of tables: 0

Number of pages: 27

Number of words in whole manuscript: 10168

Number of words in abstract: 248

Number of words in introduction: 768

Running title: Chronic cocaine and D₂ receptors

Keywords: stereotypy, locomotion, [³⁵S]GTPγS, autoradiography

Abstract

Understanding the neurobiology of the transition from initial drug use to excessive drug use has been a challenge in drug addiction. We examined the effect of chronic “binge” escalating dose cocaine administration, which mimics human compulsive drug use, on behavioural responses and the dopaminergic system of mice and compared it to a chronic steady dose (3x15 mg/kg/day) “binge” cocaine administration paradigm. Male C57BL/6J mice were injected with saline or cocaine in an escalating dose paradigm for 14 days. Locomotor and stereotypy activity were measured and quantitative autoradiographic mapping of D₁ and D₂ receptors, dopamine transporters, and D₂-stimulated [³⁵S]GTPγS binding was performed in the brains of mice treated with this escalating and steady dose paradigm. An initial sensitisation to the locomotor effects of cocaine followed by a dose dependent increase in the duration of the locomotor effect of cocaine was observed in the escalating but not the steady dose paradigm. Sensitisation to the stereotypy effect of cocaine and an increase in cocaine-induced stereotypy score was observed from 3x20 to 3x25 mg/kg/day cocaine. There was a significant decrease in D₂ receptor density, but an increase in D₂ stimulated G- protein activity and dopamine transporter density in the striatum of cocaine treated mice which were not observed in our steady dose paradigm. Our results document that chronic “binge” escalating dose cocaine treatment triggers profound behavioural and neurochemical changes in the dopaminergic system which might underlie the transition from drug use to compulsive drug use associated with addiction, which is a process of escalation.

Introduction

Cocaine, one of the most commonly abused psychostimulants induces the elevation of extracellular dopamine in the striatum by blocking the dopamine transporter (DAT). The dopaminergic system including D₁, D₂ dopamine receptors and DAT has been shown to play a key role in mediating the locomotor, rewarding, reinforcing, sensitising and stereotypic effects of cocaine (for review see Anderson & Pierce (2005)).

In addition, neurochemical data also supports the presence of interactions between cocaine and the dopaminergic system. There are multiple studies investigating the effect of cocaine on D₁ and D₂ receptor and DAT density with inconsistent findings with each other. Increases, decreases or no change in D₁ receptor binding, D₂ receptor binding (Taylor *et al.*, 1979; Goeders & Kuhar, 1987; Peris *et al.*, 1990; Volkow *et al.*, 1990; Volkow *et al.*, 1993; Berrettini *et al.*, 1994; Unterwald *et al.*, 1994; Maggos *et al.*, 1998; Nader & Czoty, 2005; Nader *et al.*, 2006; Bailey *et al.*, 2007b) and DAT binding (Kula & Baldessarini, 1991; Sharpe *et al.*, 1991; Benmansour *et al.*, 1992; Farfel *et al.*, 1992; Alburges *et al.*, 1993; Cass *et al.*, 1993; Hurd & Herkenham, 1993; Little *et al.*, 1993; Pilotte *et al.*, 1994; Staley *et al.*, 1994; Wilson *et al.*, 1994a; Wilson *et al.*, 1994b; Hitri *et al.*, 1996; Pilotte *et al.*, 1996; Kunko *et al.*, 1997; Maggos *et al.*, 1997; Little *et al.*, 1998a; Little *et al.*, 1999; Bailey *et al.*, 2007b) have been reported. The lack of concordance in the above studies may arise from differences in treatment paradigms (varying doses, schedule and route of administration), length of withdrawal of receptor measurement in relation to the last cocaine injection and/or ligands used to quantify receptors and DAT. Nonetheless, a decrease in D₂ availability as measured with positron emission tomography (PET) in cocaine abstinent humans, non human primates and rats has been repeatedly and consistently observed (Volkow *et al.*, 1990; Volkow *et al.*,

1993; Tsukada *et al.*, 1996; Maggos *et al.*, 1998; Nader & Czoty, 2005; Nader *et al.*, 2006) and is thought to be associated with vulnerability to the reinforcing effects of drugs of abuse.

The aim of our study is to characterise the behavioural and neurochemical effect of cocaine on the dopaminergic system of mice treated with an escalating dose “binge” cocaine administration paradigm which closely mimics common features of human cocaine abuse and to compare that with those of traditionally used chronic steady dose “binge” cocaine administration paradigm. A common feature of human cocaine abuse is the development, over time, of tolerance to the reinforcing effects of cocaine, which results in administration of increasing doses of cocaine (Koob & Kreek, 2007). Indeed, novel animal models of excessive drug taking in dependent animals have recently been developed which demonstrate that rats which are trained to self administer cocaine over a long time, display a number of behavioural patterns consistent with those commonly observed in human cocaine addicts, including a progressive escalation in drug consumption, elevated drug-directed behaviours during extinction/abstinence and increased susceptibility to cocaine induced relapse which are thought to be related to the transition to cocaine addiction . Recent studies have also shown dramatic dysregulation of brain reward pathways (Ahmed & Koob, 1998; , 1999; Ahmed *et al.*, 2003; Ahmed & Koob, 2004), activation of brain stress systems in the extended amygdala (Zorrilla *et al.*, 2001; Mantsch *et al.*, 2003) and profound changes in the μ - (MOP-r) and κ - (KOP-r) opioid receptors systems (Bailey *et al.*, 2005; Schlussman *et al.*, 2005; Bailey *et al.*, 2007a) in animal models of escalating “binge” cocaine administration. This study was designed in order to investigate the effects of this novel chronic escalating dose cocaine administration paradigm on locomotion and stereotypy and on the levels of DAT, D₁, D₂ receptor binding and D₂ stimulated G- protein activity in different brain regions of C57BL/6J mice brains and to compare it with traditionally used steady dose administration paradigms. The results would give us an insight of possible behavioural and neurochemical changes

taking place in the dopaminergic system which might underlie the transition from drug use to compulsive drug use associated with addiction, which is a process of escalation. In addition, this is the first study which investigated the effect of chronic cocaine use on D₂ receptor G protein activity with the use of quinerolane stimulated [³⁵S]GTPγS autoradiography. These results, which may indeed differ from those obtained with D₂ receptor binding, may provide important information on the biochemical basis of the behavioural sensitisation properties of cocaine.

