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Limbic Muscarinic Cholinergic and Benzodiazepine Receptor Changes With Chronic Intravenous Morphine and Self-Administration

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SMITH, J. E., C. CO AND J. D. LANE. *Limbic muscarinic cholinergic and benzodiazepine receptor changes with chronic intravenous morphine and self-administration.* PHARMACOL BIOCHEM BEHAV 20(3) 443–450, 1984.—Muscarinic cholinergic and benzodiazepine receptor affinities and densities were evaluated in membranes from seven brain regions of rats intravenously self-administering morphine and in littermates receiving yoked-morphine or yoked vehicle infusions to identify neuronal systems potentially involved in mediating opiate reinforcement processes. Passive morphine infusion resulted in increases in muscarinic cholinergic receptor densities in the pyriform cortex and in decreases in the cingulate cortex while benzodiazepine receptor densities were decreased in both the hippocampal formation and entorhinal-subicular cortex compared to littermates receiving passive infusions of vehicle. Morphine self-administration resulted in decreased muscarinic cholinergic receptor densities in the frontal and entorhinal-subicular cortices and increases in the amygdaloid complex compared to littermates receiving yoked passive drug. These data are in agreement with acetylcholine turnover rate measurements in these animals and support the proposed role of cholinergic innervations of the frontal and entorhinal-subicular cortices and amygdaloid complex in opiate reinforcement processes.

Muscarinic cholinergic receptors Benzodiazepine receptors Chronic intravenous morphine
Intravenous morphine self-administration Opiate reinforcement

OPIOIDS are self-administered primarily for their reinforcing properties. The neuronal circuitry responsible for these effects have been under investigation with a variety of neurobiological methodologies. Opiate receptor agonists modify the sensitivity of "endogenous reinforcement systems" demonstrated with intracranial electrical self-stimulation (ICSS) (review [19]). Opiate agonists and antagonists also affect the rates of other behaviors including feeding, drinking and sexual activity (review, [31]). These effects have led to speculation that endogenous opiate ligands may modulate general brain processes involved in euphoria and reinforcement [1]. However, others have suggested mesolimbic dopaminergic neurons to initiate neuronal activity mediating opiate reinforcement [47]. This hypothesis is based on three general findings: dopamine receptor antagonists decrease intravenous opiate self-administration [11, 30, 41]; morphine is intracranially self-administered into the ventral tegmental region (VTA) [2]; and, morphine injected into the VTA can serve as a place preference conditioning stimulus [3]. However, other data suggest the involvement of additional neuronal systems and indicate that opiate receptors outside the VTA initiate neuronal activity mediating the reinforcing properties of opiates [5, 25, 26, 43, 44]. Drugs that interfere with cholinergic [7,11], dopaminergic [11, 30, 41] and noradrenergic [8] neurons decrease intravenous morphine-self-administration. Electrolytic lesion procedures have shown lesions of the medial raphe nucleus, nucleus accumbens, septum, hippocampal formation and frontal cortex to increase intravenous morphine self-administration, while similar lesions of the substantia nigra and caudate nucleus decreases intake [9–14]. Lesions of the globus pallidus, locus coeruleus, dorsal raphe nucleus, amygdaloid complex, nucleus accumbens and olfactory tubercles have no effect. Intracranial self-administration methodologies indicate that neurons in the ventral tegmental area [2], lateral hypothalamus [43], preoptic nuclei [25,43], septum [43] and nucleus accumbens [25] support opioid self-administration. Therefore, these substances chemically initiate neuronal activity that has reinforcing properties. Neurotransmitter turnover rates measured in brain regions of rats intravenously self-administering morphine have implicated dopamine, norepinephrine, serotonin, aspartate, glutamate and gamma-aminobutyric acid containing neurons [37,38] in morphine-seeking behaviors. Specific neuronal circuits were suggested to mediate components of opiate reinforcement with several cholinergic

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pathways proposed to participate (innervations of the entorhinal-subicular and frontal cortices, hippocampal formation and amygdaloid complex) [40]. Direct assessment of the involvement of cholinergic neurons was initiated with turnover rate measurements supporting the role of cortical and amygdaloid cholinergic neurons in these processes [39]. However, these measurements utilize short pulse times (2 to 10 minutes) that may not detect major changes in cholinergic neuronal activity if the behavioral process precedes this time interval. Cholinergic receptor binding characteristics may more accurately reflect long-term net changes in acetylcholine (ACh) release.

This investigation was initiated to further identify cholinergic neuronal systems involved in opiate reinforcement by evaluating muscarinic cholinergic receptor binding to membranes prepared from brain regions of animals self-administering or receiving yoked infusion of morphine or vehicle. Benzodiazepine receptors were also evaluated since evidence has suggested potential involvement in the behavioral process [21,32] and since opiates have anxiolytic properties that could result from effects on endogenous ligands for these receptors. Binding of quinuclidinyl benzilate (a muscarinic cholinergic receptor agonist) and flunitrazepam (a benzodiazepine receptor agonist) was assessed in membranes prepared from the frontal, pyriform, cingulate and entorhinal-subicular cortices, hippocampal formation, amygdaloid complex and caudate nucleus-putamen of rats in which ACh turnover rates had been previously measured after pulse labelling with [^{3}H]-choline [39].

