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Nucleus accumbens NMDA receptor subunit expression and function is enhanced in morphine-dependent rats

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Abstract

We have previously shown, using radioligand binding studies, that *N*-methyl-D-aspartate (NMDA) NR1 and NR2A receptor subunits density was decreased in the forebrain of morphine-dependent rats. We have now determined if morphine-dependent rats display regional differences in NMDA receptor expression and whether such changes are functionally relevant. In morphine-dependent rats, the expression of NR1 and NR2A subunits protein, as determined by Western blotting with NMDA receptor subunit antibodies, were decreased in frontal cortex and hippocampus but significantly increased in the nucleus accumbens. The expression of the NR2B subunit was unchanged in all regions examined. In separate groups of morphine-dependent rats, MK-801-induced hyperactivity (thought to be mediated via modulation of nucleus accumbens dopamine release) was significantly enhanced in morphine-dependent animals. Similarly, the MK-801-induced increase of dopamine metabolism was significantly increased in the nucleus accumbens of morphine-dependent animals as compared to sham controls. Results provide both biochemical and behavioural evidence to suggest that NMDA receptor function in the nucleus accumbens, at least with respect to an interaction with the limbic dopamine system, is markedly enhanced in morphine-dependent rats. This increase in function may be associated with an enhanced expression of NMDA receptors, particularly those in the nucleus accumbens containing the NR2A subunit. Taken together, these data support several studies in the literature indicating that NMDA receptors in the nucleus accumbens are involved in the process of opiate dependence.

Keywords: NMDA; NR2A; MK-801; Morphine; Dopamine; Behaviour

1. Introduction

Chronic administration of the opiates, including heroin and morphine, results in the development of both tolerance and physical dependence, as demonstrated by a pronounced physical withdrawal syndrome following administration of opiate receptor antagonists (Maldonado et al., 1992). This latter feature may be observed in rats following prolonged exposure to morphine either through repeated dosing or the subcutaneous implantation of a slow release formulation of morphine. We have previously demonstrated that exposure to morphine in the form of a subcutaneously implanted pellet for 48 h is sufficient to induce physical dependence, demonstrated by the appearance of withdrawal behaviour upon administration of the opiate receptor antagonist naltrexone (Bristow et al., 1997).

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS) and its effects are mediated via both ionotropic and metabotropic receptors. The NMDA receptor is the most extensively characterized of the three families of ionotropic glutamate receptor and is a multimeric, ligand gated ion channel which is highly permeable to calcium and has a complex pharmacology due to the plethora of modulatory sites which exist on different subunits (see Yamakura and Shimoji, 1999 for review). The receptor complex is composed of three major subunit families, each derived from a separate gene; NR1 of which there are 8 splice variants (a–h),

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NR2 of which there are 4 subtypes (A–D) and NR3 (A and B) (Yakamura and Shimoji, 1999). While the exact composition of native NMDA receptors are as yet unknown it is thought that one or more NR1 subunits, which express the glycine co-agonist recognition site, co-assemble with various combinations of the NR2(A–D) or NR3 (A–B) subunits to form functional receptors (Stephenson, 2001). The combination of different receptor subunits may confer diverse biophysical and functional properties on these receptor subtypes which are differentially distributed within the CNS (Goebel and Poosch, 1999). However, the physiological role of individual NMDA receptor subtypes is further complicated as multiple receptor subtypes may be expressed on the same cell at least in new born neurones (Pina-Crespo and Gibb, 2002).

In the past decade, evidence has accumulated to indicate that NMDA receptors play a pivotal role in the development of tolerance and physical dependence to opiates (see Trujillo, 2000, for review). Thus all classes of NMDA receptor antagonist tested, including glycine site antagonists/partial agonists (Bristow et al., 1997; Popik et al., 1998), competitive glutamate site antagonists (Gonzalez et al., 1997) and ion channel blockers (Trujillo and Akil, 1994) block the physical signs of opiate withdrawal in rodents. Similarly, mutant mice with a deletion of the NR2A (ε1) subunit were shown to only weakly express the signs of naloxone precipitated morphine withdrawal (Miyamoto et al., 2004). Furthermore, radioligand binding and *in situ* hybridization studies have shown that NMDA receptor expression in rodents is influenced by prolonged exposure to morphine or opiates (Bhargava et al., 1995; Inoue et al., 2003; Zhu et al., 1999). However, it is unclear if there are functional consequences of such changes of NMDA receptor expression.