Materials and Methods

Animals and chronic cocaine treatment

6-8 week old C57BL/6J mice, 20-25 g, were studied. Mice were housed individually in a temperature-controlled room, with a 12 h light/dark schedule. Food and water were available ad libitum. All animals were weighed daily throughout the study. After 7 days of acclimatisation, intraperitoneal (i.p.) injections of either saline (1ml/kg) or cocaine were then administered in an escalating or steady dose “binge” paradigm to mimic a common pattern of self-administration in human cocaine abusers (Tsukada *et al*, 1996). Animals in the escalating dose group received 15x3 mg/kg/day on days 1–4, 20x3 mg/kg/day on days 5–8, 25x3 mg/kg/day on days 9–12, and 30x3 mg/kg/day on days 13 and 14. For animals treated with a chronic steady dose “binge” administration paradigm, 15 x 3 mg/kg/day were administered for 14 days. Three i.p. injections (1 h apart) were administered daily, with the first injection 60 min after the start of the light cycle. Animals were randomized into four groups of 6-12. Each group received either saline or cocaine injections for 14 days. Thirty minutes after the last injection, animals were killed by decapitation after a 15 second exposure to CO₂. Brains were rapidly removed and frozen by immersion for 30 seconds in isopentane

cooled to -35°C . All studies were performed in accordance with protocols approved by the Home Office (Animals Act 1986) UK.

Cocaine-induced locomotor activity

Locomotor activity was measured by the use of 12 motility chambers (40cm length x 20 cm wide x 20 cm height) (Linton Instrumentation, Norfolk, UK). Each cage had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 1 and 6 cm above the cage floor. The protocol for locomotor testing was a slight modification of that used by Heidbreder et al. (1998). When mice were tested in the activity chambers, they were habituated for 60 min after which they received an i.p. injection of saline or cocaine. The animals were then returned immediately to the chambers where locomotor activity was assessed for 60 min. The mice received a second and third injection of saline or cocaine 60 min and 120 min respectively after the first one and then returned immediately to the chambers where locomotor activity was assessed for 60 min. The locomotor activity for each mouse after each of the three daily injections of saline or cocaine was monitored daily for a period of 14 days. Locomotor activity was defined as the activity from measurement of sequential infrared beam breaks, recorded every 5 min, beginning immediately after placing the animals in the cage following an injection of saline or cocaine and continued for a 60 min period. For measurement of basal activity, mice were habituated for 60 min in the motility boxes prior to injections. Another group of mice were treated with 1 day acute “binge” cocaine administration protocol in order to compare the locomotor activity of mice at the end of the chronic escalating dose “binge” cocaine administration protocol with those treated acutely for 1 day with “binge” cocaine. Mice received three i.p. injections (1 h apart) of cocaine (30 x 3 mg/kg/day) for only 1 day and locomotor activity was measured for 60 min after each injection.

Cocaine-induced stereotypy

All mice receiving either saline or cocaine were videotaped twenty minutes after each injection for 30 sec. Videotapes were later rated for stereotypy by a trained observer blind as to the animal's treatment group. The rating was based on a slight adaptation of the scale used by Schlussman et al., (2003) and consisted of a graded scale of drug induced behaviours: 1) asleep, inactive; 2) alert, actively grooming; 3) increased sniffing (occasional light sniffing, often while exploring the cage); 4) intermittent rearing and sniffing (two or three rears in a 20s period, with sniffing frequently at the apex of the rear); 5) increased locomotion; 6) intense sniffing in one location (rapid sniffing, often with head down is the predominant behaviour displayed); 7) continuous pivoting and sniffing (no rearing, no locomotion); 8) intermittent rearing and sniffing (intermittent up and down rearing behaviour with locomotion); 9) maintained rearing and sniffing (animal remains up on hind legs throughout most of the observed period); 10) splayed limbs. Each animal received a single score following each injection, which corresponded to the specific stereotypic behaviour predominantly observed during the 30 sec observation period. The stereotypy behaviour of each mouse injected three times daily with saline or cocaine was scored every day for the 14 days duration of the study. The median stereotypy score of each mouse on each day was used for later analysis.

D₁, D₂ dopamine receptor and dopamine transporter (DAT) autoradiography

Animals treated with the escalating dose paradigm were killed 30 min following the last injection of saline or cocaine on day 14 of the study and the brains were immediately removed, quickly frozen in isopentane (-35 °C) and stored at -80°C until sectioning.

Quantitative autoradiography was performed as detailed previously for D₁, D₂ dopamine receptor binding (Lena *et al.*, 2004) and DAT binding (Javitch *et al.*, 1985) using the general procedures of Kitchen *et al.* (1997). Adjacent 20 µm coronal sections were cut at a interval of 300 µm from chronic “binge” saline or cocaine treated mice brains for the determination of total and non-specific binding of [³H]SCH-23390 (containing 1 µM mianserin in order to block 5-HT₂ and 5-HT_{1c} receptor binding sites), [³H]raclopride and [³H]mazindol at D₁, D₂ dopamine receptor and DAT, respectively. Ligand concentrations were 3-4 x K_d with all ligands used at a concentration of 4 nM. Non-specific binding (NSB) was defined in the presence of cis-flupenthixol (10 µM), sulpiride (10 µM), or unlabelled mazindol (10 µM) for [³H]SCH-23390, [³H]raclopride and [³H]mazindol binding respectively. Following binding for a period of 90 min, 60 min or 45 min for D₁, D₂ dopamine receptor and DAT binding respectively, and washing in ice cold buffer (6x1 min for D₁ and D₂ dopamine receptor binding and 2x1 min for DAT binding), the slides were apposed to MR film (Kodak) for 4 weeks. Sections from chronic “binge” saline and cocaine treated mice were processed together in a paired protocol. Films were developed using 50% Kodak D19 developer. Quantitative analysis of brain receptors was performed as detailed previously (Kitchen *et al.*, 1997; Lena *et al.*, 2004). Using an MCID image analyser (Image Research, Canada), brain structures were identified using the mouse brain atlas of Franklin and Paxinos (1997). The following structures were analysed by sampling 5-10 times with a box tool: cortex (8 x 8 mm) and olfactory tubercle (6 x 6 mm). All the other areas were analysed using freehand drawing tool. The regions analysed include cingulate cortex, nucleus accumbens core, nucleus accumbens shell, olfactory tubercle, dorsal endopiriform, dorsolateral caudate putamen, dorsomedial caudate putamen, ventomedial caudate putamen, ventrolateral caudate putamen, lateral septum and medial septum.

