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Morphine and Naloxone, IP or Locally, Affect Extracellular Acetylcholine in the Accumbens and Prefrontal Cortex

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RADA, P. V., G. P. MARK, K. A. M. TAYLOR AND B. G. HOEBEL. *Morphine and naloxone, IP or locally, affect extracellular acetylcholine in the accumbens and prefrontal cortex.* PHARMACOL BIOCHEM BEHAV 53(4) 809–816, 1996.—In rats with microdialysis probes in the nucleus accumbens (NAc) or prefrontal cortex (PFC), intraperitoneally (IP) delivered morphine on the 8th day of escalating doses decreased extracellular ACh in the NAc. On day 9, naloxone (5 mg/kg) precipitated withdrawal and increased the release of ACh. When morphine and methylnaloxonium were given locally into the NAc by reverse dialysis, the opiate again decreased extracellular ACh, and the opiate antagonist increased it. These effects were proportional to the dose of local infusions. Local morphine had the same ACh-lowering effect in morphine-dependent and nondependent rats, whereas local methylnaloxonium increased extracellular ACh significantly more in morphine-dependent animals. Systemic and local effects on ACh systems in the PFC were more complicated and showed some relation to locomotor activity. The results suggest that intrinsic ACh neurons in the NAc have a special relationship to opiate reinforcement such that extracellular ACh is low in response to morphine and high during withdrawal. Thus, low ACh may correlate with opiate reward, and high ACh with aversion.

Microdialysis Acetylcholine Morphine Opiates Methylnaloxonium Addiction Withdrawal Nucleus accumbens Prefrontal cortex Rats

THE NUCLEUS ACCUMBENS (NAc) receives input from limbic structures such as the hippocampus, amygdala, septal nuclei, and prefrontal cortex and projects mainly to the ventral globus pallidus and substantia nigra (9,30,52). It has been suggested that the NAc serves, in part, as an integrative area between sensory and motor systems (31) for motivated behaviors, such as obtaining positive reinforcers and getting away from negative reinforcers (21,22,27,49). The importance of dopamine (DA) in drug abuse is demonstrated by 6-OHDA lesions that are sufficient to block psychostimulant self-administration; however, morphine can reinforce behavior even in DA-depleted rats by acting directly in the NAc (34). Microdialysis studies show that most drugs of abuse induce a significant increase in extracellular DA in the NAc (16,19,35), and many such drugs including amphetamine, phencyclidine, cocaine, nicotine, and morphine act directly on the NAc to increase extracellular DA (21,35). The mechanism of opiate reinforcement in the NAc may also involve acetylcholine (ACh). Earlier studies have shown that morphine increases stores of ACh and decreases its release in various brain regions (3,6,12,17,24,28,32,33,40,50). Our in vivo microdialysis research revealed that intraperitoneal (IP) morphine not only can raise extracellular DA in the NAc but can also decrease extracellular ACh (36).

The decrease in accumbens ACh disappeared after repeated administration of equal daily doses of morphine. This raised the first question addressed here: Can extracellular ACh be kept at a low level during daily IP morphine injections if, instead of equal daily doses, the dose is progressively increased? This paradigm would mimic the escalating doses that drug addicts use to maintain subjective reward properties. Such a result might imply that addicts escalate their dose in part to keep ACh low in the NAc.

The NAc has been implicated as a neural substrate involved

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in drug withdrawal. In morphine-dependent rats, locally injected methylnaloxonium showed signs of being aversive as expressed in a conditioned place aversion paradigm (42). When a high dose of naloxone was injected systemically in morphine-dependent animals, ACh increased to abnormally high extracellular levels (37). This raised a second set of questions: Do morphine and naloxone alter cholinergic function by acting on receptors located in the NAc itself? If so, does the response of cholinergic neurons to local morphine change in rats that have experienced chronic, escalating IP morphine?

For comparison, the prefrontal cortex (PFC) was selected as a DA-rich brain area that receives a totally different ACh input from the NAc. In Experiment 1, microdialysis was used to monitor ACh in the NAc and PFC of animals receiving escalating doses of systemic morphine followed by a moderate dose of naloxone. At the same time, measures were taken of locomotor activity and withdrawal symptoms. In Experiment 2, morphine and naloxone were infused directly into the NAc and PFC by reverse dialysis while extracellular levels of ACh were simultaneously monitored.

METHODS

Surgery and Microdialysis Procedure

Adult male Sprague-Dawley rats bred and raised at Princeton University and weighing 350–450 g were housed individually on a 12 L : 12 D reversed schedule (lights off 0900–2100 h) with Purina chow pellets (BioServ, Frenchtown, NJ) and water available ad lib. For surgery, subjects were anesthetized with pentobarbital (20 mg/kg, IP) supplemented by ketamine (40 mg/kg, IP). Bilateral 21-ga stainless-steel guide shafts aimed at the posterior medial NAc or the medial PFC were stereotaxically implanted according to the atlas of Paxinos and Watson as follows: NAc: A 10.0 mm, L 1.2 mm, V 4.0 mm; PFC: A 11.7 mm, L 0.5 mm, V 1.0 mm, with reference to the interaural line, midsaggital sinus, and surface of the level skull, respectively. Guide shafts were kept patent with 26-ga stylets.

Microdialysis probes extended 5 mm beyond the guide shafts and were inserted into either the left or right NAc or PFC. Probes were constructed of silica glass tubing (37 μM i.d.; Polymicro Technologies Inc., Phoenix, AZ) inside a 26-ga stainless-steel tube with a microdialysis tip of cellulose tubing (Spectrum Medical Co., Los Angeles, CA) sealed at the end with epoxy cement (6000 mol. wt. cutoff, 0.2 mm o.d. $\times$ 2 mm long for the NAc and 3 mm long for PFC). Descriptions of this probe design have been published elsewhere (20). Probes were perfused with buffered Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.3) at a flow rate of 1.0 μl/min. Neostigmine (0.3 μM; Sigma Chemical Co., St. Louis, MO) was added to the perfusion fluid to improve basal recovery of ACh by hindering its enzymatic degradation while allowing the extracellular level to vary up or down during the drug manipulations. The outlet branch of the probe extended along a flexible cable to a 400-μl vial clipped 25 cm above the head of the rat. Samples were collected every 20 min during the experimental day. Probes were inserted and fixed in place at least 24 h before each experiment to allow neurotransmitter recovery to stabilize.

