Antagonism of NMDA Receptors Reduces the Vulnerability of the Immune System to Stress Following Chronic Morphine

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Abstract

It has been shown that morphine-tolerant animals have an altered immunologic sensitivity to stress. While the glutamatergic system has been implicated in the neuroadaptive process underlying this tolerant state, its potential role in development of the altered immunologic sensitivity consequent to chronic morphine treatment is not known. To determine this, a morphine-tolerant state was induced by 10-day administration of an escalating dose of morphine from 10 to 40mg/kg (s.c., b.i.d.) and lymphocyte proliferative response to a T-cell mitogen was measured. Morphine challenge (10mg/kg, s.c.) after days of treatment was gradually less immunosuppressive and this tolerance progression was delayed by concurrent administration of the NMDA receptor antagonist, MK-801 (0.1mg/kg; s.c.), with chronic morphine. The effect was independent of glucocorticoid level changes and was not a result of an acute interaction of the drugs or the prolonged presence of the antagonist alone. Subsequent to chronic treatment, animals were subjected to opioid withdrawal and water stress. Both stressors induced 50% immunosuppression in morphine-tolerant animals compared to saline-treated controls. Increased immunologic sensitivity to these stressors was attenuated when MK-801 was administered with chronic morphine as demonstrated by an accelerated recovery rate and lack of immunosuppression from opioid withdrawal and water stress, respectively. Together, these findings provide the first evidence that the neuroadapted state of the immune response after chronic morphine can be modified by NMDA receptor antagonism, as illustrated by a temporal deceleration of the development of immunologic tolerance during chronic treatment that is associated with an attenuation of the immunologic vulnerability of morphine-tolerant animals to stress.

Introduction

The outcome of the neuroadaptation thought to underlie opioid-induced tolerance is a modified homeostatic state such that reaction to various stimuli such as drug exposure and stress is altered (Carlson, 1977; Molina et al., 1994; Fitzgerald et al., 1996; Houshyar et al., 2001a; Houshyar et al., 2001b; Weiss et al., 2001). It has been shown that morphine-tolerant animals have an altered response to cocaine, MDMA, cannabinoid and alcohol administration (Erdtmann-Vourliotis et al., 2000; Gonzalez et al., 2002; Howd and Pryor, 1980), a modified reaction to activators of the hypothalamic-pituitary-adrenal axis such as stress and steroids (Borsook et al., 1994 Houshyar et al., 2001a; Houshyar et al., 2001b), and a weakened host defense mechanism to pathogens (Bryant et al., 1987; Rouveix, 1992; West et al., 1997). While extensively studied, the neuroadaptive process thought to be responsible for tolerance is far from elucidated. There is, however, evidence that the central glutamatergic receptor system is a major participant in the development of the tolerant state.   Several studies have found that manipulation of central glutamatergic receptors can modulate the development and outcome of tolerance to opioid-induced analgesia (Trujillo and Akil, 1991; Bespalov et al., 1994; Fundytus, 2001), behavioral sensitization (Jeziorski et al., 1994; Iijima et al., 1996) and physical dependence (Fundytus et al., 1997; Gonzalez et al., 1997; Bisaga and Popik., 2000)   The additional role of NMDA receptors as a critical factor in neuroplasticity suggests that this receptor system would also affect the resulting altered state of an animal after prolonged opioid treatment (Wang and Feng, 1992; Shors and Servatius, 1995; Krug et al., 2001).

Among the acute effects of opioids that have been shown to be diminished subsequent to chronic treatment is immunosuppression. Previous studies have found that the inhibition of natural killer cell activity (Rouveix, 1992; West et al., 1997), humoral and cellular immune responses (Rouveix, 1992; Rahim et al., 2002), and cytokine activity (Chang et al., 1998; Limiroli et al., 2002; Raghavendra et al., 2002) as a result of an acute administration of morphine is absent after a prolonged period of morphine management. Studies in this laboratory have shown that morphine administration (10mg/kg) to a naïve animal significantly suppresses blood lymphocyte proliferative response to a T cell mitogen and that this effect is mediated through central mu opioid receptors (Bayer et al., 1990; Hernandez et al., 1993; Flores et al., 1995). Following chronic morphine treatment, however, the suppressive effect of morphine administration (10mg/kg) on the proliferative response is absent (Mellon et al., 2001; Bayer et al., 1996). However, these chronically-treated animals were found to be much more sensitive to the immunosuppressive effects of restraint stress, suggesting an altered vulnerability of these animals to the stressor (Bayer et al., 1994).

Similar to other opioidergic effects, the mechanisms fundamental to the development of tolerance to this immune response to morphine is largely unknown. Surprisingly, while NMDA receptor antagonism has been shown to modulate tolerance of opioid effects such as analgesia and reinforcement, the role of the glutamatergic receptor system in the development and outcome of immunomodulatory tolerance has not been examined. The present study, therefore, endeavors to determine whether NMDA receptor activity has a role in the development of immunologic tolerance by comparing mitogen-induced blood lymphocyte proliferative activity after morphine challenge during chronic

treatment with either morphine and MK-801 or morphine alone. Additionally, it will be

determined whether opioid withdrawal and water stress applied subsequent to chronic

morphine treatment induces an altered immune response and whether this immunologic

vulnerability to these stressors is dependent on NMDA receptor activity during chronic

dosing.

Methods

Animals. Male Sprague-Dawley rats were received from Taconic Farms (Germantown, NY) weighing 200 to 225 g upon arrival. Animals were housed three per cage in polypropylene cages with micro isolator tops that were contained in a thermoregulated ($23 \pm 1^{o}C$) and light (12-h light/dark)-controlled room. Following receipt, animals were allowed to acclimate for one week before experimental manipulation. Free access to food (Purina Rat Chow; Purina, St. Louis, MO) and water was provided. In experiments requiring surgery, animals were allowed to recover for an additional week before experimentation. All animal studies have been approved by the Georgetown University Animal Care and Use Committee in accordance with the guidelines adopted by National Institutes of Health.

Drugs. Morphine sulfate was generously provided by the National Institute on Drug Abuse (Research Triangle Park, NC). MK-801 was purchased from Sigma Chemicals (St. Louis, MO). Equithesin was prepared by dissolving chloral hydrate and magnesium sulfate in a solution of propylene glycol and pentobarbital. All vehicle and drug solutions were made using sterile, 0.9% saline.

Chronic treatment. Chronic morphine treatment was initiated after acclimization period with drug administered as follows (doses in mg/kg morphine, s.c.):

Day of Treatment:	1	2	3	4	5	6	7	8	9	10
AM dose	10	10	20	20	30	30	40	40	40	sacrifice
PM dose	10	20	30	30	40	40	40	40	40	------

Groups receiving MK-801 (0.1mg/kg; s.c.) were administered drug either instead of morphine or immediately prior to each morphine injection for concurrent treatment.

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Plasma Corticosterone Measurement. Heparinized plasma was collected at time of sacrifice and stored at $-20^{\circ}C$. Samples were assayed for corticosterone levels using a solid phase ^{125}I radioimmunoassay purchased from ICN Pharmaceuticals (Costa Mesa, CA).