Quinelorane-stimulated [35 S]GTP γ S autoradiography

Quinerolane-stimulated [35 S]GTP γ S autoradiography was performed essentially as described by Newman-Tancredi et al., (2001). Coronal sections (20 μ m thick) were cut in a cryostat at an interval of 300 μ m from the same chronic “binge” escalating dose treated C57BL/6J mice or chronic saline treated mice used for the receptor binding autoradiography described in the previous section for the determination of basal, quinelorane-stimulated [35 S]GTP γ S binding, raclopride reversal of quinelorane-stimulated [35 S]GTP γ S binding, and non-specific binding. The same protocol was carried out in brains of mice treated with a chronic steady dose cocaine administration paradigm. Sections were preincubated in assay buffer (50 mM Tris/HCl, 3 mM MgCl₂.6H₂O, 0.2 mM EGTA, 100 mM NaCl, pH7.4 at 25 °C) for 10 min. Sections were then incubated in assay buffer containing 2 mM GDP, pH 7.4 at 25 °C for 15 min. D₂ receptor agonist stimulated activity was determined by incubating the sections in [35 S]GTP γ S (0.04 nM) with the selective D₂ receptor agonist quinelorane (100 μ M) for 2 hours in assay buffer containing 2 mM GDP. Basal G-protein activity was determined by incubating the sections in [35 S]GTP γ S in the presence of GDP (2mM) and in the absence of the agonist. Non-specific binding was assessed by incubating sections in [35 S]GTP γ S in assay buffer containing 2 mM GDP in the presence of 10 μ M unlabelled GTP γ S. Reversal of quinelorane-stimulated [35 S]GTP γ S binding was determined by incubating sections in [35 S]GTP γ S in the presence of quinelorane (100 μ M) and raclopride (10 μ M) in assay buffer containing 2 mM GDP. After incubation for 2 h, slides were rinsed twice for 3 min in ice-cold buffer (50 mM Tris/HCl, pH 7.4 at 0°C) followed by one rinse in de-ionised water. Slides were then dried for 2 h in a cold air stream and drying was continued for a further 2 days using anhydrous calcium sulfate (Drierite, BDHChemicals, Poole, UK). Sections from the brains of chronic cocaine treated mice and chronic saline treated mice were apposed to Kodak MR films (Amersham International plc, Buckinghamshire, UK) together with [14 C]

standards (Amersham) for 3 days. Quantitative analysis of optical density was performed as detailed previously (Schroeder *et al.*, 2003) using [^{14}C] standards. For each brain region examined, [^{35}S]GTP γ S binding was calculated by first subtracting the optical density of NSB sections from the optical density of agonist-stimulated, reversal of agonist stimulated or basal binding sections. The regions analysed include nucleus accumbens core, nucleus accumbens shell, olfactory tubercle, dorsolateral caudate putamen, dorsomedial caudate putamen, ventomedial caudate putamen, ventrolateral caudate putamen, lateral septum and medial septum.

Statistics

Cocaine-induced locomotor activity in 5 min data collection bins was analysed by two-way analysis of variance (ANOVA) (treatment and day) with repeated measures for day, followed by post hoc Newman-Keuls test. Values were expressed as mean $\pm$ S.E.M of total activity per 5 min bin. The median stereotypy score of each animal on each day was used as the raw data for subsequent analysis. Stereotypy behaviour was analysed by mean $\pm$ S.E.M of daily median stereotypy score of saline or chronic cocaine-treated mice. The statistics approach used in this case was two-way ANOVA (for factors treatment and day) with repeated measures for day, followed by post hoc Newman-Keuls test. Since the expression of behavioural stereotypy was measured with a behavioural rating scale, non parametric Mann-Whitney U tests were also used in order to confirm results obtained by the ANOVA. Two-way ANOVA (for factors region and treatment) was used for comparison of quantitative measures (fmol/mg) of D₁, D₂ receptors, DAT in brain regions of chronic “binge” saline and cocaine treated mice followed by post hoc least significant difference (LSD) test. Quinrolane-stimulated [^{35}S]GTP γ S binding activity in brain sections of mice was calculated as the increase in relative optical density (ROD) above the basal level in each brain region.

Comparison of quinerolane-stimulated [^{35}S]GTP γ S binding and reversal of this stimulation in brains of chronic saline and cocaine-treated mice was carried out using two way ANOVA for factors treatment 1 (saline vs cocaine) and treatment 2 (absence of raclopride vs addition of raclopride) followed by Newman-Keuls test. Comparison of quinerolane-stimulated [^{35}S]GTP γ S binding in the presence and absence of raclopride in each brain region of saline and cocaine-treated mice, was carried out using Student's t-test. Comparison of basal [^{35}S]GTP γ S binding in chronic saline and cocaine treated brains were carried out using two way ANOVA for factors treatment (cocaine, saline) and region followed by Newman-Keuls post hoc test. Data from all autoradiography experiments were expressed as the mean $\pm$ S.E.M of each experimental group.

Materials

Cocaine HCl was purchased from Sigma-Aldrich (Dorset, UK); [^3H]SCH-23390 (70 Ci/mmol), [^3H]raclopride (60.1 Ci/mmol), [^3H]mazindol (20.6 Ci/mmol) and [^{35}S] GTP γ S (1250 Ci/mmol) were purchased from Perkin Elmer (Bucks, UK); cis flupenthixol, sulpiride, raclopride, quinerolane, GDP, GTP γ S and mazindol were purchased from Sigma-Aldrich.

Results

Locomotor activity during chronic escalating and steady dose “binge” cocaine administration

Locomotor activity of naïve C57BL/6J mice (before saline or cocaine treatment) was measured for 60 min after placement in the locomotor chambers. The last 5 min of the 60 min duration on day 1 is shown in Fig. 1. One-way ANOVA, did not show any significant effect on basal locomotor activity between the two groups on day 1 ($P>0.05$). Basal locomotor

activity between the two treatment groups were measured during the 14 days of the study. Greater basal locomotor activity was observed in the cocaine treated group compared to saline on days 2-14 ($P<0.01$) (Fig. 1).

