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Effect of Heroin-Conditioned Auditory Stimuli on Cerebral Functional Activity in Rats

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TRUSK, T. C. AND E. A. STEIN. *Effect of heroin-conditioned auditory stimuli on cerebral functional activity in rats.* PHARMACOL BIOCHEM BEHAV **30**(4) 983–993, 1988.—Cerebral functional activity was measured as changes in distribution of the free fatty acid [1-^{14}C]octanoate in autoradiograms obtained from rats during brief presentation of a tone previously paired to infusions of heroin or saline. Rats were trained in groups of three consisting of one heroin self-administering animal and two animals receiving yoked infusions of heroin or saline. Behavioral experiments in separate groups of rats demonstrated that these training parameters impart secondary reinforcing properties to the tone for animals self-administering heroin while the tone remains behaviorally neutral in yoked-infusion animals. The optical densities of thirty-seven brain regions were normalized to a relative index for comparison between groups. Previous pairing of the tone to heroin infusions irrespective of behavior (yoked-heroin vs. yoked-saline groups) produced functional activity changes in fifteen brain areas. In addition, nineteen regional differences in octanoate labeling density were evident when comparison was made between animals previously trained to self-administer heroin to those receiving yoked-heroin infusions, while twelve differences were noted when comparisons were made between the yoked vehicle and self administration group. These functional activity changes are presumed related to the secondary reinforcing capacity of the tone acquired by association with heroin, and may identify neural substrates involved in auditory signalled conditioning of positive reinforcement to opiates.

Functional neuroanatomy Octanoate Heroin-conditioned tone Reinforcement

THE positive reinforcing properties of opiates are well known. Several studies have demonstrated that response-dependent infusions of heroin or morphine maintain complex patterns of behavior in animals and man [19]. This rewarding effect of opiates results from their interaction with opioid receptors in the brain, probably within one or more specific neuronal systems involved in the detection of reinforcing events. Various research approaches have significantly increased understanding of the location of opiate receptor populations involved, and defined the nature of central functional systems associated with opiate reinforcement processes. For example, rats will learn to self-administer opiates directly into several intracranial locations including nucleus accumbens [34] lateral hypothalamus, preoptic area [43] and ventral tegmentum [2], indicating that many opiate receptor populations relevant to reinforcement may exist within the brain. Electrolytic lesions of areas such as hippocampus and frontal cortex diminish, while lesions of the caudate or substantia nigra facilitate opiate-seeking behavior [15], suggesting that several brain systems modulate the reinforcing effects of opiates. Regional assays of neurotransmitter turnover in rats self-administering morphine compared to that in rats receiving yoked infusions indicate that at least two complex neural circuits may be active in opiate-seeking animals [39,40].

The purpose of this study was to further investigate the functional anatomy of brain systems involved in opiate reinforcement using a tracer of cerebral metabolic activity. Such measurements allow the simultaneous assessment of functional activity in several brain regions in conscious, unrestrained animals. Two major difficulties are present in attempts to measure metabolic activity in opiate-seeking animals. First, the stimulus used to induce the cerebral metabolic response under consideration is assumed to be both salient and stable during the period of tracer incorporation [42]. During the 30–45-minute period required in the 2-deoxyglucose (2DG) method, a rat in an operant chamber will be exposed to many types of stimuli, and will perform a wide

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range of motor behaviors. Although comparisons to appropriate control animals may help exclude the effects of such extraneous variables, rewarding stimuli and associated approach behaviors likely induce brief changes in functional activity which represent a small fraction of the total cerebral metabolic response measured. Such brief changes in metabolic activity may thus not be detectable. Indeed, the functional activity of ten brain regions measured with labeled 2DG in rats self-administering morphine was identical to that obtained in yoked morphine-infused rats [14]. The second difficulty concerns the presence of opiates in the animal during determination of cerebral metabolic activity. Opioid receptors are found throughout the central nervous system [1], and exogenous opiates induce many effects other than reward [27], which may mask metabolic responses associated with the behavior under investigation. Furthermore, the cerebral metabolic response induced by opiates, per se, is reportedly masked by systemic hypercapnia occurring in the course of the prolonged experimental period required in these studies of local cerebral glucose utilization (LCGU) [26]. This may explain why the administration of 8 mg/kg morphine sulfate SC or 5 mg/kg IV, doses known to have profound behavioral and physiologic effects, were recently found to have minimal effects on LCGU in much of the rat central nervous system [10,24]. In contrast, we have recently demonstrated that 0.2 mg/kg heroin IV profoundly alters (mainly increases) local cerebral blood flow when measures are made 1 min after drug administration and prior to alterations in blood chemistry [45]. In this study, these difficulties were circumvented by using [1-^{14}C]octanoate (OCTO) as a rapid tracer of cerebral functional activity in drug-free rats during brief exposure to an auditory stimulus previously associated with infusions of heroin.