METHOD

Behavioral

Fourteen litters of three adult male Fischer F-344 rats (90–150 days old) were used to identify neurochemical changes resulting from the passive or response contingent presentation of morphine. One rat in each litter intravenously self-administered morphine while a second littermate received a simultaneous identical infusion of morphine and a third littermate the equivalent volume of vehicle (saline). This experimental design permits assessment of the general effects of the drug (yoked-morphine group compared with yoked-vehicle infused group) and the reinforcing effects of the drug-taking milieu (self-administering group compared with the yoked-morphine infused group).

Surgical

Rats were implanted with chronic jugular catheters using previously described procedures [29,46]. The catheter (0.76 mm o.d.×0.25 mm i.d. polyvinylchloride tubing) was inserted into the right posterior facial vein and lowered into the right jugular vein until it terminated just outside the right atrium of the heart. The catheter was anchored to tissue in the surrounding area and continued subcutaneously to the back where it exited behind the scapulae through a plastic-stainless steel harness. The plastic portion of the harness was implanted under the skin to provide one point of attachment for a needle tubing and metal spring leash which enclosed the catheter. The catheter passed through the top of the animal chamber and attached to a leak-proof swivel [4] which was connected with polyvinylchloride tubing to one syringe in a three-syringe infusion pump. The leash and swivel were counter balanced to permit unrestrained movement by the animals. Betadine® ointment was applied daily to the skin at the point of catheter exit to prevent tissue infection.

Self-Administration

Each litter was housed together in a large sound attenuated chamber in individual self-administration cages with free access to food and water. Each of these chambers was equipped with a house light that was illuminated on a reversed 12-hr light (1700 hr to 500 hr) and 12-hr dark (500 hr to 1700 hr) cycle and a speaker which maintained a constant level of white noise to mask external auditory stimuli. All three self-administration cages in the chamber contained a lever (which was not present initially) and a stimulus light. The first two days after surgery, all three littermates received hourly infusions of 200 μl of heparinized saline. Two of the rats in each litter were then made physically dependent with hourly infusions of morphine sulfate in increasing dosages (2 days each of 1.25, 2.5, 5.0 and 10.0 mg/kg) while the third littermate received heparinized-saline. These injections (for all three littermates) were paired with a tone and light stimulus of 30-sec duration to facilitate reinforcing efficacy and later development of lever pressing by the self-administering littermate. On the eleventh day after surgery, infusions were discontinued and levers introduced into the three self-administration cages. One of the physically dependent animals was allowed to self-administer morphine (10 mg/kg in 200 μl delivered over 5.5 sec) by pressing the lever with continued pairing of the tone and light stimulus, while the other physically dependent littermate received an identical yoked-morphine infusion and the third littermate yoked-vehicle. Initially, one lever press resulted in an infusion, however, 10 lever presses were eventually required (fixed ratio 10 schedule). Both yoked-infused littermates had identical levers in their cages, but lever presses had no programmed consequences. The self-administering rat in each litter had 24-hr access to the drug from the day of initial self-administration until the pulse label period immediately preceding sacrifice.

After stable baselines of self-administration developed, an average interinjection interval was calculated from the injection record for the previous 72 hr of each self-administering rat. At 5 (n=7 litters) or 10 (n=7 litters) min prior to a predicted morphine self-infusion, 0.5 mCi [methyl-^{3}H] choline chloride (Spec. Act. 15 Ci/mmol; New England Nuclear) was injected in 100 μl of saline through the jugular catheter from outside the chamber for determination of acetylcholine specific radioactivities and calculation of turnover rates. Infusions appropriately responded for by the self-administering animals during this pulse label interval were not delivered to control for non-specific general drug effects. However, the tone and light stimuli were presented during this period. Each litter was sacrificed at a time when the self-administering animal would predictably seek another infusion of morphine. The heads were removed and stored at -70°C for future analyses. The heads were warmed to −20°C, the brains removed, cut into 0.5 mm coronal sections and micro-dissected at −20°C into the following discrete regions: frontal, pyriform, cingulate and entorhinal-subicular cortices, caudate nucleus-putamen, hippocampal formation and amygdaloid complex. The tissue samples were individually pulverized in liquid N_2 in a stainless steel mortar and stored at −70°C until after acetylcholine turnover rates were determined. Membranes were then prepared from the remaining frozen tissue.

Preparation of Membranes

Frozen tissue powder from 2 to 3 rats from the same treatment group were combined (to total a minimum of 200 mg) and homogenized in 100 volumes of 50 mM KH_2PO_4/Na_2HPO_4, pH 7.4 buffer with a Polytron® PT-10 (setting 6; 30 seconds). The same litters and amounts of tissue from each animal were pooled for each condition so that the membrane preparation accurately reflected the treatment condition. The suspensions were centrifuged at 48,000 × g for 30 minutes at 4°C. Resuspension and centrifugation of the pellets were repeated twice. The pellets were then resuspended in 100 volumes of 50 mM KH_2PO_4/Na_2HPO_4, pH 7.4 buffer, protein concentrations determined [22] and the suspension frozen and stored at −20°C.