Acetylcholine Assay

We measured ACh by reverse-phase high-performance liquid chromatography with electrochemical detection (HPLC-EC) using a single piston pump and pulse dampener (Model 222D; SSI Co., State College, PA), a 50-μl sample loop, and an amperometric detector (EG&G Princeton Applied Res. Corp., Princeton, NJ). The mobile phase contained 200 mM potassium phosphate at pH 8.0. ACh and choline were separated on an 8-cm C18 analytical column (Chrompack, Raritan, NJ) and then converted sequentially to betaine and hydrogen peroxide by an immobilized enzyme reactor (Chrompack; acetylcholinesterase and choline oxidase from Sigma Chemical Co.). The resultant hydrogen peroxide was oxidized on a platinum electrode (BAS, Inc., Lafayette, IN) set at 500 mV with respect to an Ag-AgCl reference electrode (EG&G Princeton Applied Res. Corp.). Preliminary tests used to confirm the identity of the putative ACh peak were described previously (29). The detection limit was 20 fmol of ACh/20-μl standard sample.

Probe Recovery Properties

Neurotransmitter recovery was tested in vitro by immersing probes (n = 4) in a beaker containing 1 μM ACh. The percent recovery (mean $\pm$ SEM) of ACh was 19.3 $\pm$ 0.43%.

Effective use of the reverse dialysis technique requires that any compound infused into the brain from the probe not interfere with the recovery of the transmitter being measured. To ensure that ACh recovery was not altered by this method, probe efficiency was tested in vitro during infusion of morphine sulphate and methylnaloxonium (n = 3). Neither of these infusions affected the in vitro recovery of ACh.

Experimental Design

Experiment 1: test of ip morphine and naloxone on ACh in a) NAc and b) PFC, with behavioral measures of locomotion and withdrawal.

General methods. Experiment 1 used two groups of rats: one with NAc probes (Experiment 1A) and the other with PFC probes (Experiment 1B). Locomotor activity and withdrawal symptoms were measured in both groups. All rats received repeated IP injections of morphine (NIH) in saline every 12 h (0800–2000 h) for 8 days starting with 10 mg/kg (i.e., 20 mg/kg per day) on day 1. This dose was increased by 20 mg/kg per day every other day until day 8, when rats were receiving 40 mg/kg (i.e., 80 mg/kg per day). On day 9, withdrawal was precipitated in a subgroup of rats with naloxone (5 mg/kg, IP), and the control group received an equal volume of saline (1 ml/kg, IP). This dose of naloxone was one quarter that used in the original study (37).

Experiment 1a: ACh in the NAc (dependent rats). Group 1A was tested with microdialysis in the NAc. ACh was not measured on day 1 during the first morphine injection, because the discovery of a decrease in ACh in drug-naive rats had been reported previously (36,37). Therefore, probes were inserted in the NAc on day 7, and ACh was measured during the last IP morphine injection (day 8; n = 16) and the following day during saline (n = 8) or naloxone-precipitated withdrawal (day 9; n = 7; one lost its cannulas).

Experiment 1b: ACh in the PFC (naive and dependent rats). For the PFC, group 1B (n = 10) had a microdialysis probe inserted into one side 24 h before the first morphine injection, and changes in ACh were monitored during the injection on day 1. After a week of escalating morphine injections, a microdialysis probe was inserted into the PFC on the opposite side, and 24 h later, ACh measurements were taken during the last morphine injection (day 8) and naloxone-precipitated withdrawal (day 9). Right and left brain sites were counterbalanced in both NAc and PFC rats.

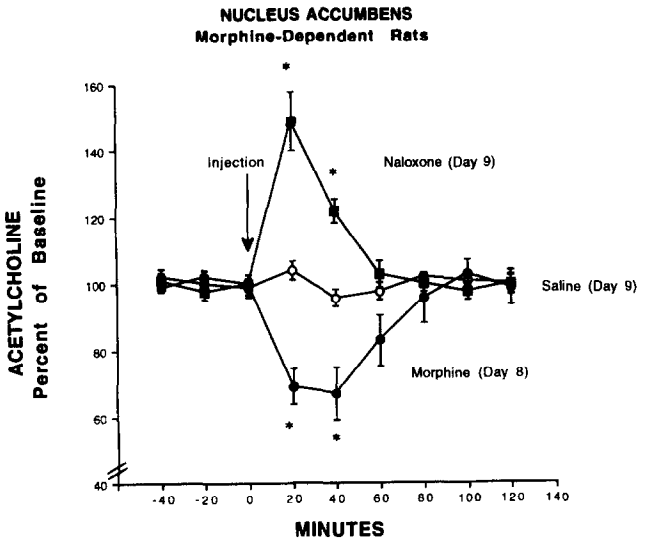

FIG. 1. Experiment 1A. Extracellular ACh in the NAc decreased significantly following morphine administration (40 mg/kg, IP) on the 8th day of escalating injections (lower curve, ●, $n = 15$). Saline injected IP on day 9 had no effect in rats that had received daily escalating morphine (○, $n = 8$), but naloxone on day 9 significantly increased ACh (5 mg/kg; upper curve, ■, $n = 7$, $*p < 0.05$).

Behavioral measurements. Motor activity was recorded during the first and last IP morphine session and during naloxone-precipitated withdrawal. The microdialysis cage was equipped with two photocell beams that divided the dialysis cage into thirds. Withdrawal symptoms were measured by two independent observers for 20 min before and 20 min after an IP injection of naloxone or saline. The observers were blind to the treatment that each rat had received. The following symptoms were scored: piloerection and diarrhea (0 = none, 1 = mild, 2 = moderate, 3 = severe), rearing, wet-dog shakes, teeth chattering, paw shakes, grooming, scratching, pronation, and writhing.

Experiment 2: Test of local infusions of morphine and methylnaloxonium on ACh in a) NAc and b) PFC.

Experiment 2a: ACh in NAC with local infusions (naive and dependent rats. ACh was monitored by microdialysis in drug-naive rats, while we infused doses of morphine sulphate (0, 1, 10, 100, and 500 μM) or methylnaloxonium (0, 10, 100, and 500 μM) into the NAc using reverse dialysis. A second group of animals was made morphine-dependent by escalating doses of IP morphine as previously described. On day 7, microdialysis probes were inserted, and on day 8 the same dose-response analysis was performed with morphine infused locally into the NAc while ACh levels were monitored. A third group of rats was made morphine-dependent in the same way. Microdialysis probes were inserted on day 8, and on day 9 the doses of methylnaloxonium were locally administered into the NAc while ACh levels were monitored.