The mesolimbic dopamine pathway arises in the ventral tegmental area and projects to several forebrain regions including the nucleus accumbens. This pathway plays an important role in the rewarding properties of drugs of abuse which increase mesocorticolimbic dopamine release and metabolism (Leone et al., 1991; Imperato et al., 1990). NMDA receptors are located in the nucleus accumbens and their blockade by ion channel antagonists such as MK-801 and phencyclidine (PCP) have been reliably demonstrated to increase locomotor activity (Carlsson and Svensson, 1990). This increase in locomotor activity is accompanied by increased dopamine transmission in the nucleus accumbens (French and Vantini, 1984) which may be, at least in part, responsible for the observed behavioural effects of such compounds. Siggins et al. (2003) reported that morphine dependence reduced the affinity of the co-agonist glycine for NMDA receptors in the nucleus accumbens and altered their desensitisation rates. It is conceivable that MK-801-induced changes of locomotor activity and nucleus accumbens dopamine metabolism may reflect the functional status of NMDA receptors in the nucleus accumbens, although as MK-801 is a nonselective NMDA receptor antagonist its effects are unlikely to provide information on the functional status of specific NMDA receptor subtypes.

Therefore, in the present study we have determined if NMDA receptor subunit expression, as determined by Western blotting and subtype-selective antibodies, is altered in the cortex, nucleus accumbens or hippocampus of morphine-dependent rats. Furthermore, we have also determined if such changes of subunit expression are functionally relevant by determining the neurochemical and behavioural effects of MK-801 in morphine-dependent rats. Part of this study was presented in abstract form (Harrison et al. 2000).

2. Materials and methods

Male Sprague Dawley rats (Bantin and Kingman, Hull, U.K.) (250–275 g) were housed in groups of five and maintained on a 12 h light:dark cycle (lights on at 07:00) with food and water available *ad libitum*. Rats were implanted subcutaneously under isofluorane anaesthesia with a pellet containing either 75 mg morphine base or incipient (Bristow et al., 1997). Forty eight hours later (experimental conditions previously shown to be sufficient for the development of physical dependence as measured by the response to naltrexone; Bristow et al., 1997) animals were either used in behavioural studies, or were humanely killed for brain tissue analyses. Brains were removed, dissected into regions and frozen on dry ice, either for the determination of NMDA NR1, NR2A and NR2B receptor subunits protein expression by Western blot analysis, or for neurochemical analysis as described below. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

2.1. Western blot analysis

2.1.1. Antibody generation

The cDNA sequences corresponding to the Ser^{14}-Leu^{76} of rat NR1, the Leu^{898}-Gln^{1209} of rat NR2A and the Arg^{27}-Thr^{76} of the rat NR2B were incorporated into pRSET5a (a T7 polymerase expression vector) and expressed in *E. coli* BL21 (DE3) Lys S, the recombinant protein purified by preparative sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and electroelution, and antisera raised in rabbits as previously described (Grimwood et al., 1996).

2.1.2. Sample preparation and gel electrophoresis

Brain regions (frontal cortex, nucleus accumbens and hippocampus) were sonicated in RIPA buffer (Tris–HCl, 50 mM, pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; phenylmethanesulphonylfluoride, 1 mM; Aprotinin, leupeptin, pepstatin, 1 μg/ml each; Na_3VO_4, 1 mM) centrifuged at 10,000 ×*g* and supernatants protein assayed using a micro BCA kit (Perbio). Samples were normalized for protein and 20 μg was run on a NuPAGE 10% Bis–Tris gel (Invitrogen) and electrophoretically transferred to a nitrocellulose membrane using a semi dry blotter and run at constant voltage (25v) for one hour. One hour incubation in 5% non-fat milk (Merck) in phosphate buffered saline (PBS)/Tween 20 (0.5%) was used to block non-specific binding before incubating the membrane with NMDA NR1, NR2A or NR2B primary antibody overnight at 4 °C. Antibody dilutions were 1:500 for NR1 and 1:250 for NR2A and NR2B antibodies.

