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Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence

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1 The contribution of various excitatory amino acid (EAA) receptors (NMDA, AMPA/kainate and metabotropic) in the brain to the development of morphine dependence was examined. This was performed by measuring the severity of the precipitated withdrawal syndrome following chronic subcutaneous (s.c.) morphine and intracerebroventricular (i.c.v.) EAA antagonist treatment.

2 Continuous subcutaneous (s.c.) treatment with morphine sulphate (36.65 μmol day^{-1}) produced an intense and reliable naloxone-precipitated withdrawal syndrome.

3 Chronic i.c.v. treatment with antagonists selective for metabotropic and NMDA receptors, but not AMPA/kainate receptors, significantly attenuated abstinence symptoms. Conversely, EAA antagonists had very little effect on non-withdrawal behaviours.

4 These results suggest that, as well as changes elicited by activation of NMDA receptors, metabotropic receptors and intracellular changes in the phosphatidylinositol (PI) second-messenger system or the cyclic adenosine 3′,5′-monophosphate (cAMP) second messenger system, to which EAA metabotropic receptors are linked, may be involved in the development of opioid dependence with chronic morphine treatment.

Keywords: Opioid; morphine; dependence; glutamate; metabotropic glutamate receptor; AMPA; kainate; NMDA; abstinence syndrome; excitatory amino acids

Introduction

Although opioid drugs such as morphine are widely used for the management of pain, their clinical usefulness is limited by the development of tolerance and dependence that occurs with their chronic use. Tolerance is indicated by a decreased efficacy of the drug with repeated administration, and results in a need to increase the morphine dose in order to achieve the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, and leads to an aversive withdrawal or abstinence syndrome when morphine administration is terminated. Recently, it has been demonstrated that co-administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists attenuates the development of morphine tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). Since the endogenous excitatory amino acid (EAA) glutamate activates NMDA receptors, it is likely that glutamate contributes to the development of these phenomena. In addition to NMDA receptors, glutamate acts at at least two other types of ionotropic receptors: receptors at which α-2-amino-3-(hydroxy-5-methylisoxazol-4yl)propanoic acid (AMPA) is a selective agonist and receptors at which kainate is a selective agonist; and metabotropic receptors (Mayer & Westbrook, 1987a; Monaghan *et al.*, 1989). While a role for the NMDA receptor has already been suggested, the specific contribution of these other receptors to behavioural indices of opioid tolerance and dependence has not been investigated.

The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+} (MacDermott *et al.*, 1986; Mayer *et al.*, 1987) and is gated in a voltage-dependent fashion by Mg^{2+}(Mayer *et al.*, 1984; Nowak *et al.*, 1984). It is the voltage-dependent Ca^{2+} permeability of the NMDA receptor that is thought to be necessary for use-dependent synaptic plasticity (Cotman *et al.*, 1988) and may be critical for the development of neuronal changes that mediate opioid tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). AMPA and kainate receptors gate cation channels that are permeable to Na^{+}, but for the most part have negligible permeability to Ca^{2+} (Mayer & Westbrook, 1987b; Murphy & Miller, 1989). However, AMPA/kainate receptors that exhibit Ca^{2+} permeability have recently been cloned (Miller, 1991; Sommer & Seeburg, 1992). Traditionally, AMPA/kainate receptors are thought to be involved in the mediation of rapid excitatory responses to EAA transmitters (Kiskin *et al.*, 1986; Jonas & Sakmann, 1992) and may contribute to neuronal plasticity by relieving the NMDA receptor of its voltage-dependent block by Mg^{2+}. Unlike ionotropic receptors, metabotropic glutamate receptors are not linked to cation channels. Instead they are coupled directly to the cell membrane by a G protein (Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987). Several subtypes of metabotropic glutamate receptors have recently been cloned. Some subtypes affect phosphatidylinositol (PI) hydrolysis (mGluR1α, mGluR1β and mGluR5), while others affect the production of adenosine 3′,5′-cyclic monophosphate (cAMP) (mGluR2, mGluR3, mGluR4) (Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the PI system activates phospholipase C, which catalyses phosphatidylinositol hydrolysis, leading to the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the cAMP second messenger system generally leads to decreased production of cAMP, although activation of mGluR1α stimulates an increase in cAMP (Schoepp & Conn, 1993). Through the increased production of intracellular messengers associated with PI hydrolysis, or decreased production of cAMP, metabotropic receptor activation may play an important role in the long-term effects mediated by glutamate (Nicoletti *et al.*, 1991), and like NMDA receptors may be critical to the development of neuronal changes mediating opioid tolerance and dependence.

