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BRIEF COMMUNICATION

Morphine Dependence and Protracted Abstinence: Regional Alterations in CNS Radioligand Binding

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CARLSON, K. R. AND D. O. COOPER. *Morphine dependence and protracted abstinence: Regional alterations in CNS radioligand binding.* PHARMACOL BIOCHEM BEHAV **23**(6) 1059–1063, 1985.—Rats (Fisher F-344) were given free access to a 10% sucrose solution containing 0.5 mg/ml morphine sulfate (controls received the sucrose vehicle only) as their sole source of fluid. Daily morphine intake averaged 119±21 mg/kg, an amount sufficient to induce physical dependence. After 18 days on this regimen, the control and dependent subjects were sacrificed. A protracted abstinence group was weaned from morphine by reducing its concentration in the vehicle by 20% over the next 5 days, followed by a 5-week drug-free period before sacrifice concurrent with the other groups. These subjects showed no signs of an abstinence syndrome. Binding assays for alpha-2 adrenergic sites (^{3}H-clonidine), beta-1/beta-2 adrenergic sites (^{3}H-dihydroalprenolol), and dopaminergic (D_2)/serotonergic (5-HT_2) sites (^{3}H-spiroperidol) were performed on tissue from frontal cortex, hippocampus, striatum, and brainstem. No alterations in ^{3}H-clonidine or ^{3}H-dihydroalprenolol binding were observed in dependence or protracted abstinence, suggesting that noradrenergic systems are well-regulated both during dependence and in protracted abstinence. ^{3}H-spiroperidol binding was significantly elevated in the striatum (D_2 sites) and hippocampus (5-HT_2 sites) during dependence. Hippocampal ^{3}H-spiroperidol binding returned to control levels in protracted abstinence, reflecting a morphine-induced change in 5-HT_2 binding sites which had normalized by 5 weeks post-drug. Striatal ^{3}H-spiroperidol binding was significantly decreased below control levels after withdrawal, suggesting that alterations of D_2 sites in this structure may play a role in protracted abstinence.

Receptor binding Chronic morphine Hippocampus Striatum

MAJOR effects of chronic opiate administration include the induction of tolerance and dependence, and, upon drug termination, an acute withdrawal syndrome followed by a period of protracted abstinence. Numerous studies have been devoted to characterizing the biochemical correlates of tolerance, dependence, and the acute withdrawal syndrome. While a state of protracted abstinence has been demonstrated behaviorally in several species including man [1, 15, 22], little attention has been paid to possible biochemical correlates of this phenomenon.

Studies of morphine dependence and withdrawal in rats have typically employed techniques such as rapid induction through multiple injection or pellet implantation, and rapid withdrawal by abrupt removal of drug or administration of naloxone. While these procedures are quick and effective, they bear little resemblance to human opiate abuse. In recognition of this, some investigators have employed procedures which are non-stressful and allow the experimental subjects to self-administer morphine by placing the drug in their drinking water (e.g., [18]). Such studies have clearly demonstrated that rats will self-administer morphine in amounts sufficient to induce physical dependence. Unfortunately, to our knowledge, no studies have been performed which allowed an equally non-stressful period of withdrawal, including graded reductions in drug dosage as is seen in clinical detoxification of opiate abusers.

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Many neurotransmitter systems have been implicated in morphine's effects. For example, roles for dopamine, noradrenalin, and serotonin have been documented through measurement of transmitter levels and turnover (for reviews see [3, 17, 33]). The advent of receptor binding techniques has prompted examination of alterations in radioligand binding during dependence and withdrawal. These studies often present conflicting results, which may be attributable to variables such as biochemical technique, rat strains employed, brain regions examined, or procedural differences in drug administration. Another problem is related to the fact that most investigators measure changes in only one neurotransmitter system, although it would be preferable to examine several binding sites in the same animals [26].

