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Limbic Acetylcholine Turnover Rates Correlated With Rat Morphine-Seeking Behaviors

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SMITH, J. E., C. CO AND J. D. LANE. *Limbic acetylcholine turnover rates correlated with rat morphine-seeking behaviors.* PHARMACOL BIOCHEM BEHAV **20**(3) 429–442, 1984.—Acetylcholine (ACh) turnover rates were measured in fourteen brain regions of rats intravenously self-administering morphine and in yoked-morphine and yoked-vehicle infused littermates to identify cholinergic neuronal pathways potentially involved in opiate reinforcement processes. Rats receiving chronic passive administration of morphine had increased ACh turnover rates in the frontal cortex and diagonal band and decreased rates in the medial septum. The significant changes in animals self-administering the drug were prominent in limbic regions with increases in the frontal cortex and decreases in the pyriform cortex, nucleus accumbens, amygdala and ventral tegmental area. Some components of opiate reinforcement may be mediated by increases in the activity of cholinergic ventral pallidal and diagonal band fibers innervating the frontal cortex and by decreases in activity of cholinergic fibers innervating the ventral tegmental area. These data and turnover rates for dopamine, norepinephrine, serotonin, aspartate, glutamate and gamma-aminobutyric acid previously determined in similarly treated animals are consistent with two neuronal circuits that may be involved in opiate seeking behaviors and opiate reinforcement processes.

Acetylcholine turnover rates Intravenous morphine self-administration Chronic intravenous morphine Opiate reinforcement

THE self-administration of opiates is thought to result primarily from the reinforcing properties of these drugs. Animal behavioral studies have shown response-contingent presentation of morphine and heroin to be reinforcing stimuli that will maintain high rates of responding on intermittent schedules of reinforcement [34,64]. At the cellular level, the reinforcing properties of opiates must result from drug interactions with brain opiate receptors initiating neuronal events in specific pathways. Most opiate receptors are probably localized on membranes of neurons that release neurotransmitters and neuromodulators other than the endogenous ligands for these receptors. Activation or inhibition of subpopulations of neurons by the opiate results in the initiation and propagation of neuronal activity indicative of the occurrence of a reinforcing event. The location and nature of these neurons have been under vigorous investigation. The involvement of cholinergic [8,16], dopaminergic [16, 41, 57] and noradrenergic [9] neurons have been implicated by decrements in self-administration observed after treatment with drugs that interfere with these neuronal systems. The locus of the catecholamine and amino acid neurotransmitter involvement has been suggested from turnover rate measurements in intravenous morphine self-administering rats [53,54]. Turnover rates of dopamine (DA), norepinephrine (NA), serotonin (5-HT), aspartate (Asp), glutamate (Glu), glycine (Gly) and gamma-aminobutyric acid (GABA) were concurrently measured in small brain regions with the results suggesting two neuronal circuits that may be involved in these reinforcement processes [56] (Fig. 1). Several cholinergic tracts were hypothesized to participate in these circuits. The present study was initiated to directly assess cholinergic involvement by measuring acetylcholine (ACh) turnover rates in brain regions of rats intravenously self-administering morphine and in yoked-morphine and yoked-vehicle infused littermates.

METHOD

Subjects

Fourteen litters of three adult male Fischer F-344 rats (90–150 days old) were used in this investigation to identify neurochemical changes that result from the reinforcing properties of morphine. One littermate in each litter was allowed to intravenously self-administer morphine, while a second littermate received a simultaneous equivalent infusion of morphine and a third littermate the equivalent volume of vehicle (saline). This experimental design permits the general effects of the drug (analgesia, physical dependence, tolerance, etc.) on brain neurotransmitters to be estimated (yoked-morphine littermate compared with yoked-vehicle infused littermate) and isolated from the reinforcing effects of the drug-taking milieu (self-administering littermate compared with the yoked-morphine infused littermate).

Surgical

The rats were implanted with chronic jugular catheters

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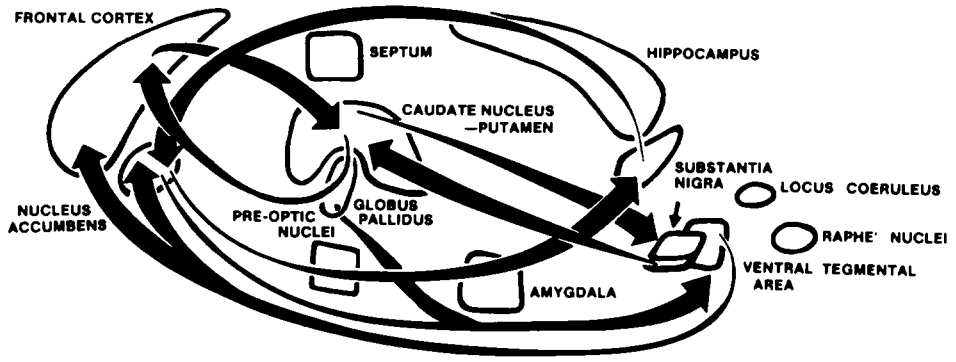

FIG. 1. Neuronal circuits proposed to mediate opiate reinforcement processes. The circuits include a hippocampal formation—nucleus accumbens—amygdaloid complex—entorhinal-subicular cortex—hippocampal formation circuit and a frontal cortex—caudate nucleus-putamen—globus pallidus—frontal cortex circuit. Activity in these circuits may be modulated by the substantia nigra, ventral tegmental area, locus coeruleus and raphe nuclei. Feedback pathways to the brainstem in turn could modulate activity in these centers. The septum and preoptic region may also be important in these neuronal circuits.

using previously described procedures [40,63]. 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 immediately behind the scapulae through a plastic-stainless steel harness. The plastic portion of the harness was implanted under the skin providing a point of attachment for a needle tubing and metal spring leash which enclosed the catheter and passed through the top of the animal chamber to a leak-proof swivel [5] which was attached to one syringe in a three-syringe infusion pump with polyvinyl chloride tubing. The leash and swivel were counter balanced to permit freedom of movement. The patency of each catheter was checked periodically (every 2 weeks and 3 days prior to sacrifice) by injecting 100 μl of 5% (w/v) sodium thiopental. Immediate anesthesia (2–3 seconds) indicated a functional catheter.

