Title: Mouse strain differences in locomotor, sensitisation and rewarding effect of heroin;
association with alterations in MOP-r activation and dopamine transporter binding

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Abstract

There is growing agreement that genetic factors play an important role in the risk to develop heroin addiction and comparisons of heroin addiction vulnerability in inbred strains of mice could provide useful information on the question of individual vulnerability to heroin addiction. This study examined the rewarding and locomotor stimulating effects of heroin in male C57BL/6J and DBA/2J mice. Heroin induced locomotion and sensitisation in C57BL/6J but not in DBA/2J mice. C57BL/6J mice developed conditioned place preference (CPP) to the highest doses of heroin, while DBA/2J showed CPP to only the lowest heroin doses, indicating a higher sensitivity of DBA/2J mice to the rewarding properties of heroin versus C57BL/6J mice. In order to investigate the neurobiological substrate underlying some of these differences, the effect of chronic “intermittent” escalating dose heroin administration on the opioid, dopaminergic and stress systems was explored. Two fold higher μ - opioid receptor (MOP-r) stimulated [35 S]GTP γ S binding was observed in the nucleus accumbens and caudate of saline treated C57BL/6J mice compared to DBA/2J. Heroin decreased MOP-r density in brain regions of C57BL/6J mice, but not in DBA/2J. Higher density of dopamine transporters (DAT) was observed in nucleus accumbens shell and caudate of heroin treated DBA/2J mice compared to heroin treated C57BL/6J. There were no effects on D₁ and D₂ binding. Chronic heroin administration decreased corticosterone levels in both strains with no effect of strain. These results suggest that genetic differences in MOP-r activation and DAT expression may be responsible for individual differences in vulnerability to heroin addiction (250 words)

Heroin is one of the widest abused opiate drug (Pouletty 2002). There is growing agreement that genetic factors play an important role in the risk to develop heroin addiction (Kreek *et al.* 2005). Inbred strains of mice have served as a valuable model to study vulnerability to drugs of abuse due to the large inter strain variability in their drug-induced behavioural phenotype and the observations clearly suggest that major genetic influences control this effect (Murphy *et al.* 2001; Schlussman *et al.* 2008).

In this regard, two of the most studied inbred strains of mice are C57BL/6J mice and DBA/2J. C57BL/6J mice are considered to be highly sensitive to the rewarding and reinforcing effects of alcohol, nicotine and amphetamine, but DBA/2J mice appear to be insensitive to amphetamine and alcohol (Belknap *et al.* 1993; Meliska *et al.* 1995; Stolerman *et al.* 1999; Cabib *et al.* 2000; Orsini *et al.* 2004; Glatt *et al.* 2009), whereas inconsistent findings have been reported for cocaine and morphine (Cunningham *et al.* 1992; Semenova *et al.* 1995; Orsini *et al.* 2005).

Although heroin is one of the widest abused opiates (Pouletty 2002), there are limited number of studies addressing the effect of genotype on heroin addictive behaviours in inbred strains of mice. There is some evidence suggesting that the pharmacological and behavioural profiles of morphine and heroin may differ in respect to analgesia, tolerance and dependence (Rady *et al.* 1991; Rossi *et al.* 1996; Klein *et al.* 2008). This study sought to determine whether the rewarding and locomotor stimulating effect of heroin are dependent on genotype by comparing the locomotor, sensitisation and CPP responses to heroin in C57BL/6J and DBA/2J mice during a paired CPP protocol.

The neurobiological substrate underlying the strain differences in opioid abuse sensitivity is not clear. While Berrettini et al., (1994) reported that strain differences in MOP-r density and regulation may be responsible for the distinct behavioural phenotypes in response to morphine, Doyle et al., (2006) failed to find any significant transcriptional regulation of MOP-r in any of these strains. Moreover, as the stimulatory effects of opiates in rodents has been attributed to an increase in dopamine release in the striatum (Di-Chiara & Imperato 1988a; 1988b), it was hypothesised that strain differences in opioid induced dopamine release could account for the distinct behavioural phenotypes. Microdialysis studies did not confirm this hypothesis (Murphy *et al.* 2001; Fadda *et al.* 2005). Nonetheless, that does not exclude the presence of differences in the sensitivity of the D₁ and D₂ receptors and dopamine transporters (DAT) between the two strains. As a result we hypothesise that strain differences in the sensitivity of MOP-r and dopaminergic system or in their regulation by opiates could account for the strain differences observed in chronic opiate mediated addictive related behaviours. We carried out MOP-r, MOP-r agonist stimulated [³⁵S] guanosine 5'-[γ-[³⁵S]thio]-triphosphate (GTPγS), D₁, D₂ and DAT autoradiography in parallel in brain sections of C57BL/6J and DBA/2J mice treated with a chronic “intermittent” escalating dose heroin administration paradigm. (495 words)

Methods

Animals

Male C57BL/6J and DBA/2J mice (B & K Universal Ltd, Hull, UK) weighing 20-25 g were used. Mice were individually housed in a temperature and humidity controlled room with a 12h light/dark schedule for at least 2 weeks before experiments were begun. Food and water were available at libitum. All animals were weighed daily throughout the study. All studies

were performed in accordance with protocols approved by the Home Office (Animals Act 1986) UK and the European Community Council Directive of the 24th Nov 1986 (86/609/EEC).

Heroin-induced conditioned place preference and sensitization

The place preference of the animals was monitored by the use of the automated CPP apparatus, Opto-Max Activity Meter v2.16 (Columbus Instruments, Columbus, OH, USA). This apparatus has been previously described by Achat-Mendes (2003). The apparatus consisted of a rectangular Plexiglas box (42 x 20 x 20 cm) divided by a removable guillotine door into two chambers of equal size (21 x 20 x 20 cm), one comprising a black floor with 4 black walls (black compartment) and the other a white floor with black vertical stripes (2 cm) with 4 white walls with black vertical stripes (2 cm) (white compartment). The apparatus was covered with a transparent Plexiglas lid perforated to allow adequate ventilation. A transparent colourless, enclosed Plexiglas waiting chamber (12 x 8 x 8 cm) was affixed to one side of the CPP cage at the junction of the black and white compartments. Mice were placed in the waiting chamber and allowed entry via a guillotine door. The cage was equipped with matching pairs of horizontal sensors mounted alongside opposing lengths (42 cm long). The black and striped compartments were scanned at a rate of 10 Hz by 7 infrared beams, spaced at 2.54 cm intervals. A null zone was assigned at the interface of the black and white zones in the centre of the box and was monitored by 2 beams.