Receptor Analysis

The density and affinity of muscarinic cholinergic and benzodiazepine receptors were assessed using Rosenthal plots [33] and Scatchard analysis [35].

Muscarinic cholinergic receptors–[^{3}H]-quinuclidinyl benzilate (QNB) was used to assess muscarinic cholinergic receptor binding [48]. The membranes were thawed, resuspended and 0.25 ml added to tubes containing 0.75 ml of 50 mM phosphate buffer (10 mM KH_2PO_4 and 40 mM Na_2HPO_4, pH 7.4)±9 μM of atropine sulfate and 0.25 ml of five to seven concentrations (0.0375 nM to 2.4 nM) of [^{3}H]-QNB (New England Nuclear, specific radioactivity 40.2 Ci/mmol). Duplicate determinations of each condition (±atropine sulfate) at each concentration were used to evaluate binding. The tubes were incubated for 60 minutes at 25°C, the incubation terminated with the addition of 3 ml of 4°C 50 mM phosphate buffer and the membranes rapidly collected on Whatman GF/B glass fiber filters by suction filtration. The filters were rapidly washed twice with 3 ml of cold phosphate buffer, transferred to counting vials, 6 ml of HP/b (Beckman) scintillation fluid added and the vials stored overnight at 4°C. Radioactivity was determined with a Searle 6872 Isocap liquid scintillation spectrometer. Specific binding was calculated as the difference between that with and that without atropine sulfate. Rosenthal plots and Scatchard analysis were used to determine binding affinities (K_D in nM) and densities (B_{max} in fmole mg protein^{-1}).

Benzodiazepine receptors—[^{3}H]-flunitrazepam was used to assess benzodiazepine binding [42]. The membrane preparations were thawed, resuspended and 0.25 ml added to duplicate tubes containing 0.75 ml of 50 mM KCl/Na_2HPO_4, pH 7.4 buffer ±3 μM clonazepam and 0.25 ml of seven concentrations (0.125 nM to 8 nM) of [^{3}H]-flunitrazepam (Amserham Searle, specific radioactivity 84.8 Ci/mmol). Duplicate determinations of each concentration were used to evaluate binding. The samples were incubated 40 minutes at 4°C, the incubation terminated by the addition of 3 ml of 4°C 50 mM KCl/Na_2HPO_4 and the membranes rapidly collected on Whatman GF/B glass fiber filters by suction filtration. The filters were rapidly washed twice with 3 ml of cold KCl/Na_2HPO_4 buffer, transferred to counting vials, 6 ml of HP/b (Beckman) scintillation fluid added and the vials stored overnight at 4°C. Radioactivity in each sample was determined by liquid scintillation spectrometry and specific binding calculated as the difference between that with and that without clonazepam. Rosenthal plots and Scatchard analysis were used to determine binding affinities (K_D in nM) and densities (B_{max} in fmole mg protein^{-1}).

RESULTS

Behavioral

Stable rates of intravenous morphine self-administration were observed by the fourth week of exposure with an average interinjection interval of 132 minutes. For 10 of the 14 litters, the first completed ratio for an injection occurred when 92±8% (mean±S.D.) of the predicted interinjection interval had elapsed (52±25% for the other four litters) indicating that the calculated interinjection intervals were generally accurate estimates and that the probability of seeking another injection increased as this time approached. Lever pressing by the yoked animals were infrequent and appeared randomly distributed as would be expected for non-consequated behavior. The mean number of lever-press responses during the three-hour interval, just prior to sacrifice, were 28.4, 8.5 and 0.3 for the self-administering, yoked-morphine and yoked-vehicle infused groups, respectively.

Receptor Binding

Membranes prepared from the brains of animals previously pulse labelled with [^{3}H]-choline (5 or 10 minutes) did not show significant background radioactivity. Non-specific binding was not different between labelled or non-labelled animals probably resulting from the short [^{3}H]-choline pulse interval (5 and 10 minutes).

Scatchard analysis revealed differences in binding in six of the seven brain regions resulting from either passive infusion or self-administration of morphine (Figs. 1 and 2). Significant differences in receptor densities (B_{max}) but not in affinities (K_D) were observed in both muscarinic cholinergic and benzodiazepine receptors (Table 1). Receptor affinities and densities in the yoked-vehicle infused group were differentially distributed in brain regions and generally in agreement with previously reported values [18, 20, 23, 24, 50]. The highest affinities and densities of benzodiazepine receptors were seen in the frontal and cingulate cortices while the pyriform and entorhinal-subicular cortices showed the lowest affinities and the latter the lowest densities. The highest affinities of cholinergic muscarinic receptors were seen in the caudate nucleus-putamen, cingulate and entorhinal-subicular cortices, while the lowest affinities were seen in the frontal cortex and amygdaloid complex with the lowest densities in the latter.