Locomotor activity of C57BL/6J mice was measured after each of the three hourly daily injections of saline or cocaine (15-30 x 3 mg/kg/day) from day 1 to day 14 of the chronic escalating dose “binge” cocaine treatment (Fig. 1a). Two-way ANOVA showed a significant main effect treatment ($P<0.0001$) and day ($P<0.0001$) and treatment x day interaction ($P<0.0001$). Mice treated with cocaine had greater locomotor activity compared to saline on all treatment days ($P<0.0001$, Newman-Keuls post hoc test) (Fig.1a). A significant increase in cocaine-induced locomotor activity was observed on day 3 (15 x 3 mg/kg/day) compared to day 1 (15 x 3 mg/kg/day) ($P<0.0001$) indicating the presence of behavioural sensitisation to the locomotor-stimulating effect of cocaine. Moreover an increase in cocaine-induced locomotor activity was observed on day 5 (20 x 3 mg/kg/day) vs day 1 ($P<0.0001$), day 9 (25 x 3 mg/kg/day) vs day 5 ($P<0.01$), and day 13 (30 x 3 mg/kg/day) vs day 9 ($P<0.01$) indicating that the increase in cocaine-induced locomotion is dose dependent. Both the magnitude and the duration of the cocaine response was enhanced on day 3 compared to day 1. However, although the magnitude of the cocaine response did not increase, a dramatic increase in the duration of cocaine-induced hyperactivity was observed following the increase of the cocaine dose from 20 to 25 mg/kg/inj and from 25 to 30 mg/kg/inj (Fig. 1a). On day 5, activity of the cocaine-injected animals returned to baseline levels 60 min after each of the three cocaine injections. However, on day 13 the activity of cocaine-injected animals remained constantly elevated at its peak magnitude during the whole 60 min after each of the three cocaine injections (Fig.1a).

Another group of mice were injected with 30 x 3 mg/kg/day cocaine at 1 h intervals for only 1 day to compare locomotor activity of mice on day 13 (30 x 3 mg/kg/day) of the escalating dose “binge” cocaine administration and after acute (1 day) “binge” cocaine administration (30 x 3 mg/kg/day). In contrast with the chronic paradigm where the duration of the peak cocaine response persisted for at least 60 min after cocaine administration, the activity of 1 day acute cocaine treated animals (3x30 mg/kg) returned to baseline 60 min after each of the three cocaine injections (Fig. 1a).

Other groups of mice were treated with a steady dose (3x15 mg/kg/day) cocaine administration protocol for 14 days (Fig. 1b). Cocaine increased locomotor activity compared to saline every day during the 14 day duration of the study ($P<0.001$, Newman-Keuls post hoc test). A significant increase in cocaine induced locomotor activity was observed on day 3 compared to day 1 ($P<0.001$) which persisted up to day 5 indicating the presence of behavioural sensitisation to the locomotor effect of cocaine (Fig. 1b). A reduction in cocaine-induced locomotor activity was observed on day 9 and day 13 vs day 5 of our chronic steady dose paradigm (Fig. 1b). In contrast to the escalating dose paradigm, the activity of cocaine injected animals returned to baseline levels 60 min after each of the three cocaine injections every day during the 14 day duration of the study (Fig. 1b).

Stereotypy behaviour during chronic escalating and steady dose “binge” cocaine administration

Stereotypy activity of C57BL/6J mice was scored 20 min after each of the three injections of saline or cocaine every day for the whole duration of the study and the median score of the three daily injections have recorded (Fig. 2). For the escalating dose group, two-way ANOVA showed a significant main effect of treatment ($P<0.0001$), day ($P<0.0001$) and

significant treatment x day interaction ($P < 0.0001$). Newman-Keuls post hoc test showed that escalating dose “binge” pattern cocaine administration resulted in a significant expression of behavioural stereotypy ($P < 0.0001$) compared to saline controls (Fig. 2a). A significant increase in cocaine-induced stereotypy was observed on day 3 (15 x 3 mg/kg/day) compared to day 1 (15 x 3 mg/kg/day) ($P < 0.0001$) indicating the presence of behavioural sensitisation to the stereotypy-stimulating effect of cocaine. Moreover an increase in cocaine-induced stereotypy behaviour was observed on day 5 (20 x 3 mg/kg/day) vs day 1 ($P < 0.0001$), day 9 (25 mg/kg/day) vs day 5 ($P < 0.05$), but not on day 13 (3 x 30 mg/kg/day) vs day 9 ($P > 0.05$). As far as the steady dose group is concerned, cocaine increased stereotypy behaviour every day during the 14 day duration of the chronic steady dose (3 x 15 mg/kg/day) cocaine administration study compared to saline control ($P < 0.001$) (Fig. 2b). A significant increase in cocaine-induced stereotypy was observed on day 3 compared to day 1 which persisted for the rest of the duration of the study indicating the presence of behavioural sensitisation to the stereotypy stimulatory effect of cocaine (Fig. 2b). In contrast to the escalating dose paradigm, no increase in cocaine-induced stereotypy behaviour was observed on day 9 vs day 5 ($P > 0.05$) (Fig. 2b).

D₁, D₂ dopamine receptor and dopamine transporter (DAT) autoradiography during chronic escalating dose “binge” cocaine administration

For D₂ dopamine receptor binding, two way ANOVA showed a significant main effect of treatment ($P < 0.001$). LSD post hoc test showed significantly lower levels of D₂ binding in the dorsolateral, dorsomedial, ventrolateral and ventromedial ($P < 0.001$) areas of the caudate putamen as well as in the nucleus accumbens core and shell ($P < 0.05$) of cocaine-treated mice compared to controls (Figs. 3, 4A). For DAT binding, two way ANOVA also showed a significant main effect of treatment ($P < 0.001$). In contrast to D₂ binding, significantly

higher levels of DAT binding were observed in the nucleus accumbens shell ($P<0.01$) and core ($P<0.01$), in the olfactory tubercle ($P<0.01$) and in all regions the caudate putamen ($P<0.05$) of chronic cocaine-treated mice compared to saline controls (Figs. 3, 4B). There was no significant treatment effect on D₁ binding (Fig. 3, 4C) ($P>0.05$).