The CPP experiments were conducted during the light phase of the light-dark cycle (lights on: 7:00 a.m) and followed by four sequential phases: habituation, preconditioning test, conditioning and postconditioning test. Throughout the experiment, the mice were brought into the test room 1 h before being tested. Following habituation (day 1), in which animals

had free-access to both compartments of the place preference chambers for 20 min, the mice were given a preconditioning test (20 min) (day 2) in which the time spent in the three compartments (black, white and null) in the last 15 min of the test was compared to establish compartment preference prior to conditioning. Animals showing strong unconditioned aversion (less than 33 % of the session time present in one compartment compared to the others) or preference (more than 67% of the session time present in one compartment compared to the others) for any compartment were discarded. On the following 4 days, two conditioning sessions were given daily. The rectangular Plexiglas box was then divided into two equal chambers separated by a removable guillotine door. At 9:00 a.m., C57BL/6J and DBA/2J mice were injected with saline (10 ml/kg, s.c.) and immediately confined to one side of the apparatus (a.m. session). Four hours later, the same animals were injected with heroin (Sigma-Aldrich, Poole, Dorset, UK) (0.5, 1, 2, 4 or 8 mg/kg, s.c.) and confined to the opposite side of the apparatus (p.m. session). Always, during each conditioning session, the mice that showed initial preference to one chamber were paired with the drug injection in the opposite chamber (drug paired with the least preferred side- non counterbalanced biased compartment assignment). Conditioning trials lasted 30 min. Control animals were included which received saline every day. As described by Szumliński (2005), locomotor activity was monitored during the first and fourth heroin/saline conditioning session when the mice were confined to one compartment to assess for strain differences in the development of heroin-induced locomotor sensitization. The day after the last conditioning session, a postconditioning test session was performed in the undrugged state by allowing free access of the mice to all chambers of the apparatus as in the preconditioning session. Time spent in each chamber was recorded for the last 15 min of the 20 min test to establish compartment preference after conditioning. The first 5 min were omitted in our preference testing in order to minimize the influence of stress that the mice would experience in their non home cage environment on CPP behaviour.

Chronic heroin administration

Following acclimatisation, subcutaneous (s.c.) injections of either saline (10 ml/kg) or heroin were then administered to C57BL/6J and DBA/2J mice in a chronic “intermittent” escalating dose paradigm to mimic a common pattern of self-administration in human heroin abusers (Kreek *et al.* 2002). Animals in the escalating dose group received 2x1 mg/kg/injection on day 1, 2x2 mg/kg/injection on days 2 and 3, 2x4 mg/kg/injection on days 4 and 5, and 2x8 mg/kg/injection on days 6 and 7. Two subcutaneous injections were given daily (5 p.m and 9 a.m) , in accordance with a protocol which has been used as an animal model to investigate heroin and morphine addictive processes (Spanagel 1995; Muller & Unterwald 2004).

Heroin-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 6 cm above the cage floor. Locomotor activity was defined as the activity from measurement of sequential infrared beam breaks, recorded every 15 min, beginning immediately after placing the animals in the cage following an injection of saline or heroin and continued for a 2 h period. For measurement of basal activity, mice were habituated for 60 min in the motility boxes prior to injections. The locomotor activity for each mouse injected with the 9 a.m injections of saline or heroin was monitored every day for the 7 days duration of the study.

μ -(MOP-r), D₁, D₂ dopamine receptor and dopamine transporter (DAT) autoradiography

Animals were killed 2 h following the last injection of saline or heroin on day 7 of the “intermittent” escalating dose heroin administration study described above, and the brains were immediately removed, snap frozen at -25 °C and stored at -80 °C until sectioning. Quantitative autoradiography was performed as detailed previously for MOP-r binding (Kitchen *et al.* 1997; Bailey *et al.* 2002; Bailey *et al.* 2005; Bailey *et al.* 2007a; Bailey *et al.* 2008), for D₁, D₂ dopamine receptor binding (Lena *et al.* 2004; Bailey *et al.* 2008) and DAT binding (Javitch *et al.* 1985; Bailey *et al.* 2008) using the general procedures of Kitchen *et al.* (1997). Adjacent 20 µm coronal sections were cut at an interval of 300 µm from chronic saline or heroin treated C57BL/6J and DBA/2J mice brains for the determination of total and non-specific binding of [³H] D-Ala²-MePhe⁴-Gly-ol⁵ enkephalin DAMGO (GE Healthcare Life Sciences, Bucks, UK), [³H]SCH-23390, [³H]raclopride and [³H]mazindol (Perkin Elmer, Bucks, UK) at MOP-r, 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 was defined in the presence of naloxone (1 µM) for [³H]DAMGO, and cis-flupenthixol (10 µM), sulpiride (10 µM), or unlabelled mazindol (10 µM) (Sigma-Aldrich, Poole, Dorset, UK) for [³H]SCH-23390, [³H]raclopride and [³H]mazindol binding respectively. Following a binding period of 60 min for MOP-r and 90 min, 60 min or 45 min for D₁, D₂ dopamine receptor and DAT respectively, and washing in ice cold buffer (3x5 min for MOP-r binding, 6x1 min for D₁ and D₂ dopamine receptor binding and 2x1 min for DAT binding), the slides were apposed to Kodak MR film (Sigma-Aldrich, Poole, Dorset, UK) for 4 weeks for D₁ and DAT binding and 3 weeks for MOP-r and D₂ binding. Sections from chronic saline and heroin treated C57BL/6J and DBA/2J mice were processed together in a paired protocol. Films were developed using 50% Kodak D19 developer (Sigma-Aldrich, Poole, Dorset, UK). 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, Ontario, Canada) and brain structures were identified using the mouse brain atlas of Franklin and Paxinos (1997).