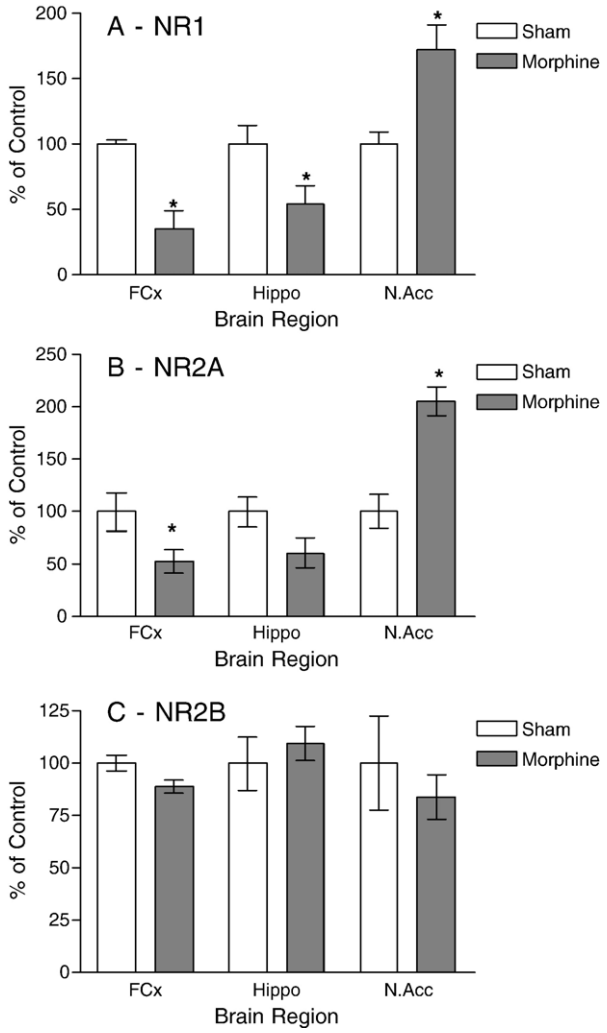

Fig. 1. Expression of NMDA receptor (A) NR1, (B) NR2A and (C) NR2B subunits, as determined by Western blotting, in frontal cortex (FCx), hippocampus (Hippo) and nucleus accumbens (N. Acc) of rats implanted with either a sham or morphine pellet for 48 h. Data are mean±S.E.M. expressed as percent change of sham treated rats, $n=5/6$ per group. Data were analyzed by two way ANOVA (with brain region and treatment as categorical predictors) followed where appropriate by Fisher's LSD post hoc test $*p<0.05$ compared with region matched sham treated rats.

Following several wash steps with PBS/Tween 20 (0.5%), membranes were incubated with a 1 in 5000 dilution of horse radish peroxidase conjugated donkey anti-rabbit secondary antibody (Amersham) for 1 h. Bands were detected by 1 min incubation with enhanced chemiluminescence (ECL) reagents (Amersham) and membrane opposed to film and quantified by densitometry using AIS 6.0 Image Analysis Software (Imaging Research Inc.).

2.2. Locomotor activity

Rats were habituated to individual activity cages (215×320×350 mm) equipped with 5 infra-red beams: 1 positioned at each end of the base of the cage to record cage crossings, i.e. consecutive beam breaks, and 3 positioned across the top of the cage to record rearing. One hour later, rats were removed and injected with either vehicle or MK-801. Photocell beam breaks were then monitored in 10 min intervals for 4 h and results expressed as the mean±S.E.M. number of total counts and cage crosses recorded. An initial experiment was performed in sham pellet implanted rats to determine the optimum dose of MK-801 required to produce the maximum increase in locomotor activity in the absence of stereotypy and ataxia. In these initial experiments, animals were habituated to activity cages as before and then injected with either saline (1 ml/kg i.p.) or MK-801 (0.05, 0.1, 0.2, 0.4 or 0.8 mg/kg, i.p.) and locomotor activity monitored for 4 h. In all subsequent experiments to examine the effects of morphine dependence, rats were administered either saline (1 ml/kg, i.p.) or MK-801 (0.2 mg/kg, i.p.).