Passive infusion of morphine (yoked-morphine infused group compared with the yoked-vehicle infused group) resulted in significant decreases in the densities of muscarinic cholinergic receptors in the cingulate cortex (21%) and increases in the pyriform cortex (11%) while benzodiazepine receptor densities were decreased in the entorhinal-subicular cortex (29%) and hippocampal formation (13%). These changes were also present in the self-administering animals and, therefore, may primarily represent alterations in neuronal activity related to the general pharmacological actions of morphine. The self-administration of morphine (self-administration group compared with the yoked-morphine infused group) resulted in significant additional increases in the densities of cholinergic muscarinic receptors in the amygdaloid complex (13%) and decreases in the frontal (29%) and entorhinal-subicular (19%) cortices.

DISCUSSION

These receptor changes indicate that chronic passive infusion or self-administration of morphine results in long du-

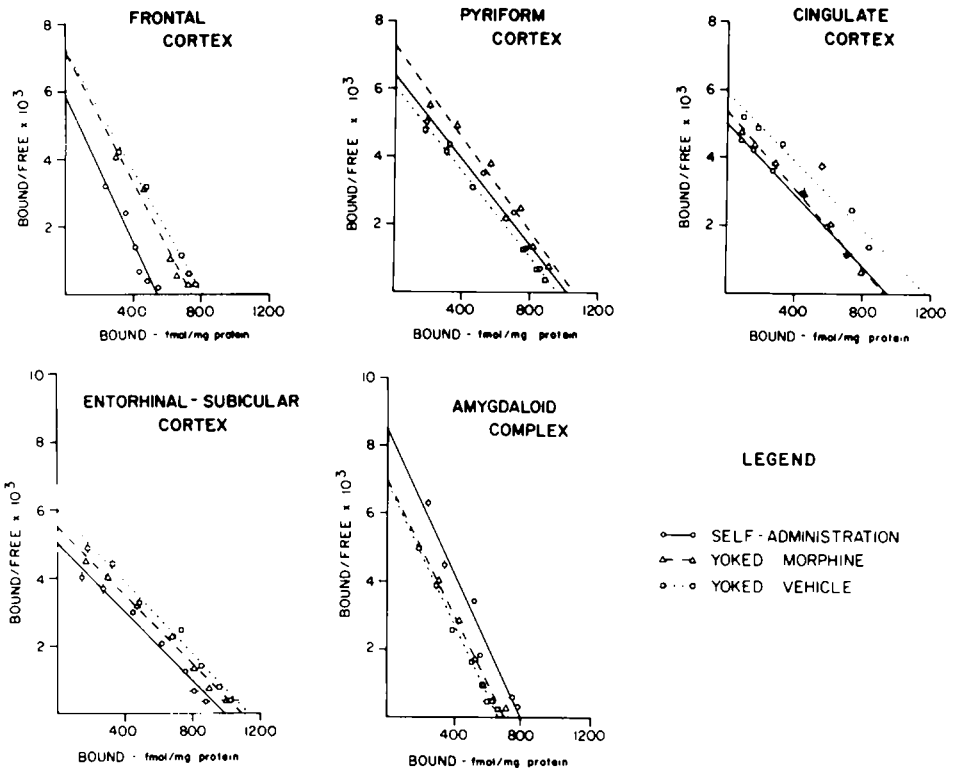

FIG. 1. Scatchard plots of the statistically significant differential saturable binding of [^{3}H]-quinuclidinyl benzilate in membranes from the frontal, pyriform, cingulate and entorhinal-subicular cortices and amygdaloid complex of rats intravenously self-administering morphine (○) and in yoked-morphine (△) and yoked-vehicle (□) infused littermates. Values are the average of four to six independent Scatchard analyses. The error measures are SEM (horizontal and vertical bars) and are indicated only if greater than the point itself.

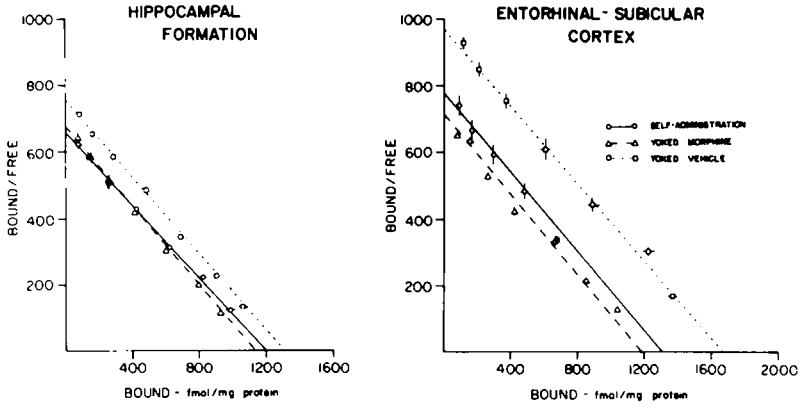

FIG. 2. Scatchard plots of the statistically significant differential saturable binding of [^{3}H]-flunitrazepam in the hippocampal formation and entorhinal-subicular cortex of groups of littermate rats intravenously self-administering morphine (○) and in yoked-morphine (△) and yoked-vehicle (□) infused littermates. Values are the average of four to six independent Scatchard analyses. The error measures are SEM (horizontal and vertical bars) and are indicated only if greater than the point itself.