μ - opioid receptor (MOP-r) stimulated [35 S]GTP γ S binding autoradiography

General procedures for MOP-r agonist-stimulated guanosine 5'-[γ - 35 S]thio]-triphosphate ([35 S]GTP γ S) autoradiography were performed essentially as described by Sim *et al.* (1996), Kirschke *et al.* (2002) and Bailey *et al.* (2004; 2007b). Adjacent 20 μ m coronal sections were cut at a interval of 300 μ m from chronic saline or heroin treated C57BL/6J and DBA/2J mice brains for the determination of basal, DAMGO-stimulated [35 S]GTP γ S and non-specific binding. Sections were preincubated in assay buffer (50 mM TrisHCl, 3 mM MgCl₂.6H₂O, 0.2 mM EGTA, 100 mM NaCl, pH 7.4 at 25 °C) for 10 min. Sections were then incubated in assay buffer containing 1 mM GDP (Sigma-Aldrich, Poole, Dorset, UK), pH 7.4 at 25 °C for 15 min and rinsed in fresh assay buffer. MOP-r agonist-stimulated activity was determined by incubating the sections in [35 S]GTP γ S (0.04 nM) (Perkin Elmer, Bucks, UK) with the selective μ - opioid receptor agonist DAMGO (5 μ M) (Tocris Bioscience, Bristol, UK) for 2 h in assay buffer containing 1 mM GDP. Basal G protein activity was determined by incubating the sections in [35 S]GTP γ S in the presence of GDP (1 mM) and in the absence of the agonist. Non-specific binding was assessed by incubating sections with [35 S]GTP γ S in assay buffer in the presence of 10 μ M unlabelled GTP γ S (Sigma-Aldrich, Poole, Dorset, UK). After incubation for 2 h, slides were rinsed twice for 3 min in ice cold buffer (50 mM TrisHCl, 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 sulphate (Drierite, BDH Chemicals, Poole, UK). Sections from chronic saline and heroin

treated C57BL/6J and DBA/2J mice were apposed in a paired protocol to Kodak MR film together with [^{14}C] standards (GE Healthcare Life Sciences, Bucks, UK) 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 non-specific binding sections from the optical density of either agonist-stimulated or basal binding sections.

Measurement of corticosterone plasma levels

Chronic “intermittent” saline and heroin treated C57BL/6J and DBA/2J mice were decapitated at the end of the treatment and trunk blood from each animal was collected in tubes placed on ice, and was spun in a refrigerated centrifuge. Plasma was separated and stored at -20°C for corticosterone measurements by radioimmunoassay. Corticosterone levels were assayed by using a rat/mouse corticosterone [^{125}I] kit (MP Biomedicals, NY, NY, USA). All corticosterone levels were determined in duplicate in a single assay.

Statistics

For the conditioned place preference experiments, three-way ANOVA for factors strain, dose (saline, 0.5, 1, 2, 4, 8mg/kg heroin) and test (pre-conditioned, post-conditioned) with repeated measures for test, followed by the Newman-Keuls post hoc test was used for comparison of pre-conditioning and post-conditioning time spent in the drug paired compartment (shift in preference). Two way ANOVA for factors dose and strain followed by the Newman-Keuls post hoc test was used for comparison of the change of time spent in heroin paired compartment. Time spent by each strain in the white and black compartments of the heroin CPP apparatus during the preconditioning phase of the CPP study was analysed using the

nonparametric Mann-Whitney *U* test. For ambulatory activity data in CPP experiments, three-way ANOVA for factors strain, dose and day (i.e. first day of conditioning and fourth day of conditioning) with repeated measures for the last variable, followed by the Newman-Keuls post hoc test was used for comparison of ambulatory activity of C57BL/6J and DBA/2J mice on the first and fourth conditioning day of our CPP paradigm. For the chronic escalating dose study, heroin-induced locomotor activity in 15 min bins was analysed by three-way analysis of variance (ANOVA) (for factors strain, treatment and day) with repeated measures for day, followed by Newman-Keuls post hoc test. Three-way ANOVA (for factors strain, treatment and region) was used for comparison of quantitative measures of MOP-r, D₁, D₂ receptors, DAT and MOP-r stimulated [³⁵S]GTPγS binding in chronic saline and heroin treated C57BL/6J and DBA/2J brains followed by LSD post hoc test. Comparison of plasma corticosterone levels in chronic “intermittent” saline and heroin treated C57BL/6J and DBA/2J mice was performed using two-way ANOVA (for factors strain and treatment) followed by post hoc LSD test. All values were expressed as mean ± S.E.M. All statistical analysis was carried out with the use of statistical package “Statistica” (Bedford, Beds, UK)

Results

Heroin-induced conditioned place preference in C57BL/6J and DBA/2J mice

Rewarding responses induced by heroin (0.5, 1, 2, 4 and 8 mg/kg, s.c.) were evaluated in C57BL/6J and DBA/2J mice using the place conditioning paradigm. One-way ANOVA revealed a similar time spent in the drug associated compartment during the pre-conditioning phase in the different groups (C57BL/6J and DBA/2J) (*P*>0.05). Moreover, neither C57BL/6J nor DBA/2J mice showed any unconditioned preference for either of the two compartments (black or white) of the apparatus used for heroin conditioned place preference