Quinelorane-stimulated [³⁵S]GTPγS autoradiography during chronic escalating and steady dose “binge” cocaine administration

Quinerolane (100 μM) stimulated [³⁵S]GTPγS autoradiography was performed in coronal brain sections of chronic saline or cocaine-treated mice in order to investigate whether there were any alterations in D₂ receptor stimulated G- protein activity after chronic escalating or steady dose “binge” cocaine administration. In saline and cocaine-treated animals, quinerolane-stimulated [³⁵S]GTPγS binding was observed in the nucleus accumbens, caudate putamen, tubercle and septum (Figs. 5 and 6). The level of stimulation was weakest in the lateral septum and medial septum (around 15% over basal) but was over 50% above basal in the caudate putamen (data not shown). In the escalating dose groups, adjacent sections were also treated with [³⁵S]GTPγS in the presence of both quinerolane (100μM) and the selective D₂ receptor antagonist raclopride (10 μM) in order to investigate whether the stimulation of G- protein binding by quinerolane is D₂ receptor dependent. Two-way ANOVA for factors treatment 1 (saline, cocaine) and treatment 2 (raclopride reversal in the presence of raclopride, no raclopride reversal) with repeated measures for the last variable, showed a significant main effect of treatment 2 ($P<0.001$) and a significant treatment 1 x treatment 2 interaction ($P<0.05$). Newman-Keuls post hoc test showed that raclopride significantly reversed the stimulation of [³⁵S]GTPγS binding in response to quinerolane (Fig.5, 6a) both in saline ($P<0.001$) and chronic escalating dose cocaine ($P<0.001$) treated groups. The significant

reversal effect of raclopride in each individual region can be observed in Fig.6a. Moreover, an overall increase in quinerolane-stimulated [35 S]GTP γ S binding was observed in escalating dose cocaine-treated brains compared to saline controls ($P<0.05$, Newman-Keuls). A significant increase in quinerolane-stimulated [35 S]GTP γ S binding was observed in the nucleus accumbens shell and the ventrolateral area of the caudate putamen of escalating dose cocaine-treated animals compared to saline controls (Fig. 6a) ($P<0.05$, Student's *t* test). No change in quinerolane-stimulated [35 S]GTP γ S binding was observed in any brain region of chronic steady dose cocaine (3 x 15 mg/kg/day for 14 days) treated mice compared to saline control ($P>0.05$) (Fig. 6b).

Two-way ANOVA for factors treatment and region of the basal [35 S]GTP γ S data for escalating dose treated animals revealed significantly higher overall levels of basal [35 S]GTP γ S binding in cocaine treated mice brains compared to saline controls ($P=0.05$) (data not shown). However, there was no significant increase in basal [35 S]GTP γ S binding in any of the individual regions analysed ($P>0.05$). Despite of this, there was still a significant increase in quinerolane stimulated [35 S]GTP γ S binding in cocaine treated brains compared to saline controls. There was no treatment effect in brains on basal G protein activity of steady dose cocaine treated mice compared to saline controls ($P>0.05$) (data not shown).

Discussion

The behavioural results obtained in this study indicate the initial presence of locomotor sensitization to the locomotor effect of cocaine, followed by a dose dependent increase in the duration but not the magnitude of the locomotor activating effect of cocaine. In our locomotor paradigm of chronic “binge” cocaine administration, behavioural sensitisation

could be reflected by the increase in the magnitude (maximum or peak response) as measured by increased amount of counts at the time point where the counts are maximal (peak counts) or by increased duration of the locomotor effect of cocaine. Indeed in our escalating dose paradigm (see Fig. 1a) an increase in the peak locomotor effect of cocaine in day 3 compared to day 1 and an increase in the duration of the cocaine effect on day 13 (without observing an increase in the peak response to cocaine) compared to day 3 were observed, which is an indication of behavioural sensitisation. However, in our steady dose paradigm, an increase in the peak locomotor effect of cocaine was observed in day 3 compared to day 1 (initial sensitisation), but a decrease in the peak locomotor effect of cocaine was observed in day 13 compared to day 3 without alteration of the duration of the locomotor effect of cocaine, which indicates a lack of sensitisation effect at that time point. These results suggest an increase of behavioural sensitisation on day 13 of our escalating dose cocaine administration paradigm but not in our steady dose paradigm. These results are in agreement with Foltin et al., (2003) who compared the effects of escalating doses of smoked cocaine with a fixed dosing group in humans. They demonstrated a dose-dependent increase in heart rate, blood pressure, ratings of positive drug effect in the escalating dose group and a maintenance of the initial cocaine effect throughout the whole study in the fixed group. Together these results demonstrate that increasing the dose of cocaine during a “binge” of cocaine use can increase the effect of cocaine, but administering the same cocaine dose maintains, but not increases the effect of cocaine suggesting the presence of different neurobiological mechanisms that underlie the transition from drug use to compulsive drug use associated with addiction, which is a process of escalation.

The initial sensitization effect of cocaine observed in our study is in agreement with the development of locomotor sensitization observed in C57BL/6J mice in our study and in others using a 14 day steady dose “binge” pattern (15 x 3 mg/kg/day) cocaine administration

paradigm (Unterwald *et al.*, 1994; Bailey *et al.*, 2007a), but in contrast with Schlussman *et al.*, (2003) who showed tolerance to the locomotor effect of cocaine in C57BL/6J mice treated the same chronic steady dose “binge” paradigm as above, in their home cage. This discrepancy is likely to be due to the different environment where the drug was administered during the chronic “binge” paradigm (home cage vs novel environment). Indeed, it has been reported that sensitisation is more robust when cocaine is repeatedly administered outside the home cage than in the home cage (Badiani *et al.*, 1995; Browman *et al.*, 1998; Uslaner *et al.*, 2003). As a result, the increase in the duration of cocaine action observed in days 9-14 of our escalating dose paradigm is unlikely to be due to a dose effect rather than sensitization as we did not observe any increase in the duration of the cocaine effect when mice were treated with an acute 3 x 30 mg/kg (Fig 1a) or during any day of the 14 day steady dose cocaine (3 x 15 mg/kg/day) administration paradigm. This is in agreement with Daunais and McGinty (1995) who found no differences in the behavioural rating scores of male Wistar rats which received 30 or 60 mg/kg of cocaine in daily “binges”, suggesting that the expression of behavioural activity has reached a maximum at the relatively high dose of 15 mg/kg/injection. As a result, other factors such as the dysregulation of the dopaminergic system may account for the locomotor effects of chronic “binge” escalating dose cocaine administration.

