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Brain Neurotransmitter Turnover Correlated with Morphine-Seeking Behavior of Rats[1]

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SMITH, J E , C CO, M E FREEMAN AND J D LANE *Brain neurotransmitter turnover correlated with morphine-seeking behavior of rats* PHARMAC BIOCHEM BEHAV **16**(3) 509–519, 1982 —Neurochemical substrates of intravenous opiate self-administration were investigated in rats using littermate controls for vehicle and passive morphine infusion The rates of turnover of the putative neurotransmitters, dopamine, norepinephrine, serotonin, gamma-aminobutyric acid, aspartate and glutamate were concurrently measured in eleven brain regions of rats intravenously self-administering morphine and in yoked-morphine or yoked-vehicle infused littermates The passive infusion of morphine resulted in significant changes in the rates of turnover of the biogenic monoamine and amino acid neurotransmitters in six brain regions with the caudate nucleus-putamen-globus pallidus showing the most changes The contingent infusion of morphine resulted in changes in utilization rates that were generally greater in both magnitude and number than the effects of the drug itself Twenty-nine significant changes were observed in the self-administereing group with most changes occurring in limbic structures The neurotransmitter turnover rate changes resulting from contingent administration suggest that the drug administration environment is an important factor that should be considered in studies of interactions between drugs and neuronal systems

Norepinephrine Dopamine Serotonin Gamma-aminobutyric acid Glutamate
Morphine self-administration Opiate reinforcement systems Neurotransmitter turnover rates

OPIATES are self-administered by man and animals for their positive reinforcing properties The central nervous system (CNS) mechanisms responsible for opiate reinforcement have been extensively investigated in animals with pharmacological and electrolytic lesion procedures Drugs that interrupt catecholaminergic and cholinergic neuronal activity decrease intravenous opiate self-administration in rats [9–11] and monkeys [57] Electrolytic lesions of the medial forebrain bundle [20,21], medial raphé nucleus [22], hippocampus, frontal cortex [23] and anterior cingulate cortex [75] antagonize the rewarding properties of morphine while lesions of the caudate nucleus [22,26] and substantia nigra [24] enhance them Increased metabolic activity [25] (demonstrated by the 2-deoxyglucose method) and elevated turnover rates of the biogenic amine and amino acid neurotransmitters have been found in the striatum of morphine self-administering rats compared to yoked-morphine infused littermates [67] This study was initiated to further investigate the role of individual neuronal systems in opiate reward processes The rates of turnover of the biogenic amine and amino acid neurotransmitters were concurrently measured in eleven brain regions of rats self-administering morphine and in yoked-morphine and yoked-vehicle infused littermates A littermate design was used to identify neurochemical changes resulting from the passive infusion or self-administration of the drug One littermate was allowed to intravenously self-administer morphine while the second and third received a simultaneous equivalent infusion of morphine or an equivalent volume of vehicle (saline) With this design, the general effects of the drug (analgesia, physical dependence, tolerance, etc) on brain neurotransmitter turnover rates can be assessed (yoked-morphine compared with yoked-vehicle infused) and separated from the rewarding effects of the drug-taking milieu (self-administration compared with the yoked-morphine infused)

METHOD

Behavioral Procedures

Eleven litters of three adult male Fisher F-344 rats (90–150 days old) were implanted with chronic jugular catheters using previously described procedures [56,82] 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 jugular vein until it terminated just outside the right ventricle The catheter was anchored to tissue in the surrounding area and continued subcutaneously to the back where it

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exited immediately behind the scapulae through a teflon-stainless steel harness The harness, of which the teflon portion was implanted under the skin, provided a point of attachment for the catheter to a leash constructed from needle tubing and metal spring that passed through the top of the animal chamber to a leak-proof swivel [3] which was attached to the three-syringe infusion pump The leash and swivel were counter balanced to permit complete freedom of movement Each litter was housed in a large conditioning chamber in individual self-administration cages Each chamber was equipped with a house light that was illuminated on a reversed 12-hour light and 12-hour 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 immediately above the lever For the first two days after surgery, the three littermates in each group received programmed hourly infusions of 200 μl of heparinized-saline from the infusion pump Two of the rats in each litter were then made physically dependent with hourly infusions of morphine sulfate in increasing doses (2 days each of 1 25, 2 5, 5 0 and 10 0 mg/kg) The third littermate continued to receive hourly heparinized-saline infusions These hourly injections were paired with a tone and light stimulus of 30 seconds duration to facilitate later development of self-administration On the eleventh day after surgery, automatic infusions were discontinued and a lever introduced into each self-administration cage One of the physically dependent animals was allowed to self-administer morphine (10 mg/kg in 200 μl over 5 5 seconds) by pressing the lever The other physically dependent littermate received a yoked infusion of morphine (identical dose) and the third littermate a yoked-vehicle infusion whenever the self-administering animal earned an infusion 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 consequences and were minimal Each self-administering rat had 24-hour 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 hours of each self-administering rat

Neurochemical Procedures

At 60 or 90 minutes prior to a predicted self-infusion, precursors to the respective neurotransmitters ((0 2 mCi D-[U-^{14}C]-glucose (I C N , Spec Act 210 mCi/mmol), 0 5 mCi L-[U-^{3}H]-trytophan (Amersham Searle, Spec Act 7 1 Ci/mmol) and 1 0 mCi L-[2,6-^{3}H]-tyrosine (Amersham Searle, Spec Act 34 Ci/mmol)) were injected in 100 μl of saline through the jugular catheter The three rats in each litter were sacrificed at the predicted infusion time by immersion in liquid N_2 until 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 microdissected at −20°C into the following regions frontal-pyriform cortex, nucleus accumbens, caudate nucleus-putamen-globus pallidus, septum, hypothalamus-preoptic area, hippocampal formation, amygdaloid complex, thalamus, motor-somatosensory cortex, entorhinal-subicular cortex and brain stem The individual tissue samples were pulverized in liquid N_2 in a stainless steel mortar stored at −70°C until extraction and assay