but spent less time in the smaller centre compartment ensuring the use of an apparatus unbiased procedure (Table 1). Shifts in preference between preconditioning and post conditioning days after heroin treatment are shown in Suppl. 1. Three-way ANOVA showed a significant dose ($F_{(5,152)}=4.3$, $P<0.01$), test ($F_{(1,152)}=101.6$, $P<0.001$), strain x test interaction ($F_{(1,152)}=4.5$, $P<0.05$) and a significant dose x test effect ($F_{(5,152)}=5.3$, $P<0.001$). The Newman-Keuls post hoc test showed that C57BL/6J mice developed CPP at 2, 4 and 8 mg/kg of heroin but not at 0.5 and 1 mg/kg (Suppl. 1). C57BL/6J mice spent significantly more time in the drug paired compartment when they were paired with 2 mg/kg heroin compared to 0.5 and 1 mg/kg heroin during the post conditioning phase (Newman-Keuls post hoc test) (Suppl. 1). However, DBA/2J mice developed CPP at 0.5, 1 and 2 mg/kg heroin ($P<0.001$), but not at 4 and 8 mg/kg ($P>0.05$) (Suppl. 1). DBA/2J mice spent significantly less time in the drug paired compartment when they were paired with 4 and 8 mg/kg heroin compared to 2 mg/kg heroin during the post-conditioning phase (Newman-Keuls post hoc test, Suppl. 1). The change in time spent in heroin paired compartments are shown in Fig. 1. Two-way ANOVA showed a significant strain ($F_{(1,154)}=4.5$, $P<0.05$) and dose ($F_{(5,154)}=5.3$, $P<0.001$) effects. The results are similar to the ones presented above. While C57BL/6J mice developed conditioned place preference to 2, 4 and 8 mg/kg of heroin but not at 0.5 and 1 mg/kg, DBA/2J mice developed conditioned place preference at 0.5, 1 and 2 mg/kg heroin, but not at 4 and 8 mg/kg (Fig. 1, Neuman Keuls post hoc test).

Locomotor responses induced by heroin (0.5, 1, 2, 4 and 8 mg/kg, s.c.) were evaluated in C57BL/6J and DBA/2J mice during the first (day 1) and the last (day 4) day of the conditioning phase of our CPP paradigm. Three way ANOVA showed a significant strain ($F_{(1,132)}=490.0$, $P<0.001$), dose ($F_{(5,132)}=53.5$, $P<0.001$), strain x dose interaction ($F_{(1,132)}=57.3$, $P<0.001$), strain x day ($F_{(1,132)}=9.7$, $P<0.01$), dose x day ($F_{(5,132)}=4.1$, $P<0.01$) and strain x dose x day ($F_{(5,132)}=5.7$, $P<0.001$) effects. Newman Keuls post hoc test showed that heroin (1,

2, 4 and 8 mg/kg, s.c.) significantly increased ambulatory activity both on day1 ($P<0.001$) and day 4 ($P<0.001$) of the conditioning phase in C57BL/6J mice, but not in DBA/2J ($P>0.05$) (Fig. 2). Moreover, repeated injections of heroin (2, 4 and 8 mg/kg, s.c.) resulted in a significant increase ($P<0.01$, Newman Keuls post hoc test) in ambulatory activity on day 4 of the conditioning phase compared to day1 in C57BL/6J mice, thus demonstrating the development of locomotor sensitisation. Such sensitization effect was not observed at 0.5 and 1 mg/kg heroin. Heroin had no locomotor or sensitisation effect in DBA/2J mice at any dose tested ($P>0.05$) (Fig. 2).

Heroin-induced locomotion in C57BL/6J and DBA/2J mice during chronic “intermittent” heroin administration

Locomotor activity of naïve C57BL/6J and DBA/2J mice was measured 45 min after placement in the locomotor chambers for a 15 min duration on day 1 (Fig. 3). One-way ANOVA, did not show any significant strain effect on basal locomotor activity ($P>0.05$).

Locomotor activity of C57BL/6J and DBA/2J mice was measured after the second of the two daily injections of saline or heroin (1-8 mg/kg) from day 1 to day 7 of the chronic “escalating” dose heroin treatment (9 a.m.) (Fig. 3). There was no significant difference in locomotor activity between saline treated C57BL/6J and DBA/2J mice at any day of the treatment protocol.

Three-way ANOVA, for factors strain (C57BL/6J and DBA/2J), treatment (saline, heroin), day (day 1 through to day 7) with repeated measures on the last variable, showed a significant main effect of strain [$F_{(1,188)}=56.85$, $P<0.0001$], treatment [$F_{(1,188)}=101.76$, $P<0.0001$], day [$F_{(6, 1128)}=80.16$, $P<0.0001$] and a significant strain x treatment x day interaction [$F_{(6,$

$_{1128})=59.66, P<0.001]$. Heroin injections significantly increased locomotor activity in C57BL/6J animals versus saline in a dose dependent manner (Fig. 3). However, heroin did not increase locomotor activity of DBA/2J mice at any day or dose used in the treatment protocol (Fig.3). The locomotor activity of heroin-treated C57BL/6J mice was significantly higher than that of DBA/2J mice on days 3-7.

MOP-r receptor autoradiography in chronic heroin treated C57BL/6J and DBA/2J mice

Quantitative analysis of MOP-r binding showed a significant treatment treatment ($F_{1,251} = 6.71, P < 0.05$), strain ($F_{1,251} = 7.52, P < 0.01$), region x strain ($F_{13,251} = 149.2, P < 0.001$) and strain x treatment interaction ($F_{1,251} = 9.10, P < 0.01$). LSD post hoc analysis showed a significant decrease in MOP-r binding in the nucleus accumbens shell ($P<0.05$), centromedial nucleus of the thalamus ($P<0.05$) and habenula ($P<0.01$) of chronic heroin treated C57BL/6J mice compared to saline controls (Fig. 4). In contrast, chronic heroin treatment did not alter the density of MOP-r in any of the brain regions of DBA/2J mice analysed (Fig. 4).

Moreover, significantly higher binding of MOP-r was observed in the nucleus accumbens core ($P<0.05$) and shell ($P<0.05$), the hypothalamus ($P<0.05$) and habenula ($P<0.001$) of chronic heroin treated DBA/2J mice compared to chronic heroin treated C57BL/6J mice (Fig. 4). Finally, there was significantly lower binding of MOP-r in the centromedial nucleus of the thalamus ($P<0.05$) and centrolateral nucleus of the thalamus ($P<0.01$) of chronic saline treated DBA/2J mice compared to saline treated C57BL/2J animals (Fig. 4).