In the present study we have investigated the contribution of various EAA receptor subtypes in the brain to the

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development of opioid dependence. This purpose was achieved by examining the effects of the intracerebroventricular (i.c.v.) administration of selective EAA receptor antagonists concurrently with the chronic subcutaneous (s.c.) administration of morphine. NMDA receptors were antagonized with the non-competitive antagonist 5-methyl-10,11-dihydro-5H-dibenzocycloheptene-5,10-imine hydrogen maleate (MK-801) (Wong *et al.*, 1986); AMPA/kainate receptors were antagonized with 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine-hydrochloride (GYKI 52466) (Donevan & Rogawski, 1993). Although distinct families of high-affinity AMPA and kainate receptors have been isolated, the functional distinction between these receptors is not entirely clear (Barnard & Henley, 1990). Consequently, pharmacological investigations with receptor antagonists are limited to investigations of non-selective AMPA/kainate receptor effects. Metabotropic receptors were antagonized with the highly selective antagonist (*S*)-4-carboxyphenylglycine [(*S*)-4C-PG] (Birse *et al.*, 1993; Eaton *et al.*, 1993) and a more commonly used, yet less selective antagonist, L-2-amino-3-phosphonopropanoic acid (L-AP3) (Schoepp *et al.*, 1990; Birse *et al.*, 1993).

Methods

Subjects and surgery

Subjects were male Long Evans rats (280–350 g). The rats were housed 2–3 per cage, on a 12:12 h light–dark cycle (lights on at 06:00 h), with food and water available *ad libitum*.

On day 0 rats were anaesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, 60 mg kg^{-1}), and 23 gauge stainless steel cannulae, attached to model 2001 Alzet osmotic mini-pumps filled with one of the EAA antagonist solutions or saline, were implanted stereotaxically in the lateral ventrical (AP = −1.3 mm and L = −1.8 mm from bregma and V = −3.8 mm from the top of the skull; Paxinos & Watson, 1986). While the rats were still under pentobarbital anaesthesia, one unprimed (i.e. not yet pumping) model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. These morphine-containing pumps started pumping the morphine solution approximately 2–4 h following implantation. On the following day, day 1, rats were briefly anaesthetized with halothane and a second unprimed model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. This two day pump implantation procedure was used to reduce the risk of mortality resulting from the accumulation of lethal systemic morphine concentrations prior to any tolerance development. To assess the effects of chronic i.c.v. EAA antagonist treatment on behaviour in rats not dependent on morphine, some rats were given vehicle or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 without concurrent morphine treatment.

Drugs

MK-801, L-AP3 and GYKI 52466 were obtained from Research Biochemicals, (Natick, MA, U.S.A.), while (*S*)-4C-PG was purchased from Tocris Neuramin (Bristol, U.K.). EAA antagonists were continuously infused at a rate of 1 μl h^{-1} in the following concentrations: 1.6 nmol day^{-1}, 8 nmol day^{-1} and 40 nmol day^{-1}. Morphine sulphate (Sabex, Montreal, Canada) was continuously delivered at a rate of 10 μl h^{-1} for a total dose of 36.65 μmol day^{-1} morphine sulphate.

Withdrawal measurement

Precipitated abstinence symptoms were assessed on the seventh day of treatment (while all pumps were still delivering antagonists and morphine) after injection of naloxone (1 mg kg^{-1} s.c.). For 10 min before and 40 min after naloxone injection, the withdrawal symptoms were assessed by measuring the amount of time spent teeth chattering and writhing, as well as by counting jumps and wet dog shakes (Bläsig *et al.*, 1973). The time spent in non-withdrawal behaviours (ambulating, rearing, grooming and resting) was also measured for comparison, for 10 min before and after the injection of naloxone, in rats treated with i.c.v. EAA antagonists either alone or with s.c. morphine.