Experiment 2b: ACh in PFC with local infusions (naive and dependent rats). The same doses of morphine and methylnaloxonium used in the NAc were locally infused in the PFC while ACh was measured. Three parallel groups were tested: naive with local morphine and methylnaloxonium, IP-dependent with local morphine, and IP-dependent with local methylnaloxonium.

Statistical Analysis and Histology

Basal recovery of ACh varied considerably between subjects. For this reason, peak heights for ACh were converted to a percent of the mean of three consecutive baseline samples ± SEM. Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures between drug infusion vs. saline (condition vs. time). Two-tailed Student's *t*-test was used to evaluate the significance of withdrawal symptoms. Linear correlations were used to test dose dependency. Histology using unstained, frozen, 40-μm brain sections was performed to verify probe placement in the NAc and PFC.

RESULTS

Absolute Amounts of ACh in the NAc and PFC

The basal amount of ACh (mean ± SEM, not corrected for probe recovery) in the NAc of nondependent rats was 0.55 ± 0.13 pmol (range 0.07–1.72) compared with 0.47 ± 0.07 pmol (range 0.08–1.31) in rats that were dependent. In the PFC, the basal level of ACh was 0.66 ± 0.08 pmol (range 0.075–1.41) in nondependent animals and 0.59 ± 0.05 pmol (range from 0.09–1.28) when they were morphine-dependent. These basal levels were not significantly different.

Experiment 1A: Opposite Effects of Systemic Morphine and Naloxone-Precipitated Withdrawal on Extracellular ACh in the Nac

Extracellular basal levels in the NAc were 67 ± 8% of baseline in the samples at 20 and 40 min after the last injection of morphine on day 8 (40 mg/kg) [$F(1, 8) = 8.5$, $p < 0.01$]. ACh levels recovered to the initial baseline concentration 80 min after the injection (Fig. 1).

Intraperitoneal naloxone (5 mg/kg) the next day (day 9) in morphine-dependent rats induced a significant increase in ACh levels to 150 ± 9% of baseline in the first 20 min after the injection. ACh levels recovered 60 min later [$F(1, 8) = 13.817$, $p < 0.01$) (Fig. 1).

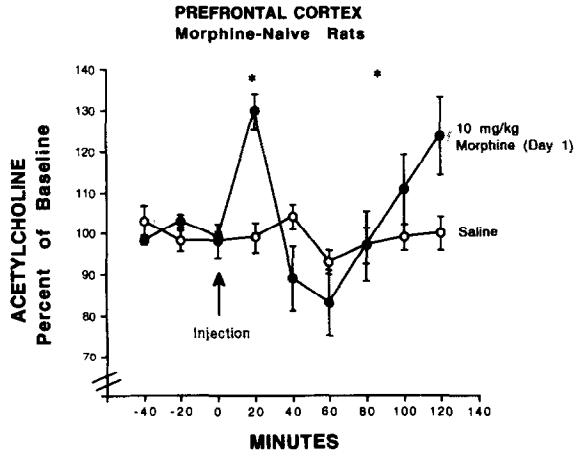

FIG. 2. Experiment 1B. Effects of the first systemic morphine injection (10 mg/kg, IP, day 1) on extracellular ACh in the prefrontal cortex of morphine-naive rats (●, $n = 10$).

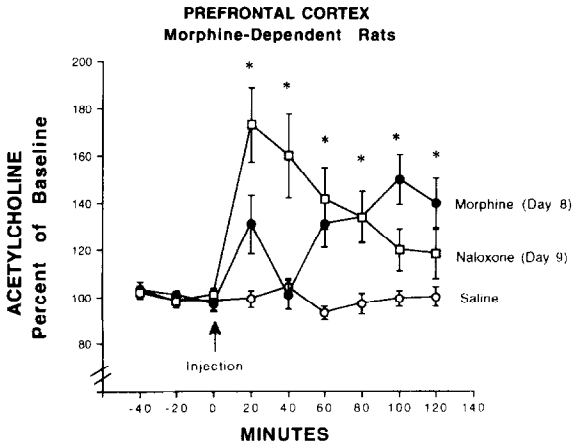

FIG. 3. Experiment 1B. Effects of systemic morphine injection on the 8th-day (40 mg/kg, IP, ●, $n = 8$), and 9th-day systemic naloxone (5 mg/kg, IP, □, $n = 7$) or saline (○, $n = 6$) on extracellular ACh in the PFC of morphine-dependent rats. (*$p < 0.05$).

Experiment 1B: Mixed Effects of Morphine Injections and Naloxone-Precipitated Withdrawal on Extracellular ACh in the PFC

Unlike the NAc, the PFC (Fig. 2) showed a triphasic response in extracellular ACh following an acute morphine injection on day 1 (10 mg/kg). There was an initial significant increase to 130 ± 4% immediately after the injection. Then, levels dropped to 83 ± 8% during the next 40 min, and finally, ACh levels rebounded to 131 ± 10% after 100 min [$F(1, 8) = 3.738, p < 0.01$].

A cyclic ACh pattern was also produced after the last injection in the escalating dose schedule of morphine on day 8 (40 mg/kg). ACh increased significantly to 131 ± 12% immediately after morphine administration, then decreased, but never dropped below baseline levels. A rebound to 150 ± 11% occurred sooner, lasted longer, and reached higher levels than on day 1 [$F(1, 8) = 5.64, p < 0.01$) (Fig. 3).

Naloxone administered on day 9 to these morphine-dependent rats induced a significant ACh increase in the PFC, to 173 ± 11% [$F(1, 8) = 7.857, p < 0.05$) (Fig. 3). Levels recovered to basal starting levels 100 min after the injection.

Behavioral Effects: Motor Activity and Withdrawal Symptoms

The first dose of morphine (day 1) produced a pronounced decrease in locomotion, to 10 ± 6% in photobeam crossings 40 min after the injection, followed by a rebound increase to 230% of initial levels [$F(1, 8) = 7.879, p < 0.01$) (Fig. 4).

The last morphine injection (day 8) induced an immediate, significant increase to 469 ± 132% of photobeam crossings [$F(1, 8) = 6.042, p < 0.01$] instead of a suppression of motor activity as seen on day 1. Naloxone during day 9 did not significantly modify motor activity (Fig. 4).

Figure 5 shows the withdrawal symptoms. After systemic injection of naloxone, significant differences appeared in the following symptoms: diarrhea and piloerection, wet-dog shakes, teeth chattering, pronation, and writhing.