D₁, D₂ dopamine receptor and dopamine transporter (DAT) autoradiography in chronic heroin treated C57BL/6J and DBA/2J mice

In the case of DAT binding, three way ANOVA showed a significant main effect of strain ($F_{1,95} = 13.3$, $P < 0.001$) and a significant strain x treatment interaction ($F_{1,95} = 8.8$, $P < 0.01$) with no significant treatment effect. LSD post hoc analysis showed significantly higher levels DAT binding in the nucleus accumbens core of chronic heroin treated DBA/2J mice compared to chronic saline treated DBA/2J mice ($P < 0.05$) (Fig. 5A, supplement 2). Post hoc analysis also showed significantly higher levels of DAT binding in the nucleus accumbens shell ($P < 0.05$), olfactory tubercle ($P < 0.001$) and caudate putamen ($P < 0.05$) of chronic heroin treated DBA/2J mice compared to chronic heroin treated C57BL/6J mice. In contrast to DBA/2J mice, no significant treatment effect was observed in any of the regions of C57BL/6J mice analysed (Fig. 5A). In contrast to DAT binding, there was no significant strain or treatment or treatment x strain interaction effect of D₁ or D₂ dopamine receptor binding (Fig. 5 B, C supplement 2) ($P > 0.05$).

μ - opioid receptor stimulated [³⁵S]GTP γ S binding autoradiography in brains of chronic saline and heroin treated C57BL/6J and DBA/2J mice

DAMGO (5 μ M) stimulated [³⁵S]GTP γ S autoradiography was performed in coronal brain sections of chronic saline or heroin treated C57BL/6J and DBA/2J mice (Fig. 6) in order to investigate whether there are any alterations in MOP-r activity after chronic heroin administration. Three-way ANOVA for factors strain, treatment and region showed a significant effect of strain ($P < 0.001$) and strain x region interaction ($P < 0.01$) with no significant treatment effect (Fig. 7). Significantly higher levels of MOP-r stimulated [³⁵S]GTP γ S binding (around 100%) was observed in the nucleus accumbens core ($P < 0.05$) and shell ($P < 0.05$) of both saline and heroin treated C57BL/6J mice compared to DBA/2J

(Fig. 7). Lower levels of MOP-r stimulated [³⁵S]GTPγS binding ($P<0.05$) was also observed in the caudate putamen of saline treated DBA/2J mice compared saline treated C57BL/6J.

Blood corticosterone levels in chronic saline and heroin treated C57BL/6J and DBA/2J mice

Two way ANOVA showed a significant treatment effect ($P<0.01$) but no strain effect.

Chronic heroin treatment decreased corticosterone levels in both C57BL/6J and DBA/2J mice ($P<0.05$, LSD post hoc test) (Fig. 8).

Discussion

The behavioural results in this study demonstrate profound differences in the sensitivity to the locomotor, sensitisation and rewarding effects of heroin, between C57BL/6J and DBA/2J mice suggesting that the sensitivity to heroin related addictive behaviours is genotype dependent. While heroin significantly increased locomotor activity in C57BL/6J, it did not increase locomotion in DBA/2J mice, even at the highest dose of 8 mg/kg of our CPP and chronic “intermittent” escalating dose heroin administration paradigm. Moreover, repeated administration of heroin at all but 0.5 and 1 mg/kg doses tested in our CPP paradigm induced locomotor sensitisation in C57BL/6J mice but not in DBA/2J, which indicates that C57BL/6J are sensitive and DBA/2J mice resistant to the locomotor and sensitisation effect of heroin. These results are consistent with studies showing that morphine induced strong locomotor and sensitisation responses in C57BL/6J mice, while DBA/2J mice are not stimulated at all (Oliverio & Castellano 1974a; 1974b; Brase *et al.* 1977; Belknap *et al.* 1989; Wenger 1989; Belknap *et al.* 1998; Orsini *et al.* 2005). The fact that the locomotion and sensitization results obtained in our study with heroin, a synthetic opiate which is metabolized to MOP-r agonists morphine and 6-monoacetylmorphine, are in complete agreement and accordance with those

obtained by other groups with the use morphine, suggest that the locomotor and sensitization effects of both heroin and morphine are most likely to be mediated via the same mechanism, via MOP-r activation. In support of this, it has been shown that both heroin and morphine lacked any kind of locomotor stimulation in MOP-r deficient mice (Matthes *et al.* 1996; Contarino *et al.* 2002). However, this is not necessarily true for other opioid effects. For instance Schuller et al., (1999) reported that heroin retained its analgesic effect in mutant mice with a targeted disruption of exon 1 of MOP-r, while morphine lost its analgesic response in these animals.

C57BL/6J and DBA/2J mice also differed in their rewarding effect of heroin as measured with CPP. Both C57BL/6J and DBA/2J mice showed significant rewarding responses to 2 mg/kg heroin, whereas high doses of 4 and 8 mg/kg heroin were only rewarding in C57BL/6J mice. At these high doses, heroin had no rewarding effect in DBA/2J mice. In contrast, at the lower doses of 0.5 and 1 mg/kg, heroin was shown to be highly rewarding in DBA/2J mice, while it did not induce reward in C57BL/6J mice. This inverted U-shaped dose response functional stimulation observed for the rewarding effect of heroin in C57BL/6J and DBA/2J mice has also been reported in C57BL/6J mice (Schlussman *et al.* 2008). This shift in the dose response curve to the left observed in our study in DBA/2J mice indicates a higher sensitivity of the DBA/2J strain to the rewarding properties of heroin compared to C57BL/6J mice which seems paradoxical considering our locomotor data.