Self-Administration

Each litter was housed in a large sound-attenuated chamber in individual self-administration cages with unlimited access to food and water. Each chamber was equipped with a house light that was illuminated on a reversed 12-hr light and 12-hr dark cycle and a speaker which maintained a constant level of white noise to mask external auditory stimuli. Each self-administration cage contained a lever (which was not present initially) and a stimulus light above the lever.

The three littermates in each group received hourly infusions of 200 μl of heparinized saline the first two days after surgery. 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, 24 infusions per day). The third littermate continued to receive hourly heparinized-saline infusions. These hourly injections 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, hourly infusions were discontinued and levers introduced into each self-administration cage. 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 presentation of the tone and light stimuli to all three littermates. The other physically dependent littermate received an identical yoked-morphine infusion and the third littermate, yoked-vehicle whenever the self-administering animal received an infusion. Initially, one lever press resulted in an infusion, however, 10 lever presses were eventually required (fixed ratio 10 schedule). A fixed ratio 10 schedule was utilized to require some minimal response cost to decrease the likelihood of adventitious reinforcement yet small enough so that no motor differences were introduced. Both yoked-infused littermates had identical levers in their cages, but lever presses had no programmed consequences. The self-administering animal 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.

Pulse Labelling

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) in 100 μl of saline was injected through the jugular catheter from outside the chamber. Infusions were not delivered during the pulse-label interval to control for non-specific general drug effects. However, the tone and light stimuli (conditioned reinforcers) were presented during this period if the response requirement was met. The three rats in each litter were sacrificed at the predicted infusion time by simultaneous immersion of the self-administration cages in liquid nitrogen for 5 minutes (completely frozen). Thus, each litter was sacrificed at a time when the self-administering animal would predictably seek an 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 1.0 mm coronal sections and micro-

dissected at −20°C into the following discrete regions: frontal cortex, pyriform cortex, cingulate cortex, motor-somatosensory cortex, entorhinal-subicular cortex, nucleus accumbens, caudate nucleus-putamen, globus pallidus, diagonal band, medial septum, hippocampal formation, amygdaloid complex, ventral tegmental area and substantia nigra. The larger tissue samples were individually pulverized in liquid N_2 in a stainless steel mortar and stored at −70°C until extraction and assay. Smaller samples were pulverized singly or pooled and pulverized immediately before extraction and assay.

Acetylcholine and Choline Assay

ACh and Choline (Ch) were extracted from 10–100 mg of pulverized frozen tissue into 1 ml 1 N formic acid/acetone (15/85:v/v). A reagent blank and tissue blanks with different levels of ACh and Ch standards added were processed in parallel. [^{3}H]-ACh or [^{3}H]-Ch were also added to the tissue blanks for correction of radioactive recovery. The formic acid/acetone extraction was repeated and the pellets saved for protein determinations [30]. Lipids and lipoproteins were removed with a wash of 3 volumes of heptane/chloroform (8/1:v/v) and the organic layer and interphase aspirated and discarded. The aqueous solutions were dried under a stream of dry nitrogen and stored at −20°C.

The dried extracts were reconstituted with 170 μl of pH 4 water, centrifuged at 1000 × g for 10 min and 160 μl transferred to a 1 ml test tube containing 5 μl 0.01 M tetramethylammonium bromide and 50 μl periodide solution (0.5 g potassium iodide + 1.0 g iodine crystals in 10 ml H_2O). The tubes were mixed thoroughly and incubated in an ice bath for 20 min, centrifuged at 2000 × g for 15 min and the supernatant discarded. The precipitates were dissolved in 100 μl pH 4 water and excess periodide removed with 400 μl ether washes. The ether washes were repeated until the aqueous solutions were colorless (2 to 3 times) and then placed in a boiling water bath for 10 min to remove excess ether and iodine. After cooling, the tubes were dried in a vacuum desiccator centrifuge and stored at −20°C.

The dried ACh and Ch extracts were reconstituted with 100 μl pH 4 water, four 15 μl and one 35 μl aliquots were transferred to separate microfuge tubes and dried again in the vacuum desiccator centrifuge. Two of the dried 15 μl portions were used for measurement of ACh and the other two for Ch. The 35 μl portion was used to separate [^{3}H]-Ch and [^{3}H]-ACh by paper electrophoresis for specific radioactivity determinations.

ACh and Ch were assayed with a previously reported radioenzymatic procedure using choline kinase and choline acetyltransferase [51]. Briefly, for ACh measurements, 30 μl of a choline kinase reaction mixture were added to one set of tubes, final concentration: 0.15 M sodium phosphate pH 8.0, 15 mM neutralized adenosine triphosphate, 15 mM $MgCl_2$ and 0.005 units choline kinase (Sigma). The tubes were incubated for 30 min at 37°C and then the ACh hydrolyzed with 20 μl of 2 N NH_4OH by allowing the samples to stand at room temperature for 20 min. The samples were taken to dryness in a vacuum desiccator centrifuge. The Ch freed by the hydrolysis of ACh was then measured by adding 25 μl of a choline acetyltransferase reaction mixture (final concentration: 60 mM sodium phosphate, pH 8.0, 0.1 mM eserine salicylate, 0.06 mM acetyl [^{14}C]-coenzyme A and 0.00045 units of choline acetyltransferase). The tubes were mixed thoroughly and incubated at 37°C for 1 hr, the [^{14}C]-ACh extracted with 100 μl of sodium tetraphenylboron in 3-butenenitrile (30 mg/ml), 85 μl of the butenenitrile solution transferred to scintillation vials, 10 ml of Aquasol-2 (New England Nuclear) added and the radioactivity in each sample determined by liquid scintillation spectrophotometry. Ch was measured in the other two samples by omitting the choline kinase and alkaline hydrolysis steps and proceeding directly to the choline acetyltransferase reaction.