Rowley and Collins [36] have previously described an autoradiographic assay of cerebral functional activity using OCTO. This free fatty acid is rapidly cleared from the blood and beta-oxidized, producing a labeled pool of glutamate. As it is known that glutamate synthetase is found predominantly within protoplasmic astrocytes rather than neurons [31], it has been posited [36] that OCTO accumulation is due to its entry into astrocytes lining cerebral capillaries and thus reflecting changes in CBF. However, unlike the freely diffusable tracer iodoantipyrene, which has more traditionally been used to measure CBF [37], and which presumably diffuses through and/or around astrocytes into nuerons and their surrounding extracellular space, labeled OCTO ultimately becomes converted to glutamine in astrocytes which results in the retention of the labeled carbon for up to 6 minutes. The sequence by which changes in astrocytes might reflect neuronal firing has been suggested by a recent report demonstrating that an increase in extracellular potassium ions following neuronal activation leads to an increase in intracellular astrocyte pH, thus suggesting a mechanism for local alterations in CBF [4]. Further, measurements as early as 60 to 90 sec are possible with this label. As such, regional OCTO labeling densities likely provide a measure of local blood flow with autoradiographic resolution surpassing that obtained with inert, freely-diffusible tracers such as iodoantipyrine. Local blood flow remains coupled to surrounding physiological activity in the brain under most conditions [42]. Despite the inability to directly quantify blood flow via OCTO metabolism, the relative regional accumulation of tracer provides a useful rapid indicator of functional activity in the brain [46].

Cerebral functional activity was measured in this study during brief exposure to a tone previously paired with heroin or vehicle infusions. Environmental stimuli repeatedly paired with opiate injections eventually serve as conditional stimuli for opiate-induced effects [25]. Several studies have demonstrated that opiate-conditioned stimuli will increase motor activity [29], reduce withdrawal symptoms [9], induce morphine-like effects on serum glucose and striatal homovanillic acid content [23], and support instrumental behavior as secondary reinforcers [32,41]. Many of the opiate-like effects of such conditioned stimuli are reversed by naloxone, suggesting that these previously neutral events acquire the ability to produce opiate actions by causing the release of endogenous opioids [8]. Thus, stimuli paired with heroin infusions may gain secondary reinforcing properties by releasing endogenous opioids within brain reward systems. In this study, we measured cerebral functional activity in rats exposed to a tone previously paired to response-dependent heroin infusions, and in rats exposed to a tone previously paired to heroin or saline infusions that were delivered independent of behavior but at a pairing rate yoked to a rat self-administering heroin. Preliminary behavioral experiments in separate groups of similarly-trained animals showed that a tone paired to response-dependent heroin infusions gains secondary reinforcing properties, while a tone paired to heroin infusions independent of behavior does not. Thus, this design allows determination of the functional activity changes related to auditory stimuli with similar training history, but different motivational properties. It is hypothesized that the behavioral-independent effects of a tone paired to heroin infusions on cerebral functional activity are evident by comparison of the yoked-vehicle and yoked-heroin trained rats, and can be separated from the heroin-conditioned secondary reinforcing property of the tone in heroin-seeking animals.