One portion of each tissue sample was used to determine content and specific radioactivity of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) by a previously reported method [30,68] Briefly, the biogenic amines were extracted into 20 volumes of ice-cold 1 N formic acid/acetone (v/v 15/85) and the tissue pellets saved for protein determination [37] The formic acid/acetone extracts were washed with three volumes of heptane/chloroform (v/v 8/1), the organic layer and lipid interphase discarded and the aqueous portion take to dryness at 37°C under dry N_2 Internal standards for fluorescent recovery were processed in parallel and tracer levels of ^{14}C-radioactive standards for DA(Amersham Searle, Spec Act 60 mCi/mmol), NE (Amersham Searle, Spec Act 67 mCi/mmol) and 5-HT (Amersham Searle, Spec Act 54 mCi/mmol) were added to each tissue extract to correct for radioactive recovery The dried extracts were dissolved in pH 4 0 water, buffered to pH 7 0 with 1 M phosphate buffer, pH 9 0 and the biogenic amines absorbed onto a 5×5 mm column of BioRex 70 cation exchange resin (BioRad Laboratories) The biogenic monoamines were eluted from the column and the samples dried at 37°C under dry N_2 The dried samples were redissolved in pH 4 0 water and small portions taken in duplicate for the determination of content of DA, NE and 5-HT by compound-specific fluorometric assays [69] A portion of the reconstituted BioRex 70 extract was used for separation of the radioactive biogenic monoamines This fraction was buffered to pH 7 5 with 1 M glycylglycine buffer, pH 9 0 and layered on a 5×5 mm activated alumina column The catecholamines were retained on the alumina, while 5-HT passed through and was collected DA and NE were eluted from the alumina with 0 5 N acetic acid and taken to dryness at 37°C under dry N_2 The dried extracts were redissolved in 95% ethanol and applied to 500 μm thick cellulose glass-backed thin layer chromatography plates (20×20 cm MN 300 cellulose, Analtech) The thin-layer chromatography plates were developed for 4 hours in a solvent system of n-butanol/ethanol/1 N acetic acid/benzene (v/v 35/10/10/5) The NE and DA spots were visualized by placing the plates in a tank of sublimed iodine vapor for a short period The individual spots were scraped, placed in counting vials, extracted with 0 1 N HCl and neutralized with 1 N NaOH The 5-HT fraction was washed with n-butanol/heptane (v/v 9/1) to remove any ^{14}C-labelled acetylcholine that might be present and labelled with the ^{14}C-glucose precursor More recently, an alternative analysis for biogenic amines was introduced Following elution from the BioRex column, the samples were taken to dryness under N_2 and dissolved in a mobile phase of 0 1 M citrate-phosphate buffer, pH 3 5, containing 0 004% sodium octyl sulfate (Eastman) and 15% methanol The samples were injected into a Bioanalytical Systems, Inc high pressure liquid chromatography system utilizing a C18 reverse phase column, and DA, NE and 5-HT were eluted over 12 minutes with the isocratic mobile phase [36] A TL-9A custom electrochemical detector cell was utilized to monitor content of each biogenic amine by peak height and allow the collection of the individual peaks for radioactivity monitoring [31] Both techniques gave comparable results The separate DA, NE and 5-HT fractions were counted for radioactivity in 10 ml of Aquasol-2 (NEN) in a Searle Isocap Model 6872 liquid scintillation spectrometer Disintegrations per minute (dpm)

were calculated from a double-label quench curve corrected for counting efficiency by automatic external standardization Recoveries of fluorescence and radioactivity were corrected and specific radioactivities (dpm/pmol) calculated

The amino acids were extracted from another portion of the tissue powder and assayed for content and specific radioactivity with a modification [19] of a previously reported procedure [65] A 3 to 10 mg portion of the tissue was extracted with 7% trichloracetic acid, the trichloracetic acid removed with four 1 ml ether washes and the samples taken to dryness in a vacuum desiccator centrifuge Internal standards of the amino acids were added to brain extracts and processed in parallel The dried samples were reconstituted in 0 1 M borate buffer, pH 10 0, added to a solution of ^{3}H-dinitrofluorobenzene (NEN, Spec Act 12 Ci/mmol) in benzene and incubated at 60°C for 30 minutes The samples were then acidified with 5 N HCl and the dinitrofluorobenzene and dinitrophenol removed with four 1 ml heptane/bromobenzene (v/v 80/20) washes The ^{3}H-dinitrophenyl-amino acid derivatives were separated with two ether extractions which were combined and take to dryness in a vacuum desiccator centrifuge The dried extracts were redissolved in 1 N HCl and the two ether extractions repeated and dried as before The ^{3}H-dinitrophenyl derivatives were separated by two dimensional thin-layer chromatography on 20×20 cm, 500 μm thick silica gel-G (E Merck) glass plates (Analtech) The plates were first developed in a solvent system of ether/methanol/7 N NH_4OH (v/v 100/20/8), rotated 90° and developed in a solvent system of ether/glacial acetic acid/H_2O (v/v 100/10/10) The individual-dinitrophenyl-amino acid spots were scraped into counting vials, eluted with 1 ml of 0 01 M $NaHCO_3$, 15 ml of Aquasol-2 was added and the radioactivity in each sample determined by liquid scintillation spectrometry Content (nmol/mg protein) was determined from the internal standards and specific radioactivity (dpm/nmol) calculated for each sample