Separation of [^{3}H]-Acetylcholine and [^{3}H]-Choline

The [^{3}H]-ACh synthesized endogenously was separated from [^{3}H]-Ch by paper electrophoresis using a previously reported precedure [10]. Briefly, 10 μl of 3 mM tetraethylammonium bromide and 10 μl ethanol were added to the dried 35 μl aliquots of the tissue extracts. The mixture was applied to a SS No. 2043-A paper electrophoresis strip (Schleicher and Schnell) and the tubes washed with 20 μl ethanol which was applied to the same spot. ACh and Ch were separated by electrophoresis for 90 min at 500 volts in a buffer of 7.5% acetic acid:2.5% formic acid. The paper strips were removed, placed in an iodine vapor tank, the tetraethylammonium bromide (which co-migrates with ACh) visualized, marked, separated from the strips and placed in scintillation vials. The Ch region determined from the tissue blanks with added [^{3}H]-Ch was also separated from the paper strips and placed in scintillation vials. [^{3}H]-ACh and [^{3}H]-Ch were eluted with 0.5 ml pH 4 water, 10 ml HP/b Ready Solv® (Beckman) added and the radioactivity measured by liquid scintillation spectrophotometry.

Calculation of Turnover Rates

Turnover rates were determined with a previously reported method with the assumption that radiolabel was disappearing from a single open pool [66] since there is no acceptable method for determining CNS intraneuronal compartmentation *in vivo*. Thus, turnover ACh = K × content ACh where the apparent fractional rate constant (K) was calculated:

$$K = \frac{\ln 2}{t1/2}$$

and the t1/2 was extrapolated from a semilogarithmic plot of the specific radioactivities (dpm nmol^{-1}) obtained at the two pulse times on the linear portion of the decay in radioactivity curve. The apparent fractional rate constants (K) were determined by calculating a grand mean from the average values obtained by comparing each specific radioactivity value at the 5 min pulse interval with each at the 10 min pulse interval. The rate constant is the mean and the error estimates the standard deviation of these average K values. The turnover rate is expressed as nmol mg protein^{-1} hr^{-1} and is the product of the rate constant (hr^{-1}) and the content values (pmol mg protein^{-1}). These turnover rates are assumed to be representative of the utilization of ACh.

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. Typical fixed ratio patterns of responding were observed in the self-

TABLE 1

CONTENT OF ACETYLCHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

Brain Area	Content (pmoles mg protein^{-1}) Morphine Self-Administration	Yoked-Morphine Infused	Yoked-Vehicle Infused
Frontal Cortex	132.5 ± 26.2	130.5 ± 23.0	115.3 ± 12.6
Pyriform Cortex	158.6 ± 59.1	145.4 ± 51.4	164.2 ± 69.5
Cingulate Cortex	73.0 ± 22.9	75.1 ± 12.1	69.0 ± 15.2
Motor-Somatosensory Cortex	73.1 ± 17.5	78.8 ± 15.5	74.7 ± 10.8
Entorhinal-Subicular Cortex	114.4 ± 24.3	116.7 ± 12.9	128.1 ± 23.2
Nucleus Accumbens	165.1 ± 60.2	205.7 ± 60.3	179.6 ± 85.4
Caudate Nucleus Putamen	556.8 ± 32.4	587.1 ± 73.0	577.8 ± 64.4
Globus Pallidus	109.6 ± 5.0*	147.0 ± 4.6	141.6†
Diagonal Band	115.4 ± 35.9	121.6 ± 39.4	114.2 ± 43.0
Medial Septum	147.2 ± 26.8*	202.6 ± 19.6	203.0 ± 23.9
Hippocampal Formation	125.2 ± 33.5	140.5 ± 17.8	126.3 ± 21.2
Amygdaloid Complex	409.2 ± 113.3	416.0 ± 117.6	435.3 ± 66.7
Ventral Tegmental Area	110.0 ± 20.1	119.5 ± 23.6	136.9 ± 42.0
Substantia Nigra	83.1 ± 7.5	98.5†	65.1 ± 0.6

Values are means ± S.D. for N=14 per treatment condition. The significance of the difference between means determined with Students' t-test were *$p<0.05$. The self-administering group was compared with the yoked-morphine infused group and the latter with the yoked-vehicle group.

†Represents value for a single pooled sample determination.

administering animals with a moderately rapid rate of successive lever presses occurring just prior to each injection. For 10 of the 14 litters, the first completed ratio for an injection occurred with 92±8% (mean±S.D.) of the predicted interinjection interval had elapsed (52±25% for the other four litters) suggesting that the calculated interinjection intervals were generally accurate estimates for most of the litters and that the probability of seeking another injection increased as this time approached. The animals were sacrificed when they were most likely to seek another infusion. 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.

Acetylcholine and Choline Content

The content of ACh in all three groups was differentially distributed throughout brain regions (Table 1); highest in the caudate nucleus-putamen and amygdala and moderate in the medial septum where the cell bodies of the cholinergic septo-hippocampal tracts originate. All other regions were similar in content except for the cingulate cortex, motor-somatosensory cortex and substantia nigra, which were all lower. Only two significant changes in ACh content were observed between the experimental treatment conditions (lower levels in the globus pallidus and medial septum of the self-administering animals). Ch content (Table 2) was highest in the cingulate cortex, caudate nucleus-putamen, diagonal band, hippocampal formation and amygdaloid complex; moderate in the frontal, pyriform, motor-somatosensory and entorhinal-subicular cortices, nucleus accumbens, medial septum and ventral tegmental area; and lowest in the globus pallidus and substantia nigra. There were no significant differences in Ch content between the experimental conditions.

Flux of Choline Through Acetylcholine

Radioactivity rapidly appears in brain ACh after intravenous pulse labelling with radiolabelled Ch with peak specific radioactivities seen at 2 to 4 min post-injection [1,22]. ACh specific radioactivity then decreases in a log-linear fashion over the next 30 min. The specific radioactivities of ACh in the fourteen brain regions at the 5 and 10 min pulse intervals (Table 3) were consistently higher at the 5 min point and were highest in the pyriform, cingulate and motor-somatosensory cortices, nucleus accumbens and diagonal band and lowest in the caudate nucleus-putamen, globus pallidus, medial spetum, amygdaloid complex, ventral tegmental area and substantia nigra. Ch specific radioactivities (Table 4) were lower than ACh except in the caudate nucleus-putamen, globus pallidus, amygdaloid complex and ventral tegmental area. Values were similar in the experimental treatment groups, except in the ventral tegmental area where lower specific radioactivities were seen in the