METHOD

Animals

Thirty-one male Sprague Dawley-derived rats (Holtzman Co., Madison, WI) weighing 250–300 g were individually housed in Plexiglas cages with food and water available ad lib. Animals were kept on a reversed light/dark cycle with lights off between 0700 and 1900 hr. All experiments were conducted during the dark phase. Chronic catheters were implanted into the right external jugular vein of each rat under Chloropent® anesthesia using standard procedures [47]. Catheters continued subcutaneously to exit between the scapulae and through the center of a 1/4–20 nylon screw with a 3 cm circle of nylon mesh glued to the base. The nylon mesh was implanted under the skin and the protruding screw provided an attachment point for a metal spring leash described below. Rats were allowed at least 3 days of recovery before training.

Apparatus

Experiments were conducted in 16×50×34 cm black Plexiglas chambers with metal grid floors housed in plywood sound-attenuated boxes. A metal spring leash protecting a 40 cm length of PE20 connected to a fluid commutator was suspended over the center of each chamber by a counterbalanced arm. Fluid commutators [3] were attached to syringes mounted on a Harvard Apparatus infusion pump (Model 975). All intravenous infusions were presented over

1–2 seconds in 0.05–0.1 ml volume. Heroin (3,6-diacetylmorphine HCl) was dissolved in normal saline (0.9%) vehicle, and doses are expressed as the salt. Each chamber was equipped with a speaker which maintained a constant level of white noise and presented the auditory stimulus used in conditioning, and two levers (2×7 cm) separated by 16 cm mounted in the center of the opposite chamber wall. The auditory stimulus was a pulsating 12 KHz tone that was on 700 msec and off 300 msec during each second of presentation. A microcomputer counted lever responses, controlled auditory stimuli presentations, and operated the infusion pump.

Procedures

Behavioral experiments. Two behavioral experiments tested for conditioned reinforcing capacity of the tone paired to heroin and saline infusions, results of which determined the training conditions used in the OCTO experiment described below. In the training phase of the OCTO study, the tone was paired to response-contingent heroin infusions, or to either heroin or saline infusions independent of behavior. Thus, these preliminary studies were designed to test the ability of the tone to function as a conditioned reinforcer following similar tone-infusion pairing parameters.

Behavioral experiment 1 measured the behavioral significance of a tone paired with response-contingent heroin administration in 4 rats. Each animal was trained to self-administer heroin (0.06 mg/kg) on a continuous reinforcement schedule in 5-hr sessions over 5 days. A 5-sec tone was paired with each heroin infusion. During all self-administration and extinction sessions, responses on the second chamber lever were counted and had no consequence. On the sixth day, heroin was replaced with normal saline and self-administration responding was allowed to extinguish. Lever responses during this 5-hr extinction session produced the tone for 2 rats, and did not produce the tone for the others. On days 7–11, rats were retrained to self-administer heroin with the tone once again paired to drug infusions. Self-administration responding was again extinguished in a 5-hr session on day 12. The lever response consequence (tone presence or absence) for each rat was reversed from that experienced in the first extinction session. An elevation of extinction response rate with tone present above that obtained with tone absent would indicate the ability of the tone to maintain responding as a conditioned reinforcer.