Statistical analysis

Timed withdrawal behaviours (teeth chattering, writhing) were analysed using ANOVA, followed by *post hoc* tests on significant main effects. Counted withdrawal behaviours (number of jumps and wet dog shakes) were analysed using a Kruskal–Wallis ANOVA for non-parametric data, followed by Mann–Whitney *U*-tests on significant main effects.

The effect of EAA antagonist treatment on non-withdrawal behaviours (ambulating, rearing, grooming and resting) was assessed by comparing the first two time blocks (i.e. 10 min prior to naloxone injection and 10 min after naloxone injection) for rats in each treatment group. Planned comparisons were used to analyse differences in the proportion of time spent in each timed non-withdrawal and the two timed withdrawal behaviours during these two time blocks across the different treatment conditions.

Results

Administration of 36.65 μmol day^{-1} s.c. morphine sulphate by Alzet pump produced an intense and reliable naloxone-precipitated abstinence syndrome which was evidenced by the occurrence of teeth chattering, writhing, jumping and wet dog shaking. As indicated in Figure 1a, the metabotropic receptor antagonists (*S*)-4C-PG and L-AP3 significantly decreased the occurrence of timed abstinence symptoms (teeth chattering and writhing). The effect for (*S*)-4C-PG appeared to be dose dependent, with the highest dose, 40 nmol day^{-1}, producing the greatest reduction in teeth chattering and writhing. L-AP3 was most effective at 8 nmol day^{-1}. Figure 1b shows the amount of time spent teeth chattering and writhing for rats treated with MK-801 and GYKI 52466. The NMDA receptor antagonist MK-801 significantly decreased the time spent in withdrawal at all doses used. The AMPA/kainate receptor antagonist GYKI 52466 did not affect the amount of time spent in withdrawal at any of the doses used.

Figure 2 illustrates the frequency of the counted abstinence symptoms, jumps and wet dog shakes. Although MK-801 tended to *increase* the number of jumps and wet dog shakes at the high dose (40 nmol day^{-1}), none of the i.c.v. EAA antagonist treatments *significantly* affected the number of jumps and wet dog shakes.

Figure 3 shows the percentage of time spent in each of the timed behaviours during the 10 min prior to naloxone administration and during the 10 min following naloxone for rats in each i.c.v. treatment group either with or without concurrent morphine treatment. As can be seen in Figure 3a and b, prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being more grooming in L-AP3 treated rats than in saline treated rats. In addition, saline-treated rats that were also given morphine reared more that rats given i.c.v. saline alone. Although activity levels (ambulating and rearing) were lower and resting was generally higher after the injection of naloxone, rats given i.c.v. EAA antagonists without morphine still behaved very similarly to rats given i.c.v. saline without morphine. The only differences observed were an increase in grooming in L-AP3-treated rats and increased activity in GYKI 52466-

treated rats, as evidenced by increased ambulating, rearing and grooming and decreased resting. As expected, rats dependent on morphine showed significantly more naloxone-precipitated withdrawal, with a resultant decrease in non-withdrawal behaviours, than rats given i.c.v. treatments alone. In morphine-dependent rats, time in withdrawal was significantly less in L-AP3-, (*S*)-4C-PG- and MK-801-treated rats, which coincided with an increase in ambulation in (*S*)-4C-PG- and MK-801-treated rats.