These results are in agreement with morphine-induced CPP studies showing that DBA/2J mice display stronger CPP in response to morphine than C57BL/6J (Cunningham *et al.* 1992; Semenova *et al.* 1995) and in contrast with Orsini et al., (2005) who showed higher sensitivity for morphine in C57BL/6J mice compared to DBA/2J. As differences between results could occur due to differences in experimental protocol, our study was designed to measure

locomotor and CPP behaviours during the same CPP session in the same animals. Although different methodologies were used between our study and others (e.g. different apparatus bias, conditioning time), it is unlikely that the discrepancies observed are due to differences in the CPP protocol. However, differences in environment conditions (e.g. stress) experienced by our DBA/2J mice compared to DBA/2J mice used by other groups may account for the discrepancies observed. Indeed, it has been well demonstrated that behavioural effects of DBA/2J mice in response to amphetamine are highly dependent on environmental experiences (Cabib *et al.* 2000) and social isolation has also been shown to attenuate morphine induced CPP in male NMRI mice (Coudereau *et al.* 1997). In our study, both C57BL/6J and DBA/2J mice were individually housed for at least 2 weeks before the CPP experiment begun, whereas in all the aforementioned experiments, the mice were multiply housed in groups (4-12 animals per cage). Moreover, acute opioid administration is well known to induce a stress response by activating the hypothalamic-pituitary-adrenal (HPA) axis activity in rodents (Buckingham & Cooper 1984; Fuertes *et al.* 2000). As a result, we explored whether behavioural strain differences observed in response to heroin in our study are due to differential effect of heroin on HPA axis activity in the two strains of mice. The stress hormone corticosterone was measured in the plasma of chronic heroin treated C57BL/6J and DBA/2J mice but no strain differences were observed. Chronic heroin administration significantly decreased corticosterone in both C57BL/6J and DBA/2J mice which is a indication of the development of tolerance to the opioid stimulating effect on endocrine secretion which is consistent with the findings of Fuertes et al., (2000). This result suggests that stress may not have influenced the behavioural response to heroin in DBA/2J mice.

The lack of correlation between the rewarding and locomotor responses to heroin in C57BL/6J and DBA/2J mice supports the hypothesis that reward and locomotion are

distinctly separate responses regulated by distinct mechanisms. The same may be true for other MOP-r mediated effects. Interestingly, while DBA/2J mice are more sensitive to the analgesic responses of morphine (Semenova *et al.* 1995), C57BL/6J mice showed greater morphine consumption compared to DBA/2J mice (Crabbe & Belknap 1992) suggesting that the analgesic and self administration effect of opioids are also regulated by distinct mechanisms.

As profound differences were observed in the behavioural effects of heroin in C57BL/6J and DBA/2J mice, we carried out further studies in order to investigate the neurobiological basis that might underlie some of these differences. Interestingly, different effects were observed between MOP-r and MOP-r stimulated G protein activity in our study. While a profound strain differences in MOP-r activity was observed in C57BL/6J mice compared to DBA/2J with a two fold reduced MOP-r activity observed in the nucleus accumbens and caudate putamen of saline treated DBA/2J mice vs C57BL/6J, this was not accompanied by a decrease in MOP-r density. However, C57BL/6J and DBA/2J mice differed in their regulation of MOP-r by heroin which is in accordance with Pertuzzi *et al.*, (1997) who reported similar results for morphine. Heroin decreased MOP-r's binding in the nucleus accumbens shell, centromedial thalamus and habenula of C57BL/6J mice, but not in DBA/2J. No heroin-induced regulation of MOP-r G protein activity was observed in any brain regions of C57BL/6J or DBA/2J mice. These results show the importance of determining functional activity of receptor systems, demonstrating that downregulation in receptor density does not necessarily translate into loss of functional activity which is consistent with our recent study where a decrease in D2 dopamine receptor density was accompanied by an increase in D2 receptor activity in the striatum of mice treated with an escalating dose "binge" cocaine administration protocol (Bailey *et al.* 2008). The substantially reduced levels of MOP-r stimulated G protein activity observed in the caudate putamen, which is a region well known

to be linked with locomotor activity (Angulo & McEwen 1994) of DBA/2J mice compared to C57BL/6J mice, could partially explain the strain differences in locomotor stimulating phenotype in response to opiates. Maher et al., (2005) showed that even a 30 % decrease in MOP-r stimulated [³⁵S]GTPγS binding produced a 7.5 fold increase in the concentration of heroin necessary to produce this level of G protein activation. Different expression of G protein subunits between strains may account for differences in MOP-r G protein activation observed in our study. Indeed, a recent microarray study revealed differences in G protein subunit expression between the nucleus accumbens of C57BL/6J and DBA/2J mice (Grice *et al.* 2007). Overall, our results imply that the ability of heroin to induce MOP-r activity in specific brain regions might be an important factor in individual's vulnerability to develop heroin addiction. This is in complete agreement with Bond et al., (1998) and Kroslak et al., (2007) who showed that A118G human variant of MOP-r which has been associated with opioid addiction (LaForge *et al.* 2000) has significantly higher affinity and potency to activate G-protein coupled inward rectifying potassium channels by opioids compared to the prototype receptor.

Higher density of DAT binding was observed in the caudate putamen, the olfactory tubercle and the nucleus accumbens shell of chronic heroin treated DBA/2J mice compared to heroin treated C57BL/6J, indicating a strain difference in the regulation of DAT following chronic heroin treatment. By removing extracellular dopamine and recycling it back into the neuron, DAT plays a key role in regulating neurotransmission in the dopaminergic system (Gulley & Zahniser 2003). Mice lacking DAT have been shown to be hyperactive and have enhanced dopaminergic neurotransmission and increased extracellular dopamine levels in the striatum (Giros *et al.* 1996; Jones *et al.* 1998; Spielowoy *et al.* 2000). As a result, our data suggests that C57BL/6J mice may have enhanced striatal dopaminergic neurotransmission and extracellular dopamine levels in response to heroin compared to DBA/2J mice, due to

differences in striatal DAT density. Differences in dopamine neurotransmission have been observed following acute morphine administration in C57BL/6J and DBA/2J mice. Microdialysis studies showed that although no strain differences in basal extracellular dopamine levels were observed, C57BL/6J mice had higher increases in ventral and dorsal striatal extracellular dopamine levels in response to morphine compared to DBA/2J mice (Murphy *et al.* 2001; Fadda *et al.* 2005). However, as the effect of drugs of abuse on the dopaminergic system are well known to differ when the drug is administered acutely compared to chronic administration (Unterwald *et al.* 1994; Maisonneuve *et al.* 1995), it is difficult to make conclusions on the state of dopaminergic neurotransmission in chronically heroin treated C57BL/6J and DBA/2J mice, based on the acute microdialysis study alone. However, the increase of locomotor effect of heroin in C57BL/6J mice compared to DBA/2J mice, which is generally thought to be under striatal dopaminergic control (Murphy *et al.* 2001), together with the differential regulation of DAT by chronic heroin treatment in C57BL/6J mice vs DBA/2J, clearly indicates an enhancement of limbic dopamine neurotransmission in chronically heroin treated C57BL/6J vs DBA/2J mice. As a result, it is likely that the strain differences in the regulation of DAT by chronic heroin treatment may partially be responsible for some of the behavioural strain differences (at least the chronic locomotor behavioural effects). This possibility though requires further investigation.