TABLE 2

CONTENT OF CHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

	Content (pmoles mg protein^{-1})		
Brain Area	Morphine Self-Administration	Yoked-Morphine Infused	Yoked-Vehicle Infused
Frontal Cortex	426.9 ± 97.4	425.4 ± 93.4	435.3 ± 76.7
Pyriform Cortex	701.8 ± 66.2	630.1 ± 149.4	638.2 ± 79.8
Cingulate Cortex	849.0 ± 156.0	748.6 ± 123.7	744.1 ± 257.4
Motor-Somatosensory Cortex	551.4 ± 152.0	424.6 ± 172.2	523.3 ± 149.2
Entorhinal-Subicular Cortex	553.8 ± 157.6	593.2 ± 96.7	599.1 ± 126.8
Nucleus Accumbens	592.9 ± 76.2	554.1 ± 174.4	549.6 ± 93.4
Caudate Nucleus Putamen	860.1 ± 156.2	829.9 ± 125.9	850.6 ± 146.5
Globus Pallidus	228.3 ± 7.9	249.2 ± 30.8	275.8*
Diagonal Band	918.7 ± 133.5	903.8 ± 119.0	826.5 ± 196.6
Medial Septum	558.4 ± 32.9	596.3 ± 89.6	606.8 ± 35.9
Hippocampal Formation	812.3 ± 134.0	790.1 ± 120.7	814.2 ± 184.5
Amygdaloid Complex	934.7 ± 108.9	894.3 ± 162.0	921.9 ± 129.1
Ventral Tegmental Area	555.8 ± 185.5	420.5 ± 145.0	390.7 ± 95.0
Substantia Nigra	271.6 ± 60.5	217.2*	307.9 ± 35.9

Values are means ± S.D. for N = 14 per treatment condition.
*Represents value for a single pooled sample determination.

self-administering group at the 5 min pulse time than in the yoked-morphine infused group. Therefore, any changes in turnover rates are not likely to result from differences in the availability of labelled precursor except possibly in the ventral tegmental area. However, whether this lower specific radioactivity represents increased turnover of ACh or decreased availability of precursor is unclear since Ch is both precursor and product of ACh.

Acetylcholine Turnover

The fractional turnover rates (that fraction of the pool turning over per hour, K × 100) in the vehicle-infused group were highest in the medial septum, pyriform cortex and nucleus accumbens (750% hr^{-1}) with moderate rates (400–500% hr^{-1}) seen in all other areas except the caudate nucleus-putamen, diagonal band, ventral tegmental area and substantia nigra, which had rates less than 300% hr^{-1} (Table 5). The turnover rates for ACh in the yoked-vehicle infused group were differentially distributed in brain regions (Table 6). The highest turnover rates were observed in the amygdaloid complex, caudate nucleus-putamen and medial septum (1.5 nmoles mg protein^{-1} hr^{-1}). Moderate rates (1.0 nmoles mg protein^{-1} hr^{-1}) were seen in the pyriform cortex and nucleus accumbens with lowest rates (0.5 nmoles mg protein^{-1} hr^{-1}) in the frontal, cingulate, motor-somatosensory and entorhinal-subicular cortices, globus pallidus, diagonal band, hippocampal formation, ventral tegmental area and substantia nigra. Chronic passive morphine infusion (comparing the yoked-morphine with the yoked-vehicle infused group) did not affect the steady-state levels of ACh or Ch, but did result in significant increases in ACh turnover in the frontal cortex (78%) and diagonal band (114%) and in a significant decrease in the medial septum (−59%). The self-administration of morphine (self-administering group compared with the yoked-morphine infused group) resulted in significant changes in ACh turnover in five brain regions; decreases in the pyriform cortex, nucleus accumbens, amygdaloid complex (−46%) and ventral tegmental area (to undetectable levels) and an increase in the frontal cortex (44%).

DISCUSSION

The significant turnover rate changes support the involvement of cholinergic pathways in the neuronal circuits suggested to mediate components of opiate reinforcement [56]. The ACh turnover rate changes correlated with self-administration showed directional changes consistent with the involvement of specific cholinergic pathways. Increases in turnover were observed in the pallidal frontal cortex pathway while decreases were seen in the preoptic pathway innervating the pyriform cortex, nucleus accumbens and amygdala and in the pathway innervating the ventral tegmental area. Investigations of the neurobiological basis of behavior are fraught with difficulties, particularly studies that involve assessment of neurotransmitter changes. These studies often produce information that is not consistent with other findings which is a direct result of the inherent characteristics of neurotransmitter systems. Brain neurotransmitters have been hypothesized to exist in multiple pools. Generally, the total neurotransmitter in a neuron is thought to be divided into a large, firmly bound pool that is not readily releasable and a functional pool. Response demands on the

TABLE 3

SPECIFIC RADIOACTIVITIES OF ACETYLCHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

	Specific Radioactivities (DPM pmole^{-1})					
	Morphine Self-Administration		Yoked-Morphine Infused		Yoked-Vehicle Infused	
Brain Area	5′ pulse	10′ pulse	5′ pulse	10′ pulse	5′ pulse	10′ pulse
Frontal Cortex	4.32 ±0.78	2.44 ±0.85	4.80 ±0.57	3.11 ±0.64	4.59 ±1.25	3.57 ±0.86
Pyriform Cortex	4.27 ±2.00	4.23 ±1.57	6.74 ±2.71	3.25 ±0.98	5.90 ±3.48	3.74 ±1.11
Cingulae Cortex	5.76 ±0.80	4.39 ±0.92	5.68 ±1.32	4.50 ±1.06	6.76 ±0.93	4.62 ±1.01
Motor-Somatosensory Cortex	6.33 ±1.26	4.12 ±0.53	5.96 ±1.45	4.20 ±0.95	6.48 ±1.08	4.62 ±1.17
Entorhinal-Subicular Cortex	3.74 ±0.63	2.58 ±0.50	4.01 ±1.21	2.66 ±0.70	3.80 ±0.63	2.61 ±0.35
Nucleus Accumbens	5.43 ±1.67	4.41 ±1.66	4.36 ±1.07	2.57 ±0.57	6.61 ±2.02	3.78 ±0.82
Caudate Nucleus Putamen	1.50 ±0.25	1.38 ±0.28	1.61 ±0.26	1.42 ±0.26	1.76 ±0.14	1.49 ±0.25
Globus Pallidus*	2.14	1.40	1.74	1.20	2.43	1.74
Diagonal Band	5.99 ±0.28	4.08 ±1.40	6.02 ±2.33	4.17 ±1.43	5.78 ±1.66	4.64 ±0.80
Medial Septum	2.07 ±0.51	1.59 ±0.20	1.68 ±0.12	1.31 ±0.27	2.43 ±0.65	1.26 ±0.14
Hippocampal Formation	3.06 ±0.64	2.43 ±0.64	2.86 ±0.60	2.14 ±0.64	3.67 ±1.06	2.52 ±0.64
Amygdaloid Complex	1.10 ±0.25	0.99 ±0.19	1.16 ±0.44	1.32 ±0.54	1.25 ±0.23	0.99 ±0.32
Ventral Tegmental Area	1.27 ±0.17	1.46 ±0.03	1.36 ±0.01	1.09 ±0.15	1.27 ±0.13	1.02 ±0.19
Substantia Nigra*	1.92	0.95	2.40	1.14	2.17	2.27

Values are means ± S.D. for N=7 at each of the pulse intervals.
*Represents value for a single pooled sample determination.