TABLE 1

BINDING PARAMETERS FOR [^{3}H]-FLUNITRAZEPAM AND [^{3}H]-QUINUCLIDINYL BENZILATE IN THE FRONTAL, PYRIFORM, CINGULATE AND ENTORHINAL-SUBICULAR CORTICES, HIPPOCAMPAL FORMATION, AMYGDALOID COMPLEX AND CAUDATE NUCLEUS PUTAMEN OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE AND LITTERMATES RECEIVING YOKED INFUSIONS OF MORPHINE OR VEHICLE

		[^{3}H]-Flunitrazepam		[^{3}H]-Quinuclinidnyl Benzilate	
		K_D (nM)	B_{max} (fmole/mg Protein)	K_D (nM)	B_{max} (fmole/mg Protein)
Frontal Cortex§	SA¶	2.39	1928.6 ± 356.0	0.09	546.5 ± 30.1‡
	YM¶	2.36	1928.5 ± 173.2	0.11	745.5 ± 36.3
	YV¶	2.38	1864.5 ± 231.2	0.11	808.0 ± 51.3
Pyriform Cortex	SA	1.68	1494.4 ± 337.3	0.16	1022.0 ± 57.6
	YM	1.63	1460.2 ± 198.6	0.15	1062.8 ± 24.7†
	YV	1.69	1529.9 ± 170.4	0.16	957.8 ± 49.0
Cingulate Cortex	SA	2.11	1596.1 ± 470.5	0.19	947.3 ± 54.3
	YM	2.17	1568.2 ± 151.4	0.17	928.0 ± 90.7†
	YV	2.07	1610.3 ± 376.0	0.20	1171.3 ± 49.0
Entorhinal-Subicular Cortex	SA	1.70	1310.5 ± 54.3	0.20	989.0 ± 50.6*
	YM	1.66	1191.3 ± 84.4†	0.20	1085.6 ± 27.4
	YV	1.73	1669.5 ± 155.1	0.19	1131.5 ± 66.8
Hippocampal Formation	SA	1.81	1201.2 ± 92.1	0.16	798.8 ± 89.2
	YM	1.69	1132.0 ± 27.5†	0.15	762.2 ± 25.7
	YV	1.72	1304.7 ± 73.6	0.15	812.0 ± 53.8
Amygdaloid Complex§	SA	[a]	[a]	0.09	782.5 ± 43.0*
	YM	[a]	[a]	0.10	693.5 ± 33.8
	YV	[a]	[a]	0.10	653.5 ± 41.5
Caudate Nucleus-Putamen	SA	[a]	[a]	0.21	1174.1 ± 81.6
	YM	[a]	[a]	0.23	1162.1 ± 54.6
	YV	[a]	[a]	0.22	1150.7 ± 25.1

Values are means ± S.D. for 4 to 7 separate Scatchard analysis using 5 to 7 concentrations of the ligand. Significant differences between means determined with Student's t-test were: *$p<0.05$; †$p<0.01$; ‡$p<0.001$. The self-administering group was compared with the yoked-morphine group and the latter with the yoked-vehicle infused group.

§Represents 2 separate Scatchard analysis at 7 different concentrations of ligand.

¶SA = morphine self-administration; YM = yoked morphine infused; YV = yoked vehicle infused.

[a]No measurements because of insufficient sample.

ration changes in cholinergic neuronal activity and possibly in the activity of cells releasing endogenous ligands for benzodiazepine receptors. The differences in receptor densities in the passive-morphine infused animals compared with the vehicle infused group are considered to result primarily from the general effects of chronic administration of the drug, while the differences between the self-administering animals and passive morphine infused group are considered to result primarily from the reinforcing properties of the drug. These animals were sacrificed at a time when the self-administering rats were most likely to seek a drug infusion. This drug-seeking behavior is assumed to be an integral part of the reinforcement processes and may result in neuronal changes that are even more specific than those occurring following the presentation of the reinforcer which would include alterations in neurons resulting from other pharmacological actions of the drug. Although it is true that the presentation of morphine to the yoked-passively infused animals may be adventitiously reinforcing, there is no programmed contingency and, therefore, these animals cannot control the delivery of the reinforcer. While it is probable that some of the changes in this group may result from reinforcement, the changes seen in the self-administering animals are assumed to be more indicative of reinforcement processes.

Receptor dynamics interpretation of the data would conclude that an increase in density of receptors resulted from a net decrease in neurotransmitter release and a decrease resulted from a net increase in release. Thus, chronic passive morphine infusions would be assumed to have resulted in an increase in acetylcholine turnover in the cingulate cortex and a decrease in the pyriform cortex. However in neither of these regions was the turnover of ACh different from vehicle control [39]. Muscarinic cholinergic receptors have been previously shown to respond dynamically to agonists and/or antagonists for these receptors [6, 16, 24]. Reports of changes in cholinergic neuronal activity in response to chronic morphine treatment include pharmacological demonstrations of decreased sensitivity to cholinergic antagonists [49], neurophysiological demonstration of supersensitivity of cortical neurons to iontophoresed ACh [34] and behavioral demonstrations of supersensitivity to cholinergic agonists [5,45]. The increases in muscarinic cholinergic re-