The purpose of the present exploratory study was to examine dopaminergic D_2, adrenergic alpha-2 and beta-1/beta-2, and serotonin-2 (5-HT_2) binding sites in several CNS regions associated with the rewarding properties of opiates [14], abstinence phenomena [36], and altered sensitivity to dopaminergic agonists following chronic opiate treatment [6, 7, 28]. Accordingly, rats were allowed free access to morphine in their drinking water and were then withdrawn by gradual reductions in drug dosage. These techniques are akin to voluntary consumption and clinical withdrawal in humans. The withdrawn group was allowed a five week drug-free period so that binding alterations might be assessed during protracted abstinence.

METHOD

Subjects and Drug Administration

Male F-344 Fisher rats (Charles River) were randomly assigned to the control and the two experimental groups (N=5 or 6 per group). The subjects weighed 250–300 g at the onset of experimentation and were housed individually in a climate-controlled room with a 0700 on—1900 off light cycle. Subjects in both experimental groups were given free access to Purina rat chow and to a 10% sucrose solution containing 0.5 mg/ml morphine sulfate (Mallinckrodt). This procedure has been shown to induce physical dependence within 5 days [18]. Control animals received food and 10% sucrose vehicle. All subjects were kept on this regimen for 18 days, during which no differences were observed in food or fluid intake. The average daily morphine intake was 119±21 mg per kg.

At the end of this period, the control and dependent subjects were sacrificed. Subjects in the protracted abstinence group were weaned from morphine by reducing its concentration in the sucrose vehicle by 20% per day over a 5-day period, after which they were maintained on a normal water and food diet for a further 5 weeks before sacrifice. No abstinence signs (e.g., weight loss, wet dog shakes) were observed during the withdrawal period.

The experimental schedule was such that all subjects were sacrificed on the same day.

Binding Assays

Immediately following sacrifice by decapitation, subjects' brains were quickly dissected over ice into frontal cortex, hippocampus, striatum, and brainstem (pons-medulla). Brain regions were frozen in aluminum foil on dry ice and kept at −70°C until assay.

Frontal cortex and hippocampus were assayed using all three ligands, striatum using ^{3}H-spiroperidol, and brainstem using ^{3}H-clonidine and ^{3}H-dihydroalprenolol. Each brain region from each subject was assayed separately with the appropriate ligand(s), i.e., tissues from different subjects were not pooled. Tissue was homogenized (Brinkmann Polytron, setting 7, two 5-sec bursts) in ice-cold 50 mM Tris buffer, pH 7.4, at a concentration of 10 mg wet weight per ml (resulting in approximately 1 mg protein per assay tube). The homogenate was then centrifuged at 48,000 × g for 10 minutes and the supernatant was discarded. The pellet was resuspended in the same volume of fresh Tris and rehomogenized and re-centrifuged as above. The final pellet was once again resuspended and re-homogenized for assay.

Binding assays were performed in duplicate in 12×75 mm culture tubes. Total binding tubes contained 0.1 ml of radioligand and 0.1 ml Tris. Non-specific binding tubes contained 0.1 ml radioligand and 0.1 ml of the appropriate displacing agent. Ligands and displacing agents for each binding assay were: (1) alpha-2 adrenergic [34]: ^{3}H-clonidine (S.A. 23.8 Ci/mMol, New England Nuclear-NEN) final concentration 3.0 nM displaced by 0.5 mM levarterenol; (2) Beta-adrenergic [5]: ^{3}H-dihydroalprenolol (S.A. 49.0 Ci/mMol, DHA, NEN) 1.0 nM displaced by 1.0 μM dl-propranolol; (3) 5-HT_2 and D_2: ^{3}H-spiroperidol (S.A. 23.8 Ci/mMol, SPD, NEN) 0.3 nM displaced by 1.0 μM d-lysergic acid diethylamide (LSD). The SPD/LSD assay system labels both dopamine-2 (D_2) and 5-HT_2 binding sites, the proportion being dependent on the brain region, i.e., primarily D_2 in the striatum [12,27] and 5-HT_2 in frontal cortex and hippocampus [11,31].