Discussion

The present results demonstrate that concurrent treatment of rats with various EAA antagonists and chronic morphine leads to a decrease in various symptoms of morphine withdrawal. The NMDA receptor antagonist MK-801 and the metabotropic receptor antagonists (*S*)-4C-PG and L-AP3 were all effective at decreasing the amount of time spent exhibiting the withdrawal symptoms of teeth chattering and writhing, while the AMPA/kainate receptor antagonist GYKI 52466 had no effect. Although all but the AMPA/kainate receptor antagonist significantly reduced the timed withdrawal symptoms, none of the EAA antagonists significantly affected the counted withdrawal symptoms (i.e. jumping and wet dog shakes). It has been suggested that withdrawal symptoms such as jumping are mediated primarily by structures around the fourth ventricle, as evidenced by focal brain micro-injections of naltrexone and levallorphan in morphine-dependent rats (Laschka *et al.*, 1976; Koob *et al.*, 1992). In the present study, EAA antagonists were infused in very small volumes into the lateral ventricle, thus it is possible that the counted symptoms were not affected because the drugs were unable to diffuse to the appropriate brain structures around the fourth ventricle.

There was very little effect of i.c.v. EAA antagonist treatment on non-withdrawal behaviours, except for a general increase in grooming in rats given L-AP3 and increased activity in GYKI 52466-treated rats given naloxone. In general, rats given i.c.v. treatment alone rested more during the second 10 min (i.e. after naloxone), with concurrent decreases in ambulation and rearing. This is probably *not* an effect of naloxone, but rather because they had adequately explored the test box and were comfortable in the environment (a phenomenon common in untreated rats). Rats treated chronically with morphine exhibited withdrawal following the injection of naloxone, which caused a subsequent decrease in other timed behaviours. L-AP3, (*S*)-4C-PG and MK-801 all decreased withdrawal compared with saline in morphine-treated rats, with a resultant increase in ambulation in rats given MK-801 + morphine and (*S*)-4C-PG + morphine. The decrease in withdrawal allows for more time to be spent in other behaviours.

Although we have no explanation for why L-AP3 would increase grooming, it appears to be a robust effect since it occurred in every condition except in rats going through withdrawal (i.e. morphine + naloxone). The increase in activity produced by GYKI 52466 appears to be less robust since it occurred only in the group also treated with naloxone. Nonetheless, it is not expected that these behavioural effects of L-AP3 or GYKI 52466 alter our conclusions about the effects of EAA antagonists on withdrawal behaviour. GYKI 52466 did not significantly affect withdrawal, and the effects of the metabotropic receptor antagonist L-AP3 were confirmed by another metabotropic antagonist (*S*)-4C-PG, which did not significantly influence non-withdrawal behaviours. Therefore, we propose that EAA antagonist treatment affects the development of dependence directly, and not indirectly by interfering with the measurement of the withdrawal behaviours.

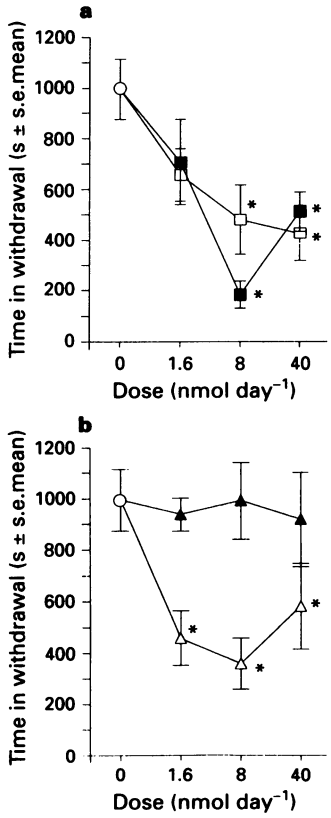

Figure 1 Mean time spent exhibiting withdrawal (teeth chattering and writhing) during the 40 min withdrawal period in rats given chronic s.c. morphine and i.c.v. treatment with the metabotropic receptor antagonists (*S*)-4C-PG (□) ($n = 5-10$ per dose) and L-AP3 (■) ($n = 4-10$ per dose) (a), the NMDA receptor antagonist MK-801 (△) ($n = 4-10$ per dose) (b), and the AMPA/kainate receptor antagonist GYKI 52466 (▲) ($n = 4-10$ per dose) (b). ANOVA indicated significant effects of (*S*)-4C-PG [$F_{(3,22)} = 5.124$, $P < 0.001$], L-AP3 [$F_{(3,23)} = 12.107$, $P < 0.001$] and MK-801 [$F_{(3,21)} = 6.222$, $P < 6.222$, $P < 0.01$], but not GYKI 52466 [$F_{(3,20)} = 0.071$, $P > 0.05$]. Significant differences from the control group are indicated by asterisks (*$P < 0.05$, LSD *t*-test).