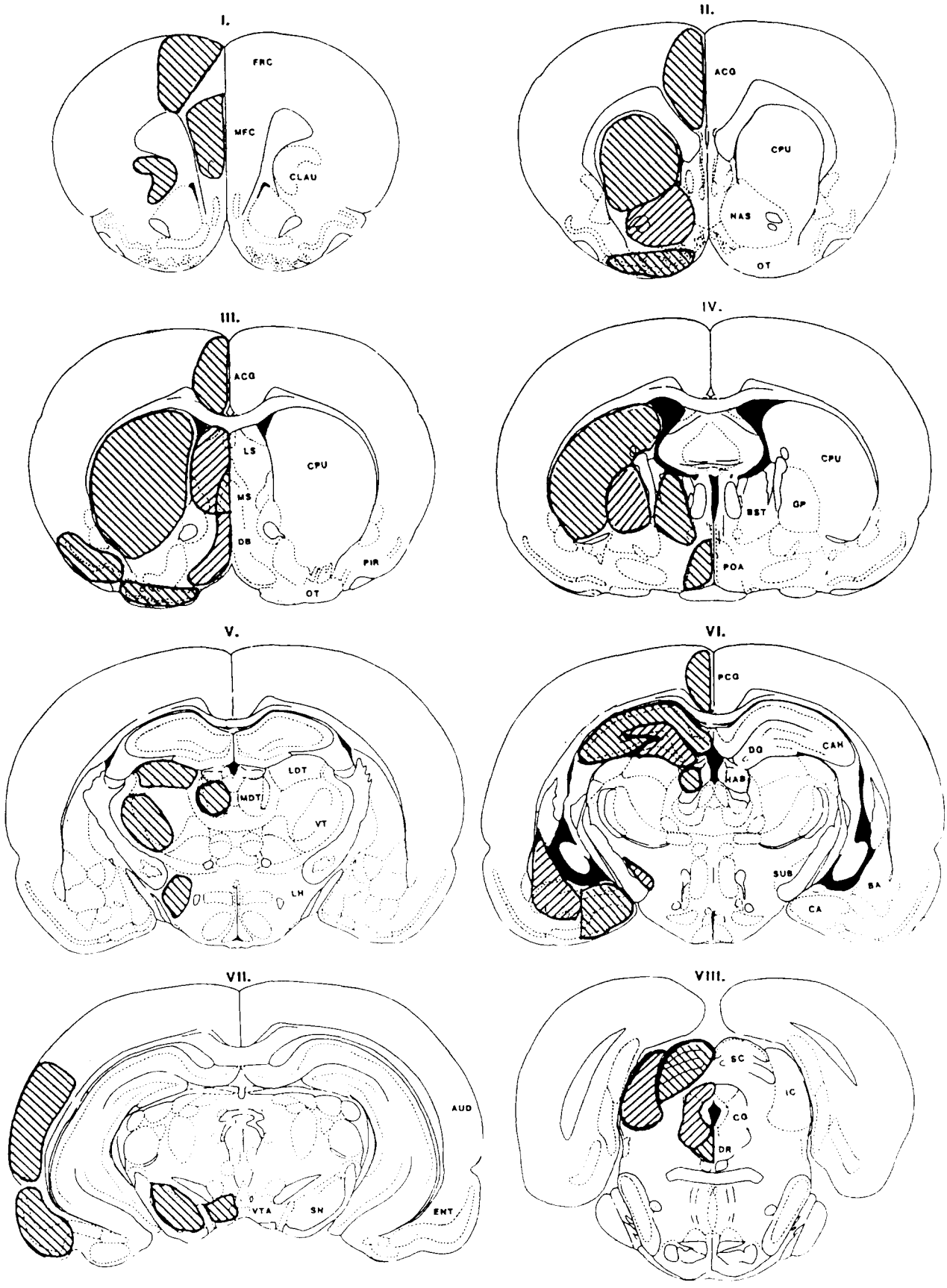
Behavioral experiment 2 examined the behavioral significance of a tone paired to heroin or saline infusions without response contingency in 12 naive animals. Rats were first adapted to the infusion leash and experimental chamber with the levers removed for 1 hr. On day 2, responses on either lever had no consequence and lever preference was determined by counting responses in a 1-hr session. The pretraining phase consisted of 5 daily 1-hr sessions during which responses on the nonpreferred lever produced a tone (5 sec) and responses on the preferred lever had no consequence. During the following 5 day training phase, levers were removed and rats were given 25 behaviorally noncontingent pairings of tone (5 sec) and heroin (0.06 mg/kg; n=6) or tone and normal saline (n=6) each day. Tone-infusion pairings were presented randomly over 5 hours with an average interpairing interval of 10 minutes. Each rat received a total of 125 pairings. The conditions of the pretraining phase were then reinstated, and lever responses were counted in a single 1-hr session. An elevation of tone-lever responding above that on the inconsequential lever, and greater than the operant rate determined in pretraining, would indicate an ability of the tone to reinforce lever pressing. The conditioned reinforcement quality of the tone would presumably result from contiguous pairing with heroin, a primary reinforcer.

OCTO experiment. In order to investigate those neuronal structures involved with heroin-induced reward, the following autoradiographic study was performed.