Binding assays were begun by addition of 0.8 ml tissue suspension to prepared total and non-specific binding tubes over ice. After mixing, the tubes were allowed to incubate at 22°C for 30 minutes (clonidine and DHA) or at 37°C for 15 minutes (SPD). Following incubation, the contents of the tubes were poured over Whatman GF/B filters (pre-wetted with 2 ml Tris) under vacuum. The filters were than washed with 5 ml Tris. Incubation tubes were filled with 3 ml Tris and emptied once again over the filters. The filters received a final 5 ml Tris wash and were placed in 10 ml scintillation counting fluor (Ready-solv, Beckman). Filters were allowed to resorb in the counting fluid for 48 hours, after which they were counted with ^3H_2O standards to assess counting efficiency (average 32%). Results were expressed as fmol of specific binding (total minus non-specific) ±S.E.M. per mg protein, as assessed by the method of Lowry [21] using a bovine serum albumin standard curve.

Data for each ligand in each brain region were analyzed by a one-way analysis of variance. Significant F values were further analyzed by the post-hoc Neuman-Keuls test to determine which individual comparisons were responsible for overall significance [35].

RESULTS

The results are presented in Table 1. No significant alterations in either ^{3}H-clonidine or ^{3}H-DHA binding were found during dependence or in protracted abstinence.

With respect to ^{3}H-SPD binding, analysis of variance revealed a highly significant morphine effect on striatal binding, $F(2,14)=39.6$, $p<0.001$. Binding was increased by 34% over control levels during dependence ($p<0.01$), and, in contrast, decreased by 26% from control levels in protracted abstinence ($p<0.01$). These alterations are thought to represent changes primarily in D_2 binding sites [12,27].

In the hippocampus, morphine also produced a significant effect on ^{3}H-SPD binding, $F(2,14)=10.6$, $p<0.001$. In this

TABLE 1

AMOUNT OF ^{3}H-LIGAND SPECIFICALLY BOUND (MEAN ± SEM, fm/mg PROTEIN)

		Morphine-Dependence State		
^{3}H-Ligand	Brain Area	Control	Dependent	Withdrawn
Spiroperidol	Striatum	199.94 ± 4.24	268.14 ± 14.02*	148.09 ± 9.54*
	Cortex	91.99 ± 7.53	94.11 ± 7.53	98.45 ± 5.38
	Hippocampus	22.82 ± 1.52	45.20 ± 7.01*	23.05 ± 2.47
Clonidine	Cortex	17.52 ± 1.75	19.45 ± 2.48	17.07 ± 1.46
	Hippocampus	25.49 ± 2.91	24.44 ± 2.74	24.13 ± 2.53
	Brainstem	10.94 ± 1.05	11.83 ± 1.88	11.10 ± 0.88
Dihydroal-prenolol	Cortex	25.08 ± 1.67	29.30 ± 0.82	25.39 ± 1.37
	Hippocampus	17.26 ± 1.12	16.38 ± 2.10	16.32 ± 1.65
	Brainstem	9.21 ± 1.09	9.38 ± 1.06	8.96 ± 1.25

*Different from Control group, $p<0.01$.

case, however, the overall significance was attributable solely to a 98% increase over control levels during dependence ($p<0.01$); binding was no different from control in protracted abstinence. ^{3}H-SPD binding in frontal cortex was unaltered by morphine. In these two areas ^{3}H-SPD is considered to bind primarily to 5-HT_2 sites [11,31].

DISCUSSION

In the first investigation of 5-HT_2 binding as a function of morphine treatment, we recently reported that hippocampal binding was elevated during dependence [10]. The present study replicates that finding, and supports suggestions that 5-HT mechanisms [37] and the hippocampus [14] may be involved in the effects of opiates. In addition, we found that binding returns to control levels after an extended period of withdrawal. Examination of these binding sites at earlier time points might reveal a time course of reduction consistent with alleviation of the acute withdrawal syndrome, since evidence exists relating serotonergic mechanisms with behavioral phenomena such as "wet dog shakes" [36].