2.3. Neurochemical studies

Rats (implanted subcutaneously with either a sham or morphine pellet) were injected with either saline (1 ml/kg, i.p.) or MK-801 (0.2 mg/kg, i.p.) and returned to their home cages. Animals were humanely killed 60 min later and brain regions (medial prefrontal cortex and nucleus accumbens) were dissected, frozen on dry ice and stored at −80 °C until ready for analysis. Dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by reversed phase HPLC using electrochemical detection as previously described (Hutson et al., 1991). Briefly, brain regions were homogenized in 0.4 M perchloric acid containing 0.1% sodium metabisulphate, 0.01% sodium EDTA, 0.01% cysteine and centrifuged at 3000 ×*g* for 10 min. An aliquot was used for the determination of dopamine, DOPAC and HVA. The HPLC system comprised an ultrasphere 3 μm ODS reverse phase column (4.6 mm×7.5 cm) (HPLC Technology Ltd). The mobile phase consisted of 0.07 M KH_2PO_4, 0.0035% EDTA, 0.023% octyl sodium sulphate and 12% v/v methanol pH 2.75, filtered (0.2 μm) and degassed with helium before use at a flow rate of 1 ml/min. The detector was an ESA Coulochem model 5100A with a dual electrode analytical cell (5011). Electrode 1 was set at −0.04 V and electrode 2 at +0.42 V. The ratio [DOPAC+HVA]/[dopamine] provided an index of dopamine metabolism.

2.4. Drugs and chemicals

MK-801 ((5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine) was supplied by Sigma (Cat No. M107). Morphine and Sham pellets were supplied by the PR&D group, MSD Hoddesdon.

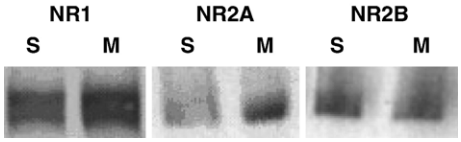

Fig. 2. Illustrative Western blotting bands showing N. Acc NR1 (A), NR2A (B) and NR2B (C) protein bands. In each case left hand band represents sham (S) and right hand band morphine (M) pelleted animals.

3. Results

3.1. NMDA subunit expression

NMDA NR1 subunit expression was significantly altered by 48 h morphine pellet implant and there were regional differences in these effects ($F_{(2,22)}=16.4$, $p<0.001$). In medial prefrontal cortex and hippocampus NMDA NR1 subunit expression was significantly (65% and 46%, respectively) decreased after 48 h exposure to morphine, which is sufficient to induce physical dependence (Bristow et al., 1997). Conversely, the expression of the NMDA NR1 subunit was significantly (172% of vehicle) increased in the nucleus accumbens (Fig. 1A). A similar pattern of expression was found for the NR2A subunit in morphine-dependent rats, where again there was a significant effect of treatment and brain region ($F=_{(2,19)}=14.9$, $p<0.001$). NR2A was decreased by 48% and 40% in frontal cortex and hippocampus, respectively, although the latter did not achieve significance (Fig. 1B). However, there was a pronounced (205% of vehicle controls) and significant ($p<0.05$) increase of NR2A subunit expression in nucleus accumbens (Fig. 1B). In contrast to these marked changes of NR1 and NR2A subunits expression observed in morphine-dependent rats, the expression of the NR2B subunit was relatively unaffected by treatment or brain region examined ($F_{(2,24)}=0.63$, $p=0.54$) (Fig. 1C). Illustrative Western blotting bands are shown in Fig. 2.

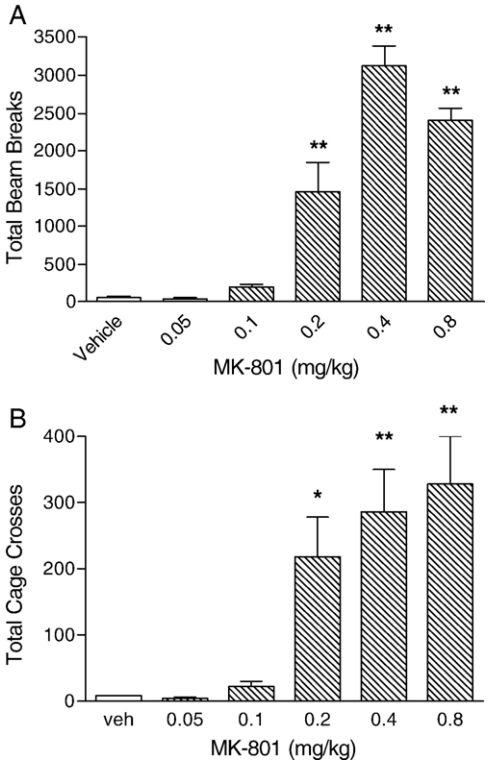

Fig. 3. Effect of increasing doses of MK-801 (0.05–0.8 mg/kg i.p.) on locomotor activity in naive rats expressed as (A) total beam breaks or (B) total crosses, over a 2 h period. Data are mean±S.E.M., $n=5/6$ per group. Data were analysed by one way ANOVA followed by Dunnett's post hoc test, $*p<0.05$, $**p<0.01$ as compared to vehicle treated animals.