TABLE 4

SPECIFIC RADIOACTIVITIES OF CHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

	Specific Radioactivities (DPM pmole^{-1})					
	Morphine Self-Administration		Yoked-Morphine Infused		Yoked-Vehicle Infused	
Brain Area	5′ pulse	10′ pulse	5′ pulse	10′ pulse	5′ pulse	10′ pulse
Frontal Cortex	3.29 ±0.37	1.98 ±0.55	3.12 ±1.34	2.28 ±0.53	3.44 ±1.00	1.97 ±0.45
Pyriform Cortex	2.14 ±0.44	1.33 ±0.28	1.91 ±0.49	1.71 ±0.32	2.79 ±0.90	1.52 ±0.57
Cingulate Cortex	2.26 ±0.73	1.46 ±0.43	2.25 ±0.43	1.71 ±0.32	2.02 ±0.45	1.32 ±0.39
Motor-Somatosensory Cortex	3.27 ±1.23	2.22 ±1.18	2.77 ±0.81	2.27 ±0.72	3.15 ±0.78	2.38 ±1.18
Entorhinal-Subicular Cortex	2.41 ±0.67	1.76 ±0.81	2.04 ±0.78	1.55 ±0.34	2.14 ±0.67	1.63 ±0.54
Nucleus Accumbens	2.21 ±0.45	2.02 ±0.36	2.79 ±0.97	1.91 ±0.73	2.42 ±0.69	2.25 ±0.31
Caudate Nucleus Putamen	1.78 ±0.28	1.49 ±0.33	1.75 ±0.51	1.69 ±0.49	1.90 ±0.51	1.65 ±0.30
Globus Pallidus*	2.93	1.94	3.69	1.82	—	1.81
Diagonal Band	1.32 ±0.18	1.00 ±0.35	1.28 ±0.22	1.09 ±0.24	1.26 ±0.29	0.97 ±0.16
Medial Septum	1.61 ±0.26	1.27 ±0.09	1.72 ±0.15	1.43*	1.90 ±0.33	1.03*
Hippocampal Formation	1.54 ±0.38	1.15 ±0.43	1.39 ±0.24	1.07 ±0.18	1.29 ±0.69	1.20 ±0.44
Amygdaloid Complex	1.38 ±0.25	0.97 ±0.13	1.21 ±0.23	1.22 ±0.33	1.16 ±0.25	1.20 ±0.30
Ventral Tegmental Area	1.39 ±0.23	1.32 ±0.14	2.21 ±0.88	1.25 ±0.45	2.28 ±0.52	1.70 ±0.42
Substantia Nigra*	2.02	1.55	—	1.66	2.45	1.79

Values are means ± S.D. for N=7 at each of the pulse intervals.
*Represents value for a single pooled sample determination.

TABLE 5

FRACTIONAL RATE CONSTANTS FOR ACETYLCHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

	Fractional Rate Constants (hour $^{-1}$)		
Brain Area	Morphine Self-Administration	Yoked-Morphine Infused	Yoked-Vehicle Infused
Frontal Cortex	7.64 ± 1.82	5.35 ± 1.37	3.87 ± 2.38
Pyriform Cortex	4.46 + 2.49	9.11 · 4.11	7.00 ± 4.47
Cingulate Cortex	3.89 ± 1.28	3.72 + 1.96	4.94 ± 1.38
Motor-Somatosensory Cortex	5.06 + 2.38	4.72 ± 2.19	5.00 ± 1.89
Entorhinal-Subicular Cortex	4.92 ± 1.42	5.76 ± 2.57	4.64 ± 1.73
Nucleus Accumbens	4.24 ± 2.36	6.34 ± 2.89	6.60 ± 3.58
Caudate Nucleus Putamen	2.52 ± 1.17	2.45 + 1.24	2.76 ± 0.31
Globus Pallidus*	5.14	4.43	4.02
Diagonal Band	5.17 ± 0.55	6.31 ± 2.63	3.14 ± 2.01
Medial Septum	3.61 ± 2.22	3.16 + 0.85	7.68 ± 3.22
Hippocampal Formation	4.19 ± 1.21	4.89 + 1.96	5.26 ± 2.57
Amygdaloid Complex	2.41 + 1.24	4.45 ± 2.70	4.50 ± 1.37
Ventral Tegmental Area	†	2.74 ± 0.09	3.11 + 0.78
Substantia Nigra*	8.49	8.90	†

Values are means ± S.D. Fractional rate constants (K) were calculated as:

$$K = \frac{\ln 2}{t1/2}$$

and the t1/2 was obtained from a semilogarithmic plot of the specific radioactivities at the two pulse times on the linear portion of the decay in radoactivity curve. The K values in this table were determined by calculating a grand mean fractional rate constant (K) from average values obtained by comparing each specific radioactivity value at the short pulse interval with those at the long pulse interval. The fractional rate constant is the mean and the error estimates the S.D. of these individual average K values.

*Represents values from a single pooled sample determination.