Lymphocyte Proliferation. Animals were decapitated and trunk blood collected in 50ml polypropylene tubes containing 0.2ml heparin (1000 U/ml). Whole blood was diluted 1:5 in sterile RPMI 1640 cell culture media (Gibco, Grand Island, MY) supplemented with 5% fetal bovine serum and 1% gentamacin. Diluted blood (100 μl) was incubated with increasing concentrations of T-cell specific mitogen concanavalin A (ConA at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 μg/well) for 96 hours at 37^0C with 8% CO_2. Samples were pulsed with 0.5μCi of [methyl-^{3}H] thymidine (6.7mmol; New England Nuclear, Boston, MA) in a 20μl volume and incubated for an additional 24 h at 37^0C with 8% CO_2. Samples were lysed with distilled water and harvested onto glass fiber filters using a 96-well cell harvester (Brandel, Gaithersburg, MD). The amount of labeled DNA was determined via liquid scintillation spectrophotometry (Beta Plate; L.K.B. Pharmacia).

Maximum proliferative response was calculated from the ConA dose response curves generated. To allow comparisons from experiment-to-experiment, all data were expressed as a percentage of the response of the saline-treated control group from each individual experiment. All experiments were repeated at least 2-3 times.

Withdrawal. After 10 days of chronic treatment, as described above, all drug injections were replaced with sterile saline s.c. Body weights were measured at 12-hour intervals up to 72 hrs. after initiation of drug withdrawal. Separate groups of animals were

sacrificed after 24 and 72 hours of withdrawal for corticosterone and blood lymphocyte proliferation analysis.

Water Stress Assay. After 10 days of chronic treatment, as described above, animals were individually placed in polypropylene cages (standard mouse cage) containing 2cm water (room temperature) or regular bedding for 1h. Animals were sacrificed and trunk blood collected for plasma corticosterone and peripheral blood lymphocyte proliferation analysis.

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Results

NMDA receptor antagonism alters the rate of development of immunologic tolerance during chronic morphine treatment.   In order to determine whether NMDA receptor antagonism alters the rate of development of morphine-induced tolerance, immune responses were analyzed at various timepoints throughout chronic treatment with either morphine alone or morphine and MK-801. It has been previously shown in this laboratory that naïve animals challenged with acute morphine showed a suppression of lymphocyte proliferation response to a T-cell mitogen compared to saline-treated control animals and that tolerance develops to this immunosuppressive effect after chronic treatment (Bayer et. al, 1990 and Bayer et al. 1994). In animals receiving escalating doses of morphine, an initial 75% inhibition of T-cell proliferative ability after a morphine challenge (10mg/kg, s.c.) after one day of chronic treatment was gradually less apparent such that peripheral lymphocytes were 70, 80 and 100% responsive to mitogen after 2, 4 and 10 days of treatment (Fig 1a). Similarly, lymphocyte proliferative response during combined administration of chronic morphine and MK-801 was initially significantly suppressed, however this suppression was prolonged and responses continued to be suppressed by 75% even after 4 days of treatment ($p<0.01$). After 6 days of treatment, animals receiving either concurrent morphine and MK-801 or morphine alone showed lymphocyte activity that was not significantly different from controls (Fig. 1a).

The development of tolerance to steroid elevation during chronic morphine with or without concurrent NMDA receptor antagonism was also studied at these different time points. In animals receiving escalating doses of morphine, plasma corticosterone concentrations were 300% over control levels on the first day of treatment but gradually

decreased so that by Day 10 of the treatment regime, plasma corticosterone

concentrations were not different from those measured in saline-treated control animals.

Antagonism of NMDA receptors with MK-801 during chronic morphine did not alter this

development of tolerance to the corticosterone elevation caused by morphine challenge.

Animals receiving both morphine and MK-801 chronically exhibited plasma

corticosterone concentrations that were 250% over control levels with a gradual decrease

in levels such that they were not longer significantly different from control animals after

10 days of chronic treatment (1b).

Antagonism of NMDA receptors does not alter the immunosuppressive effect

of acute morphine. As previously shown in our lab, acute morphine significantly

suppresses lymphocyte proliferation in naïve animals. Whether NMDA receptor

modulation would affect lymphocyte proliferation alone or alter morphine's acute effects

is not known. Therefore, in order to determine whether the effect of MK-801 on

immunologic tolerance is related directly to an acute interaction of the drug with

morphine, animals were treated with morphine (10mg/kg, s.c.) and MK-801 (0.1mg/kg;

s.c.) for two hours. Administration of MK-801 did not suppress the proliferative

response of lymphocytes to mitogen, nor did it affect the immunosuppressive effect of

acute morphine (Fig. 2).

Immunosuppression by acute morphine is not modified by chronic treatment

with MK-801.   The effect of prolonged antagonism of NMDA receptors on the immune

system is unknown. It may be that the chronic antagonism of glutamate receptors alone

is sufficient to bring about a delay in immunologic tolerance development during chronic

of morphine. Therefore, the acute effect of morphine in animals chronically treated with

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MK-801 was studied. Surprisingly, animals receiving twice-daily injections of MK-801 (0.1mg/kg) alone showed a potentiated mitogenic T cell response as compared to control animals. Lymphocyte proliferative response to mitogen in animals administered 10 days of chronic morphine was not suppressed 2 hrs after a morphine challenge (10mg/kg). In contrast, a similar dose of morphine in animals that received chronic MK-801 (0.1mg/kg) for 10 days was still significantly suppressive (p<0.001). Confirming previously shown data (Fig. 1), animals administered both morphine and MK-801 chronically showed no immunosuppression to a morphine challenge dose (10mg/kg) after 10 days of treatment (Fig 3).

Morphine withdrawal induces profound weight loss that is unaffected by antagonism of NMDA receptors during chronic treatment. Recent evidence has shown that the deprivation of morphine following chronic exposure is likened to a stress response, resulting in significant immunosuppression and a significant elevation of steroid levels (Ignar and Kuhn, 1990; Houshyar et al., 2001b). In order to determine whether NMDA receptor antagonism would alter the effects and rate of recovery from the effects of opioid withdrawal, animals were treated chronically with morphine or morphine and MK-801 as previously described, after which opioid withdrawal was induced by replacing all drug injections with saline. The withdrawal syndrome was characterized by a dramatic weight loss that peaked after 24 hours of drug deprivation (p<0.001) and a return to control levels after 48 hours. This characteristic weight loss observed during morphine withdrawal was not altered when MK-801 was administered during chronic morphine treatment (Fig 4).

Rate of recovery from opioid withdrawal-induced immunosuppression, but not plasma steroid elevation, is accelerated by NMDA receptor antagonism during chronic treatment. In addition to its impact on animal body weight, opioid withdrawal had a significant effect on plasma steroid elevation and mitogenic response of peripheral T-cells. Lymphocytes isolated from morphine-withdrawing animals had a severe loss of proliferative ability. Mitogen-induced proliferation after 24 and 72 hours of opioid withdrawal was 40% and 70% suppressed compared to non-withdrawing controls, respectively. However, animals receiving MK-801 during chronic morphine treatment exhibited an altered immune profile. T-cell proliferation in animals receiving both morphine and MK-801 chronically was significantly suppressed after 24 hours of withdrawal. However, unlike animals treated with morphine alone, lymphocyte responses in these animals returned to control levels after 72 hours of drug deprivation (Fig.5b).

Additionally, animals undergoing opioid withdrawal showed an elevation of plasma corticosterone that reached maximum levels after 24 hours of withdrawal (p<0.001) and returned to control values after 72 hours of abstinence from drug. In contrast to the modulation of the immune response, changes in plasma steroid levels during morphine withdrawal were unaffected by NMDA receptor antagonism during chronic morphine treatment (Fig. 5a).