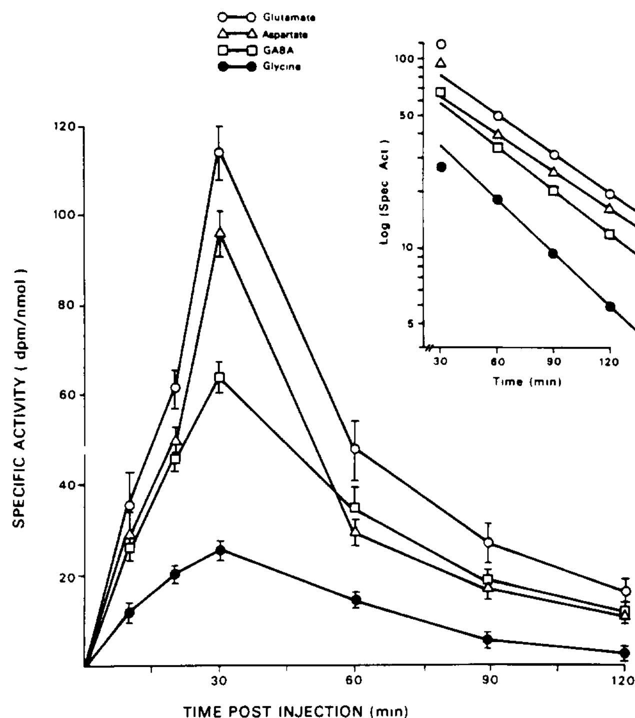

FIG 1 Glucose incorporation into amino acids The specific radioactivities of aspartate, glutamate, glycine and gamma-aminobutyric acid in the cerebral cortex at various times after the intravenous administration of 0 2 mCi D-[U-^{14}C]-glucose Each point is the mean±S D of five rats

Turnover Rate Calculation Procedures

Turnover rates were determined with the assumption that radiolabel from each neurotransmitter was disappearing from a single open pool since there is no acceptable method for determining CNS intraneuronal compartmentation *in vivo* Thus, $\text{turnover}_A = K \times \text{content}_A$ where the apparent fractional rate constant (K) = ($\ln 2/t^1/_2$) and $t^1/_2$ was obtained from a semilogarithmic plot of the specific radioactivities (dpm $pmol^{-1}$ or dpm $nmol^{-1}$) obtained at the two pulse times on the linear portion of the decay in radioactivity curve for each neurotransmitter The fractional rate constant (K) and error values are determined by calculating a grand mean K value from average values obtained by comparing each specific radioactivity value at the short pulse interval with each at the long pulse interval The fractional rate constant is the mean and the error estimate the S D of these individual average K values The turnover rate is expressed as pmol mg $protein^{-1}$ hr^{-1} or nmol mg $protein^{-1}$ hr^{-1} and is the product of the rate constant (hr^{-1}) and the content values (pmol mg $protein^{-1}$ or nmol mg $protein^{-1}$) These turnover rates are assumed to be representative of the utilization of the respective neurotransmitters

This interpretation of turnover is simplified since it disregards potential compartmentation of the neurotransmitters In most regions where biogenic amine neurons serve major functions, the disappearance of radiolabel from an open compartment has been used to evaluate rates of utilization [30,68] resulting in apparent fractional rate constants that range from 20 to 100 percent per hour which are consistent with previous reports Unfortunately, similar interpretation of amino acid neurotransmitter utilization is complicated by several factors Glucose preferentially labels neuronal pools of amino acids, pool(s) which are readily releasable in the presence of depolarizing stimuli [7, 14, 42, 78, 79], although it does not distinguish intraneuronal compartmentation This may not be a problem in the forebrain, where a majority of synapses are thought to release amino acids such as Glu, Asp and GABA, strongly suggesting that most of the amino acid pool(s) are dedicated to neurotransmitter function Therefore, when large pool(s) of Asp, Glu and GABA are labelled from glucose, the contributions of these amino acids to non-transmitter functions (metabolic or synthetic) are probably small and can be ignored without resulting in significant error However, these changes in turnover may represent general changes in neuronal activity, but if this is the case, this procedure would still identify those groups of neurons which utilize the respective amino acids as neurotransmitters The 60 and 90 minute pulse lengths were selected because they are on the linear portion of the decay in radioactivity curves (Fig 1), are coordinated with the administration of monoamine precursors, and because they insure that the animals are not stressed or stimulated near the predicted self-administration time However, one may be concerned as to whether neurotransmitter utilization is being monitored at these extended time points During short pulse intervals after

TABLE 1

CONTENT OF DOPAMINE, NOREPINEPHRINE, SEROTONIN, ASPARTATE, GLUTAMATE GLYCINE, AND GAMMA-AMINOBUTYRIC ACID CONCURRENTLY MEASURED IN ELEVEN BRAIN REGIONS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