Experiment 2A: Opposite Effects of Local Morphine and Methylnaloxonium Infusion on Extracellular ACh in the NAc of Morphine-Naive and Dependent Rats

Local morphine infusion in the NAc mimicked the effect reported earlier with a systemic injection. In a group of rats that had not yet received IP morphine, the addition of 1, 10, and 100 μM morphine sulphate to the microdialysis perfusate dose-dependently decreased extracellular levels of ACh ($y = -.383x + 76.88, R^2 = .298; F = 9.77; p < 0.005$) to 70 ± 9%, 53 ± 8%, and 41 ± 6% of basal levels, respectively [$F(3, 7) = 7.122, p < 0.01$) (Fig. 6, top).

Local methylnaloxonium (10, 100, and 500 μM) in the NAc of morphine-naive rats had the opposite effect by dose-dependently increasing ACh ($y = .148x + 129.3; R^2 = .433; F = 17.6; p < 0.01$) to 143 ± 5%, 175 ± 14%, and 193 ± 13% of basal levels, respectively [$F(3, 7) = 8.45, p < 0.01$) (Fig. 6, bottom).

As shown in Fig. 7, morphine infusion (1 μM) into the NAc of dependent rats on the 8th day after 7 days of escalating IP morphine treatment significantly decreased ACh levels to 67 ± 2.2% of their baseline. Higher doses (10 and 100 μM) decreased ACh to 47 ± 4% of basal levels [$F(3, 7) = 10.1, p < 0.01$) (Fig. 7, top).

Local infusion of 10, 100, and 500 μM methylnaloxonium in morphine-dependent animals produced a dose-dependent increase in extracellular ACh concentrations in the NAc ($y = .406x + 144.362; R^2 = .77; F = 73.68; p < 0.01$) (Fig. 7, lower). ACh levels rose to 170 ± 11% (10 μM), 208 ± 16% (100 μM), and 342 ± 27% (500 μM), respectively. This increase was significantly greater than that observed in nondependent rats [$F(1, 7) = 5.69, p < 0.01$].

Experiment 2B: Effects of Local Morphine and Methylnaloxonium on ACh in the PFC When Given Locally

Local infusion of 10 and 100 μM morphine did not modify extracellular levels of ACh in the PFC; however, 500 μM significantly increased ACh to 123 ± 3% during the infusion and to 175 ± 16% in the next sample [$F(3, 6) = 7.60, p < 0.01$) (Fig. 8, top). This was different from the effect of morphine in the NAc (Fig. 7, top).

Infusion of 10 and 100 μM methylnaloxonium also had no effect on ACh levels, but 500 μM significantly increased ACh to 426 ± 42% of basal levels [$F(3, 6) = 29.6, p < 0.01$) (Fig. 8, bottom).

In morphine-dependent rats, infusions of 10, 100, and 500 μM morphine into the PFC had no effect (data not shown).

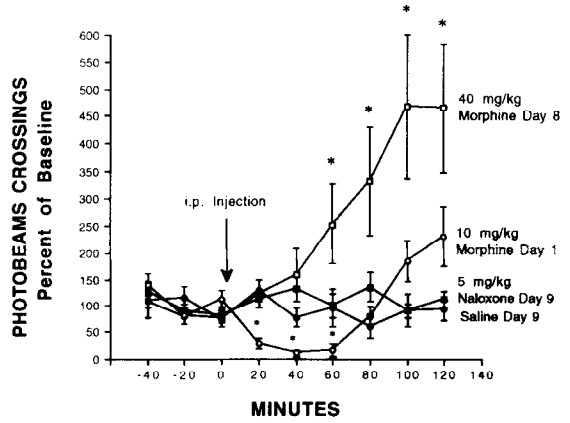

FIG. 4. Photobeams crossings (normalized to percentage of baseline) following morphine on day 1 (10 mg/kg, IP, ○, $n = 10$) and chronic morphine on day 8 (40 mg/kg, IP, □, $n = 10$), saline on day 9 (■, $n = 10$), and naloxone on day 9 in morphine-dependent rats (5 mg/kg, IP, ■, $n = 10$, *$p < 0.05$).

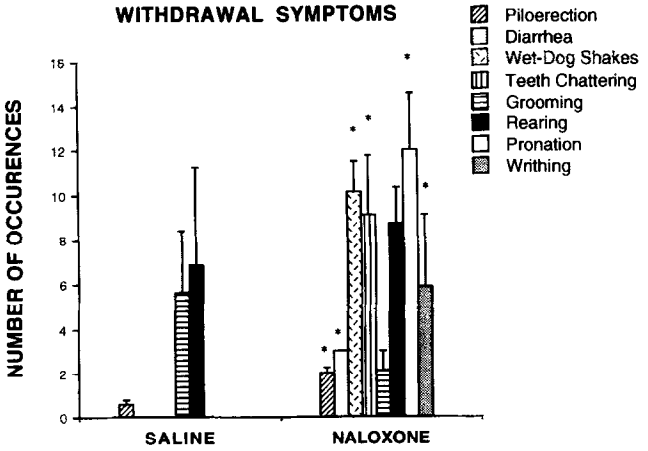

FIG. 5. Ocurrence of withdrawal symptoms following saline (1 ml/kg, $n = 7$) or naloxone (5 mg/kg, $n = 7$) on day 9 in morphine-dependent rats (*$p < 0.05$, one-tailed Student's t-test).

Only a local infusion of 500 μM methylnaloxonium significantly increased ACh to 284 $\pm$ 29% of basal levels [$F(3, 7) = 9.819$, $p < 0.01$) (data not shown). This increase was significantly lower than that observed in nondependent rats [$F(1, 7) = 10.64$, $p < 0.01$].

DISCUSSION

The main result of this study was a decrease in extracellular ACh in the NAc of morphine-dependent rats given systemic or local morphine. An earlier in vivo microdialysis study from this laboratory showed that the effect of morphine on accumbens ACh disappeared after 1 week (37), but in the present study, which used an escalating dose paradigm, morphine retained its capacity to lower extracellular ACh significantly in the NAc. Naloxone and methylnaloxonium had the opposite effect of morphine, causing an increase in ACh in the Nac.

Opiate modulation of ACh release has been described previously, although the exact nature of this modulation is debated and varies among structures. Most studies until now have focused on changes in ACh turnover or release from the striatum, parietal cortex, and occipital cortex. In an early study, Sharkawi (40) observed a significant decrease in ACh release from cortical slices following treatment with morphine. Similar results were obtained in parietal and occipital cortices by other investigators (3,6,12,17,24,28,32,33,50). Conflicting results have been obtained in the striatum. Several authors (11,12,50) found that μ-agonists did not affect striatal ACh turnover; however, others have reported a decrease in ACh release in striatal slices (3,17,18,28) and microdialysates (43).