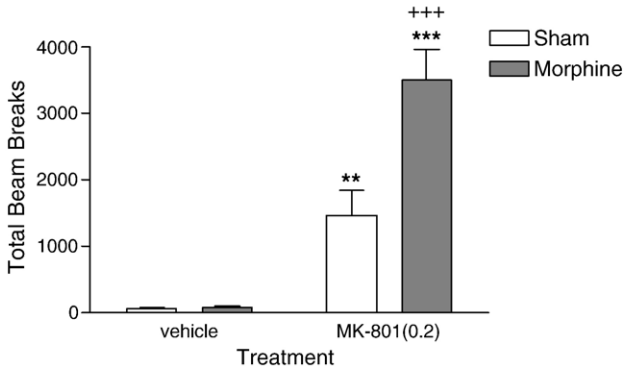

Fig. 4. Effect of either vehicle (0.9% saline, 1 ml/kg i.p.) or MK-801 (0.2 mg/kg, i.p.) on locomotor activity in rats implanted with either sham or morphine pellet for 48 h on total beam breaks over a 2 h period. Data are mean±S.E.M., $n=5/6$ per group. Data were analyzed by two way ANOVA (with implant and drug treatment as categorical predictors) followed where appropriate by Fisher's LSD post hoc test $**p<0.01$, $***p<0.001$ compared with implant matched vehicle treated rats, $++p<0.01$, $+++p<0.001$ compared with drug treatment matched MK-801 treated rats.

3.2. Locomotor activity

In sham pellet implanted animals, MK-801 (0.05–0.8 mg/kg, i.p.) dose-dependently increased locomotor activity as measured using total beam breaks or total cage crosses with a

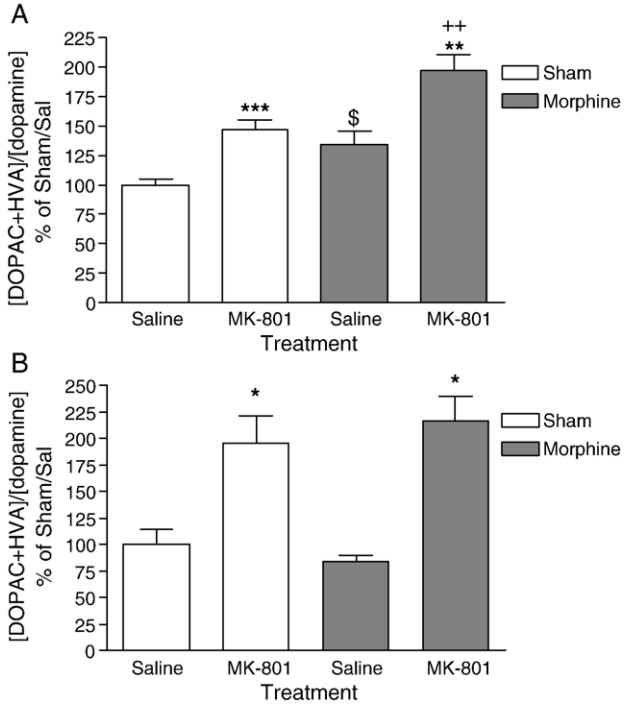

Fig. 5. Effect of either vehicle (0.9% saline, 1 ml/kg, i.p.) or MK-801 (0.2 mg/kg, i.p.) on dopamine metabolism (as indicated by [DOPAC]+[HVA]/[Dopamine]) in (A) nucleus accumbens (B) medial prefrontal, in rats implanted with either a sham or morphine pellet for 48 h. Data are mean±S.E.M., $n=5/6$ per group. Data were analyzed by Student's t-test. $*p<0.05$,$**p<0.01$, $***p<0.001$ compared with vehicle treated rats, $++p<0.01$ compared with MK-801 in sham pellet implanted rats and $\$p<0.05$ compared to sham pellet implanted rats following saline treatment.