		pmoles mg Protein [1]			nmoles mg Protein [1]			
		DA	NE	5-HT	Asp	Glu	Gly	GABA
Frontal-Pyriform Cortex	S-A[1]	58 7 ± 22.3	26 9 ± 8 2	42 2 ± 7 5	35 1 ± 5.3	138 2 ± 22.6	7 5 ± 0 5	26 7 ± 4 1
	Y-M[1]	53 8 ± 10 7	25.5 ± 6 3	38 6 ± 7 1	31 0 ± 3 9	120 4 ± 22.9	7 1 ± 0 7	25 6 ± 7 1
	Y V[1]	56 3 ± 16 1	24 4 ± 5 7	40 0 ± 10 6	31 5 ± 7 0	117 0 ± 28 9	6 7 ± 0 9	22 8 ± 4 4
Nucleus Accumbens[2]	S-A	347 3 ± 38 0	14 8 ± 1 6	47 9 ± 14 0	30 0 ± 7 1	130 7 ± 36 8	7 8 ± 2.8	42 8 ± 15 2
	Y-M	345 9 ± 25 1	14 8 ± 4 0	38 7 ± 13 8	29 9 ± 7 7	119 3 ± 39 5	7 7 ± 3 0	48.8 ± 11 9
	Y-V	377 0 ± 43 2	15 8 ± 2.4	46 0 ± 12.5	30 9 ± 9 9	122.1 ± 36 4	7 1 ± 3 3	60 6 ± 12.2
Caudate N - Putamen-Globus Pallidus	S-A	544 5 ± 77 5	34 2 ± 9 6	45 6 ± 5 9	20 1 ± 3 6	86.8 ± 10.5	6 0 ± 1 3	25 3 ± 4 9
	Y M	524 1 ± 76 5	35.5 ± 7 4	44 9 ± 5 7	22.2 ± 3 4	93 8 ± 10 3	6 8 ± 1 4	29 8 ± 5 8
	Y-V	549 8 ± 85 0	36 1 ± 8 2	45 8 ± 5 0	21 4 ± 4 3	88 0 ± 8 0	6 5 ± 1 5	27 1 ± 7 1
Septum[2]	S-A	27 6 ± 6.8	27 9 ± 2.9	40.3 ± 8 1	24 0 ± 6 3	106 3 ± 25 7	6 6 ± 2 0	45 4 ± 15 0
	Y-M	24.8 ± 5 4	24 8 ± 2.9	34 1 ± 14 2	24 1 ± 6 4	100 4 ± 31 4	6 7 ± 2 3	55 8 ± 21 2
	Y-V	30 6 ± 5.6	26.1 ± 0 2	33 8 ± 21 3	22 8 ± 5 7	102.8 ± 27 4	6 1 ± 1 9	36 2 ± 13 9
Hypothalamus[2]	S-A	52.8 ± 18 3	104 1 ± 55.1	51 9 ± 12.1	28 4 ± 4 1	84 9 ± 17 9	8 6 ± 2.2	62.6 ± 9 7
	Y-M	58 8 ± 28 5	103 1 ± 48.0	56 0 ± 15.5	29 5 ± 5 6	92.1 ± 15 5	9 4 ± 1 7	68 9 ± 15.3
	Y V	53 4 ± 32 6	107 3 ± 54 9	60 6 ± 5 4	28 9 ± 7 3	92.2 ± 23 2	8 5 ± 2 4	62 3 ± 18 4
Hippocampal Formation	S-A	9 6 ± 4 5	26 9 ± 12.7	5 7 ± 2.7	20 8 ± 2.6	91 0 ± 9 6	6 4 ± 1 2	14 9 ± 1 9
	Y-M	6 2 ± 3 2	25 3 ± 10 6	6 2 ± 3 0	22 2 ± 6 3	91 6 ± 14 4	7 1 ± 1 1	15 8 ± 1 7
	Y-V	7 7 ± 3.2	28 6 ± 11 4	6 6 ± 2.3	21 9 ± 2.6	89 4 ± 12.4	6 5 ± 1 1	14 3 ± 2.1
Amygdaloid Complex	S-A	18 6 ± 10 7	16 5 ± 1 1	36 3[3]	30 2 ± 8 7	97 1 ± 10 4	6 0 ± 1 2	17 2 ± 2.7
	Y M	18 8 ± 7 5	17 3 ± 2.1	36 1 ± 3 1	27 6 ± 9 5	96 5 ± 30 8	6 0 ± 1 8	18 6 ± 7 0
	Y-V	15 2 ± 2.3	16 8 ± 2.9	38 5[3]	27 2 ± 5 0	104 4 ± 14 8	6 5 ± 1 1	17 3 ± 2.8
Thalmus	S-A	7 1 ± 2.7	28 1 ± 5 6	40.5 ± 5 1	29 2 ± 7 6	102 7 ± 13 3	8 3 ± 1 6	28.8 ± 9 9
	Y M	9 3 ± 7 8	30 1 ± 8 1	39 6 ± 7 2	32 3 ± 9 0	111 1 ± 31 3	8 6 ± 1 9	29 6 ± 9 2
	Y-V	10 2 ± 6 6	31 9 ± 10 0	40 8 ± 7 6	29 8 ± 3 3	100 4 ± 14 0	8 3 ± 1 1	27 2 ± 4 4
Motor-Somato-Sensory Cortex	S-A	7 7 ± 1 8	21 8 ± 5 1	27 7 ± 7 0	49 1 ± 11 2	145 4 ± 15 7	7 7 ± 1 5	17 1 ± 5 0
	Y-M	10 2 ± 5 5	23.0 ± 3 7	27 8 ± 10 1	53 9 ± 20 0	140 7 ± 23 3	7 6 ± 1 7	17 0 ± 4 4
	Y V	5 4 ± 2.3	21 6 ± 3 9	25 1 ± 5 6	41 6 ± 10 9	128 9 ± 18 6	6 3 ± 1 0	16 3 ± 4 6
Entorhinal-Subicular Cortex	S-A	1 3 ± 0 4	9 1 ± 0 9	17 8 ± 10 4	31 2 ± 4 7	117 3 ± 12.8	7 1 ± 1 9	16 8 ± 2 5
	Y M	1 2 ± 0 6	8 1 ± 1 7	13 0 ± 7 3	31 9 ± 5 6	117 5 ± 17 3	7 4 ± 1 2	16 7 ± 2.2
	Y-V	1 2 ± 0 5	8 8 ± 1 7	17 5 ± 9 2	32 5 ± 3 9	121 6 ± 11 5	7 2 ± 0 9	17 6 ± 2.4
Brain Stem	S-A	12.7 ± 5 3	29 4 ± 13 7	56 6 ± 8 2	35 3 ± 6 1	85 4 ± 22 4	30 6 ± 3 9	29 7 ± 9 3
	Y M	12 3 ± 5 3	28 9 ± 15 6	52.7 ± 2.0	40 2 ± 5 3	96 1 ± 8 5*	32 7 ± 4 2	32 5 ± 4 2†
	Y V	9 1 ± 2.0	28 2 ± 13 5	56 9 ± 2.9	35 3 ± 5 3	83 7 ± 12 8	30 5 ± 3 0	26 8 ± 3 9

Values are means±standard deviation for N=11 in each treatment group The significance of the difference between means as determined with Students' t-test was $^*p<0.05$, $^\dagger p<0.01$ The self-administration group was compared with the yoked-morphine group and the latter was compared with the yoked-vehicle group

[1] S-A=morphine self-administration, Y-M=yoked morphine infused, Y-V=yoked-vehicle infused

[2] Values are means=standard deviation for eleven samples pooled into two fractions