Few studies have considered the effect of opiates on ACh release or turnover in the NAc. Costa and co-workers (12) demonstrated a decrease in ACh turnover following morphine in brain homogenates from NAc and parietal cortex, and not from striatum. The NAc result was later confirmed using in vivo microdialysis by Rada et al. (36,37). In a recent publication, Hejna et al. (18) reported a decrease in K^+-induced ACh release from NAc slices of naive rats. Thus, three laboratories with different techniques have found an opiate-induced decrease in ACh turnover in the NAc. Systemic naloxone had the opposite effect of morphine by increasing ACh, showing that blockage of opiate receptors somewhere in the brain disinhibits ACh interneurons in the NAc (36,37).

The present report demonstrates that the decrease in accumbens ACh following systemic morphine is, in part, locally mediated. Local infusion of morphine into the NAc dose-dependently decreased ACh in both naive (Fig. 6) and morphine-dependent rats (Fig. 7). Heijna (18) suggested that the decrease in ACh release from NAc slices is mediated specifically by μ and δ receptors thought to be on cholinergic terminals. Methylnaloxonium dose-dependently increased extracellular ACh in the NAc of morphine-naive rats. This suggests that accumbens cholinergic neurons are normally under tonic inhibition by an opiate system.

Opiate effects on ACh release or turnover in the PFC have not been reported previously. Wood and co-workers used frontal cortex homogenates but did not find changes in ACh turnover (50). A study by Smith et al. (41) found that muscarinic binding was decreased in frontal cortex during morphine self-administration, suggesting that a persistent increase in ACh release might have downregulated muscarinic receptors.

Our results show a PFC effect very different from what

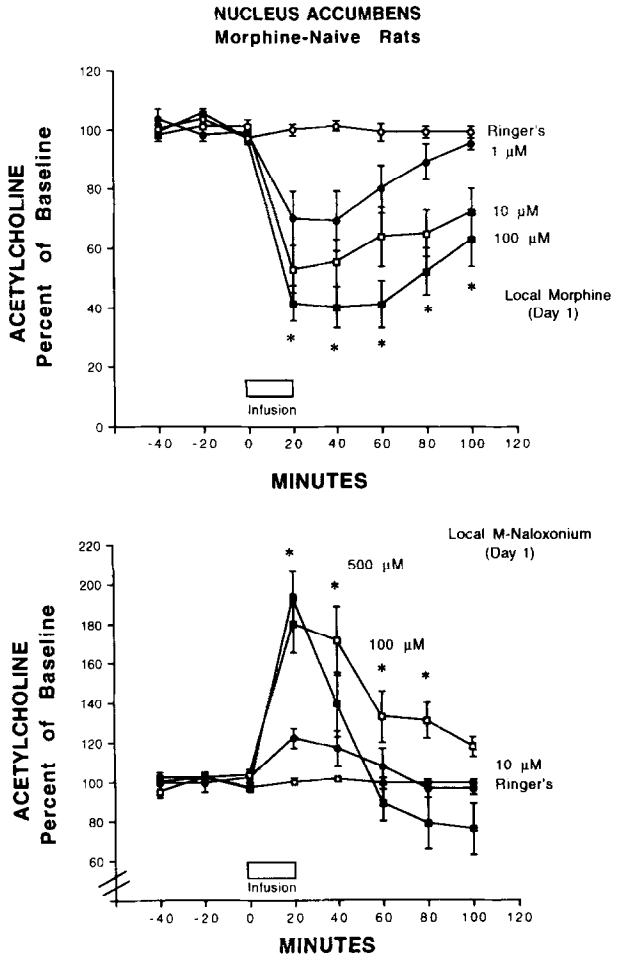

FIG. 6. Local morphine decreases ACh; methylnaloxonium increased it in morphine-naive rats. (Top) Dose-dependent decrease in extracellular levels of ACh in the NAc during local infusion by reverse microdialysis of 0, 1, 10, and 100 μM morphine ($n = 6, 6, 7$, and 6, respectively). (Bottom) Locally infused nethylnaloxomium, 0 μM ($n = 6$), 10 μM ($n = 6$), 100 μM ($n = 8$), and 500 μM ($n = 5$), dose-dependently increased ACh in the NAc ($p < 0.05$).

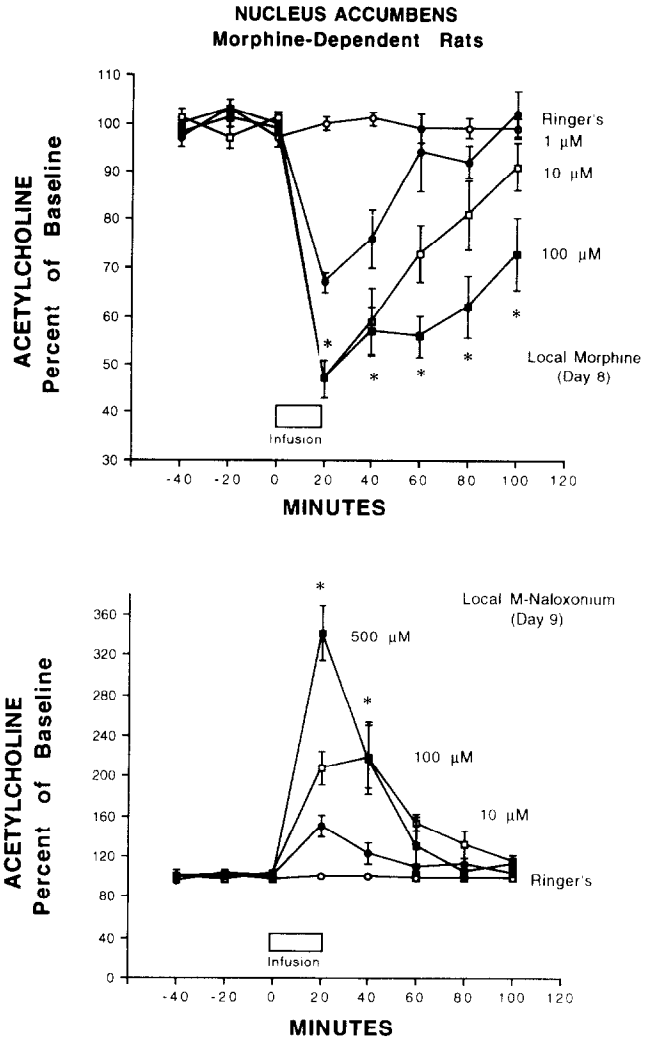

FIG. 7. Effects of local infusion in morphine-dependent rats. (Top) ACh decreased in the NAc in response to local infusion of 0, 1, 10, and 100 μM morphine (n = 6/group) on day 8 of escalating IP injections. (Bottom) Local infusion into the NAc of 0, 10, 100, and 500 μM methylnaloxonium (n = 6, 6, 6, and 5, respectively) on day 8 significantly increased ACh levels ($*p < 0.05$). This ACh increase in morphine-dependent rats was significantly greater than the increase in morphine-naive rats (Fig. 6, bottom).