Training phase. The catheter of each rat was flushed with heparinized saline immediately prior to training sessions to ensure catheter patency and to familiarize the animal with manual infusion procedures. Fifteen rats were divided into three training groups. On the first day each group was allowed 1 hr of adaptation to the experimental chambers while lever presses were counted to determine lever preference. During a 3-hr session on the second day, each response on the originally nonpreferred lever resulted in a 2-sec infusion of normal saline. Auditory stimuli were not presented during this session. Any animals demonstrating saline self-administration during this session were subsequently placed in one of the yoked-infusion groups. All remaining training sessions were 5 hours in duration. One animal in each group was allowed to self-administer heroin (0.06 mg/kg) by a single press of the originally nonpreferred lever (treatment group SA). Responses on the other lever were counted but had no consequence. A second animal received a yoked infusion of heroin (0.06 mg/kg; group YH), and the third received a yoked infusion of saline vehicle (group YV) whenever the self-administering rat received an infusion. Responses on both levers in the yoked animals' chambers were also counted and without consequence. All drug and vehicle infusions in these sessions were accompanied by a 5-sec auditory stimulus presentation. Training continued daily for a minimum of 5 days until the response rate of the self-administering rat varied less than 20% over 3 consecutive days.

Autoradiography. Relative regional cerebral functional activity was measured with the OCTO method [36] during presentation of the conditioned auditory stimulus on the day immediately following establishment of criterion self-administration behavior. Heroin was not administered to any of the animals during this procedure. Due to time constraints, the three rats of each group were treated sequentially. The catheter of each rat was flushed with heparinized saline and 16 μCi/100 g body weight OCTO (53 mCi/mmol, New England Nuclear) suspended in 0.1 ml normal saline was rapidly infused. The catheter was closed and the animal was placed in its respective experimental chamber with leash attached. The auditory stimulus was started by the experimenter and continued for 2 min. Rats were then immediately removed from the chamber and decapitated. The lower jaw and skin were rapidly removed and brains were frozen in skull in 2-methylbutane (-40°C) for 5 minutes, removed from skull in a cryostat at -20°C, and stored at -70°C. Brains were cut into 20 μm sections at -20°C in a cryostat (IEC, Model CTI). Every fourth section was thaw-mounted onto glass slides, dried on a warming tray and apposed to x-ray film (Kodak SB-5) in standard cassettes (Wolf) for at least 15 days. Tissue sections were stained with thionin following film development.

Analysis. Autoradiograms were analyzed with a computer-assisted image analyzer (Model 850, Spatial Data Systems), and the optical density of brain regions of interest were nor-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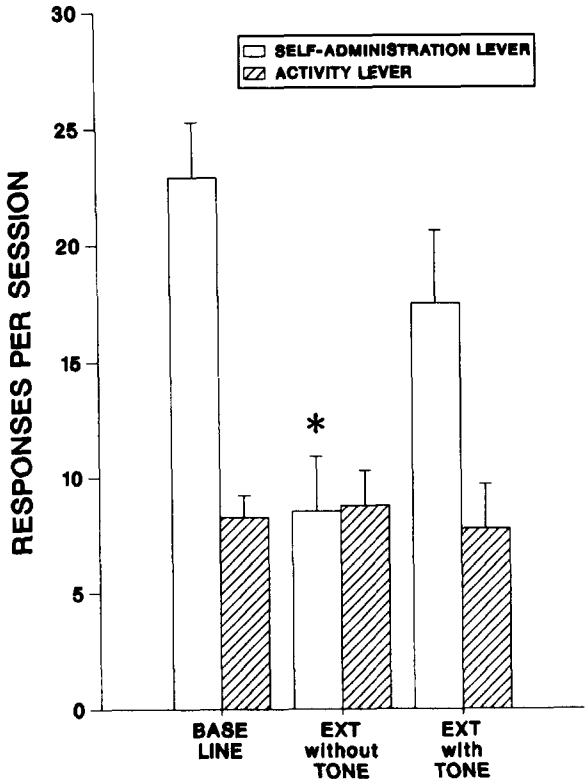