[3] Represents one determination

glucose administration, Bertilsson *et al* [2] observed a high flux of label from glucose into Glu and GABA, and using the method of Neff *et al* [47], calculated turnover rates for the first 7 minutes post-injection, finding a trend toward decreasing fractional rate constants for GABA This was attributed to the progressive recycling of radiolabel through the amino acids which is likely occurring in the present study, but does not reduce the usefulness of the observations Even if recycling of radiolabel is occurring, the flux through the amino acids is a useful measure for deriving an apparent rate constant since any up- or down-regulation of neuronal activity would merely accelerate or diminish the contributions of recycling, and thus not effect the interpretation Irrespective of these concerns, the turnover rate comparisons are always conducted within a stringent experimental protocol so that observed differences likely reflect changes in utilization resulting from the experimental manipulations Even considering these potential shortcomings, the turnover of putative amino acid neurotransmitters can be monitored in discrete CNS regions after behavioral and/or pharmacological perturbation, with reasonable confidence that increased turnover rates represent elevated neuronal activity

RESULTS

Stable baselines of morphine self-administration were ob-

TABLE 2

SIGNIFICANT CHANGES IN THE TURNOVER RATE OF DOPAMINE IN BRAIN AREAS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE AND IN YOKED-MORPHINE OR YOKED-VEHICLE INFUSED LITTERMATES

Brain Area	Treatment	Turnover Rate (pmoles mg Protein^{-1} hr^{-1})
Frontal Cortex	S-A§	34 1 ± 16 4*
	Y-M§	9 2 ± 4 3
	Y-V§	15 2 ± 9 6
Nucleus Accumbens	S-A	17 4 ± 7 3‡
	Y-M	139 6 ± 10 0†
	Y-V	104 5 ± 12 1
Septum	S-A	39 7 ± 6 1†
	Y-M	18 8 ± 4 1
	Y-V	23 0 ± 12 2
Caudate Nucleus Putamen	S-A	287 0 ± 42 6‡
	Y-M	117 8 ± 17 6‡
	Y-V	46 6 ± 7 7

Values are means ± standard deviation and are calculated as the product of the fractional rate constants (K) and content values $Turnover_A = (K)(content_A)$ The fractional rate constant (K) and error values are determined by calculating a grand mean K value from average values obtained by comparing each specific radioactivity value at the short pulse interval with each at the long pulse interval The fractional rate constant is the mean and the error estimate the S D of these individual average K values The significance of the difference between means as determined with Student's t-tests were $*=p<0\,05$, $\dagger=p<0\,01$, $\ddagger=p<0\,001$ The self-administration group was compared with the yoked-morphine infused group and the latter with the yoked-vehicle infused group

§S-A = morphine self-administration, Y-M = yoked-morphine infused, Y-V = yoked-vehicle infused

tained within three to four weeks (mean of 24 days) The average interinjection interval for the 11 litters was 122±35 minutes (all values are mean±standard deviation) Neither the total days of self-administration nor average interinjection interval differed between 60 and 90 minute pulse label groups

Content values for the seven putative neurotransmitters are shown in Table 1 Only two significant changes were observed out of 330 comparisons Levels of glutamate (Glu) and gamma-aminobutyric acid (GABA) in the brain stem of the yoked-vehicle infused group were significantly lower than in the yoked-morphine infused group These few changes in content support the hypothesis that neuronal systems are capable of maintaining sufficient neurotransmitter stores to meet functional needs within normal physiological limits Such an interpretation would indicate that the effects of the drug and the behavioral requirements in this study did not exceed these normal functional limits

Numerous changes in rates of neurotransmitter turnover were observed as a result of both the passive and contingent administration of morphine (Tables 2–5) The passive infusion of the drug (the yoked-morphine infused rats compared to the yoked-vehicle infused rats) resulted in fifteen signifi-

TABLE 3

SIGNIFICANT CHANGES IN THE TURNOVER RATE OF NOREPINEPHRINE IN BRAIN AREAS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE AND IN YOKED-MORPHINE OR YOKED-VEHICLE INFUSED LITTERMATES

Brain Area	Treatment	Turnover Rate (pmoles mg Protein^{-1} hr^{-1})
Nucleus Accumbens	S-A‡	0 7 ± 0 7†
	Y-M‡	8 3 ± 2 2
	Y-V‡	9 3 ± 1 4
Septum	S-A	42 7 ± 4 4†
	Y-M	16 6 ± 1 9
	Y-V	16 7 ± 0 1
Amygdaloid Complex	S-A	7 4 ± 2 8
	Y-M	5 5 ± 6 4*
	Y-V	17 3 ± 3 5

Values are means ± standard deviation and are calculated as the product of the fractional rate constants (K) and content values $Turnover_A = (K)(content_A)$ The fractional rate constant (K) and error values are determined by calculating a grand mean K value from average values obtained by comparing each specific radioactivity value at the short pulse interval with each at the long pulse interval The fractional rate constant is the mean and the error estimate the S D of these individual average K values The significance of the difference between means as determined with Student's t-tests were $*=p<0\,05$, $\dagger=p<0\,001$ The self-administration group was compared with the yoked-morphine infused group and the latter with the yoked-vehicle infused group

‡S-A = morphine self-administration, Y-M = yoked-morphine infused, Y-V = yoked-vehicle infused

TABLE 4

SIGNIFICANT CHANGES IN THE TURNOVER RATE OF SEROTONIN IN THE NUCLEUS ACCUMBENS AND ENTORHINAL-SUBICULAR CORTEX OF RATS SELF-ADMINISTERING MORPHINE AND IN YOKED-MORPHINE OR YOKED-VEHICLE INFUSED LITTERMATES

Brain Area	Treatment	Turnover Rate (pmoles mg Protein^{-1} hr^{-1})
Nucleus Accumbens	S-A‡	27 3 ± 8 0*
	Y-M‡	40 6 ± 6 6
	Y-V‡	46 9 ± 13 4
Entorhinal-Subicular Cortex	S-A	12 6 ± 7 1
	Y-M	4 7 ± 1 3†
	Y-V	30 6 ± 8 2