occurs in the NAc. In the PFC, systemic morphine in drug-naive rats caused an initial increase in extracellular levels of ACh, followed by a decrease and then a rebound. In morphine-dependent rats, morphine on day 8 or naloxone on day 9 both increased ACh (Fig. 3). In the PFC, locally applied morphine or methylnaloxonium in naive rats increased ACh only when the highest doses were used. Morphine's effect occurred one sample after the infusion of the drug, suggesting that it probably affected receptors located at a distance from the infusion site, or that at high doses it might have simulated other types of opiate receptors. This result may partially explain the complex cholinergic response observed in the PFC after systemic morphine. It is surprising that morphine and methylnaloxonium both increased ACh levels. This response suggests that morphine and its antagonist (at high doses) may have affected multiple systems. In morphine-dependent rats, local morphine had no detectable effect in the PFC even at the highest doses. Methylnaloxonium, on the other hand, produced a robust increase in extracellular ACh.

The effect of chronic morphine administration on receptor regulation is still controversial. Some authors have suggested that there is downregulation of the opiate system with a decrease in binding sites in opiate-dependent rats (44,46); whereas others have shown an increase in binding sites and affinity after repeated exposure to opiates (1,39). Chronic administration of opiate antagonists have been shown to upregulate opiate receptors (45,51). Other experimenters have found no changes in binding affinities. For example, DeVries et al. (15) reported that repeated morphine administration did not change μ-receptor binding, although it did upregulate a second messenger system. Van Vliet et al. (48) suggested upregulation of the adenylate cyclase system by chronic activation during

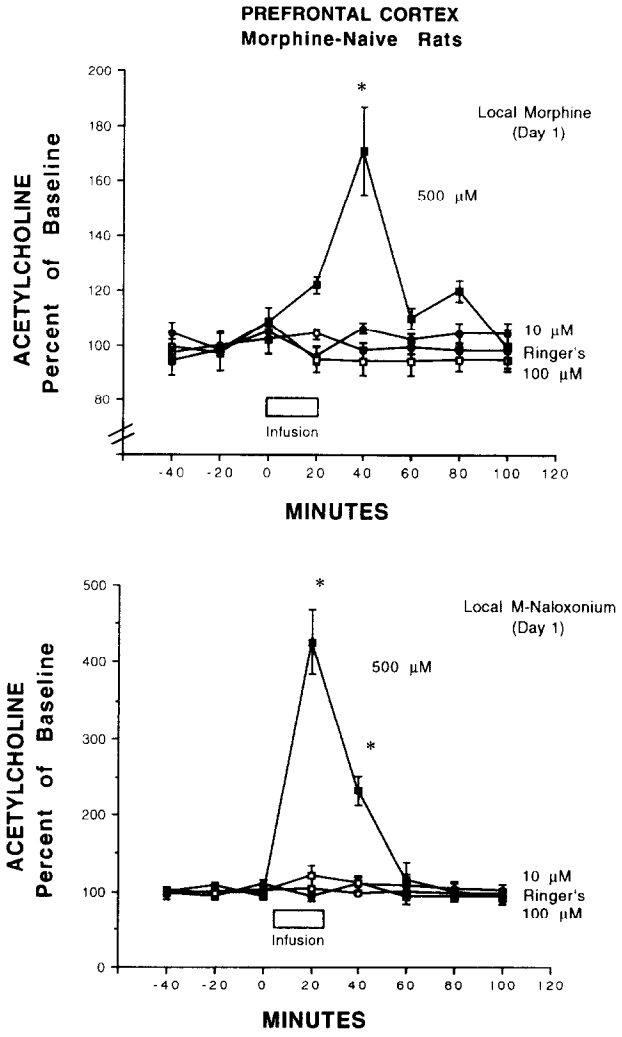

FIG. 8. (Top) In the PFC of morphine-naive rats, locally infused morphine significantly increased extracellular ACh levels at a dose of 500 μM (n = 5, $*p < 0.05$). (Bottom) Local infusion of methylnaloxonium (500 μM) also increased ACh levels (n = 5, $*p < 0.05$). Other doses were ineffective.

the development of opiate tolerance and dependence. Recently, Ronken et al. (38) showed no desensitization of tritiated norepinephrine release from locus coeruleus neurons following chronic opiate activation; instead, an enhanced release occurred upon opioid withdrawal. We find that local morphine infused into the NAc produced approximately the same change in ACh levels in naive and dependent animals. This suggests that chronic morphine treatment did not induce receptor desensitization manifested in ACh release. On the other hand, an augmented ACh response was observed upon local administration of methylnaloxonium in the NAc, showing that the opioid/ACh system had in fact been affected by chronic morphine treatment, probably by upregulation of opiate receptors.

The motor activity pattern after acute and chronic morphine and naloxone-precipitated withdrawal reported here replicates previous findings (4,5). Acute morphine injection caused an initial locomotor depression followed by a delayed excitatory effect. Cortical ACh levels seemed to follow this motor activity pattern, but only after the first 20 min. After chronic opiate treatment, systemic morphine on day 8 increased locomotion, and the PFC cholinergic response again appeared partially to correlate with photobeam counts. On the other hand, naloxone-precipitated withdrawal had no effect on locomotion compared with control rats, despite a significant increase in extracellular ACh in both NAc and PFC.