TABLE 2

PERCENT AND DIRECTION OF SIGNIFICANT CHANGES IN ACETYLCHOLINE TURNOVER RATES AND MUSCARINIC CHOLINERGIC RECEPTOR DENSITIES IN SEVEN BRAIN REGIONS AS A RESULT OF PASSIVE INTRAVENOUS MORPHINE INFUSION OR INTRAVENOUS MORPHINE SELF-ADMINISTRATION

	Passive Morphine		Self-Administration	
	Muscarinic Cholinergic Receptors	Acetylcholine Turnover*	Muscarinic Cholinergic Receptors	Acetylcholine Turnover
Frontal Cortex	—	78↑	27↓	44↑
Pyriform Cortex	11↑	—	—	46↓
Cingulate Cortex	21↓	—	—	—
Entorhinal-Subicular Cortex	—	—	9↓	—
Hippocampal Formation	—	—	—	—
Amygdaloid Complex	—	—	13↑	46↓
Caudate Nucleus-Putamen	—	—	—	—

*The acetylcholine turnover rates from which these values were derived are published elsewhere [39].

ceptor densities observed in the pyriform cortex is consistent with this supersensitivity but the changes in the cingulate cortex are not. The receptor dynamics interpretation would also conclude that an increase in turnover of the endogenous ligands for the benzodiazepine receptors occurred in the entorhinal-subicular cortex and hippocampal formation in response to that passive infusion of morphine. Benzodiazepine receptors have been demonstrated to be altered in pathological conditions and stressful environments. Benzodiazepine receptors were decreased in the brains of an "emotional" strain of mice [32], in the cerebellum of "nervous" mutant mice [36] and in the cerebral cortex of rats whose food responding was suppressed [21], while increases were seen in the cerebral cortex of rats after experimentally induced seizures [28]. Benzodiazepine receptors were decreased in the hippocampal formation and entorhinal-subicular cortex in both morphine-treated groups which may suggest the locus of some of the anxiolytic effects of this drug. The direction of the change would suggest that chronic morphine either directly or indirectly increased release of endogenous ligands for the benzodiazepine receptor in these important limbic regions.

Similarly, the chronic long-term self-administration of morphine would be assumed to have resulted in a decrease in ACh turnover in the amygdala and increases in the frontal and entorhinal-subicular cortices. Cholinergic receptors have been previously demonstrated to be altered with pathological and environmental conditions. Muscarinic cholinergic receptors were increased in the posterior hypothalmus of hypertensive rats [17] and decreased in the cerebral cortex of rats whose food responding was suppressed by the presentation of a stimulus previously paired with unavoidable foot shock [21]. The decreases in muscarinic cholinergic binding in the frontal and entorhinal-subicular cortices and increases in the amygdala of the self-administering rats implicate cholinergic involvement in behavioral processess and demonstrate the heterogeniety of this neuronal system. Subtle changes in the mode of administration of identical drug dosages (passive vs contigent infusion) resulted in significant and long duration changes in the activity of specific cholinergic fiber systems.

Turnover rates of ACh were determined in fourteen brain regions of these animals [39] with the results not completely in agreement with the classic interpretation of neurohumor turnover-receptor dynamics. Compensatory changes in receptors in the opposite direction of changes in turnover rates were seen only in the frontal cortex and amygdaloid complex of the self-administering group (Table 2). In three other regions, receptor changes were observed where turnover rate changes were not, and in two regions (frontal cortex of yoked-morphine infused group and pyriform cortex of the self-administering group) large turnover rate changes were not accompanied by changes in receptors. This is somewhat disconcerting unless the parameters and limitations of turnover rate methodologies are considered. Acetylcholine turnover is determined with relatively short pulse lengths (2 to 10 minutes) with the rates representative only of neuronal events occurring during this interval. The average interinjection interval of the self-administering animals was 132 minutes. The pulse label periods were 5 or 10 minutes just prior to a predicted self-infusion. The major direct effects of the drug probably occurred shortly after administration which was 122 to 127 minutes earlier. For these reasons, cholinergic receptor densities may be more representative than turnover rates of long duration net changes in cholinergic neuronal activity. Prior to the pulse label interval, overall cholinergic neuronal activity was probably elevated in the cingulate cortex and decreased in the pyriform cortex of the passively infused animals resulting in the receptor density changes. Since these same receptor changes were also present in the self-administering animals, it is possible that cholinergic innervations of the cingulate cortex may be involved in the cortical effects of opiates on pain perception. Humans often report the presence of pain that just does not bother them as much, suggesting such cortical involvement which could include these cholinergic innervations. The increased turnover of acetylcholine in the frontal cortex [39] of the passive morphine infused group appears to be transient since receptor densities were not altered.

The receptor changes indicate that the opportunity to self-administer morphine appears to have resulted in two significant changes in cholinergic neuronal activity—increases in the frontal cortex and decreases in the amygdaloid complex. Turnover changes were also seen in these

areas [39] which represent significant long duration net fluctuations in activity since compensatory changes in receptor densities occurred. The decrease in turnover in the pyriform cortex is likely transient and/or of short duration since receptor densities were not altered. The decrease in receptors in the entorhinal-subicular cortex suggest that a net increase in cholinergic neuronal activity occurred in this region in the self-administering animals, but prior to the pulse label period since no change in turnover rate was detected [39].