Values are means ± standard deviation and are calculated as the product of the fractional rate constants (K) and content values $Turnover_A = (K)(content_A)$ The fractional rate constant (K) and error values are determined by calculating a grand mean K value from average values obtained by comparing each specific radioactivity value at the short pulse interval with each at the long pulse interval The fractional rate constant is the mean and the error estimate the S D of these individual average K values The significance of the difference between means as determined with Student's t-tests were $*=p<0\,05$, $\dagger=p<0\,01$ The self-administration group was compared with the yoked-morphine infused group and the latter with the yoked-vehicle infused group

‡S-A = morphine self-administration, Y-M = yoked-morphine infused, Y-V = yoked-vehicle infused

TABLE 5

TURNOVER RATES OF THE AMINO ACID PUTATIVE NEUROTRANSMITTERS CONCURRENTLY MEASURED IN ELEVEN BRAIN REGIONS OF RATS INTRAVENOUSLY SELF-ADMINISTERING MORPHINE AND IN YOKED-MORPHINE AND YOKED-VEHICLE INFUSED LITTERMATES

Brain Area	Treatment	Turnover Rate (nmoles mg Protein^{-1} hr^{-1}) Asp	Glu	GABA
Frontal-Pyriform Cortex	S-A§	48 4 ± 7 4†	156 7 ± 29 0‡	25 4 ± 7 5*
	Y-M§	26 4 ± 15 8	75 9 ± 38 5	17 9 ± 5 4
	Y-V§	30 6 ± 8 2	91 3 ± 33 9	17 3 ± 5 9
Nucleus Accumbens	S-A	25 5 ± 13 8	85 0 ± 32 7	38 5 ± 6 0‡
	Y-M	26 9 ± 5 4	90 7 ± 17 9*	71 2 ± 13 2‡
	Y-V	23 5 ± 5 3	58 6 ± 37 9	27 8 ± 3 6
Caudate Nucleus Putamen	S-A	28 1 ± 4 2†	94 6 ± 12 2‡	28 3 ± 4 8‡
	Y-M	14 4 ± 8 4*	44 1 ± 18 8†	18 2 ± 0 9‡
	Y-V	23 8 ± 8 1	74 8 ± 17 6	11 9 ± 1 6
Septum	S-A	16 8 ± 11 5	64 8 ± 20 2	8 6 ± 2 6‡
	Y-M	14 2 ± 4 3*	65 3 ± 19 6†	2 8 ± 1 4‡
	Y-V	10 3 ± 1 1	31 7 ± 14 3	23 2 ± 1 8
Hypothalamus	S-A	21 3 ± 12 2	58 6 ± 22 1	72 6 ± 21 9†
	Y-M	15 6 ± 4 7	58 0 ± 17 4	43 4 ± 13 0‡
	Y-V	12 7 ± 4 6	36 0 ± 26 7	17 4 ± 5 2
Hippocampal Formation	S-A	25 6 ± 4 6‡	85 5 ± 14 6‡	16 1 ± 3 0†
	Y-M	13 3 ± 4 4	41 2 ± 17 4	8 5 ± 4 0
	Y-V	16 6 ± 7 0	51 9 ± 26 8	11 3 ± 4 1
Amygdaloid Complex	S-A	33 5 ± 4 2‡	92 2 ± 15 5†	16 2 ± 3 6†
	Y-M	18 2 ± 6 9*	45 4 ± 26 1	8 7 ± 5 0
	Y-V	26 9 ± 9 2	69 9 ± 23 0	11 6 ± 3 8
Thalamus	S-A	38 5 ± 9 6*	106 8 ± 32 9	44 9 ± 8 1†
	Y-M	21 6 ± 15 5	71 1 ± 50 0	22 5 ± 13 9
	Y-V	17 6 ± 9 5	59 2 ± 29 1	15 5 ± 6 8
Motor-Somatosensory Cortex	S-A	81 5 ± 28 5*	164 3 ± 48 0‡	22 6 ± 5 1†
	Y-M	48 0 ± 16 7	81 6 ± 19 7	11 9 ± 5 8
	Y-V	45 8 ± 9 6	91 6 ± 33 5	15 0 ± 4 1
Entorhinal-Subicular Cortex	S-A	34 3 ± 11 9	106 7 ± 44 6	19 3 ± 8 6
	Y-M	33 2 ± 17 9	97 5 ± 51 7	24 7 ± 11 7*
	Y-V	32 8 ± 10 4	93 6 ± 35 3	13 9 ± 6 7
Brain Stem	S-A	40 6 ± 6 7*	95 6 ± 26 5	32 4 ± 8 0*
	Y-M	24 9 ± 16 1	65 3 ± 44 2	22 4 ± 10 7
	Y-V	31 1 ± 15 5	69 5 ± 23 4	21 7 ± 8 3

Values are means ± standard deviation and are calculated as the product of the fractional rate constants (K) and content values $Turnover_A = (K)(content_A)$ The fractional rate constants (K) and error values are determined by calculating a grand mean K value from average values obtained by comparing each specific radioactivity value at the short pulse interval with each at the long pulse interval The fractional rate constant is the mean and the error estimate the S D of these individual average K values The significance of the difference between means as determined with Student's t-tests were $*p<0\ 05$, $\dagger=p<0\ 01$, $\ddagger=p<0\ 001$ The self-administration group was compared with the yoked-morphine infused group and the latter with the yoked-vehicle infused group

§S-A = morphine self-administration, Y-M = yoked-morphine infused, Y-V = yoked-vehicle infused

cant ($p<0$ 05 to $p<0$ 001) changes in turnover The caudate-putamen-globus pallidus had the greatest number of changes DA (153%) and GABA (53%) turnover was elevated in the morphine infused group, while Asp (−43%) and Glu (−41%) decreased In the septum, Asp (38%) and Glu (106%) utilization increased while GABA (−88%) decreased In the nucleus accumbens DA (34%), Glu (55%) and GABA (156%) turnover was increased while amygdala NE (−68%) and Asp (−32%) decreased The entorhinal-subicular cortex showed a decrease in 5-HT utilization (−85%) and an increase in GABA (78%) A significant elevation in GABA utilization (149%) was also seen in the hypothalamus