We assessed morphine dependency in this work by measuring withdrawal symptoms such as teeth chattering, piloerection, wet-dog shakes, diarrhea, writhing, and pronation. Systemic naloxone after escalating doses of morphine precipitated a moderate withdrawal syndrome that correlated with the increase in extracellular ACh in both NAc and PFC. These results are in agreement with those of other researchers who have found a significant increase in ACh release in other brain areas during naloxone-precipitated withdrawal (2,6,7,8,11,13, 25). Some of the symptoms of naloxone-precipitated withdrawal have been mimicked by administration of cholinergic agents (26,47), and ACh may enhance the appearence and severity of withdrawal in rats (13,25). Cholinergic antagonists such as atropine can block morphine-associated reinforcement during self-administration (14), and a muscarinic M_2 antagonist, 4-diphenylacetoxy-*N*-methylpiperidine, is capable of partial blockade of behavioral withdrawal symptoms (10,23). There is no reason to suggest that the increases in ACh we observed are causally related to the peripheral physiologic syndrome observed. It may be, however, that ACh, particularly in the NAc, causes a central state that is aversive or dysphoric. The NAc was one of the most sensitive regions in the brain for producing a conditioned place aversion in response to the local infusion of methylnaloxonium in morphine-dependent animals (42). Conceivably, the rise in extracellular ACh observed in the NAC during local methylnaloxonium engenders some of the aversive aspects of withdrawal.

If it is true that extracellular ACh combined with low DA is in some sense aversive, then it follows that the opposite condition, low ACh with high DA, might contribute to the enablement of positive reinforcement or positive sensations. This is the neurochemical state caused by morphine, as seen in Fig. 1.

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REFERENCES

1. Abdelhamid, E. E.; Takemori, A. E. Characteristics of mu and delta binding sites in striatal slices of morphine-tolerant and -dependent mice. Eur. J. Pharmacol. 198:157-163; 1991.
2. Antonelli, T.; Beani, L.; Bianchi, C.; Rando, S.; Simonato, M.; Tanganelli, S. Cortical acetylcholine release is increased and gamma-aminobutyric acid outflow is reduced during morphine withdrawal. Br. J. Pharmacol. 89:853-860; 1986.
3. Arenas, E.; Alberch, J.; Sanchez, R.; Marsal, J. Effect of opioids on acetylcholine release evoked by K^+ or glutamic acid from rat neostriatal slices. Brain Res. 523:51-56; 1990.
4. Bartoletti, M.; Gaiardi, M.; Gubellini, G.; Bacchi, A.; Babbini, M. Long-term sensitization to the excitatory effects of morphine. Neuropharmacology 22:1193-1196; 1983.
5. Bartoletti, M.; Gaiardi, M.; Gubellini, C.; Bachi, A.; Babbini, M. Effects of buprenorphine on motility in chronically morphine treated rats. Neuropharmacology 32:865-868; 1993.
6. Beani, L.; Bianchi, C.; Siniscalchi, A. The effect of naloxone on opioid-induced inhibition and facilitation of acetylcholine release in brain slices. Br. J. Pharmacol. 76:393-401; 1982.
7. Bhargava, H. N.; Way, E. L. Acetylcholinesterase inhibition and morphine effects in morphine tolerant and dependent mice. J. Pharmacol. Exp. Ther. 183:31-40; 1972.
8. Bhargava, H. N.; Way, E. L. Brain acetylcholine and choline following acute and chronic morphine treatment and during withdrawal. J. Pharmacol. Exp. Ther. 194:65-73; 1975.
9. Brog, J. S.; Salyapongse, A.; Deutch, A. Y.; Zahm, D. S. The patterns of afferent innervation of the core and shell in the accumbens part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. J. Comp. Neurol. 338:255-278; 1993.
10. Buccafusco, J. J. Inhibition of the morphine withdrawal syndrome by a novel muscarinic antagonist (4-DAMP). Life Sci. 48: 749-756; 1991.
11. Cheney, D. L.; Trabucchi, M.; Racagni, G.; Wang, C.; Costa, E. Effects of acute and chronic morphine on regional rat brain acetylcholine turnover rate. Life Sci. 15:1977-1990; 1974.
12. Costa, E.; Cheney, D. L.; Racagni, G.; Zsilla, G. An analysis at synaptic level of the morphine action in striatum and n. accumbens: Dopamine and acetylcholine interactions. Life Sci. 17:1-8; 1975.
13. Crossland, J.; Ahmed, K. Z. Brain acetylcholine during morphine withdrawal. Neurochem. Res. 9:351-366; 1984.
14. Davis, M.; Smith, S. Central cholinergic influence on self-administration of morphine and amphetamine. Life Sci. 16:237-246; 1975.
15. De Vries, T. J.; Tjon Tien Ril, G. H. K.; Van der Laan, J. W.; Mulder, A. H.; Schoffelmeer, A. N. M. Chronic exposure to morphine and naltrexone induces changes in catecholaminergic neurotransmission in rat brain without altering mu-opioid receptor sensitivity. Life Sci. 52:1685-1693; 1993.
16. Di Chiara, G.; Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. USA 85: 5274-5278; 1988.
17. Ennis, C.; Wyllie, M. G. Evidence for functionally distinct μ receptors modulating acetylcholine release. Neuropeptides 5:109-112; 1984.
18. Heijna, M. H.; Hogenboom, F.; Mulder, A. H.; Schoffelmeer, N. M. Opioid receptor-mediated inhibition of [^{3}H]-dopamine and [^{14}C]-acetylcholine release from rat nucleus accumbens slices. Naunyn-Schmied. Arch. Pharmacol. 345:627-632; 1992.
19. Hernandez, L.; Hoebel, B. G. Food reward and cocaine increase extracellular dopamine in the nucleus accumbens as measured by microdialysis. Life Sci. 42:705-712; 1988.
20. Hernandez, L.; Stanley, B. G.; Hoebel, B. G. A small, removable microdialysis probe. Life Sci. 39:2629-2637; 1986.