In accordance with the locomotor activity data, behavioural sensitization to the stereotypy inducing effect of cocaine, followed by an increase in magnitude of stereotypy score on day 9 compared to day 5, but not on day 13 vs day 9 where the stereotypy effect of cocaine seems to reach saturation, was observed in our escalating dose study. An initial sensitisation was observed on day 3 of our steady dose paradigm which persisted throughout the rest of the duration of the study. These findings are in contrast with studies showing a lack of sensitisation to the stereotypy behaviour during an identical 14 day “binge” escalating dose cocaine administration paradigm in rats (Schlussman *et al.*, 2005) and during a 14 day steady

dose (15 x 3 mg/kg/day) “binge” cocaine administration in rats (Schlussman *et al.*, 2005) and C57BL/6J mice (Schlussman *et al.*, 2003; Bailey *et al.*, 2007a). This divergence is again likely to be due to the different environment where the drug was administered during the chronic “binge” paradigm reinforcing the importance of home cage vs novel environment.

As discussed above, there is a profound difference in the behavioural effect of cocaine when administered in a chronic “binge” escalating dose (15-30 x 3 mg/kg/day) paradigm compared to a chronic “binge” steady dose (15 x 3 mg/kg/day) paradigm. We hypothesised that these changes might be due to profound alterations in the dopamine neurotransmission during escalation of cocaine administration which may not take place during steady dose administration. In support of this, Ahmed *et al.*, (2003) showed that rats which escalate their intake of cocaine maintain greater levels of dopamine in the nucleus accumbens and are more sensitive to the actions of the selective dopamine antagonist cis-flupenthixol than rats which had limited access to the drug (steady dose) and that this phenomenon is not associated with a change in cocaine pharmacokinetics or efficacy. The above findings support the hypothesis that a decrease in dopamine neurotransmission contributes to escalation of cocaine self administration.

Although there have been numerous studies investigating the effect of chronic cocaine administration on D₁, D₂ dopamine receptor and DAT with conflicting results (see introduction), this is the first study which has investigated the effects of cocaine administered in a chronic “binge” escalating dose pattern which more closely mimics human drug abuse, on D₁, D₂ dopamine receptor, DAT binding and D₂ stimulated G protein activity. A significant decrease in D₂ receptor density was observed in all regions of the striatum of cocaine-treated mice compared to saline controls. This is in agreement with the consistent finding of a decrease in D₂ receptor availability and binding as measured by PET and in vitro

autoradiography in the brains of cocaine abstinent humans, non human primates and rats (Volkow *et al.*, 1990; Volkow *et al.*, 1993; Moore *et al.*, 1998; Nader *et al.*, 2002; Nader & Czoty, 2005; Nader *et al.*, 2006) and could reflect receptor downregulation from the exposure to a higher extracellular dopamine concentration due to chronic blockade of dopamine transporters. Moreover, and in accordance with Ahmed *et al.*, (2004), this decrease in D₂ receptors may be indicative of a “hypodopaminergic” system which is in need of escalating doses of cocaine to maintain increased levels of dopamine in the nucleus accumbens. Interestingly, our results are in contrast with those from our group (Bailey *et al.*, 2007b) and others (Unterwald *et al.*, 1994) who showed a lack of decrease in D₂ receptor binding when using a 14 day steady dose “binge” cocaine (15 x 3 mg/kg/day) administration paradigm, suggesting that cocaine-induced changes in D₂ receptors is dependent on the paradigm used (steady vs escalating). This is in agreement with Nader and Czoty, (2005) who showed that duration of exposure and dose of the drug are important factors which affect D₂ receptor density in brains of monkeys self administering cocaine.

In order to investigate whether the decrease in D₂ receptor binding was of any functional significance, we measured D₂ receptor G protein activity with the use of quinerolane stimulated [³⁵S]GTPγS autoradiography in the brains of the same animals in which D₂ receptor binding was measured, in order to obtain a completely paired result. In contrast to the D₂ receptor density data, an overall significant increase in quinerolane stimulated [³⁵S]GTPγS binding was observed in the brains of chronic “binge” escalating dose cocaine-treated mice compared to saline controls, with a marked regional significant increase in the nucleus accumbens shell and the ventromedial area of the caudate putamen. No change was observed in quinerolane-stimulated [³⁵S]GTPγS binding brains of steady dose cocaine treated mice compared to saline controls in any regions analysed. These results show the importance

of determining functional activity of receptor systems, demonstrating that downregulation of receptors does not necessarily translate into loss of functional coupling. Indeed, we showed that while no change in μ -opioid receptor (MOP-r) density was observed in brains of morphine withdrawn mice deficient in the A_{2A} receptor compared to wild types, there was an enhancement in MOP-r stimulated [35 S]GTP γ S binding in the nucleus accumbens of these mice (Bailey *et al.*, 2004). The increase in D_2 receptor coupling to G protein activity in the brains of chronic “binge” escalating cocaine treated mice suggests that chronic cocaine treatment increases D_2 receptor sensitivity by increasing D_2 receptor coupling rather than increasing D_2 receptor surface receptors. This is accordance with studies showing that locomotor and stereotypy responses to D_2 receptor agonists are greater in cocaine-sensitized rats than in saline treated controls (Ujike *et al.*, 1990; De Vries *et al.*, 2002). In addition our results are in agreement with Seeman *et al.* (2007) who showed a 2.2 fold increase in the guanine-nucleotide-sensitive component of D_2 receptor binding in amphetamine sensitized rats. This confirms the increase in D_2 receptor G protein activity in psychostimulant sensitized animals with another psychostimulant, by a different method and ligands and in different species, even though the total D_2 receptor density is significantly decreased. The maintained elevated locomotor activity observed on day 13 of the escalating dose paradigm could partially be explained by the increase in D_2 receptor activity and thus the increase in D_2 receptor sensitivity observed in these mice. This is the first study which provides biochemical evidence for the increase of sensitivity of D_2 receptors following chronic escalating dose “binge” cocaine treatment despite the decrease of D_2 receptor density.