†Fractional rate constants below 1.0 hour^{-1}.

neurons that are within normal physiological limits likely utilize the functional pool exclusively while extraordinary demands result in utilization of the firmly-bound pool. The functional pool may represent only 10–15% of the total neurotransmitter present. Because of the survival value of an adequate functional pool, most behavioral manipulations are probably within normal limits of the response capability of the neuronal population. It is likely that only the functional pool is normally involved in neuronal transmission requirements for sensory input, sensory integration, information processing and response output. Therefore, in studies correlating neurotransmitter content with behavior, only changes in a small portion of the total neurotransmitter pool is involved. Even if a 50% decrease in the size of the functional pool occurs, this may only represent a 5–7% decline in content and, therefore, may not be of sufficient magnitude to be distinguished from experimental error. These changes may also be detectable for only a few seconds since synthetic processes would rapidly reach homeostatic conditions. For these reasons, it is not surprising that few changes in the levels of neurotransmitters have been reported to correlate with behavior [21, 32, 48, 53]. Such investigations must employ methodologies that detect modulations in the neurotransmitter functional pool without artifactually modifying the observed behavior. Therefore, this study utilized measurement of neurotransmitter turnover rates after pulse labelling with a radioactive precursor to investigate cholinergic involvement in opiate reinforcement.

The significant differences in ACh turnover are assumed to result from changes in the activity of cholinergic neurons involved in morphine-seeking behaviors. The litters were sacrificed at a time when the self-administering rat would most likely seek another 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 seen in this group may result from reinforcement, the changes seen in the self-administering animals are assumed to be more indicative of reinforcement processes. It is also possible that

TABLE 6

TURNOVER RATES FOR ACETYLCHOLINE IN FOURTEEN BRAIN REGIONS OF GROUPS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE (10 mg/kg) AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

	Turnover Rates (nmoles mg protein^{-1} hour^{-1})		
Brain Area	Morphine Self-Administration	Yoked-Morphine Infused	Yoked-Vehicle Infused
Frontal Cortex	1.01 ± 0.24‡	0.70 ± 0.18†	0.45 ± 0.27
Pyriform Cortex	0.71 ± 0.39†	1.32 ± 0.60	1.15 ± 0.73
Cingulate Cortex	0.28 ± 0.09	0.28 ± 0.15	0.34 ± 0.10
Motor-Somatosensory Cortex	0.37 ± 0.17	0.37 ± 0.17	0.37 ± 0.14
Entorhinal-Subicular Cortex	0.56 ± 0.16	0.67 ± 0.30	0.59 ± 0.22
Nucleus Accumbens	0.70 ± 0.39*	1.30 ± 0.59	1.19 ± 0.64
Caudate Nucleus Putamen	1.40 ± 0.65	1.44 ± 0.73	1.59 ± 0.18
Globus Pallidus§	0.56	0.65	0.57
Diagonal Band	0.60 ± 0.06	0.77 ± 0.32†	0.36 ± 0.23
Medial Septum	0.53 ± 0.33	0.64 ± 0.17*	1.56 ± 0.65
Hippocampal Formation	0.52 ± 0.16	0.69 ± 0.28	0.66 ± 0.32
Amygdaloid Complex	0.99 ± 0.50*	1.85 ± 1.12	1.96 ± 0.60
Ventral Tegmental Area	¶	0.33 ± 0.01	0.43 ± 0.11
Substantia Nigra§	0.71	0.88	¶

Values are means ± S.D. for N = 14 per treatment condition. The turnover rates were calculated as the product of the content and the fractional rate constants (K) and the error values the product of the content and the fractional rate constant error values. The significance of the difference between means determined with Students' *t*-tests were: *$p<0.05$; †$p<0.01$; ‡$p<0.001$. The self-administration group was compared with the yoked morphine and the latter with the vehicle infused group.

§Represent values for a single pooled sample determination.

¶Turnover rate below 0.1 nmole mg protein^{-1} hour^{-1}.

some input-output systems may be identified in the self-administering animals and attributed to reinforcement processes. Such erroneously designated pathways can be differentiated with intracranial self-administration procedures [20]. Input-output systems should not sustain intracranial self-administration, since neuronal activity indicative of the occurrence of a reinforcing stimulus should not be transmitted by activation of these neurons. Such studies can be initiated after neuronal systems potentially involved are identified with turnover rate measurements.

Investigation of the involvement of cholinergic systems in behavior is a difficult challenge for the neuroscientist. Neurochemical methodologies are routinely employed that include procedures incompatible with behavioral studies. Focused microwave fixation is considered the optimum method of tissue fixation for neurochemical studies of ACh. This procedure requires placement and confinement of the animal in a small restraining apparatus which introduces stress artifacts [11]. It also introduces a delay between the behavioral events and tissue fixation since it requires handling and removal of the animal from the behavioral environment. If neuronal activity is indeed closely tied to behavior, then this procedure would also produce neurochemical artifacts, possibly obscuring those correlated directly with the behavioral process of interest. Neurochemical investigations of behavior require procedures that are conducive to both the neurochemical and the behavioral process. In many instances, a compromise methodology must be adopted. Total freezing was used in this study because it is the most acceptable method for rapid tissue fixation that is compatible with the behavioral methodologies. However, it is thought to result in changes in cholinergic parameters. Both methods result in artifacts, but of a different nature. Methods of sacrifice other than microwave fixation have been reported to result in decreases in ACh content (decapitation) or changes in Ch (near freezing). However, ACh content values obtained here (Table 1) are in agreement with those found with microwave fixation [11, 42–45, 52, 58].

ACh turnover rates in the vehicle control group agree with previously published values for most brain regions obtained with microwave fixation and non-isotopic methods [11,50] or with radioisotopic procedures [6, 42–45, 65, 67]. However, in several brain regions (caudate nucleus-putamen, hippocampus and nucleus accumbens) values are three to eight times higher than those presented here. One could conclude that this results from postmortem decreases with total freezing. It is also possible that the method of restraint for microwave fixation and for intravenous administration of precursor were stressful and resulted in increased ACh turnover as previously discussed [11]. Our methods of intravenous administration of labelled precursor involves minimal, if any stress and no restraint. If hypothetical fluc-

TABLE 7

PERCENT AND DIRECTION OF CHANGE IN TURNOVER RATES OF DOPAMINE, NOREPINEPHRINE, SEROTONIN, ASPARTATE, GLUTAMATE AND GAMMA-AMINOBUTYRIC ACID IN ELEVEN BRAIN REGIONS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE COMPARED TO YOKED-MORPHINE INFUSED LITTERMATES