In contrast to DAT and MOP-r, there was no strain or treatment effect on D₁ and D₂ dopamine receptor binding which suggests that the chronic locomotor behavioural strain differences observed in response to heroin are neither due to differences in D₁ or D₂ expression between strains nor to differences in regulation of D₁ or D₂ receptors by heroin.

Interestingly, although strain differences were observed in DAT binding of chronic heroin treated mice, no strain difference in DAT, D₁ or D₂ binding were observed between chronic

saline treated mice. This is in contrast with De Jong *et al.*, (2008) who showed higher levels of DAT mRNA and D₁ receptor binding levels and lower D₂ receptor binding levels in brain regions of drug naïve DBA/2J mice compared to C57BL/6J mice. These discrepancies are likely to be caused by differences in animal treatment protocol (chronic saline treatment vs treatment naïve mice) or also by differences in the autoradiography protocol (e.g. different radioligands used, mRNA vs binding levels *ect*).

Finally, the possibility that the interstrain behavioural and neurochemical variations observed in response to chronic heroin treatment in the present study could be due to pharmacokinetic differences between the two strains is worthy of consideration. Although reports vary, up to 40% higher concentrations of the heroin metabolite morphine have been found in brains of C57BL/6J mice compared to DBA/2J mice following peripheral administration (Brase *et al.* 1977; Gwynn & Domino 1984; Belknap *et al.* 1989). However, studies covering large numbers of varied strains drew no correlation between brain concentration and morphine sensitivity to the thermal analgesia (hot plate) and locomotor activity properties of morphine in these strains of mice (Belknap *et al.* 1998). Moreover, an explanation of the strain differences in heroin-induced behaviours based only on pharmacokinetic differences is further undermined by the fact that an 8 fold increase in heroin dose had absolutely no locomotor stimulatory effect in DBA/2J mice in our study.

In conclusion, the present results demonstrate that C57BL/6J and DBA/2J mice differ considerably in their sensitivity to the locomotor, sensitisation and rewarding effect of heroin indicating that the pharmacological effects of heroin is genotype dependent. The profound strain differences observed in MOP-r G protein activity and in heroin induced regulation of DAT expression may be responsible for some of these behavioural differences (at least the chronic locomotor behavioural effects). As a result these strains of mice may be useful in

identifying factors which would influence individuals' vulnerability to develop opiate addiction.

Table. 1

Time spent by C57BL/6J and DBA/2J mice in the black, white and null zone compartments of the heroin conditioned place preference apparatus during the last 15 min of a 20 min exposure to the entire apparatus (preconditional test)

Strain	Black	White	null
C57BL/6J	343.8±38.4	422.5±47.2	135.2±19.3
DBA/2J	416.3±39.0	395.9±53.7	89.1±12.1

Values represent mean±S.E.M of 83-85/strain.

Legend to Figure 1. Change in time spent in heroin paired compartment in C57BL/6J and DBA/2J mice. Place conditioning was induced by the administration of 0.5 mg/kg heroin (C57BL/6J, $n = 16$; DBA/2J, $n = 11$), 1 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 9$), 2 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 14$), 4 mg/kg heroin (C57BL/6J, $n = 16$; DBA/2J, $n = 25$) and 8 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 13$) (s.c.). Saline controls (C57BL/6J, $n = 10$; DBA/2J, $n = 7$) were included. All values are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs 0 mg/kg heroin

Legend to figure 2. Locomotor sensitization in C57BL/6J and DBA/2J mice. The locomotor effect of repeated saline (C57BL/6J, $n = 10$; DBA/2J, $n = 7$), 0.5 mg/kg heroin (C57BL/6J, $n = 16$; DBA/2J, $n = 11$), 1 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 9$), 2 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 14$), 4 mg/kg heroin (C57BL/6J, $n = 16$; DBA/2J, $n = 25$) and 8 mg/kg heroin (C57BL/6J, $n = 15$; DBA/2J, $n = 13$) (s.c.) was evaluated in C57BL/6J and DBA/2J mice during the first (Day 1) and the last day (Day 4) of the conditioning phase of our CPP paradigm. ** $P < 0.01$ vs Day 1 (Newman Keuls post hoc test).

Legend to Figure 3. Locomotor activity in C57BL/6J and DBA/2J mice after an injection of saline or heroin during a chronic “intermittent” escalating dose administration paradigm. The locomotor data were collected daily in 15 min bins following the second (i.e. 9 a.m.) of the two injections of an “intermittent” escalating dose (1-8 mg/kg, s.c.) pattern of heroin or saline. Responses are shown on day 1 through to day 7. Basal locomotor activity for 15 min duration after 45 min placement of mice in the locomotor chamber is shown at time 0 for each group of mice during every day of the treatment protocol. The arrow indicated the time of injection of saline or heroin. Values are mean $\pm$ S.E.M of 6 animals in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs saline C57BL/6J on the same day

Legend to Figure 4. A. Quantitative autoradiography of MOP-r binding in coronal brain sections from chronically “intermittent” escalating dose heroin or saline treated C57BL/6J and DBA/2J mice. Data are expressed as the mean specific binding (fmol/mg) $\pm$ S.E.M of 6 determinants (n=6) of [3 H]DAMGO in brain regions of chronically heroin and saline treated C57BL/6J and DBA/2J mice. Regional determinations were made from both left and right sides of the sections. The labelling was carried out on sections from chronically heroin and saline treated C57BL/6J and DBA/2J mice in a completely paired protocol * $P < 0.05$, ** $P < 0.01$ vs saline C57BL/6J; # $P < 0.05$, ### $P < 0.001$ vs C57BL/6J heroin; (LSD post hoc test).