The increase in D_2 receptor coupling to G protein in our study is unlikely to be due to increase in pertussis toxin sensitive G protein ($G_{i/o}$) which couples to D_2 receptors as it has been shown that cocaine decreases $G_{i/o}$ in the nucleus accumbens of rats (Nestler *et al.*, 1990). Alterations of other factors downstream of the receptor affecting $G_{i/o}$ coupling to D_2 receptors in response

to chronic cocaine administration might play an important role. For instance alterations in G-protein coupled receptor kinase 6 (GRK6), which is known to be involved in D₂ receptor desensitisation, might be a possible candidate, as enhanced G protein coupling to D₂ receptors and augmented locomotor responses to cocaine was observed in mice deficient in GRK6 (Gainetdinov *et al.*, 2003). The mechanism involved in the increase in D₂ receptor coupling in escalating dose cocaine treated mice is not clear. It could be the result of a homeostatic response to the decreased D₂ receptor numbers observed after chronic cocaine administration. Another possibility is that the increase in presynaptic D₂ receptor coupling would result in reducing dopamine release and thus contribute to the “hypodopaminergic” state which is indicative of escalating dose cocaine administered animals.

As quinerolane has been shown to bind to D₂ receptors as well as D₃ receptors with high affinity (Gackenhimer *et al.*, 1995), there is a possibility that the increase in quinerolane stimulated [³⁵S]GTPγS binding reflects increases of not only D₂ receptor G protein activity but also of D₃ receptors. However, the fact that quinerolane-induced stimulation was observed in regions of known high D₂ receptor density and not in regions of high D₃ receptor density (islands of Calleja, the bed nucleus of stria terminallis), and that quinerolane stimulation was completely abolished by the highly selective D₂ receptor antagonist raclopride, indicate that the increase in quinerolane stimulated [³⁵S]GTPγS binding observed in our study is attributed to D₂ receptors rather than D₃ receptors. In support of this, previous behavioural and biochemical data showed that selective D₃ and D₄ antagonists did not block quinerolane induced turning behaviour and [³⁵S]GTPγS binding in the rat (Newman-Tancredi *et al.*, 2001).

In addition to the opposite changes in D₂ receptor binding and activity observed in our study, a significant increase in DAT binding was observed in the striatum of mice treated with a

chronic “binge” escalating dose cocaine administration paradigm. This is in agreement with studies showing increases in the DAT binding and/or dopamine uptake in brains of human cocaine abusers (Staley *et al.*, 1994; Little *et al.*, 1998b; Little *et al.*, 1999; Mash *et al.*, 2002), in rodents which self administer cocaine (Wilson *et al.*, 1994a) and in rodents which have been chronically treated with cocaine (Pilotte *et al.*, 1994; Claye *et al.*, 1995; Hitri *et al.*, 1996). This increase in the numbers of DAT following chronic cocaine administration might reflect a compensatory change to modify synaptic dopamine concentration in the striatum and to maintain dopaminergic neurotransmission at more normal levels. Indeed, as cocaine elevates dopamine by blocking reuptake into the presynaptic terminal, one way to compensate for the effect of cocaine would be to enhance the uptake and transport of dopamine across the dopaminergic presynaptic membrane by increasing DAT density. Such increases in DAT might not only contribute to the dopaminergic deficit believed to occur in the brain of escalating dose cocaine self administration (Ahmed *et al.*, 2003; Ahmed & Koob, 2004), but might also contribute to cocaine withdrawal symptoms that cocaine abusers experience such as depression and suicidability and thus might sustain the rewarding effect of cocaine bingeing (Little *et al.*, 1999).

A possible mechanism by which chronic escalating dose cocaine administration might increase DAT density is by the activation of the D₂ receptors observed in our study. While Parson *et al.*, (1993) demonstrated that D₂ receptor antagonists prevents the upregulation of dopamine uptake that results from chronic intermittent cocaine administration, Mayfield and Zahniser., (2001) found that activation of D₂ receptors via the G_{i/o} coupling results in an increased number of DAT sites. Interestingly, in contrast to our data, a lack of change in DAT density was observed in C57BL/6J mice treated with a 14 day “binge” steady dose (15 x 3 mg/kg/day) administration protocol (Bailey *et al.*, 2007b).

In contrast with DAT and D₂ receptor density and activity, there was no change in D₁ receptor density in the brains of escalating dose cocaine treated mice compared to saline controls.

Although there is a large body of literature suggesting an important role for D₁ receptors in the expression of cocaine sensitisation (for review see Anderson and Pierce (2005), Spanagel (1995) and Unterwald et al., (1994), the lack of D₁ receptor changes in our study suggests that D₁ receptor numbers might not play a role in our escalating pattern of cocaine administration. These results are in accordance with a previous study in this lab (Bailey *et al.*, 2007b) which failed to find any significant change in D₁ receptor binding in a 14 day steady dose “binge” cocaine administration paradigm. However, a lack of change in D₁ receptor numbers does not exclude changes in D₁ receptor G protein coupling and in second messenger transmission downstream from the receptor. Moreover, D₁ receptors might play an important role in the initiation (early stage) of sensitisation, but not in the later stage of compulsive escalating drug abuse.

Finally, the lack of changes in D₂, D₂ stimulated [³⁵S]GTPγS and DAT binding in the steady dose cocaine treated mice together with the profound changes observed in D₂, D₂ stimulated [³⁵S]GTPγS and DAT binding in the brains of escalating dose cocaine treated mice, suggest the presence of different neurobiological mechanisms that underlie the transition from drug use to compulsive drug use associated with addiction, which is a process of escalation.

Furthermore, our results together with others indicate that our chronic “binge” escalating dose cocaine administration paradigm is a more appropriate model to study drug abuse as both the behavioural and biochemical alterations observed in this model more closely mimics those observed in human cocaine abusers.

Legend to Figure 1. Locomotor activity in C57BL/6J mice after saline or cocaine treatment during a chronic “binge” a) escalating dose and b) steady dose administration paradigm. The

locomotor data were collected daily in 5 min bins following each of the three injections of a 14 day “binge” escalating dose (15-30 x 3 mg/kg/day, i.p.) or steady dose (15 x 3 mg/kg/day, i.p.) pattern of cocaine or saline. Representative responses are shown from day 1, day 3, day 5 and day 13. The locomotor activity of one group of C57BL/6J mice treated for one day with an acute “binge” cocaine administration paradigm (30 x 3 mg/kg/day) is seen in the bottom right corner of the figure. Values are mean $\pm$ S.E.M of 6-12 animals in each group. Two-way ANOVA showed a significant main effect of treatment ($P<0.0001$), day ($P<0.0001$) and treatment x day interaction ($P<0.0001$) for both escalating and steady dose paradigms. There was an increase in basal locomotion in the cocaine treated groups vs saline control on days 2-14 ($P<0.01$) on the treatment paradigm in the escalating dose group. Significant asterisks were omitted from the graph for clarity of presentation.