More and generally larger changes in turnover rates were seen as a consequence of the contingent administration of morphine (self-administration rats compared to the yoked-morphine infused rats) Twenty-nine significant changes were observed, which were predominantly in limbic structures The caudate nucleus-putamen-globus pallidus, frontal cortex and nucleus accumbens had the most changes In the striatum, increases in the utilization of DA (144%), Asp (95%), Glu (115%) and GABA (55%) were seen that were similar to changes in the frontal-pyriform cortex (DA (271%), Asp (53%), Glu (106%), and GABA (42%)) The nucleus accumbens showed only decreases (DA (−88%), NE (−92%), 5-HT (−33%), and GABA (−46%)) while in the septum DA (111%), NE (157%) and GABA (207%) turnover was elevated The increases in hippocampal amino acid neurotransmitter utilization (Asp (92%), Glu (108%), and GABA (89%)) were similar to increases observed in the amygdala (Asp (84%), Glu (103%) and GABA (86%)) Elevated turnover of Asp (70%), Glu (101%), and GABA (90%) were also seen in the motor-somatosensory cortex In the thalamus increased utilization of Asp (78%) and GABA (86%) were found that were similar to increases in the brain stem (Asp (63% and GABA (45%)), while only GABA turnover was elevated in the hypothalamus (67%)

DISCUSSION

The most significant findings of this study are the changes in utilization rates of the putative neurotransmitters resulting from the mode of administration of morphine Few would hypothesize that such large differences in neuronal activity (as monitored by neurotransmitter turnover) would exist between animals self-administering a drug compared to littermates given identical and simultaneous passive infusions These changes are likely independent of the apparent differences resulting from the response requirements of the schedule of morphine reinforcement During the pulse-label interval, neither morphine nor vehicle were delivered to the litter even if appropriate responding occurred This safeguard was introduced to prevent confounding of the non-specific general neurochemical effects of morphine with the specific effects of the drug-seeking behavior However, the stimulus light and tone were presented if an appropriate FR 10 response was emitted, resulting in conditioned morphine-like stereotypical behavior (weaving, bobbing, circling, etc) in both morphine-dependent rats Approximately one-half the self-administering animals responded for an injection during the pulse interval with the most responses occurring near the predicted injection time, suggesting that these estimates are an accurate approximation of the time when the self-administering animal will seek another injection When they did occur, these lever presses were a small percentage of the total motor activity during the pulse interval Therefore, neurochemical differences between the morphine self-administering and the yoked-morphine infused animals would be unlikely to result from the purely motor effects of these lever presses, but may well partially result from the motivational effects of non-reinforcement (extinction) However, the neurochemical consequences of non-reinforcement have not been directly assessed

Recent advances in the knowledge of neuronal pathways in the brain have led to a better understanding of the actions of neuronal systems in brain function The location and distribution of neuronal pathways and fibers utilizing specific neurotransmitters (i e , acetylcholine [26,54], serotonin [18], catecholamines [17, 26, 32, 54, 77], substance P [8, 33, 34] and enkephalins [64, 76, 81]) and studies of neurotransmitter localization in specific brain regions (i e , hippocampus [72] and cortex [18]) allows one to interpret changes in neurotransmitter utilization as resulting from changes in activity of specific populations of neurons with discrete connections and projections Measurement of turnover rates of multiple neurotransmitters in small brain regions of the same animal permit one to set forth hypotheses concerning the involvement of specific neuronal circuits in brain function This approach has been used to interpret the data collected in this study

Effects of Morphine

The neurochemical consequences of the passive injection of morphine have been exhaustively researched [35] The effects of morphine on the turnover of GABA have been previously reported [44] However, the effects of Asp and Glu turnover have not GABA is thought to be the neurotransmitter for most intrinsic neurons in the cortex [28,59], nucleus accumbens [80], striatum [5,40], lateral septum [39] and hippocampal formation [72] The passive infusion of morphine resulted in significant increases in GABA turnover in two of these areas (nucleus accumbens and striatum), which could represent increased efferent and afferent neuronal activity If this interpretation is correct, then changes in utilization of neurotransmitters in known inputs to and outputs from these regions to projection areas should also occur The caudate-putamen-globus pallidus, which received Glu efferents from the motor-somatosensory and entorhinal-subicular cortices [16,41], was found to have decreased Glu utilization Turnover of GABA in the nucleus accumbens (representing primarily intrinsic neuronal activity) is similar to the turnover rates of Glu which probably represent activity in the cortical and hippocampal Glu efferents in this structure Passive chronic morphine infusions resulted in decreases in the turnover of NE in the amygdala and increases in DA utilization in the striatum which has been previously observed [27] However, this is not a general stimulation of forebrain dopamine neurons since similar changes in turnover were observed only in the nucleus accumbens and not in other mesolimbic structures receiving DA innervation

Six of the fifteen changes in turnover rate observed in the passively infused morphine group were identical in the self-administering group and could represent changes in activity in neuronal pathways important in mediating the central effects of this analgesic (i e , blunted affect toward chronic pain) The increases in Glu turnover in the nucleus accumbens and septum, Asp in the septum, GABA in the entorhinal-subicular cortex and the decreases in the utilization of NE in the amygdala and 5-HT in the entorhinal-

subicular cortex could represent changes in input to and output from the hippocampus The hippocampus sends Glu efferents to the nucleus accumbens which account for 50% of the high affinity uptake for this amino acid in that structure and to the lateral septum where Schaffer's collaterals terminate and are thought to be glutamatergic [16,72] The elevated turnover of Glu in these two areas [55% and 106%, respectively) in the morphine treated groups could result from increased excitatory output from the hippocampus Opiates are initially inhibitory (generally decrease neuronal activity) at most brain sites except in the hippocampus [85] Excitation in this structure is thought to result from morphine's effects on inhibitory GABA interneurons The passively infused morphine group showed a small decrease in GABA turnover that was not significant but could be indicative of a decrease in GABA inhibition in the hippocampus, thus resulting in increased glutamatergic excitatory output to the septum and nucleus accumbens The changes in 5-HT and GABA turnover in the entorhinal-subicular cortex could also be related to changes in hippocampal activity These turnover changes in these limbic structures could indeed be responsible for such analgesic effects as alterations in affect toward pain However, only direct measurements in animals exposed to chronic pain can test such involvement