maximal effect at 0.4 mg/kg, i.p. (Fig. 3A and B). The observed decline in the number of beam breaks at the higher dose can be explained by the appearance of stereotyped behaviour such as repetitive lateral head swaying and circling. There was a significant effect of both pellet implant and MK-801 treatment ($F_{(1,28)}=11.25$, $p=0.02$) in terms of total beam breaks. In rats implanted with a sham pellet, MK-801 (0.2 mg/kg, i.p.) produced a significant increase of total beam breaks similar in magnitude to that found in the pilot study (Fig. 4). In contrast, administration of MK-801 (0.2 mg/kg, i.p.) to rats implanted with a morphine pellet caused a marked increase of locomotor activity which was significantly greater than that seen in sham operated rats (Fig. 4).

3.3. Neurochemical studies

Analysis of neurochemical studies by two way ANOVA showed that although there was a significant effect of both morphine implant ($F_{(1,17)}=17.7$, $p<0.001$) and MK-801 treatment ($F_{(1,17)}=45.3$, $p<0.01$), there was no significant interaction between the two ($F_{(1,17)}=2.2$, $p=0.15$). However, further analysis of the individual group data, not taking into account multiple comparisons, using individual Students *t*-test, revealed that in the nucleus accumbens of sham pellet implanted rats, MK-801 (0.2 mg/kg, i.p.) significantly ($p<0.001$) increased dopamine metabolism (as indicated by the ratio [DOPAC+HVA]/[dopamine]) by 146%, compared with vehicle administration (Fig. 5A). However, the same dose of MK-801 increased dopamine metabolism by 159%, compared with vehicle controls, which was significantly different from the increase observed in sham pellet implanted rats administered MK-801 ($p<0.01$,Fig. 5A). Interestingly, there was a small (124% of vehicle control, $p<0.05$) increase of nucleus accumbens dopamine metabolism in vehicle treated rats implanted with a morphine pellet, compared with vehicle treated rats implanted with a sham pellet, indicating a small effect of morphine alone (Fig. 5A). In medial prefrontal cortex, MK-801 (0.2 mg/kg, i.p.) markedly (195 and 257% of vehicle control, respectively) and significantly ($p<0.01$) increased dopamine metabolism to a similar extent in rats implanted with sham and morphine pellets. There was no significant difference between the MK-801-induced increase of cortical dopamine metabolism in sham or morphine pellet implanted rats ($p=0.45$, Fig. 5B).

4. Discussion

Results in the present study demonstrated that NMDA receptor subunit expression, as determined by Western blot analysis, was significantly changed in rats implanted with a morphine pellet, at a dose and time (48 h) previously demonstrated to induce physical dependence (Bristow et al., 1997). Thus NR1 protein expression was significantly increased in the nucleus accumbens and significantly decreased in the frontal cortex and hippocampus of morphine-dependent rats when compared with rats implanted with a sham pellet. These data are consistent with several studies that have reported increased NMDA NR1 expression in nucleus accumbens (and other regions not examined in the present study, including the periaqueductal grey area and ventral tegmental area) in morphine-dependent rodents (Inoue et al., 2003). However, Zhu et al. (1999) failed to observe any changes of NMDA receptor subunit mRNA in rats infused intracerebroventricularly for 3 days with morphine.

The expression of NR2A was significantly increased in nucleus accumbens, when compared to sham controls. These data are consistent with Inoue et al. (2003), who showed that NR2A protein expression in morphine-tolerant mice was increased in the nucleus accumbens but not hippocampus. Interestingly, apart from a small though statistically significant decrease of NR2B expression in frontal cortex, the expression of this subunit was essentially unaffected in any of the brain regions examined in morphine-dependent rats. The preferential effect of morphine dependence on NR2A versus NR2B containing receptor expression and function in the nucleus accumbens is consistent with *in vitro* pharmacological and biophysical studies in nucleus accumbens neurones suggesting that morphine dependence leads to an increased expression or function of NR2A over NR2B subunits (Martin et al., 2004).