NMDA receptor antagonism alters immunologic, but not steroidal, stress sensitivity after chronic morphine. We have previously shown that animals receiving chronic morphine exhibit an altered sensitivity to restraint stress, suggesting that the immune response to stressors after chronic treatment is altered (Bayer et al., 1994).

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However, it is not known whether antagonism of glutamate receptors during chronic

morphine treatment would alter this vulnerability to stressors. In order to determine this,

animals were chronically treated with morphine or morphine and MK-801 as previously

described and underwent a water stress (at room temperature) for 1 hr. Characteristic of a

stressor, steroid levels of animals that were stressed were elevated irrespective of drug

treatment. Animals receiving chronic saline, chronic morphine or chronic morphine and

MK-801 all showed a significant increase of plasma corticosterone levels as compared to

their non-stressed controls ($p \leq 0.01$) (Fig 6b).   In contrast to this steroid response, the

water stress protocol did not cause a significant suppression of lymphocyte proliferation

in saline-treated control animals. However, water-stressed animals that were chronically

treated with morphine showed a significantly inhibited lymphocyte response ($p < 0.05$),

suggesting an altered immunologic vulnerability to the stressor. Conversely, when

animals were given concurrent MK-801 during chronic morphine, lymphocyte

proliferation was not suppressed and was not significantly different from saline-treated

controls, suggesting that NMDA receptor antagonism during chronic morphine treatment

did modulate the development of an altered state of sensitivity to the stressor (6a).

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Discussion

The glutamatergic system has been shown to impact the development of tolerance to opioidergic effects. Until now, there has been no evidence to indicate that NMDA receptors are involved in immunosuppressive tolerance after chronic morphine treatment. In the present study, rate of development and expression of immunologic tolerance, as measured by mitogen-activated peripheral blood lymphocyte proliferation, was examined after 10-day chronic treatment with morphine with or without the NMDA receptor antagonist, MK-801. We find that presence of MK-801 with morphine administration delayed the rate of immunologic tolerance progression. Furthermore, we present evidence that NMDA receptor antagonism concurrent to chronic morphine attenuates increased immunologic sensitivity of morphine-tolerant animals to two different stressors.

Animals administered twice-daily injections of an escalating morphine dose showed a development of tolerance to the immunosuppressive and steroid-elevating effect of a morphine challenge. This hallmark of tolerance is observed for many other effects of acute morphine (Donovan et al., 1977; Mucha et al., 1978; Milne et al., 1989; Detweiler et al., 1995; Ouellet and Pollack, 1995; Kest and Hopkins, 2001; Bardin et al., 2003). As previously shown, a dose of morphine (10mg/kg, s.c.) that significantly suppresses mitogen-activated lymphocyte proliferation in naïve animals had no such effect in chronic morphine-treated animals (Bayer et al., 1996; Mellon et al., 2001). Data presented in this study demonstrates a gradual tolerance progression, occurring throughout the course of chronic morphine. Addition of chronic MK-801 with morphine delayed the rate of tolerance development to the morphine challenge. The question arises

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as to whether MK-801 itself is altering mitogen-induced T cell function. Our data

indicated that administration of acute (Fig 2) and chronic (Fig 3) MK-801 was unable to

modulate the suppressive effect of acute morphine (10mg/kg) on lymphocytic

proliferation response, suggesting that alteration in the rate of tolerance progression

requires the prolonged presence of both morphine and MK-801 and is not due to the

actions of MK-801 alone.

While the presence of an NMDA receptor antagonist delayed the rate of

immunologic tolerance development, it did not alter steroid responses throughout the

course of chronic morphine treatment. Similar to other effects of morphine, elevation of

plasma steroid in response to morphine challenge was gradually diminished over the

course of chronic treatment. Unlike immunologic tolerance, this effect was not altered by

administration of MK-801. This suggests a role of NMDA receptors in development of

opioid immunologic tolerance that is not dependent on actions of the HPA axis, the

pathway mediating release of plasma corticosterone. Additionally, the impact of

morphine on proliferative responses of lymphocytes to a T-cell mitogen has been shown

to be selectively mediated through central mu opioid receptors, most likely those located

in the paraventricular nucleus (Mellon et al., 2001). Evidence of NMDA receptors

modulating this centrally mediated immune response, but not altering the peripheral

effect of steroid level changes, supports the idea that the minimized tolerant state of the

immune system is not likely mediated by direct peripheral actions of the glutamatergic

system. Rather, the effect of NMDA receptor antagonism during chronic morphine on

lymphocyte proliferative tolerance could be the result of an altered neuroadapted state

caused by perturbation of the central interaction of these receptor systems. Similar to

opioidergic effects such as analgesia, reinforcement and physical dependence ((Fundytus,

2001) Iijima et al., 1996; Fundytus et al., 1997), it may be that the central glutamatergic

receptor system is also a participant in the development of the immunologic tolerant state.

It has been reported that subsequent to chronic opioid treatment, response to

noxious stimuli or "stressors" is significantly altered. We have previously shown that

animals undergoing restraint stress were immunologically vulnerable if they were

morphine-tolerant (Bayer et al., 1994; Mellon et al., 2001). Neuroadaptation that occurs

during chronic opioid administration is thought to involve changes in levels of

neurotransmitters such as norepinephrine and GABA (Jolas et al., 2000; Van Bockstaele

et al., 2001), various receptors systems such as orphanin FQ, CCK, and aminopeptidases

(Ding and Bayer; 1993; Laorden et al., 1997; Milanes et al., 1997; Yuan et al., 1999;

Brundege and Williams, 2002; Irazusta et al., 2003) and expression of major second

messenger signaling factors such as PKC, adenylate cyclase, GPCR's, and CREB

(Parolaro et al., 1993; Lane-Ladd et al., 1997; Bernstein and Welch, 1998; Shen et al.,

2000; Tso et al., 2000; Ma et al., 2001; Shichinohe et al., 2001; Ammer and Christ, 2002;

Irazusta et al., 2003). It has been theorized that these central neuroadaptations may

mediate sensitized response to stressors and that relapse to drug-taking behavior may be

due to this stress vulnerability (Self and Nestler, 1998; Houshyar et al., 2001a; Mutasa,

2001; Sarnyai et al., 2001; Weiss et al., 2001) . Therefore, to extend the idea that NMDA

receptors are involved in the immune effect of chronic morphine, we found that

immunologic sensitivity of morphine-tolerant animals is amplified in response to opioid

withdrawal and water stress and this vulnerability is altered by antagonism of NMDA

receptors.

Following chronic treatment with morphine or morphine and MK-801, opioid withdrawal was induced by replacing drug injections with saline. It has been suggested that opioid withdrawal is likened to a "stressor" due to its significant physical ramifications including body weight loss and plasma steroid elevation (Mioduszewski et al., 1982; Houshyar et al., 2001b; Sarnyai et al., 2001). In animals chronically receiving morphine with or without MK-801, drug deprivation induced considerable weight loss and plasma corticosterone elevation (Fig 4, Fig 5b). However, animals receiving chronic morphine and MK-801 showed an accelerated rate of recovery from the immunosuppressive effect of opioid withdrawal as compared to animals treated with morphine alone (Fig 5a). These data correlate with previous findings suggesting that administration of an NMDA receptor antagonist during chronic morphine treatment has a "protective" effect by attenuating the severity of several physical manifestations of opioid withdrawal (Tanganelli et al., 1991; Trujillo and Akil, 1991; Fundytus and Coderre, 1994; Herman et al., 1995; Gonzalez et al., 1997; Bisaga et al., 2001).