With respect to striatal dopamine binding sites, the 34% increase during dependence is consistent with the 36% increase we found previously in that strain [10]. We are aware of only one other such study in which no change was found at the end of a high-dose, 4-day period of morphine treatment [23]. It is possible that the duration was not long enough to produce the increases which we have observed. Many researchers have measured dopamine binding one or two days after abrupt drug discontinuation, and with a few exceptions [9,29] have found increased affinity [2, 29, 30] or density [13]. The present results may represent changes which persist into early withdrawal, rather than changes originating during the withdrawal syndrome. Thus, the receptor alteration presumed to underly behavioral hypersensitivity to dopaminergic agonists seen shortly after chronic opiate treatment [6, 7, 28] may be established during dependence.

In earlier work, we found no striatal dopamine receptor density changes after 15 days' withdrawal from chronic opiate treatment [8]. This time point may have been one at which binding values were apparently "normal" on their way to a lower than control level, since in the present study protracted abstinence was associated with a rebound decrease in binding. In this connection, it is interesting that we found that subjects were subsensitive to the stereotypy-inducing effects of apomorphine at 5 and 6 weeks following chronic treatment with morphine or methadone [7]. The dopaminergic nature of this effect is underscored by the fact that the same behavioral subsensitivity can be seen 5 weeks after chronic haloperidol [6]. It is possible that our biochemical finding of a rebound decrease in striatal dopamine binding sites may be related to the behavioral subsensitivity observed in the other studies. Such a relationship remains to be confirmed within the same subjects. Further, the relative contributions of changes in affinity (K_D) and density (B_{max}) to the observed alterations should be assessed. Such saturation experiments were not possible within the constraints of this preliminary study, since up to three ligands were used with small amounts of tissue.

No alterations in adrenergic binding sites were found in the present study. Some investigators have found such alterations in dependence [20,32], but not in abstinence precipitated with naloxone [16]. In contrast, others have found binding to be altered only during withdrawal [19], or to be unchanged in either state [23,24]. Discrepancies in these studies could be attributed to a number of different procedural variations. Assessment of alpha-2 and beta adrenergic binding at earlier time points during morphine withdrawal will provide a more complete picture, and may demonstrate alterations consistent with a role for adrenergic systems in acute withdrawal, since administration of clonidine has been shown to alleviate some aspects of withdrawal symptomatology [4]. No alterations in adrenergic binding were found during protracted abstinence, however, suggesting that this neurotransmitter system either adapts relatively early in withdrawal, or does not change.

If the lack of alteration in adrenergic binding reflects the ability of this system to rapidly adjust, it is possible that development of tolerance and dependence may be associated with an appropriate homeostatic adjustment in adrenergic systems which renders undetectable alterations in binding. Changes in a secondary messenger system [25], for example, might represent such an adjustment.

Receptor binding is a powerful tool with which to examine neurotransmitter dynamics. Caution must be exercised, however, when attaching a significance to binding changes if the appropriate functional parameters have not also been measured [26]. In this context, we have shown that increases in striatal ^{3}H-SPD binding during dependence and rebound decreases in protracted abstinence correlate chronologically

with behavioral sensitivity to apomorphine. While this evidence is certainly not conclusive, it is highly suggestive and worthy of further investigation. Finally, it would be interesting to compare neurochemical changes occurring under stressful vs. non-stressful induction and termination of opiate dependency.