The changes in utilization rates of the biogenic monoamine and amino acid neurotransmitters resulting from passive chronic morphine treatment is not surprising when the pattern of distribution of opiate receptors [1] and enkephalin neurons [81] is considered Opiate receptors are dense in the caudate nucleus, nucleus accumbens, septum, and amygdala [1] These receptors may be located on postsynaptic cells innervated by enkephalin neurons since such fibers have been recently demonstrated [81] As previously stated, acute administration of opiates appears to decrease neuronal activity in most brain regions (except in the hippocampus) Chronic treatment results in a decrease in the number of cells responding to inhibition and an increase in the number of cells responding to excitation [85] These increases in turnover rates could represent tolerance to the inhibitory effects, or more specifically, receptor adaptation to the repeated presentation of drug to the same population of receptors

Effects of the Self-Administration Milieu

The contingent self-administration of morphine has a substantial effect upon neurotransmitter turnover The brain structures important in mediating self-administration and morphine reinforcement appear to be the hippocampus, nucleus accumbens, septum, striatum, amygdala and frontal-pyriform cortex In the hippocampus, the self-administering rats were found to have substantially elevated utilization rates of Asp, Glu and GABA suggesting the presence of increased neuronal activity Glu is thought to be a neurotransmitter in the intrahippocampal mossy fiber system [6], the interhippocampal commisural fiber system [45,46], hippocampal-subicular efferents [49,74] and the entorhinal perforant pathway [45, 73, 83] Intrinsic neurons in the hippocampus are believed to primarily release Asp [45,46] and GABA [72] The glutamatergic CA3 pyramidal cells which innervate the intrinsic hippocampal GABA neurons could be responsible for the similar increase in turnover rates of GABA, Asp and Glu observed in this area Lesions of the hippocampus decrease the reinforcing properties of morphine [23], which along with these increases in turnover, suggest an important role for this structure in morphine reinforcement processes The utilization of GABA was decreased in the nucleus accumbens of the self-administration animals In contrast, passive morphine infusion resulted in increases in inhibitory neuronal activity (GABA) The decrease in 5-HT utilization in the self-administering group in the nucleus accumbens may also be important since microinjections of 5-HT into this area attenuate intracranial electrical self-stimulation (ICSS) [48,58] This decrease in turnover of 5-HT could represent decreased activity in serotonergic neurons that attenuate reward processes The importance of the nucleus accumbens in opiate reinforcement is further supported by the observation that rats will self-administer morphine and respond for electrical self-stimulation from electrodes implanted in this structure [51] These hippocampal-nucleus accumbens innervations could have a central role in opiate reward processes

The septum, frontal cortex and striatum are also important in opiate reinforcement Lateral septal NE, DA and 5-HT inputs modulate activity in the medial septo-hippocampal cholinergic fiber systems through interneurons NE has an excitatory effect while both DA and 5-HT appear to be inhibitory to hippocampal acetycholine (ACh) synthesis [61–63] Turnover rates of DA and NE in the septum of self-administering animals were double that of the morphine and vehicle infused rats This increase in NE utilization could be partly responsible for the increased hippocampal neuronal activity resulting from changes in the cholinergic septo-hippocampal neurons The frontal cortex is also important in brain reward systems [43] and learning [4] Electrolytic lesions of the frontal cortex decrease the reinforcing properties of morphine [23] while 6-hydroxydopamine lesions result in deficits of reversal learning of rats [66] and in delayed alternation learning of monkeys [4] The increase in DA turnover in the self-administration group in the frontal cortex suggests that this structure could be involved in mediating the rewarding properties of morphine The striatum may also be important since electrolytic lesions increase the reinforcing properties of opiates [20,24] and morphine self-administering rats show a specific increase in metabolic activity (2-deoxyglucose method) in this region compared to yoked-morphine infused controls [25]) The increase in utilization of DA, Asp, Glu and GABA in the striatum of self-administering animals [67] further support a role for this structure in opiate reward mechanisms

Dopaminergic neurons are important in reward processes [84] and their involvement in opiate reinforcement is suggested by these data Frontal cortex dopaminergic innervation is thought to send collaterals to the septum [15] which is supported by the increased turnover seen in these two areas (271% and 111%, respectively) However, other neuronal systems may also be involved Norepinephrine appears to be important since noradrenergic blocking agents decrease opiate self-administration and inhibit the development of secondary-reinforcing properties of stimuli associated with morphine infusion [13] Activity in noradrenergic innervations of the nucleus accumbens and septum are significantly different in the self-administering rats Drugs that antagonize cholinergic activity also attenuate the reinforcing properties of morphine [12] It would appear that DA, NE and ACh neurons may all be involved in opiate reinforcement processes

These limbic structures involved in opiate reinforcement may also be important in other reward processes ICSS occurs in most of these regions [50] The recent demonstration

of ICSS in areas of the entorhinal cortex that have sparse dopaminergic innervation [53] or areas with near total DA depletion [55] would support the involvement of other neurons in reinforcement processes, since stimulation of non-dopaminergic components could result in transmission of a rewarding stimulus Common components of neuronal circuits mediating ICSS and opiate reward have been demonstrated Morphine lowers while naloxone raises ICSS thresholds [29] Naloxone also modulates feeding [70], drinking [71] and sexual behavior [52] The reinforcing properties of other drugs may have neuronal components in common with opiate reward pathways since 6-hydroxydopamine lesions in the nucleus accumbens modify cocaine [60] and amphetamine [38] self-administration The involvement of these pathways implicated in opiate reward in other reinforcement processes are currently under investigation

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