In order to determine if this "protective" effect is primarily on withdrawal alone, or if altered sensitivity of morphine-tolerant animals generalizes to other types of stressors, animals were subjected to water stress subsequent to chronic treatment with morphine and MK-801 or morphine alone. Plasma corticosterone was elevated whether animals were treated chronically with saline, morphine or morphine and MK-801 (Fig 6b). While there was a lack of an effect of water stress on mitogen-induced lymphocyte proliferation in control animals, suppression of blood lymphocyte response was observed in morphine-tolerant animals subjected to water stress that was absent when MK-801 treatment was included with chronic morphine (Fig 6a). This again suggests a protective

effect of NMDA receptor antagonism on immunologic vulnerability induced by chronic

morphine that corresponds to the ability of MK-801 treatment to decrease peripheral

blood lymphocyte sensitivity to the suppressive effect of opioid withdrawal.

At the time that stressors were applied, or subsequent to 10 days of chronic drug

treatment, there was no perceived difference in lymphocyte proliferative response

between animals treated with morphine or morphine and MK-801. If NMDA receptor

antagonism had altered the "tolerant state" that these animals are in, such that

neuroadaptative events occurring during chronic treatment is distorted by MK-801 as

evidenced by the delayed rate of tolerance progression, decreased immunologic

vulnerability to stressors is not surprising. It has been shown that in addition to its effect

on physical indicators, administration of glutamate receptor antagonists concurrent to

chronic opioid can alter neurochemical consequences of opioid dependence and

withdrawal and thereby altering the neuroadapted state (Kogan and Aghajanian, 1995;

Ozawa et al., 2001; Tokuyama et al., 2001; Vekovischeva et al., 2001; Watanabe et al.,

2002). It should also be noted that corresponding to lack of an effect of NMDA receptor

antagonism on progression of plasma steroid tolerance during chronic morphine

treatment, plasma corticosterone elevation triggered by opioid withdrawal or water stress

were resistant to alteration by the presence of MK-801 with chronic morphine (Fig 1b,

Fig 5a, Fig 6a), thereby discounting a steroid-dependent mechanism by which NMDA

receptor antagonism alters these stress responses. The exaggerated immune response to

opioid withdrawal and water stress and alteration of this response by concurrent MK-801,

therefore, is suggestive of a role of neuroplastic changes that occur as a result of chronic

morphine treatment. The relevance of this "protection" from stress vulnerability is

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profound in the context of drug addiction treatment. Stress has been shown to increase

the risk of relapse of drug abuse following detoxification from alcohol and other drugs,

such as opioids (O'Brian et al., 1977; Childress et al., 1986; O'Brien et al., 1998) and it

has been suggested that an altered responsivity to stress may predispose animals to self-

administration of drugs of abuse ((Shaham and Stewart, 1994; Ortiz et al., 1996). If

NMDA receptor antagonism can avert the increased sensitivity of morphine-tolerant

individuals to stress, it may also be able to convey a protection against initiation of drug

abuse behavior or prevent relapse following drug abstinence.

Overall, it can be suggested that modification of the morphine-tolerant "state" by

MK-801 is manifested as a delay in rate of immunologic tolerance progression and a

subsequent immunologic insensitivity to stressors. This ability to alter the tolerant state

induced by chronic opioid treatment by NMDA receptor antagonism serves to further

elucidate the neuroplastic events underlying tolerance of immunologic, as well as other,

opioidergic effects. Further studies addressing central mechanisms by which NMDA

receptors mediate these immune responses need to be carried out in order to fully clarify

this modulation of morphine-induced tolerance by the NMDA receptor system.

References

Ammer H and Christ TE (2002) Identity of adenylyl cyclase isoform determines the G protein mediating chronic opioid-induced adenylyl cyclase supersensitivity. *J Neurochem* **83**:818-827.

Bardin L, Tarayre JP, Malfetes N, Koek W and Colpaert FC (2003) Profound, Non-Opioid Analgesia Produced by the High-Efficacy 5-HT(1A) Agonist F 13640 in the Formalin Model of Tonic Nociceptive Pain. *Pharmacology* **67**:182-194.

Bayer BM, Brehio RM, Ding XZ and Hernandez MC (1994) Enhanced susceptibility of the immune system to stress in morphine-tolerant rats. *Brain Behav Immun* **8**:173-184.

Bayer BM, Hernandez MC and Ding XZ (1996) Tolerance and crosstolerance to the suppressive effects of cocaine and morphine on lymphocyte proliferation. *Pharmacol Biochem Behav* **53**:227-234.

Bernstein MA and Welch SP (1998) mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Brain Res Mol Brain Res* **55**:237-242.

Bespalov A, Zvartau EE, Dumpis MA and Piotrovskii LB (1994) [Tolerance to opiate analgesia: a complex effect of antagonists of excitatory amino acid receptors]. *Biull Eksp Biol Med* **117**:494-496.

Bisaga A, Comer SD, Ward AS, Popik P, Kleber HD and Fischman MW (2001) The NMDA antagonist memantine attenuates the expression of opioid physical dependence in humans. *Psychopharmacology (Berl)* **157**:1-10.

Bryant HU, Bernton EW and Holaday JW (1987) Immunosuppressive effects of chronic morphine treatment in mice. *Life Sci* **41**:1731-1738.

Carlson KR (1977) Supersensitivity to apomorphine and stress two years after chronic methadone treatment. *Neuropharmacology* **16**:795-798.

Chang SL, Wu GD, Patel NA, Vidal EL and Fiala M (1998) The effects of interaction between morphine and interleukin-1 on the immune response. *Adv Exp Med Biol* **437**:67-72.

Childress AR, McLellan AT and O'Brien CP (1986) Role of conditioning factors in the development of drug dependence. *Psychiatr Clin North Am* **9**:413-425.

Detweiler DJ, Rohde DS and Basbaum AI (1995) The development of opioid tolerance in the formalin test in the rat. *Pain* **63**:251-254.

Donovan MP, Thomas JA and Ling GM (1977) Morphine analgesia and tolerance: a possible role of cyclic AMP. *Bull Narc* **29**:9-12.

Fitzgerald LW, Ortiz J, Hamedani AG and Nestler EJ (1996) Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J Neurosci* **16**:274-282.

Fundytus ME (2001) Glutamate receptors and nociception: implications for the drug treatment of pain. *CNS Drugs* **15**:29-58.

Fundytus ME and Coderre TJ (1994) Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence. *Br J Pharmacol* **113**:1215-1220.

Gonzalez P, Cabello P, Germany A, Norris B and Contreras E (1997) Decrease of tolerance to, and physical dependence on morphine by, glutamate receptor antagonists. *Eur J Pharmacol* **332**:257-262.

Herman BH, Vocci F and Bridge P (1995) The effects of NMDA receptor antagonists and nitric oxide synthase inhibitors on opioid tolerance and withdrawal. Medication development issues for opiate addiction. *Neuropsychopharmacology* **13**:269-293.

Houshyar H, Cooper ZD and Woods JH (2001a) Paradoxical effects of chronic morphine treatment on the temperature and pituitary-adrenal responses to acute restraint stress: a chronic stress paradigm. *J Neuroendocrinol* **13**:862-874.