Legend to Figure 2. Daily stereotypy scores in C57BL/6J mice following chronic “binge” a) escalating dose and b) steady dose cocaine administration. The stereotypy score assigned to each animal was based on its median stereotypy score of the three daily injections of “binge” pattern cocaine or saline administration recorded 20 min after each of injection. Values are mean $\pm$ S.E.M of 6 animals in each group. Two way ANOVA showed a significant main effect of treatment ($P<0.0001$), day ($P<0.0001$) and significant treatment x day interaction ($P<0.0001$) for both escalating and steady dose treatment. Cocaine administration resulted in a significant expression of behavioural stereotypy ($P<0.0001$)

*** $P<0.001$ vs day 1, # $P<0.05$ vs day 5 (Newman-Keuls post hoc test)

Legend to Figure 3. Computer-enhanced autoradiograms of coronal brain sections from chronic “binge” escalating dose cocaine or saline treated C57BL/6J mice. The adjacent sections shown are from the level of the caudate (Bregma 1.10 mm). D₁, D₂ dopamine receptor and dopamine transporters (DAT) were labelled with [³H]SCH-23390 (4 nM),

[³H]raclopride (4 nM) and [³H]mazindol (4 nM), respectively. Non-specific binding (NSB) was defined in the presence of cis-flupenthixol (10 μM), sulpiride (10 μM), or unlabelled mazindol (10 μM) for [³H]SCH-23390, [³H]raclopride and [³H]mazindol binding respectively. The colour bar represents a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Sections cut from chronically cocaine and saline treated C57BL/6J brains were processed in parallel.

Legend to Figure 4. Quantitative autoradiography of D₁, D₂ dopamine receptor and dopamine transporters binding in coronal brain sections from chronic “binge” escalating dose cocaine and saline treated C57BL/6J mice. Data are expressed as the mean specific binding (fmol/mg) ± S.E.M of 5-10 determinants of A) [³H]raclopride, B) [³H]mazindol and C) [³H]SCH-23390 in brain regions of chronically cocaine or saline treated C57BL/6J mice.

The labelling was carried out on sections from chronically cocaine or saline treated C57BL/6J mice in a completely paired protocol. Specific binding was >80 % in all regions analysed.

Two way ANOVA showed a significant main effect of treatment ($P < 0.001$) for [³H]mazindol binding and [³H]raclopride binding, but not for [³H]SCH-23390 binding.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs saline controls (LSD post hoc test). Abbreviations:

CgCx, Cingulate cortex; AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; Tu, Olfactory tubercle; Den, Dorsal endopiriform; DLCPu, Dorsolateral caudate putamen; DMCPu, Dorsomedial caudate putamen; VMCPu, Ventomedial caudate putamen; VLCPu, Ventrolateral caudate putamen; LS, Lateral septum; MS, Medial septum

Legend to Figure 5. Computer-enhanced autoradiograms of coronal brain sections from chronically “binge” escalating dose cocaine and saline treated C57BL/6J mice. The sections shown are from the level of the caudate (Bregma 1.10 mm) Sections were preincubated with

2 mM GDP and then incubated for 2 h with [³⁵S]GTPγS (0.04 nM) and 2 mM GDP (basal), [³⁵S]GTPγS (0.04 nM) and 2 mM GDP in the presence of quinerolane (100 μM) (quinerolane stimulated [³⁵S]GTPγS), [³⁵S]GTPγS (0.04 nM) and 2 mM GDP in the presence of quinerolane (100 μM) and raclopride (10 μM) (reversal of quinerolane stimulated [³⁵S]GTPγS) or with [³⁵S]GTPγS (0.04 nM) and 2 mM GDP in the presence of 10 μM unlabelled GTPγS (non-specific binding, NSB). The colour bar represents a pseudo-colour interpretation of relative black and white film images. Sections cut from chronically “binge” saline and cocaine treated mice brains were processed in parallel.

Legend to Figure 6. Quinerolane stimulated [³⁵S]GTPγS binding in coronal brain sections from chronically “binge” a) escalating dose and b) steady dose cocaine and saline treated C57BL/6J mice. Data are expressed as mean ± S.E.M (n=6-12) of [³⁵S]GTPγS stimulation expressed as increase in relative optical density (ROD) above basal. Two way ANOVA showed a significant main effect of raclopride (reversal) ($P < 0.001$) and a significant raclopride x treatment (saline, cocaine) interaction ($P < 0.05$) in the escalating dose group. There was an overall increase in quinerolane stimulated [³⁵S]GTPγS binding in the escalating dose cocaine treated mice compared to saline controls ($P < 0.05$). No cocaine effect was observed in the steady dose group.

$P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs D₂ stimulated [³⁵S]GTPγS binding in the cocaine group; * $P < 0.05$ vs D₂ stimulated [³⁵S]GTPγS binding in the saline group (Student's t test).

Abbreviations: AcbC, accumbens core; AcbSh, accumbens shell; Tu, olfactory tubercle; DLCPu, Dorsolateral caudate putamen; DMCPu, Dorsomedial caudate putamen; VMCPu, Ventomedial caudate putamen; VLCPu, Ventrolateral caudate putamen; LS, Lateral septum; MS, Medial septum

Acknowledgements: Supported by the European Commission EC (LSHM-CT-2004-005166), the Korea Science and Engineering Foundation (2005-215-E00003) and Merck, Sharp and Dohme.

Abbreviations: AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; ANOVA, analysis of variance; CgCx, Cingulate cortex; DAT, dopamine transporter; Den, Dorsal endopiriform; DLCPu, Dorsolateral caudate putamen; DMCPu, Dorsomedial caudate putamen, $G_{i/o}$, G- protein; KOP-r, κ - opioid receptors, LS, Lateral septum; MOP-r, μ - opioid receptors, MS, Medial septum; NSB: non specific binding, PET, positron emission tomography; Tu, Olfactory tubercle; VMCPu, Ventomedial caudate putamen; VLCPu, Ventrolateral caudate putamen

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Fig. 1 a) Escalating dose

b) Steady dose

Fig. 2

a) Escalating dose

b) Steady dose

Fig.3

Fig.4.

Fig. 5.

Fig6

a. Escalating dose

b. Steady dose