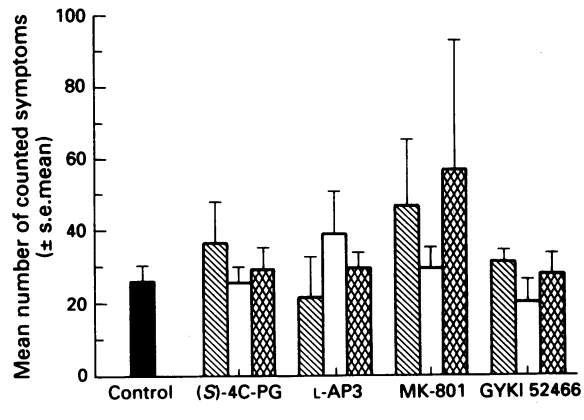

Figure 2 Frequency of counted symptoms (jumps and wet dog shakes) during the 40 min withdrawal period for rats given chronic s.c. morphine and i.c.v. treatment with the metabotropic receptor antagonists (*S*)-4C-PG ($n = 5-10$ per dose) or L-AP3 ($n = 4-10$ per dose), the NMDA receptor antagonist MK-801 ($n = 4-10$ per dose) or the AMPA/kainate receptor antagonist GYKI 52466 ($n = 4-10$ per dose). Kruskal-Wallis ANOVA for non-parametric data revealed no significant effects of any of the EAA antagonists. (*S*)-4C-PG, $H_{(3,22)} = 0.803$, $P > 0.05$; MK-801, $H_{(3,21)} = 0.747$, $P > 0.05$; GYKI 52466, $H_{(3,20)} = 2.580$, $P > 0.05$; L-AP3, $H_{(3,23)} = 2.142$, $P > 0.05$. Solid columns, 0 nmol day^{-1}; diagonal hatched columns, 1.6 nmol day^{-1}; open columns, 8 nmol day^{-1}; cross-hatched columns, 40 nmol day^{-1}.