Brain Area	DA	NE	5-HT	Asp	Glu	GABA
Frontal-Pyriform Cortex	270% ↑	—	—	83% ↑	106% ↑	42% ↑
Nucleus Accumbens	87% ↓	92% ↓	33% ↓	—	—	71% ↓
Caudate Nucleus Putamen	144% ↑	—	—	95% ↑	115% ↑	55% ↑
Septum	111% ↑	157% ↑	—	—	—	207% ↑
Hippocampal Formation	—	—	—	92% ↑	108% ↑	89% ↑
Amygdaloid Complex	—	—	—	84% ↑	103% ↑	86% ↑
Hypothalamus	—	—	—	—	—	67% ↑
Thalamus	—	—	—	78% ↑	—	100% ↑
Motor-Somatosensory Cortex	—	—	—	69% ↑	101% ↑	90% ↑
Entorhinal-Subicular Cortex	—	—	—	—	—	—
Brainstem	—	—	—	63% ↑	—	45% ↑

The turnover rates from which these percentages were derived have been previously published (Smith *et al.* [53,54].

tuations in the Ch precursor pool did occur, they would be unlikely to affect the significant differences found between the experimental groups for several reasons. First of all, values for ACh and Ch content and specific radioactivity were not different in the three treatment groups (Tables 1–4). Secondly, the turnover rate changes obtained may reflect only relative and not absolute values. However, relative values are significant in investigations of brain function when appropriate control groups are utilized that permit accurate and relevant conclusions to be made from the data.

Another possible reason for these differences is the method of turnover calculation. The log-linear decay in specific radioactivity of a neurotransmitter can be used to calculate turnover rates [13, 25–27, 53–55, 66] and was used to calculate the turnover rates shown in Table 6. Alternative methods have also been employed. Non-isotopic procedures utilize drugs that inhibit Ch uptake with the resulting decline in ACh content used to calculate rate constants, yielding turnover rates that are similar to those presented here [11,50]. Values obtained with pulse labelling procedures and the finite difference method of calculation [33] are sometimes in agreement and sometimes higher [29] than the values calculated from the log-linear decay curve. Another method using constant perfusion with [^{13}C]-labelled phosphorylcholine results in values that are identical to those calculated with the finite difference method [42]. Both of these calculations utilize the specific radioactivity or rate constant for Ch which is both product and precursor to ACh as well as a constituent of other biochemical pathways. However, the Ch pool appears to be independent of the ACh pool. First of all, Ch loading may not alter ACh turnover rates [6]. Secondly, there is no correlation between Ch specific radioactivity and ACh specific radioactivity or with cholinergic neuronal activity [1]. Thirdly, this postmortem increase is independent of cholinergic neurons since it is still seen in the hippocampus after removal of cholinergic innervations with septal lesions [12]. Fourthly, ACh specific radioactivity correlates with cholinergic neuronal activity while Ch specific radioactivity does not [1]. Fractional rate constants calculated with the finite difference method from the data presented in Tables 1–4 are three to five times greater than those shown in Table 5, and result in ACh turnover rates three to five times higher than those shown in Table 6, thus making the values more in agreement. This would not be a problem except this calculation procedure also results in significant differences in turnover between treatment groups in several regions where the specific radioactivity of ACh is not different and, thus, totally the result of differences in Ch specific radioactivity. Methods of calculation of fractional rate constants for ACh that utilize Ch parameters in brain regions where it has major metabolic roles other than neurotransmitter precursor could result in erroneous turnover estimates. This error occurs when GABA turnover is calculated with the finite difference method using glutamate as precursor [2]. Such calculations result in GABA turnover rates that are 10 to 20 times higher than those obtained by using the log-linear decay after pulse labelling with glucose [13, 27, 53, 54], the rise in GABA after gamma-aminobutyric acid transaminase inhibition [7, 24, 31, 61] or the postmortem increase in GABA specific radioactivity [39]. In brain regions where glutamate has metabolic functions other than precursor (i.e., a neurotransmitter role), fractional rate constants calculated by the finite difference method would result in errors in turnover estimates. This would be similar to using DA specific radioactivity in the calculation of NA turnover which would clearly result in errors in regions where DA has a role other than precursor to NA. The same error appears to occur when Ch is used to calculate ACh turnover. The use of a rate constant or specific radioactivity values for a precursor that could and does have other biochemical functions, whose

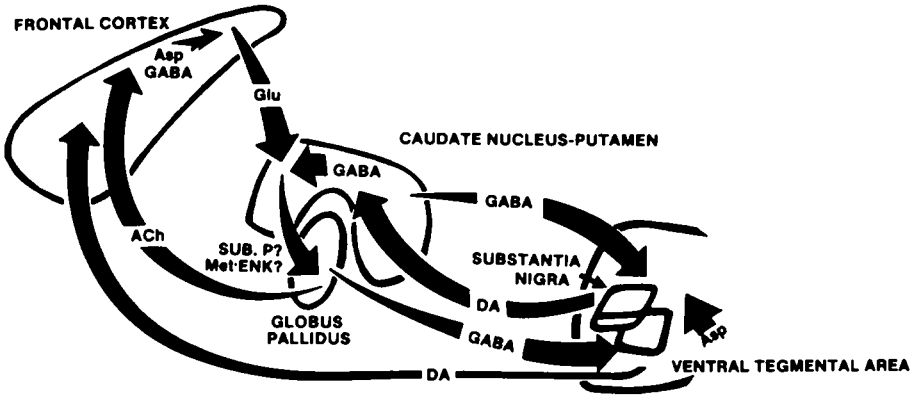

FIG. 2. Frontal cortex circuit proposed to mediate opiate reinforcement. This circuit includes a glutamatergic efferent from the frontal cortex to the caudate nucleus-putamen, an enkephalin or substance P efferent from the caudate nucleus-putamen to the globus pallidus, a cholinergic efferent from the globus pallidus to the frontal cortex and intrinsic aspartatergic and gamma-aminobutyric acid neurons in the frontal cortex. Activity in this circuit may be modulated by the biogenic monoamines at the level of the caudate nucleus-putamen and the frontal cortex. These brainstem centers could be modulated by gamma-aminobutyric acid and possibly substance P feedback pathways from the caudate nucleus-putamen.

specific activity does not correlate with the specific activity of the neurotransmitter or with the activity in neurons releasing that neurotransmitter [1] would appear to have questionable utility. Especially, when it alone is responsible for significant differences and since the decay in radioactivity of the neurotransmitter itself can be used to accurately determine turnover rates [66]. However, whether the turnover rates obtained here are accurate estimates of absolute rates is not crucial to the relevance of the significant changes between the experimental groups. The control groups make meaningful interpretation of the data possible.