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REFERENCES

1. Babbini, M., M. Gaiardi and M. Bartoletti. Persistence of chronic morphine effects upon activity in rats 8 months after ceasing the treatment. *Neuropharmacology* **14:** 611–614, 1975.
2. Bhargava, H. N. Binding of [^{3}H]spiroperidol to striatal membranes of rats treated chronically with morphine: Influence of pro-leu-gly-NH_2 and cyclo(leu-gly). *Neuropharmacology* **22:** 1357–1361, 1983.
3. Brase, D. A. Roles of serotonin and GABA in opioid effects. *Adv Biochem Psychopharmacol* **20:** 409–428, 1979.
4. Buccafusco, J. J., D. C. Marshall and R. M. Turner. A comparison of the inhibitory effects of clonidine and guanfacine on the behavioral and autonomic components of morphine withdrawal in rats. *Life Sci* **35:** 1401–1408, 1984.
5. Bylund, D. B. and S. H. Snyder. Beta-adrenergic receptor binding in membrane preparations from mammalian brain. *Mol Pharmacol* **12:** 568–580, 1976.
6. Carlson, K. R. and J. Almasi. Behavioral supersensitivity to apomorphine following chronic narcotic treatment in the guinea pig. *Psychopharmacology (Berlin)* **57:** 273–277, 1978.
7. Carlson, K. R. and J. Almasi. Time course of dopaminergic hypersensitivity following chronic narcotic treatment. *Pharmacol Biochem Behav* **11:** 283–287, 1979.
8. Carlson, K. R. and T. F. Seeger. Interaction of opiates with dopamine receptors: Receptor binding and behavioral assays. *Pharmacol Biochem Behav* **16:** 119–124, 1982.
9. Christie, M. J. and D. H. Overstreet. Sensitivity of morphine-tolerant rats to muscarinic and dopaminergic agonists: Relation to tolerance or withdrawal. *Psychopharmacology (Berlin)* **65:** 27–34, 1979.
10. Cooper, D. O., K. R. Carlson and J. W. McKearney. Comparison of regional CNS ligand binding in two inbred rat strains: Effects of chronic morphine. *Pharmacol Biochem Behav*, **23:** 349–354, 1985.
11. Creese, I. and S. H. Snyder. ^{3}H-spiroperidol labels serotonin receptors in rat cerebral cortex and hippocampus. *Eur J Pharmacol* **49:** 201–202, 1978.
12. Creese, I., R. Schneider and S. H. Snyder. ^{3}H-spiroperidol labels dopamine receptors in pituitary and brain. *Eur J Pharmacol* **46:** 377–381, 1977.
13. de la Baume, S., G. Patey, H. Marcais, P. Protais, J. Costentin and J.-C. Schwartz. Changes in dopamine receptors in mouse striatum following morphine treatments. *Life Sci* **24:** 2333–2342, 1979.
14. Glick, S. D. and R. D. Cox. Changes in morphine self-administration after tel-diencephalic lesions in rats. *Psychopyarmacology (Berlin)* **57:** 283–288, 1978.
15. Goldberg, S. R. and C. R. Schuster. Nalorphine: Increased sensitivity of monkeys formerly dependent on morphine. *Science* **166:** 1548–1549, 1969.
16. Hamburg, M. and J. F. Tallman. Chronic morphine administration increases the apparent number of alpha-2 adrenergic receptors in rat brain. *Nature* **291:** 493–495, 1981.
17. Iwamoto, E. T. and E. L. Way. Opiate actions and catecholamines. *Adv Biochem Psychopharmacol* **20:** 357–407, 1979.
18. Khavari, K. A. and M. E. Risner. Opiate dependence produced by ad lib drinking of morphine and water, saline, and sucrose vehicles. *Psychopharmacologia* **30:** 291–302, 1973.
19. Kuriyama, K., M. Muramatsu, M. Aiso and E. Ueno. Alterations in beta-adrenergic receptor binding in brain, lung and heart during morphine and alcohol dependence and withdrawal. *Neuropharmacology* **20:** 659–666, 1981.
20. Llorens, C., M. P. Martres, M. Baudry and J. C. Schwartz. Hypersensitivity to noradrenaline in cortex after chronic morphine: Relevance to tolerance and dependence. *Nature* **274:** 603–605, 1978.
21. Lowry, O., N. Rosebrough, A. Farr and J. Randall. Protein measurement with the folin reagent. *J Biol Chem* **193:** 265–275, 1951.
22. Martin, W. R. and D. R. Jasinski. Physiological parameters of morphine dependence in man. Tolerance, early abstinence, and protracted abstinence. *J Psychiatry Res* **7:** 9–17, 1969.
23. Mennini, T., E. Poggesi, S. Cotecchia, A. De Blasi and R. Saminin. Changes in serotonin, but not catecholamine receptor binding in the brain of morphine dependent rats. *Mol Pharmacol* **20:** 237–239, 1981.
24. Minneman, K. P. and S. G. Holtzman. Morphine dependence and withdrawal without alterations in cerebral beta-adrenergic receptor density. *Biochem Pharmacol* **33:** 2331–2333, 1984.
25. Mishra, R., A. Janowsky and F. Sulser. Action of mianserin and zimelidine on the norepinephrine receptor coupled adenylate cyclase system in brain: Subsensitivity without reduction in beta-adrenergic receptor binding. *Neuropharmacology* **19:** 983–987, 1980.
26. Overstreet, D. H. and H. I. Yamamura. Receptor alterations and drug tolerance. *Life Sci* **25:** 1865–1878, 1979.
27. Pedigo, N. W., T. D. Reisine, J. Z. Fields and H. I. Yamamura. ^{3}H-spiroperidol binding to two receptor sites in both the corpus striatum and frontal cortex of rat brain. *Eur J Pharmacol* **50:** 451–453, 1978.
28. Puri, S. K. and H. Lal. Effect of dopaminergic stimulation or blockade on morphine-withdrawal aggression. *Psychopharmacologia* **32:** 113–120, 1973.
29. Puri, S. K., T. C. Spaulding and C. R. Mantione. Dopamine antagonist binding: A significant decrease with morphine dependence in the rat striatum. *Life Sci* **23:** 637–642, 1978.