These data suggest that activity in the ventral pallidal-frontal cortex, diagonal band-amygdaloid complex and amygdaloid complex—entorhinal-subicular cortex cholinergic tracts were altered for substantial periods in the self-administering rats, further supporting the role of these pathways in two neuronal circuits proposed to mediate some components of opiate reinforcement [40]. The dopaminergic hypothesis for the initiation of these processes [47] is likely an oversimplification of a complex process in which dopamine participates. Opiate receptors outside the VTA initiate neuronal activity of a reinforcing nature [15, 25, 26, 43, 44] which may involve the three cholinergic fiber tracts pinpointed with these changes in muscarinic cholinergic receptors.

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REFERENCES

1. Belluzzi, J. D. and L. Stein. Enkephalin may mediate euphoria and drive-reduction reward. *Nature* **266:** 556–558, 1977.
2. Bozarth, M. A. and R. A. Wise. Intracranial self-administration of morphine into the ventral tegmental area in rats. *Life Sci* **28:** 551–555, 1981.
3. Bozarth, M. A. and R. A. Wise. Localization of the reward-relevant opiate receptors. In: *NIDA Research Monograph Problems of Drug Dependence, 1981*, edited by L. S. Harris. Rockville, MD: Committee on Problems of Drug Dependence, Inc., 1982, pp. 158–164.
4. Brown, Z. W., Z. Amit and J. R. Weeks. Simple flow-thru swivel for infusions into unrestrained animals. *Pharmacol Biochem Behav* **5:** 363–365, 1976.
5. 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:** 27–35, 1979.
6. Costa, L. G., B. W. Schwab and S. D. Murphy. Muscarinic receptor alterations following neostigmine treatment. *Eur J Pharmacol* **80:** 275–278, 1982.
7. Davis, W. M. and S. G. Smith. Central cholinergic influence on self-administration of morphine and amphetamine. *Life Sci* **16:** 237–246, 1975.
8. Davis, W. M., S. G. Smith and J. H. Khalsa. Noradrenergic role in the self-administration of morphine or amphetamine. *Pharmacol Biochem Behav* **3:** 477–484, 1975.
9. Glick, S. D. Changes in sensitivity to the rewarding properties of morphine following lesions of the medial forebrain bundle or caudate nucleus in rats. *Arch Int Pharmacodyn* **212:** 214–220, 1974.
10. Glick, S. D. and A. D. Charap. Morphine dependence in rats with medial forebrain bundle lesions. *Psychopharmacologia* **30:** 343–348, 1973.
11. Glick, S. D. and R. D. Cox. Dopaminergic and cholinergic influence on morphine self-administration in rats. *Res Commun Chem Pathol Pharmacol* **12:** 17–24, 1975.
12. Glick, S. D. and R. D. Cox. Changes in morphine self-administration after brainstem lesions in rats. *Psychopharmacology (Berlin)* **52:** 151–156, 1977.
13. Glick, S. D. and R. D. Cox. Changes in morphine self-administration after tel-diencephalic lesions in rats. *Psychopharmacology (Berlin)* **57:** 283–288, 1978.
14. Glick, S. D., R. D. Cox and A. M. Crane. Changes in morphine self-administration and morphine dependence after lesions of the caudate nucleus in rats. *Psychopharmacologia* **41:** 219–224, 1975.
15. Goeders, N. E., J. D. Lane and J. E. Smith. Self-administration of methionine-enkephalin into the nucleus accumbens. *Pharmacol Biochem Behav* **20:** 451–455, 1984.
16. Grimm, V. E. and M. Hershkowitz. The effect of chronic diazepam treatment on discrimination performance and ^{3}H-flunitrazepam binding in the brains of shocked and nonshocked rats. *Psychopharmacology (Berlin)* **74:** 132–136, 1981.
17. Hershkowitz, M., S. Eliash and S. Cohen. The muscarinic cholinergic receptors in the posterior hypothalamus of hypertensive and normotensive rats. *Eur J Pharmacol* **86:** 229–236, 1983.
18. Kobayashi, R. M., M. Palkovits, R. E. Hruska, R. Rothschild and H. I. Yamamura. Regional distribution of muscarinic cholinergic receptors in rat brain. *Brain Res* **154:** 13–23, 1978.
19. Kornetsky, C. and G. Bain. Effects of opiates on rewarding brain stimulation. In: *The Neurobiology of Opiate Reward Processes*, edited by J. E. Smith and J. D. Lane. Amsterdam: Elsevier, 1983, pp. 237–256.
20. Kuhar, M. J. and H. I. Yamamura. Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. *Brain Res* **110:** 229–243, 1976.
21. Lane, J. D., C. M. Crenshaw, G. F. Guerin, D. R. Cherek and J. E. Smith. Changes in biogenic amine and benzodiazepine receptors correlated with conditioned emotional response and its reversal by diazepam. *Eur J Pharmacol* **83:** 183–190, 1982.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
23. Mackerer, C. R., R. L. Kochman, B. A. Bierschenk and S. S. Bremer. The binding of [^{3}H]-diazepam to rat brain homogenates. *J Pharmacol Exp Ther* **206:** 405–413, 1978.
24. Mohler, H. and T. Okada. Benzodiazepine receptor: Demonstration in the central nervous system. *Science* **198:** 849–851, 1977.
25. Olds, M. W. Hypothalamic substrate for the positive reinforcing properties of morphine in the rat. *Brain Res* **168:** 351–360, 1982.
26. Olds, M. E. Reinforcing effects of morphine in the nucleus accumbens. *Brain Res* **237:** 429–440, 1982.
27. Overstreet, D. H. and H. I. Yamamura. Minireview—Receptor alterations and drug tolerance. *Life Sci* **25:** 1865–1878, 1979.
28. Paul, S. M. and P. Skolnick. Rapid changes in brain benzodiazepine receptors after experimental seizures. *Science* **202:** 892–894, 1978.
29. Pickens, R. and J. Dougherty. A method for chronic intravenous infusion of fluid into unrestrained rats. Reports from Res. Labs., Dept. Psychiat., Univ. MN. No. PR-72-1, 1972.
30. Pozuelo, J. and F. W. Kerr. Suppression of craving and other signs of dependence in morphine-addicted monkeys by administration of alpha-methyl-para-tyrosine. *Mayo Clin Proc* **47:** 621–628, 1972.