Houshyar H, Galigniana MD, Pratt WB and Woods JH (2001b) Differential responsivity of the hypothalamic-pituitary-adrenal axis to glucocorticoid negative-feedback and corticotropin releasing hormone in rats undergoing morphine withdrawal: possible mechanisms involved in facilitated and attenuated stress responses. *J Neuroendocrinol* **13**:875-886.

Ignar DM and Kuhn CM (1990) Effects of specific mu and kappa opiate tolerance and abstinence on hypothalamo-pituitary-adrenal axis secretion in the rat. *J Pharmacol Exp Ther* **255**:1287-1295.

Irazusta J, Larrinaga G, Agirregoitia N, Varona A and Casis L (2003) Effects of morphine administration and its withdrawal on rat brain aminopeptidase activities. *Regul Pept* **110**:225-230.

Kest B and Hopkins E (2001) Morphine tolerance after chronic intracerebroventricular injection in male and female mice. *Brain Res* **892**:208-210.

Kogan JH and Aghajanian GK (1995) Long-term glutamate desensitization in locus coeruleus neurons and its role in opiate withdrawal. *Brain Res* **689**:111-121.

Krug M, Brodemann R and Wagner M (2001) Simultaneous activation and opioid modulation of long-term potentiation in the dentate gyrus and the hippocampal CA3 region after stimulation of the perforant pathway in freely moving rats. *Brain Res* **913**:68-77.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK and Nestler EJ (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J Neurosci* **17**:7890-7901.

Limiroli E, Gaspani L, Panerai AE and Sacerdote P (2002) Differential morphine tolerance development in the modulation of macrophage cytokine production in mice. *J Leukoc Biol* **72**:43-48.

Ma W, Zheng WH, Powell K, Jhamandas K and Quirion R (2001) Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. *Eur J Neurosci* **14**:1091-1104.

Mellon RD, Noori NE, Hernandez MC and Bayer BM (2001) Altered T-cell responsiveness in morphine "tolerant" rats: evidence for a potential role of the paraventricular nucleus of the hypothalamus. *Adv Exp Med Biol* **493**:177-185.

Milne RJ, Gamble GD and Holford NH (1989) Behavioural tolerance to morphine analgesia is supraspinally mediated: a quantitative analysis of dose-response relationships. *Brain Res* **491**:316-327.

Mioduszewski R, Zimmermann E and Critchlow V (1982) Effects of morphine dependence, withdrawal and tolerance on prolactin and growth hormone secretion in the rat. *Life Sci* **30**:1343-1348.

Molina VA, Heyser CJ and Spear LP (1994) Chronic variable stress or chronic morphine facilitates immobility in a forced swim test: reversal by naloxone. *Psychopharmacology (Berl)* **114**:433-440.

Mucha RF, Niesink R and Kalant H (1978) Tolerance to morphine analgesia and immobility measured in rats by changes in log-dose-response curves. *Life Sci* **23**:357-364.

Mutasa HC (2001) Risk factors associated with noncompliance with methadone substitution therapy (MST) and relapse among chronic opiate users in an Outer London community. *J Adv Nurs* **35**:97-107.

O'Brian CP, Testa T, O'Brien TJ, Brady JP and Wells B (1977) Conditioned narcotic withdrawal in humans. *Science* **195**:1000-1002.

O'Brien CP, Childers SR, Ehrman RN and Robbins SJ (1998) Conditioning factors in drug abuse: can they explain the compulsion? *J Psychopharmacol* **12**:15-22.

Ortiz J, Fitzgerald LW, Lane S, Terwilliger R and Nestler EJ (1996) Biochemical adaptations in the mesolimbic dopamine system in response to repeated stress. *Neuropsychopharmacology* **14**:443-452.

Ouellet DM and Pollack GM (1995) A pharmacokinetic-pharmacodynamic model of tolerance to morphine analgesia during infusion in rats. *J Pharmacokinet Biopharm* **23**:531-549.

Ozawa T, Nakagawa T, Shige K, Minami M and Satoh M (2001) Changes in the expression of glial glutamate transporters in the rat brain accompanied with morphine dependence and naloxone-precipitated withdrawal. *Brain Res* **905**:254-258.

Parolaro D, Rubino T, Gori E, Massi P, Bendotti C, Patrini G, Marcozzi C and Parenti M (1993) In situ hybridization reveals specific increases in G alpha s and G alpha o mRNA in discrete brain regions of morphine-tolerant rats. *Eur J Pharmacol* **244**:211-222.

Raghavendra V, Rutkowski MD and DeLeo JA (2002) The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J Neurosci* **22**:9980-9989.

Rahim RT, Adler MW, Meissler JJ, Jr., Cowan A, Rogers TJ, Geller EB and Eisenstein TK (2002) Abrupt or precipitated withdrawal from morphine induces immunosuppression. *J Neuroimmunol* **127**:88-95.

Rouveix B (1992) Opiates and immune function. Consequences on infectious diseases with special reference to AIDS. *Therapie* **47**:503-512.

Sarnyai Z, Shaham Y and Heinrichs SC (2001) The role of corticotropin-releasing factor in drug addiction. *Pharmacol Rev* **53**:209-243.

Self DW and Nestler EJ (1998) Relapse to drug-seeking: neural and molecular mechanisms. *Drug Alcohol Depend* **51**:49-60.

Shaham Y and Stewart J (1994) Exposure to mild stress enhances the reinforcing efficacy of intravenous heroin self-administration in rats. *Psychopharmacology (Berl)* **114**:523-527.

Shen J, Benedict Gomes A, Gallagher A, Stafford K and Yoburn BC (2000) Role of cAMP-dependent protein kinase (PKA) in opioid agonist-induced mu-opioid receptor downregulation and tolerance in mice. *Synapse* **38**:322-327.

Shichinohe S, Ozawa H, Hashimoto E, Tatschner T, Riederer P and Saito T (2001) Changes in the cAMP-related signal transduction mechanism in postmortem human brains of heroin addicts. *J Neural Transm* **108**:335-347.

Shors TJ and Servatius RJ (1995) Stress-induced sensitization and facilitated learning require NMDA receptor activation. *Neuroreport* **6**:677-680.

Tanganelli S, Antonelli T, Morari M, Bianchi C and Beani L (1991) Glutamate antagonists prevent morphine withdrawal in mice and guinea pigs. *Neurosci Lett* **122**:270-272.

Tokuyama S, Zhu H, Oh S, Ho IK and Yamamoto T (2001) Further evidence for a role of NMDA receptors in the locus coeruleus in the expression of withdrawal syndrome from opioids. *Neurochem Int* **39**:103-109.

Trujillo KA and Akil H (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* **251**:85-87.

Tso PH, Yung LY and Wong YH (2000) Regulation of adenylyl cyclase, ERK1/2, and CREB by Gz following acute and chronic activation of the delta-opioid receptor. *J Neurochem* **74**:1685-1693.

Vekovischeva OY, Zamanillo D, Echenko O, Seppala T, Uusi-Oukari M, Honkanen A, Seeburg PH, Sprengel R and Korpi ER (2001) Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. *J Neurosci* **21**:4451-4459.

Wang JH and Feng DP (1992) Postsynaptic protein kinase C essential to induction and

maintenance of long-term potentiation in the hippocampal CA1 region. *Proc Natl Acad Sci U S A* **89**:2576-2580.