The functional consequences of altered NMDA receptor subunit expression are poorly understood, particularly with respect to morphine dependence, and due to a lack of subtype-selective antagonists have relied on the effects in mutant mice with targeted deletions of individual subunits. Thus, NR1 (ζ) subunit knockdown and NR2A (ε1) subunit knockout mutant mice (Mohn et al., 1999; Miyamoto et al., 2004) displayed increased locomotor activity similar to mice or rats treated with MK-801 or PCP, presumably reflecting the role of NMDA receptors in the mesolimbic dopamine system. Conversely, mutant mice over-expressing the NR2B subunit showed enhanced learning, even though NR1 expression was normal (Tang et al., 1999), indicating that both NR1 and NR2 subunits can influence receptor function. Therefore, it could be reasoned from the present findings, that morphine-dependent rats would have reduced NMDA receptor function (including NR1/NR2A and NR1/NR2B) in the hippocampus and cortex. i.e. disrupted cognitive function and enhanced NMDA receptor function in nucleus accumbens and enhanced modulation of mesocortico-limbic dopamine function. Clearly the latter was not apparent in morphine-dependent rats before pharmacological challenge with MK-801, perhaps reflecting the moderate changes of subunit expression observed in these animals. Thus, the hyperactive mesolimbic dopamine phenotype of the NR1 knockdown (which is in excess of 90%) and NR2A knockout was apparent in unchallenged mice (Mohn et al., 1999; Miyamoto et al., 2004), but the deletion of these subtypes was considerably more extensive compared with the observed reduction of subunit expression in the present study.

Nonsubtype-selective NMDA receptor antagonists, including MK-801, induce a characteristic behavioural profile including pronounced locomotor activity and stereotypy at higher doses (Tricklebank et al., 1989). The increased locomotor activity is primarily dependent on increased dopamine release and metabolism in the nucleus accumbens

(French and Vantini, 1984), although it is clear that other anatomical and neurochemical substrates are involved (Loscher and Honack, 1992; Bristow et al., 1997). In the present study, we therefore compared the behavioural and neurochemical effects of a submaximal dose of MK-801 in sham and morphine-treated rats in an attempt to determine the functional status of the NMDA receptors in the nucleus accumbens. As expected, and consistent with several reports in the literature in normal animals (Tricklebank et al., 1989), MK-801 increased locomotor activity and dopamine metabolism in the nucleus accumbens and medial prefrontal cortex of control rats implanted with a sham pellet. However, in rats implanted with a morphine pellet the effects of MK-801 on locomotor activity were markedly enhanced, as was the increase of nucleus accumbens dopamine metabolism. These observations are consistent with the view that NMDA receptor function in the nucleus accumbens is increased and is commensurate with the Western blot studies showing an increased expression of NR1 and NR2A protein in that region. Interestingly, NMDA subunit (NR1 and NR2A) expression in cortex was decreased in morphine-dependent rats and yet the effect on dopamine metabolism in cortex was similar to that in rats with a sham pellet. This may be due to the limited NMDA receptor reduction observed in the cortex, which was not sufficient to influence the neurochemical effects of MK-801. These behavioural and neurochemical findings suggest that the increased effect of the non-specific NMDA receptor antagonist MK-801 may be associated with enhanced functional sensitivity or an increased number of functional NMDA receptors in the nucleus accumbens in morphine-dependent rats. The present results suggest that NMDA receptors containing the NR2A subunit in the nucleus accumbens may play a role in the rewarding effects of morphine and the development of tolerance and dependence, although it is possible that other NMDA receptor subtypes e.g. NR2C or NR2D are also implicated.

Taken together, these results demonstrate firstly that NR1 and NR2A subunits are differentially regulated, in a tissue specific manner, in morphine-dependent rats and secondly, that the expression of NR2B containing NMDA receptors is not regulated by morphine. Additionally, it should be remembered that other NMDA receptor subunits (NR2C, NR2D and NR3A and NR3B) and brain regions which were not examined in this study may also be involved. For example, the amygdala and ventral tegmental area, are also thought to be important in the development of opiate dependence. The present results indicate that glutamatergic transmission, particularly through NR2A-containing NMDA receptors in the nucleus accumbens, probably contributes to the development of opiate dependence and confirms the suggestion that subtype-selective NMDA receptor antagonists may be beneficial in the treatment of opiate dependence and withdrawal.

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