21. Hoebel, B. G.; Hernandez, L.; Mark, G. P.; Pothos, E. Microdialysis in the study of psychostimulants and the neural substrate for reinforcement: Focus on dopamine and serotonin. In: Frascella, J.; Brown, R., eds. Neurobiological approaches to brain-behavior interaction. Washington, DC: National Institute of Drug Abuse; 1992:124:1-34.
22. Hoebel, B. G.; Rada, P.; Mark, G. P.; Hernandez, L. The power of integrative peptides to reinforce behavior by releasing dopamine. In: Strand, F. L.; Beckwith, B.; Chronwall, B.; Sandman, C. A., eds. Models of neuropeptide action. New York: Annals of the New York Academy of Sciences; 1994:739:36-41.
23. Holland, L. N.; Shuster, L. C.; Buccafusco, J. J. Role of spinal and supraspinal muscarinic receptors in the expression of morphine withdrawal symptoms in the rat. Neuropharmacology 32: 1387-1395; 1993.
24. Jackisch, R.; Geppert, M.; Brenner, A. S.; Illes, P. Presynaptic opioid receptors modulating acetylcholine release in the hippocampus of the rabbit. Naunyn-Schmied. Arch. Pharmacol. 332: 156-162; 1986.
25. Jhamandas, K.; Sutak, M. Modification of brain acetylcholine release by morphine and its antagonists in normal and morphine-dependent rats. Br. J. Pharmacol. 50:57-62; 1974.
26. Katz, J. L.; Valentino, R. J. The opiate quasiwithdrawal syndrome in monkeys: Comparison of naloxone-precipitated withdrawal to effects of cholinergic agents. Psychopharmacology 84: 12-15; 1984.
27. Koob, G. F.; Bloom, F. E. Cellular and molecular mechanisms of drug dependence. Science 242:715-721; 1988.
28. Lapchak, P. A.; Araujo, D. M.; Collier, B. Regulation of endogenous acetylcholine release from mammalian brain slices by opiate receptors: Hippocampus, striatum and cerebral cortex of guinea pig and rat. Neuroscience 31:313-325; 1989.
29. Mark, G.; Rada, P.; Pothos, E.; Hoebel, B. G. Effects of feeding and drinking on acetylcholine release in the nucleus accumbens, striatum and hippocampus of freely behaving rats. J. Neurochem. 58:2269-2274; 1992.
30. Meredith, G. E.; Blanf, B.; Groenewegen, H. J. The distribution and compartmental organization of the cholinergic neurons in nucleus accumbens. Neuroscience 31:327-345; 1989.
31. Mogenson, G. J.; Jones, D. L. and Yim, C. Y. From motivation to action: Functional interface between the limbic system and the motor system. Prog. Neurobiol. 14:69-97; 1980.
32. Mulder, A. H.; Wardeh, G.; Hogenboom, F.; Frankhuyzen, A. L. Kappa and delta-opioid receptor agonists differentially inhibit striatal dopamine and acetylcholine release. Nature 308:278-280; 1984.
33. Mulder, A. H.; Wardeh, G.; Hogenboom, F.; Frankhuyzen, A. L. Selectivity of various opioid peptides towards delta, kappa, and mu-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the brain. Neuropeptides 14:99-104; 1989.
34. Pettit, H. O.; Ettenberg, A.; Bloom, F. E.; Koob, G. F. Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. Psychopharmacology 84:167-173; 1984.
35. Pothos, E.; Rada, P.; Mark, G. P.; Hoebel, B. G. Microdialysis evidence that dopamine in the nucleus accumbens is involved in morphine withdrawal and its treatment with clonidine. Brain Res. 566:348-350; 1991.
36. Rada, P.; Mark, G. P.; Pothos, E.; Hoebel, B. G. Systemic morphine simultaneously decreases extracellular acetylcholine and increases dopamine in the nucleus accumbens of freely moving rats. Neuropharmacology 30:1133-1136; 1991.
37. Rada, P.; Pothos, E.; Mark, G. P.; Hoebel, B. G. Microdialysis evidence that acetylcholine in the nucleus accumbens is involved in morphine withdrawal and its treatment with clonidine. Brain Res. 561:354-356; 1991.
38. Ronken, E.; Mulder, A. H.; Schoffelmeer, A. N. M. Chronic activation of mu- and kappa-opioid receptors in cultured catecholaminergic neurons from rat brain causes neuronal supersensitivity without receptor desensitization. J. Pharmacol. Exp. Ther. 268:595-599; 1994.
39. Rothman, R. B.; Long, J. B.; Bykov, V.; Xu, H.; Jacobson, A. E.; Rice, K. C.; Holaday, J. W. Upregulation of the opioid receptor complex by the chronic administration of morphine: A biochemical marker related to the development of tolerance and dependence. Peptides 12:151-160; 1991.
40. Sharkawi, M.; Schukman, M. Inhibition by morphine of the release of [^{14}C]acetylcholine from rat brain cortex slices. J. Pharm. Pharmacol. 21:546-547; 1969.
41. Smith, J. E.; Co, C.; Lane, J. D. Limbic muscarinic cholinergic and benzodiazepine receptor changes with chronic intravenous morphine and self-administration. Pharmacol. Biochem. Behav. 20:443-450; 1984.
42. Stinus, L.; Le Moal, M.; Koob, G. F. Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. Neuroscience 37:767-773; 1990.
43. Taguchi, K.; Hagiwara, Y.; Suzuki, Y.; Kubo, T. Effects of morphine on release of acetylcholine in the rat striatum: An in vivo microdialysis study. Naunyn-Schmied. Arch. Pharmacol. 347:9-13; 1993.
44. Tao, P. L.; Seybold, V. S.; Loh, H. H. Autoradiographic evidence for decrease in binding of mu- and delta-opioid receptors after subchronic {D-Ala, D-Leu}enkephalin treatment in rats. Eur. J. Pharmacol. 231:145-149; 1993.
45. Tempel, A.; Crain, S. M.; Peterson, E. R.; Simon, E. J.; Zukin, R. S. Antagonist-induced opiate receptor upregulation in cultures of fetal mouse spinal cord-ganglion explants. Brain Res. 390:287-292; 1986.
46. Tempel, A.; Habas, J.; Paredes, W.; Barr, G. A. Morphine-induced downregulation of mu-opioid receptors in neonatal rat brain. Dev Brain Res. 41:129-133; 1988.
47. Valentino, R. J.; Aston-Jones, G. Activation of locus coeruleus neurons in the rat by a benzazocine derivative (UM 1046) that mimics opiate withdrawal. Neuropharmacology 22:1363-1368; 1983.
48. Van Vliet, B. J.; Dotman, C. H.; Wardeh, G.; Mulder, A. H.; Schoffelmeer, A. N. M. Differential effects of chronic agonist administration on mu-opioid receptors and muscarinic receptor-regulated adenylate cyclase in rat striatal neurons. Life Sci. 51: 89-94; 1992.
49. Wise, R. A. Opiate reward: Sites and substrates. Neurosci. Biobehav. Rev. 13:129-133; 1989.
50. Wood, P. L.; Stotland, L. M. Actions of enkephalin, μ and partial agonist analgesics on acetylcholine turnover in rat brain. Neuropharmacology 19:975-982; 1980.
51. Yoburn, B. C.; Duttaroy, A.; Shah, S.; Davis, T. Opioid antagonist-induced receptor upregulation: Effects of concurrent agonist administration. Brain Res. Bull 33:237-240; 1993.
52. Zahm, D. S.; Brog, J. S. On the significance of subterritories in the "accumbens" part of the rat ventral striatum. Neuroscience 50:751-767; 1992.