Watanabe T, Nakagawa T, Yamamoto R, Maeda A, Minami M and Satoh M (2002)

Involvement of glutamate receptors within the central nucleus of the amygdala in

naloxone-precipitated morphine withdrawal-induced conditioned place aversion

in rats. *Jpn J Pharmacol* **88**:399-406.

Weiss F, Ciccocioppo R, Parsons LH, Katner S, Liu X, Zorrilla EP, Valdez GR, Ben-

Shahar O, Angeletti S and Richter RR (2001) Compulsive drug-seeking behavior

and relapse. Neuroadaptation, stress, and conditioning factors. *Ann N Y Acad Sci* **937**:1-26.

West JP, Lysle DT and Dykstra LA (1997) Tolerance development to morphine-induced

alterations of immune status. *Drug Alcohol Depend* **46**:147-157.

Figure Legends

Fig. 1 NMDA receptor antagonism alters the rate of development of tolerance to the immunologic, but not steroidal effect of chronic morphine treatment. Animals were chronically treated with morphine (□) or morphine and MK-801 (▼) as described in Methods. Animals were sacrificed and trunk blood collected 2 hrs after a morphine challenge (10mg/kg, s.c.) on treatment Days 2, 4, 6, and 10. (a) Peripheral blood lymphocytes were utilized in an *in vitro* proliferation assay. Lymphocyte proliferation data was expressed as % of mean maximum proliferation $\pm$ S.E.M of the saline-treated control group for that test day (49,163 $\pm$ 3,469cpm, n=8/group). (a) Plasma corticosterone levels were measured and data expressed as % of mean corticosterone level $\pm$ S.E.M of the saline-treated control group (68.5 $\pm$ 22.8ng/ml, n=8/group). *$p \leq 0.05$ versus chronic morphine group of respective test day as determined by one-way ANOVA, Newman-Keuls post-test.

Fig. 2 Mitogen-induced lymphocyte proliferative response to acute morphine is not altered by acute MK-801. Animals were subcutaneously treated with saline (Ctrl) or acute morphine (10mg/kg, s.c.) (aMS) for 2 hrs. Half the animals from each group concurrently received an acute dose of MK-801 (0.1mg/kg, s.c.) at the time of acute morphine administration (+MK-801). Trunk blood was collected for lymphocyte proliferation analysis. Data were expressed as % of the mean maximum proliferation $\pm$ S.E.M. of the saline-treated control group (32,582 $\pm$ 5,608cpm). *$p \leq 0.05$ versus saline-treated control as determined by one-way ANOVA, Newman-Keuls post-test.

Fig. 3 Continual MK-801 treatment does not affect immunosuppression by morphine challenge. Chronic treatment with saline (Ctrl), morphine (cMS), MK-801 (cMK) or morphine and MK-801 (cMS+cMK) was carried out for 10 days as previously described. On test day,

groups on right side of graph (+ Morphine) were administered morphine (10mg/kg, s.c.) and

sacrificed 2 hrs later. Trunk blood was collected for a lymphocyte proliferation assay. Data were

expressed as % of mean maximum proliferation $\pm$ S.E.M. of the saline-treated control group

(Ctrl) (33.479 $\pm$ 3258cpm, n=6-8/group). *p$\leq$0.05 versus saline-treated control as determined by

one-way ANOVA, Newman-Keuls post-test.

Fig. 4 Weight loss precipitated by morphine withdrawal is not altered by concurrent

treatment with MK-801 during chronic morphine. Animals were chronically treated for 10

days with saline ($\bullet$), morphine ($\square$), or morphine and MK-801 ($\blacktriangledown$) as previously described.

Withdrawal was induced by replacing all drug injections with sterile saline. Body weight was

measured every 12 hours. Data were expressed as a mean of the percent body weight change

compared to starting body weight $\pm$ S.E.M. *p$\leq$0.05 versus saline-treated control group as

determined by one-way ANOVA, Newman-Keuls post-test.

Fig. 5 NMDA receptor antagonism during chronic treatment accelerated the rate of

recovery of immunosuppression, but not steroid elevation, induced by morphine

withdrawal. Animals were chronically treated with morphine (cMS), or morphine and MK-801

(cMS+cMK) as previously described. Withdrawal was induced by replacing all drug injections

with sterile saline. (a) Trunk blood was collected for analysis of blood lymphocyte proliferation

after 0, 24 and 72 hrs of withdrawal. Data were expressed as a percentage of the mean maximum

proliferative response of the saline-treated control group (37,671 $\pm$ 5,486 cpm; n=7/group). (b)

Plasma corticosterone levels were measured after chronic drug treatment and after 24 and 72

hours of withdrawal. Data were expressed as % of mean corticosterone level $\pm$ S.E.M of the

saline-treated control group (57.9 $\pm$ 10.2 ng/ml, n=8/group). *p$\leq$0.05 versus non-withdrawing

group of similar drug treatment; [a]p<0.05 versus cMS group at 72 hrs as determined by one-way

ANOVA, Newman-Keuls post-test.