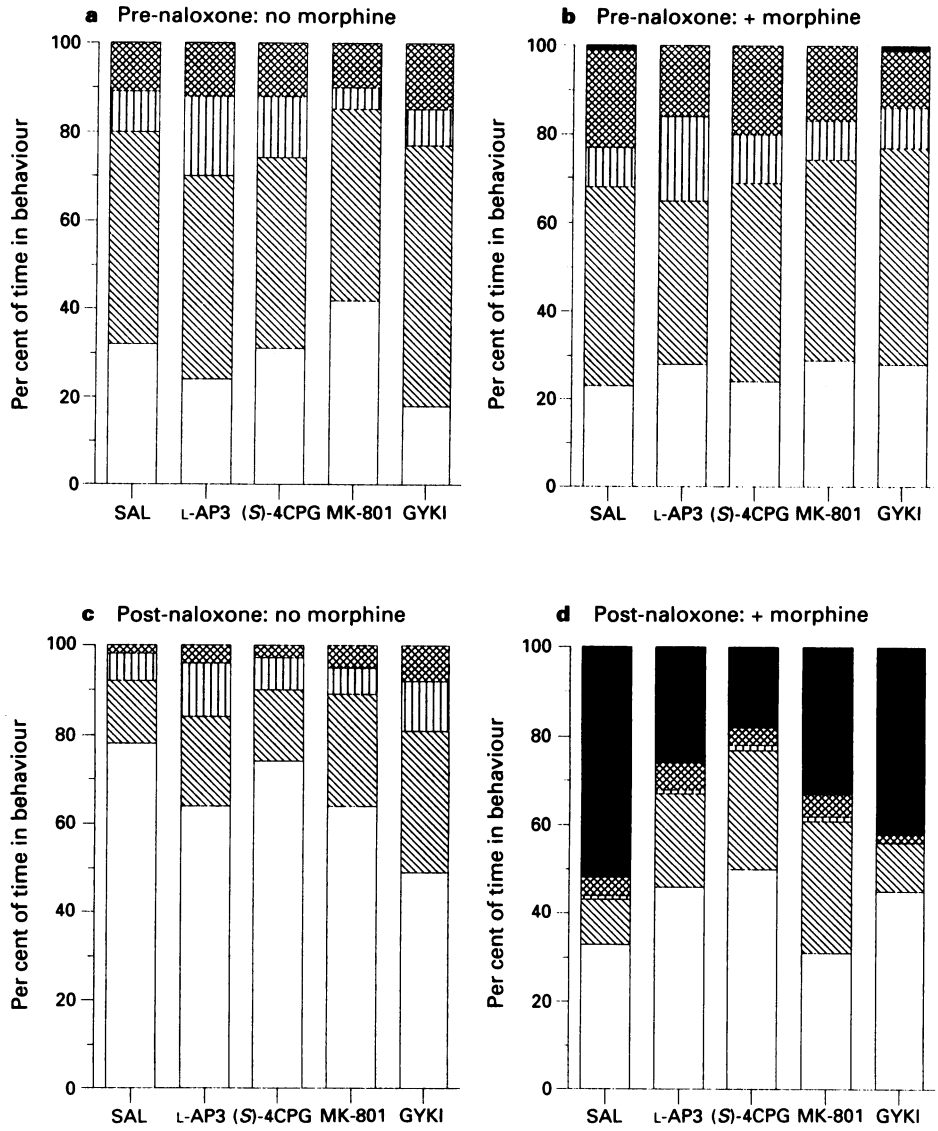

Figure 3 Percentage of time in non-withdrawal and withdrawal behaviours in rats treated chronically with either saline or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 (EAA antagonists) i.c.v. alone (i.e. no s.c. morphine; $n = 6-8$ per i.c.v. treatment) (a) or with s.c. morphine ($n = 4-10$ per i.c.v. treatment) (b) during the 10 min *prior* to the injection of naloxone; and in rats given chronic i.c.v. saline or EAA antagonists alone (c) or with s.c. morphine (d) during the 10 min *after* the injection of naloxone. Prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being increased grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (a and b). With the addition of morphine, saline-treated rats exhibited more rearing than rats not treated with morphine (planned comparison$_{(1,52)}$, $P<0.05$) (b). After the injection of naloxone, rats not dependent on morphine generally rested more than in the first 10 min. Non-dependent rats in all i.c.v. treatment groups behaved similarly, with the only differences being more grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (c) and more activity in GYKI 52466-treated rats as evidenced by significantly more ambulating (diagonal hatched columns), rearing (cross-hatched columns) and grooming (vertically hatched columns) and significantly less resting (open columns) (planned comparison$_{(1,52)}$, $P<0.05$) (c). Morphine-dependent rats showed a significant increase in withdrawal behaviours (solid columns) regardless of i.c.v. treatment after the injection of naloxone (planned comparison$_{(1,52)}$, $P<0.05$) (d). However, L-AP3, (*S*)-4C-PG and MK-801 all significantly decreased the percentage of time spent in withdrawal behviours, with a concurrent increase in ambulation in (*S*)-4C-PG- and MK-801-treated rats (planned comparison$_{1,52)}$, $P<0.05$) (d).

Previously, it has been observed that concurrent treatment of rats with the non-selective EAA antagonist kynurenic acid (Marek *et al.*, 1991a) or the non-competitive NMDA antagonist MK-801 (Marek *et al.*, 1991b; Trujillo & Akil, 1991) with daily injections of morphine attenuated the development of tolerance to morphine's analgesic effects. MK-801 also alleviated the severity of some symptoms of the precipitated withdrawal syndrome (Trujillo & Akil, 1991). Furthermore, some investigators have found that acute treatment with kynurenic acid and MK-801 only on the day of testing (i.e. not concurrently with morphine) is effective in decreasing the severity of some withdrawal symptoms (Rasmussen *et al.*, 1991a,b; Tanganelli *et al.*, 1991), while others did not find this acute administration effective (Trujillo & Akil, 1991). While each of these above studies assessed the effects of *systemically* administered EAA antagonists, in additional experiments (unpublished data), we have found that acute *i.c.v.* injections of EAA antagonists on day 7 prior to precipitation of withdrawal failed to attenuate severity of abstinence symptoms, lending support to the latter group.