B) Computer-enhanced autoradiograms of coronal brain sections from chronically “intermittent” escalating dose heroin or saline treated C57BL/6J and DBA/2J mice. The adjacent sections shown are from the level of the caudate (Bregma 1.34 mm). The colour bar represents a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Sections cut from chronically heroin and saline treated C57BL/6J and DBA/2J mice brains were processed in parallel. See list of abbreviations.

Legend to Figure 5. Quantitative autoradiography of D₁, D₂ dopamine receptor and dopamine transporters binding in coronal brain sections from chronically “intermittent” escalating dose heroin and saline treated C57BL/6J and DBA/2J mice. Data are expressed as the mean specific binding (fmol/mg) $\pm$ S.E.M of 5-6 determinants of A) [³H]mazindol, B) [³H]raclopride and C) [³H]SCH-23390 in brain regions of chronically heroin or saline treated C57BL/6J and DBA/2J mice. Regional determinations were made from both left and right sides of the sections. The labelling was carried out on sections from chronically heroin or saline treated C57BL/6J and DBA/2J mice in a completely paired protocol. Specific binding was >80 % in all regions analysed. * $P < 0.05$ vs saline DBA/2J ; # $P < 0.05$, ### $P < 0.001$ vs heroin C57BL/6J, (LSD post hoc test). See list of abbreviations.

Legend to Figure 6. Computer-enhanced autoradiograms of coronal brain sections from chronic “intermittent” escalating dose heroin and saline treated C57BL/6J and DBA/2J mice. The sections shown are from the level of the caudate (Bregma 1.10 mm) (columns 1 and 2) and from the level of the hippocampus (Bregma -1.58 mm) (columns 3 and 4). Sections were preincubated with 1 mM GDP and then incubated for 2 h with [³⁵S]GTP γ S (0.04 nM) and 1 mM GDP (basal) or with [³⁵S]GTP γ S (0.04 nM) and 1 mM GDP in the presence of DAMGO (5 μ M) (MOP-r stimulated [³⁵S]GTP γ S). The colour bar represents a pseudo-colour interpretation of relative black and white film images. Sections cut from chronic “intermittent” escalating dose heroin and saline treated C57BL/6J and DBA/2J mice brains were processed in parallel

Legend to Figure 7. DAMGO stimulated [³⁵S]GTP γ S binding in coronal brain sections from chronically heroin and saline treated C57BL/6J and DBA/2J mice. Data are expressed as mean $\pm$ S.E.M (n=6-11) of increase of [³⁵S]GTP γ S binding from basal (KBq/mg). * $P < 0.05$ vs

saline treated C57BL/6J mice, [#]P<0.05; ^{##}P<0.01 vs heroin treated C57BL/6J mice (LSD post hoc test). See list of abbreviations.

Legend to Figure 8. Effect of chronic “intermittent” heroin administration on plasma corticosterone levels of C57BL/6J and DBA/2J mice. Data are expressed as mean ± S.E.M (n=6). A significant treatment effect was observed (P<0.01) but no strain effect. * P<0.05 vs Saline (LSD post hoc test)

Legend to Supplement 1. Shift in time in the heroin-paired compartment during the post conditioning day compared to the preconditioning day in C57BL/6J and DBA/2J mice as measured in the CPP paradigm. Place conditioning was induced by the administration of 0.5 mg/kg heroin (C57BL/6J, n = 16; DBA/2J, n = 11), 1 mg/kg heroin (C57BL/6J, n = 15; DBA/2J, n = 9), 2 mg/kg heroin (C57BL/6J, n = 15; DBA/2J, n = 14), 4 mg/kg heroin (C57BL/6J, n = 16; DBA/2J, n = 25) and 8 mg/kg heroin (C57BL/6J, n = 15; DBA/2J, n = 13) (s.c.). Saline controls (C57BL/6J, n = 10; DBA/2J, n = 7) were included. All values are expressed as mean ± SEM. * P<0.05, ** P<0.01, *** P<0.001 vs preconditioning; [#] P<0.05, ^{###} P<0.001 vs 2 mg/kg heroin post-conditioning); ^T P<0.05, ^{TT} P<0.01, ^{TTT} P<0.001 vs saline postconditioning (Newman Keuls post hoc test).

Legend to Supplement 2. Computer-enhanced autoradiograms of coronal brain sections from chronically “intermittent” escalating dose heroin or saline treated C57BL/6J and DBA/2J mice. The adjacent sections shown are from the level of the caudate (Bregma 1.34 mm). Dopamine transporters (DAT), D₂ and D₁ dopamine receptor were labelled with [³H]mazindol (4 nM), [³H]raclopride (4 nM) and [³H]SCH-23390 (4 nM) respectively. The colour bar represents a pseudo-colour interpretation of black and white film images in fmol/mg tissue

equivalent. Sections cut from chronically heroin and saline treated C57BL/6J and DBA/2J mice brains were processed in parallel.

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Abbreviations: FCx; prefrontal cortex; CgCx, Cingulate cortex; AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; Tu, Olfactory tubercle; Den, Dorsal endopiriform; CPu, Caudate putamen; MS, medial septum; VDB, vertical limb diagonal band; Re, Reuniens; CMTh, central medial thalamic nuclei; CLTh, central lateral thalamic nuclei; Hy, Hypothalamus; Amy, Amygdala; Ha, habenula; PrLCx, Prelimbic cortex; SN, Substantia nigra; STh, Subthalamic nucleus; MCx, motor cortex; ANOVA, analysis of variance; MOP-r, μ - opioid receptors; DAT, dopamine transporter; $G_{i/o}$, G- protein; CPP: Conditioned Place; Preference; GTP γ S, guanosine 5'-[γ -[35 S]thio]-triphosphate; DAMGO, D-Ala²-MePhe⁴-Gly-ol⁵ enkephalin; s.c, subcutaneously; HPA, hypothalamic pituitary adrenal axis

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Figure 1

Figure 2

Figure. 3.

Figure. 4

A

B

Figure 5

Figure 6

Figure 7

Figure 8

Supplement 2

Supplement 2