When turnover rate changes for all the neurotransmitters monitored in the two experiments (Table 7) were integrated with information of neurotransmitter specific pathways [28] and with studies of the parameters of opiate self-administration using lesions [14–15, 17–19], neuronal blocking agents [8, 9, 16, 41, 57] and intracranial self-administration mapping procedures [3, 4, 20, 35–38, 59, 60] two neuronal circuits were identified that may be involved in opiate reinforcement (Fig. 1). The two neuronal systems include a frontal cortex-caudate nucleus putamen-globus pallidus-frontal cortex circuit (Fig. 2) and a hippocampal formation-nucleus accumbens-amygdaloid complex-entorhinal-subicular cortex-hippocampal formation circuit (Fig. 3). The significant increase in ACh turnover in the frontal cortex of the self-administering animals is consistent with the involvement of the pallidal cholinergic fibers innervating this region [23]. The dopaminergic innervation of the caudate nucleus-putamen which showed increased turnover rates in the self-administering rats are inhibitory to cholinergic interneurons and would be expected to decrease ACh turnover, but this was not observed. This suggests that some other excitatory system innervating these neurons is negating this inhibitory effect (Glu releasing fibers from cortical neurons?). DA neurons innervating the frontal cortex are thought to be under tonic inhibition by cholinergic fibers innervating the ventral tegmental area [29]. The increase in DA turnover in the frontal cortex [54] and decrease in ACh turnover in the ventral tegmental area support involvement of these two pathways in opiate reinforcement. Opiates could directly initiate activity in this circuit at the caudate nucleus-putamen, globus pallidus or brain stem DA centers, resulting in the transmission of information indicative of the occurrence of a reinforcing event. Interaction of opiates with receptors in the globus pallidus from the caudate nucleus-putamen enkephalinergic pathway [62] might directly modulate the activity of the pallidal cholinergic pathway innervating the frontal cortex.

The other neuronal system consistent with these data is a hippocampal formation-nucleus accumbens-amygdaloid complex-entorhinal subicular cortex-hippocampal formation circuit. Cholinergic fibers from the medial septum innervating the hippocampal formation, from the amygdaloid complex innervating the entorhinal-subicular cortex and from the preoptic region innervating the amygdaloid complex were proposed to be involved in this circuit. Involvement of cholinergic innervations of the amygdaloid complex is supported by the decrease in ACh turnover observed in the self-administration group. However, hippocampal formation cholinergic activity was not altered. The hippocampal formation was assayed *in toto* which may have masked regional changes in turnover. DA and NA turnover in the septum were elevated in the self-administering rats [54]. Septal DA input is inhibitory and NA input excitatory to hippocampal cholinergic turnover [46, 47, 49]. It is possible that both increases and decreases in activity of subpopulations of hippocampal cholinergic neurons may have occurred as a result of these septal biogenic amine innervations since increases in Asp, Glu and GABA turnover rates were seen [54]. The data do not support the proposed cholinergic innervation of the entorhinal-subicular cortex. No turnover changes in any of the eight neurotransmitters in this structure were found to correlate with morphine self-administration [54]. However, the increased turnover of Glu in the hippocampal formation suggests that perforant pathways from the entorhinal-subicular cortex may be activated. If this is true, the mechanism is not obvious from the turnover rates that have been measured.

The ACh turnover rate changes seen with self-administration support the role of cholinergic neurons in

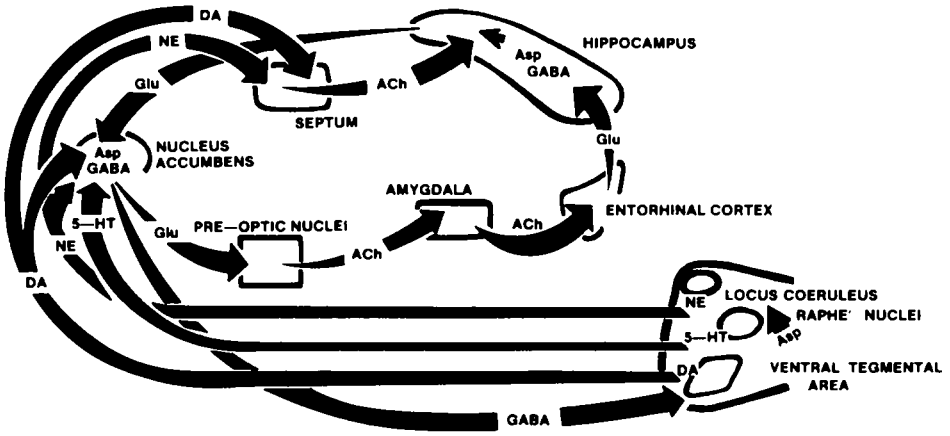

FIG. 3. Hippocampal circuit proposed to partially mediate opiate reinforcement. This includes a glutamatergic efferent from the hippocampal formation to the nucleus accumbens, intrinsic gamma-aminobutyric acid neurons in the nucleus accumbens, glutamatergic efferents from the nucleus accumbens to the preoptic nuclei, a cholinergic efferent from the preoptic nuclei to the amygdaloid complex, an efferent from the amygdaloid complex to the entorhinal-subicular cortex (cholinergic ?), a glutamatergic efferent from the entorhinal-subicular cortex to the hippocampal formation and intrinsic aspartatergic and gamma-aminobutyric acid neurons in the hippocampal formation. Brainstem biogenic monoamine neurons may modulate activity in this circuit at the nucleus accumbens and septum. In turn, activity in these brainstem centers could be modulated by gamma-aminobutyric acid feedback pathways from the nucleus accumbens.

opiate reinforcement demonstrated by the attenuation of these processes with systemic administration of cholinergic blocking agents [8,16] and by involvement in the reinforcement circuits presented here. The role of neuronal pathways outlined in these reinforcement circuits are currently under investigation in our laboratories using intracranial self-administration methodologies and neurotoxin lesion methodologies to selectively destroy discrete neuronal populations and determine the effect upon concurrent opiate, food and water reinforced responding.

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