Fig 6 Chronic morphine exposure results in enhanced sensitivity to stress that is prevented by MK-801. Animals were chronically treated with saline (Ctrl), morphine (cMS), or morphine and MK-801 (cMS+cMK) as previously described. On test day, half of the animals from each group were placed in 1 inch of room temperature water for 1 hr. before sacrifice and trunk blood was collected. (a) Blood lymphocytes were assayed and proliferation expressed as % of the mean maximum proliferative response$\pm$ S.E.M of the saline-treated, unstressed group ($37,671 \pm 5,486$ cpm; n=7/group). *$p \leq 0.05$ versus non-stressed group of similar drug-treatment as determined by one-way ANOVA. (b) Plasma corticosterone levels were determined and expressed as % mean corticosterone $\pm$ S.E.M. (180.8 ± 20.5 ng/ml, n=6/group) of the saline-treated unstressed group.
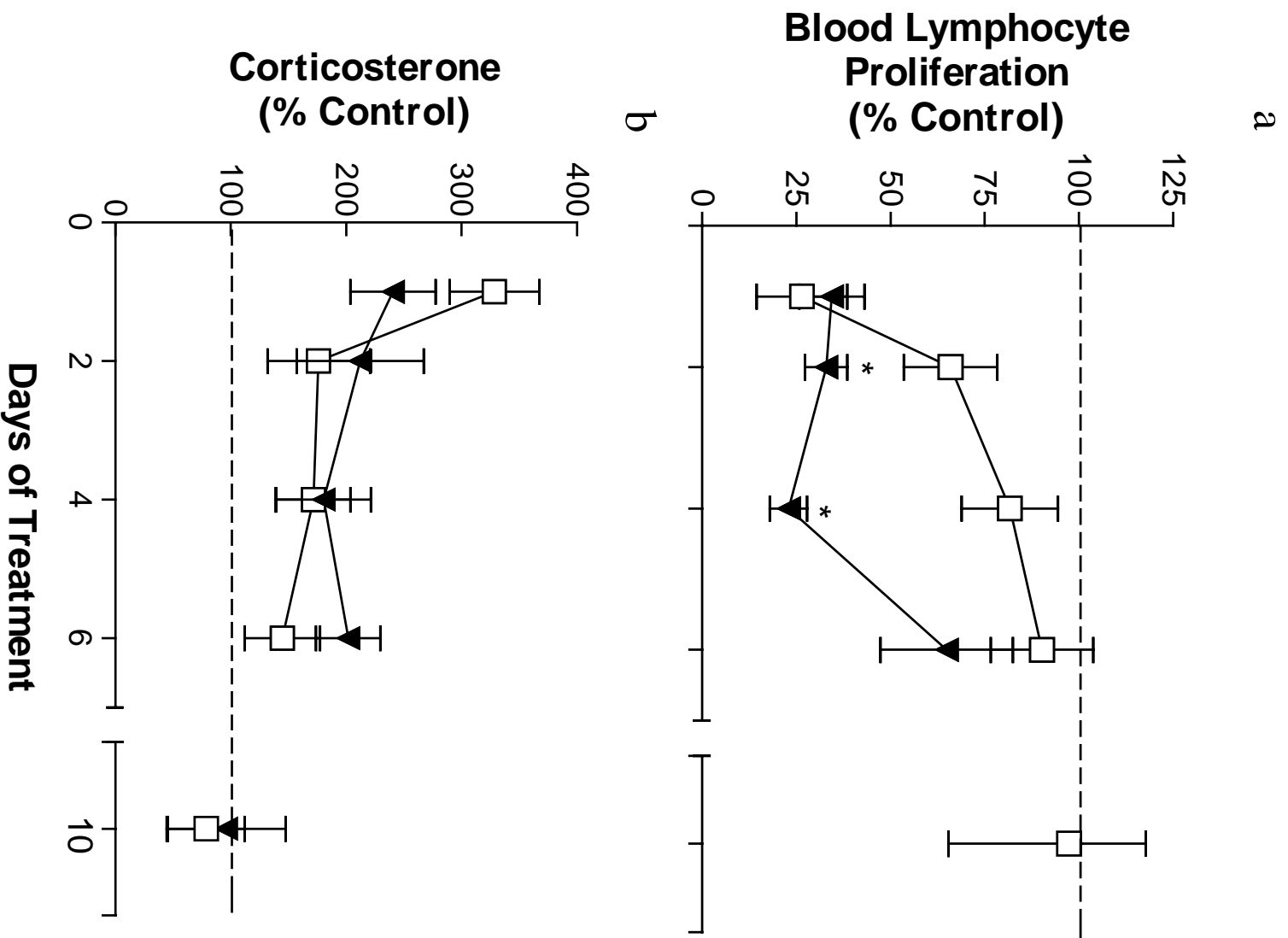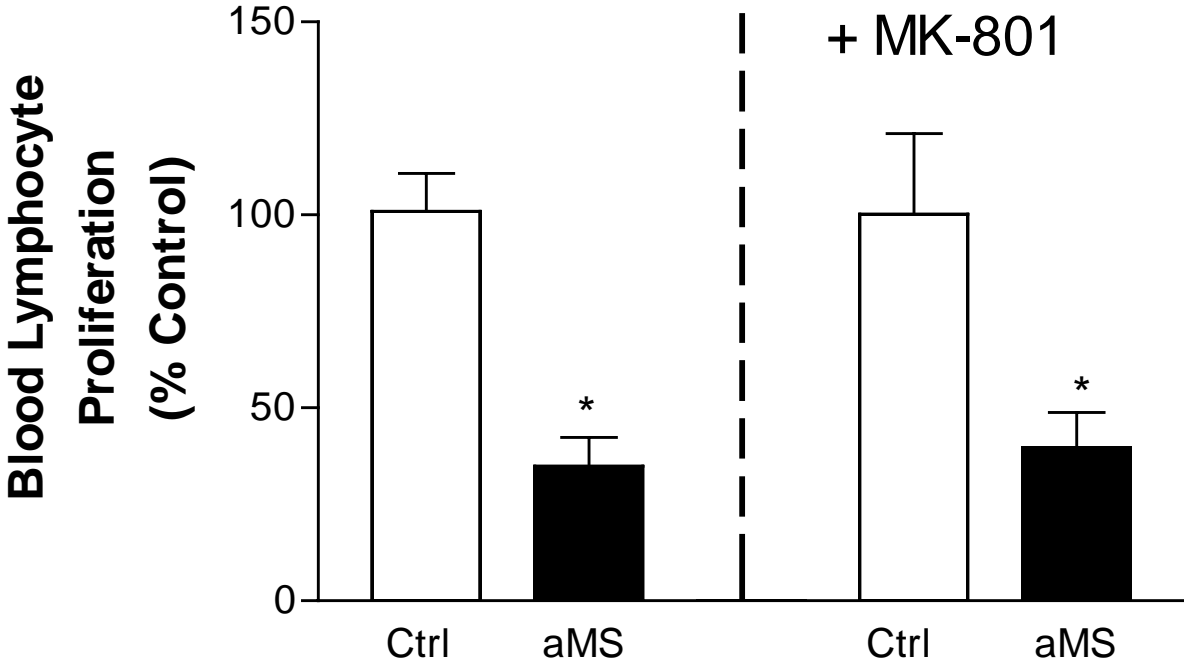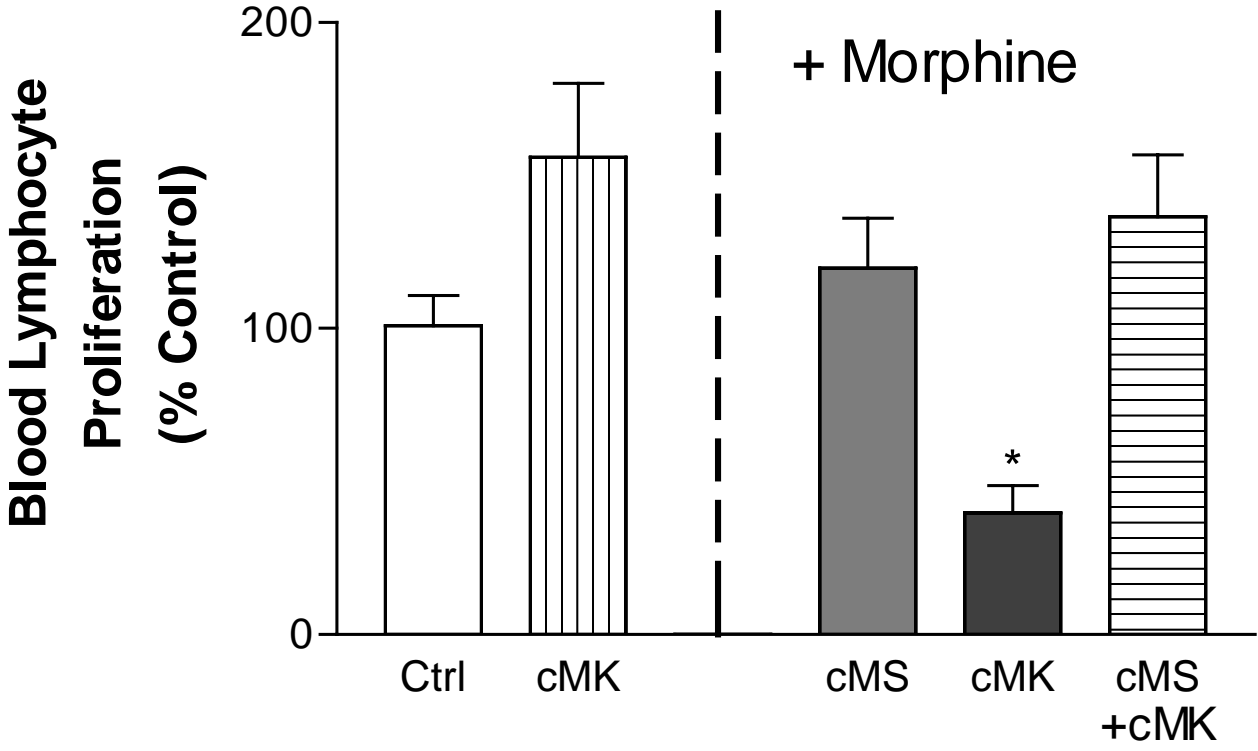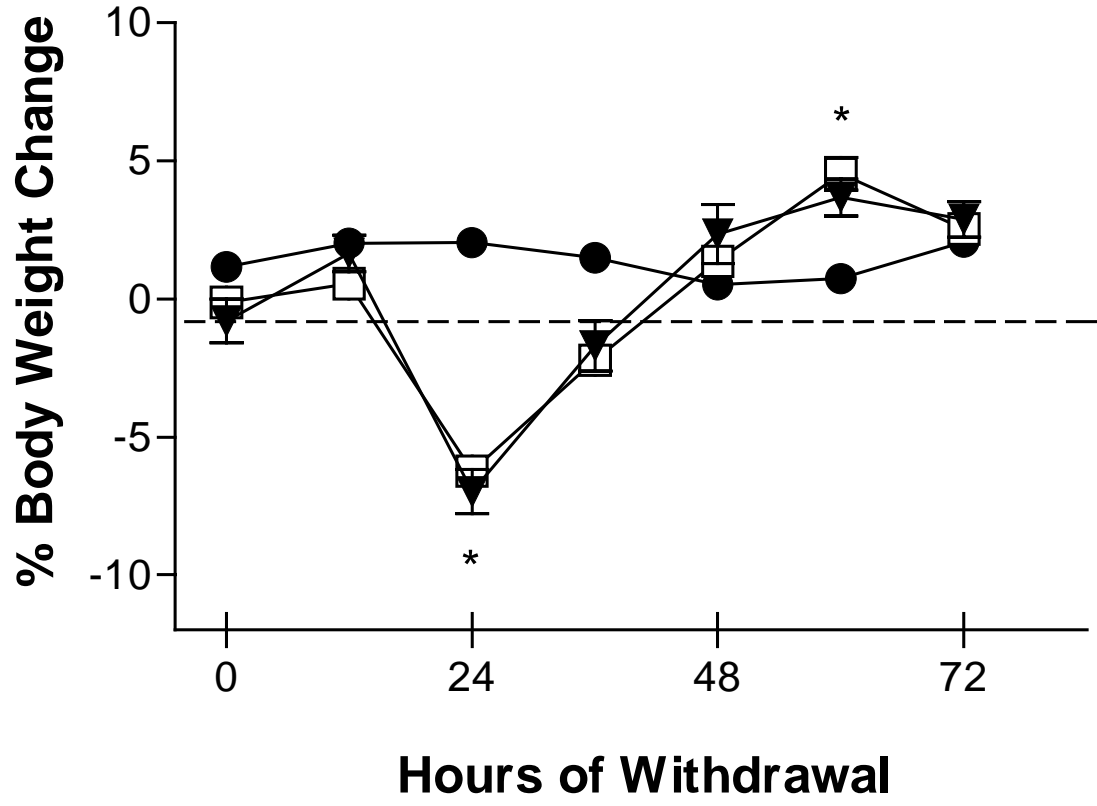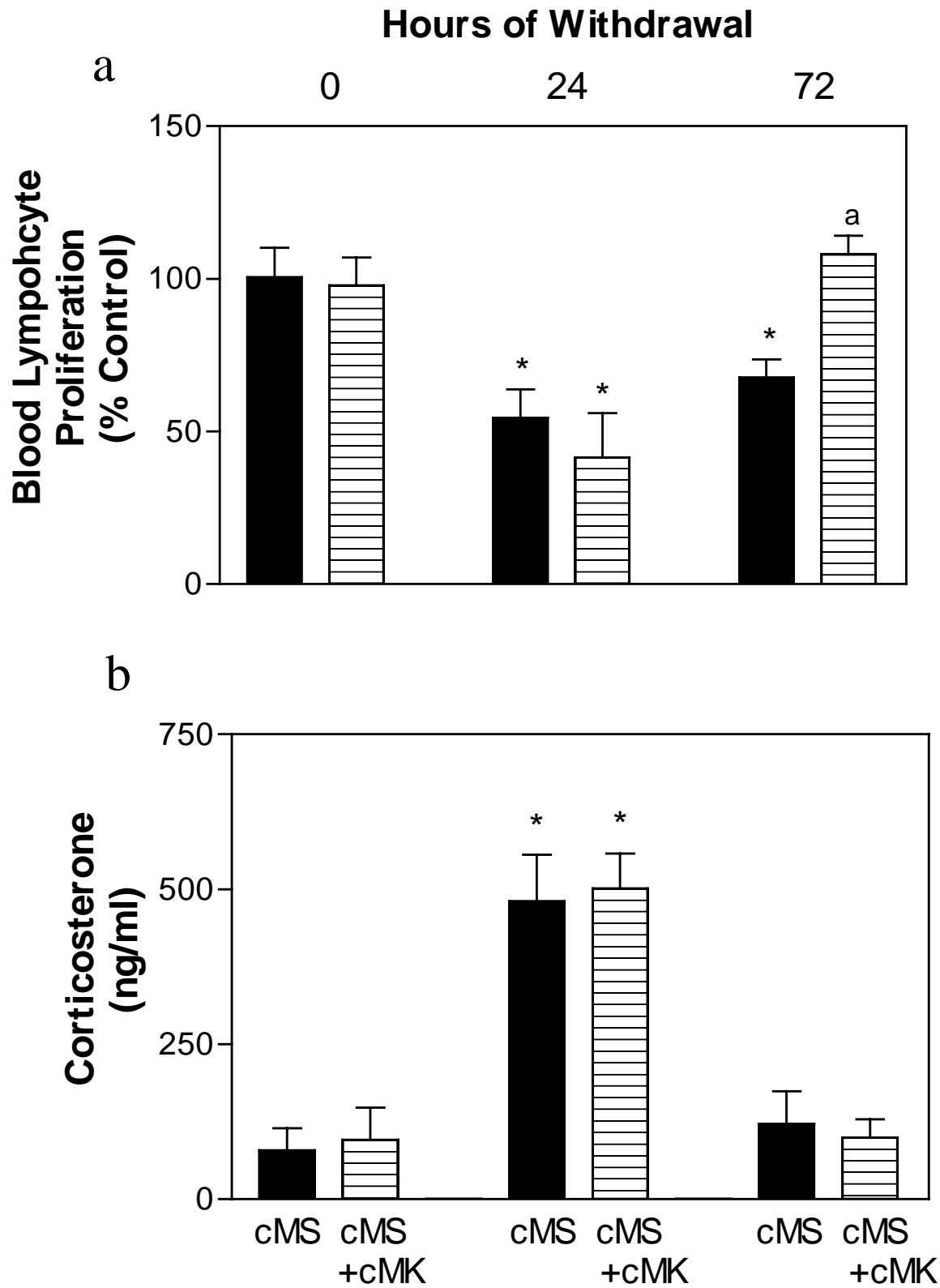
Figure 1

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