31. Reid, L. D. and S. M. Siviy. Administration of opiate antagonists reveals endorphinergic involvement in reinforcement processes. In: *The Neurobiology of Opiate Reward Processes*, edited by J. E. Smith and J. D. Lane. Amsterdam: Elsevier, 1983, pp. 257–279.
32. Robertson, H. A. Benzodiazepine receptors in "emotional" and "non-emotional" mice: Comparison of four strains. *Eur J Pharmacol* **56:** 163–166, 1979.
33. Rosenthal, H. E. A graphic method for the determination and presentation of binding parameters in a complex system. *Anal Biochem* **20:** 525–532, 1967.
34. Satoh, M., W. Zieglganzberger and A. Herz. Supersensitivity of cortical neurons of the rat to acetylcholine and L-glutamate following chronic morphine treatment. *Naunyn Schmiedebergs Arch Pharmacol* **293:** 101–103, 1976.
35. Scatchard, G. The attractions of proteins for small molecules or ions. *Ann NY Acad Sci* **51:** 660–672, 1949.
36. Skolnick, P., P. J. Syapin, B. A. Paugh and S. M. Paul. Reduction in benzodiazepine receptors associated with Purkinje cell degeneration in "nervous" mutant mice. *Nature* **277:** 397–399, 1979.
37. Smith, J. E., C. Co, M. E. Freeman and J. D. Lane. Brain neurotransmitter turnover correlated with morphine-seeking behavior of rats. *Pharmacol Biochem Behav* **16:** 509–519, 1982.
38. Smith, J. E., C. Co, M. E. Freeman, M. P. Sands and J. D. Lane. Neurotransmitter turnover in rat stiratum is correlated with morphine self-administration. *Nature* **287:** 152–153, 1980.
39. Smith, J. E., C. Co and J. D. Lane. Limbic acetylcholine turnover rates correlated with rat morphine-seeking behaviors. *Pharmacol Biochem Behav* **20:** 429–441, 1983.
40. Smith, J. E. and J. E. 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. Amsterdam: Elsevier, 1983, pp. 361–402.
41. Smith, S. G. and W. M. Davis. Haloperidol effects morphine self-administration: Testing for pharmacological modification of the primary reinforcement mechanisms. *Psychol Rec* **23:** 215–221, 1973.
42. Squires, R. F. and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* **266:** 732–734, 1977.
43. Stein, E. A. and J. Olds. Direct intracerebral self-administration of opiates in the rat. *Soc Neurosci Abstr* **3:** 302, 1977.
44. Stein, E. A. and J. Zerneskie. Is reward behavior mediated by an endogenous opiate system? *Soc Neurosci Abstr* **5:** 573, 1979.
45. Vasquez, B. J., D. H. Overstreet and R. W. Russell. Psychopharmacological evidence for increase in receptor sensitivity following chronic morphine treatment. *Psychopharmacologia* **38:** 287–302, 1974.
46. Weeks, J. R. Experimental morphine addiction: Methods for automatic intravenous injections in unrestrained rats. *Science* **138:** 143–144, 1962.
47. Wise, R. A. and M. A. Bozarth. Brain substrates for reinforcement and drug self-administration. *Prog Neuropsychopharmacol* **5:** 467–474, 1981.
48. Yamamura, H. I. and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc Natl Acad Sci USA* **71:** 1725–1729, 1974.
49. Yarbrough, G. G. Actions of acetylcholine and atropine on cerebral cortical neurons in chronically morphine-treated rats. *Life Sci* **15:** 1523–1529, 1974.
50. Young, W. S., III and M. J. Kuhar. Radiohistochemical localization of benzodiazepine receptors in rat brain. *J Pharmacol Exp Ther* **212:** 337–346, 1980.