Hyperactivity in the locus coeruleus (LC) has been shown to be correlated with the morphine withdrawal syndrome (Aghajanian, 1978; Valentino & Wehby, 1989). Central administration of non-specific, NMDA-selective and AMPA/

kainate-selective EAA antagonists into either the lateral ventricle or the locus coeruleus has been found to decrease the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Akaoka & Aston-Jones, 1991), with the best effects produced by the non-specific EAA antagonist. Conversely, systemic administration of selective NMDA receptor antagonists was unable to affect the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Rasmussen *et al.*, 1991a).

The present data indicate that chronic i.c.v. administration of the selective metabotropic EAA receptor antagonists (*S*)-4C-PG and L-AP3 was at least as effective in attenuating the severity of the morphine withdrawal syndrome as antagonists selective for the NMDA receptors. It is noteworthy that the most effective treatment was 8 nmol day^{-1} L-AP3. This strong effect of L-AP3 may have resulted from a possible additive effect of L-AP3 at both metabotropic and NMDA receptors. There is evidence that L-AP3 may have non-selective effects at the NMDA receptor as well as its proposed major action at the metabotropic receptor (Birse *et al.*, 1993). The significant dose-dependent effects of the highly selective metabotropic receptor antagonist (*S*)-4C-PG, however, do suggest an important role of metabotropic glutamate receptors in the development of morphine dependence. The failure of GYKI 52466 to influence precipitated withdrawal suggests that AMPA/kainate receptors do not play an important role in morphine dependence. Although it is possible that GYKI 52466's ineffectiveness could be explained by rapid metabolism *in vivo*, this is unlikely because the drug was chronically infused. Furthermore, it has been demonstrated, using an osmotic pump infusion method similar to that used in the present study, that GYKI 52466 is as effective at attenuating excitatory amino acid induced seizures on the 14th day of infusion as it is on the third day of infusion (Steppuhn & Turski, 1993).

Activity at specific metabotropic receptor subtypes stimulates phosphatidylinositol (PI) hydrolysis and leads to the production of the intracellular messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Chronic opioid use may alter production of these intracellular messengers and thus elicit long-term changes which contribute to opioid tolerance and dependence. There is evidence that acute morphine treatment stimulates PI hydrolysis (Raffa & Martinez, 1992), while chronic morphine treatment inhibits PI hydrolysis (Dixon *et al.*, 1990). (*S*)-4C-PG and L-AP3 antagonism of the metabotropic glutamate receptor during morphine treatment may prevent cellular changes associated with persistent phosphatidylinositol hydrolysis, and consequently reduce withdrawal symptoms that are associated with these cellular changes. Other metabotropic receptor subtypes inhibit production of cAMP (Schoepp & Conn, 1993). It is well established that both acute and chronic opioid use also affect the production of cAMP (Collier, 1980, 1983; Sharma *et al.*, 1975). Thus it is possible that (*S*)-4C-PG and L-AP3 antagonism of metabotropic receptors prevented changes in the cAMP system associated with chronic opioid use. Currently, we are determining whether changes in the PI system or changes in the cAMP system associated with activation of metabotropic receptors are important for the development of tolerance and dependence with chronic opioid use.

Thus, the present study indicates that both NMDA and metabotropic glutamate receptors may be involved in the development of dependence with chronic morphine use. Both NMDA and metabotropic glutamate receptors are associated with changes in intracellular second messenger systems. It is therefore hypothesized that NMDA and metabotropic glutamate antagonists are effective because they prevent changes in second-messenger systems associated with chronic opioid use.

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