30. Ritzmann, R. F., J. M. Lee and J. Z. Fields. Modification of morphine-induced changes in striatal [^{3}H]-spiroperidol binding and stereotype behavior by cyclo(leu-gly). *Life Sci* **30:** 1573–1580, 1982.
31. Seeman, P., K. Westman, D. Coscina and J. J. Marsh. Serotonin receptors in hippocampus and frontal cortex. *Eur J Pharmacol* **66:** 179–191, 1980.
32. Smith, C. B., P. J. Hollingsworth, J. J. Geer and H. C. Moises. Changes in alpha-2 adrenoreceptors in various brain regions after long-term administration of mu and kappa opiate agonists. *Life Sci* **33:** Supl I, 369–372, 1983.
33. Smith, J. E. and J. D. Lane. Brain neurotransmitter turnover correlated with morphine self-administration. In: *The Neurobiology of Opiate Reward Processes,* edited by J. E. Smith and J. D. Lane. New York: Elsevier, 1983, pp. 361–402.
34. U'Pritchard, D. C., W. D. Bechtel, B. M. Rouot and S. H. Snyder. Multiple apparent alpha noradrenergic receptor binding sites in rat brain: Effect of 6-OH dopamine. *Mol Pharmacol* **16:** 47–60, 1979.
35. Winer, B. J. *Statistical Principles in Experimental Design.* New York: McGraw-Hill, 1971.
36. Yap, C. Y. and D. A. Taylor. Involvement of 5-HT_2 receptors in the wet-dog shake behavior induced by 5-hydroxytryptophan in the rat. *Neuropharmacology* **22:** 801–804, 1983.
37. Zeuchner, J., L. Ronnback, L. Rosengren, A. Wronski and S. O. Ogren. Decreased morphine intake by opiate addicted rats administered zimelidine, a 5-HT uptake inhibitor. *Psychopharmacology (Berlin)* **82:** 30–35, 1984.