FIG. 2. Mean (±SE) lever response rates of heroin self-administration rats (n=4) on heroin-tone lever (open bars) and non-effective activity lever (hatched bars) in baseline sessions immediately preceding extinction (EXT), and in EXT sessions with heroin-paired tone absent or present. EXT response rate on the self-administration lever without tone was significantly lower than when responding was extinguished with tone presentations (*$p<0.01$).

malized to a relative optical density index (ROD) as described by Gallistel *et al.* [12,13]. Briefly, each autoradiogram was digitized into 150,000 to 200,000 optical density points from a range of 256 gray scale values. The frequency distribution of gray scale values for the entire brain slice (excluding background, ventricular spaces and tissue folds) was converted to a cumulative gray scale histogram, and the ROD of each gray scale value was derived as the rank order of its position within the cumulative histogram. ROD is expressed as a value between 0 and 100, and an ROD value of 80 is defined as a gray scale value darker than 80% of the density points found in a particular brain slice. The ROD normalization procedure reduces variance due to factors acting on the overall darkness of an autoradiographic image, is not affected by the nonlinear relationship between radioactivity and optical density and yields data less sensitive to between section and between brain measurement error when compared to gray-to-white ratio normalization [12,13].

Thirty-seven brain regions, as defined by Paxinos and Watson [35], were chosen for analysis based on implications of their involvement in opiate reinforcement and neurophysiological conditioning studies (see Fig. 1). Each region was outlined on a video image of the stained histological section aligned to its corresponding autoradiogram. The average ROD for the density points contained in the regional outline was obtained from 3–5 sections from each side of each brain. Thus, depending on the size of a given structure, 30–50 separate data points contributed to each anatomic regional ROD for each treatment. Since ROD is a proportion, an arcsin transformation was performed prior to evaluation of treatment effects in each brain region by individual one-way analysis of variance (ANOVA). Comparisons between groups were made with the Newman-Keuls test where appropriate [22]. To provide a more stringent analysis, ROD differences were considered significant at $p<0.01$.

RESULTS

Behavioral Data

Average session response rates prior to, and during, extinction (EXT) for the 4 rats trained to self-administer heroin in Study 1 are shown in Fig. 2. Baseline response rates on the self-administration and activity levers are the mean calculated over the three training sessions preceding EXT. Rats pressed the self-administration lever at a rate 2–3 times above that on the noneffective activity lever, and each rat self-administered between 20–25 heroin infusions per 5-hr training session. During the EXT session in which responses on the infusion lever did not result in tone presentation, response rate on the self-administration lever decreased rapidly and was not different from the session response rate on the activity lever. In contrast, responding on the self-administration lever decrease only slightly from baseline levels when EXT responses produced the tone associated with heroin infusions in training. The mean number of EXT responses on the infusion lever when the tone was absent was significantly lower than when the tone was present, $t(6)=-4.6$, $p<0.01$, demonstrating the ability of the tone to maintain self-administration behavior despite replacement of the primary reinforcer (heroin) with saline.

Figure 3 shows the effect of response-independent pairing of tone with heroin or tone with saline on subsequent tone- versus activity-lever preference in Study 2. In the 5 sessions prior to tone-infusion pairing, rats pressed the tone-lever less often than the noneffective activity lever (7.6 to 10.6 responses per session, respectively), but this difference in lever preference was not statistically significant. In the lever preference test following tone-saline conditioning, rats continued to respond more often on the activity lever. This difference was also not significant. Following tone-heroin pairing, response rate on both levers was also equivalent. Response-independent pairing of tone to heroin infusions at the same delivery rate observed in self-administration rats (Study 1) was thus not sufficient to influence lever preference and suggests that the tone failed to acquire conditioned reinforcer properties under these conditions. Thus, response-contingent tone-heroin pairings were used in the subsequent OCTO study.

Figure 4 shows the mean lever response rate of the 15 rats in the training phase of the OCTO study during the three sessions prior to the day of tracer injection. Lever 1 in the

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FIG. 1. Drawings of coronal sections of rat brain indicating different regions analyzed in OCTO autoradiograms.

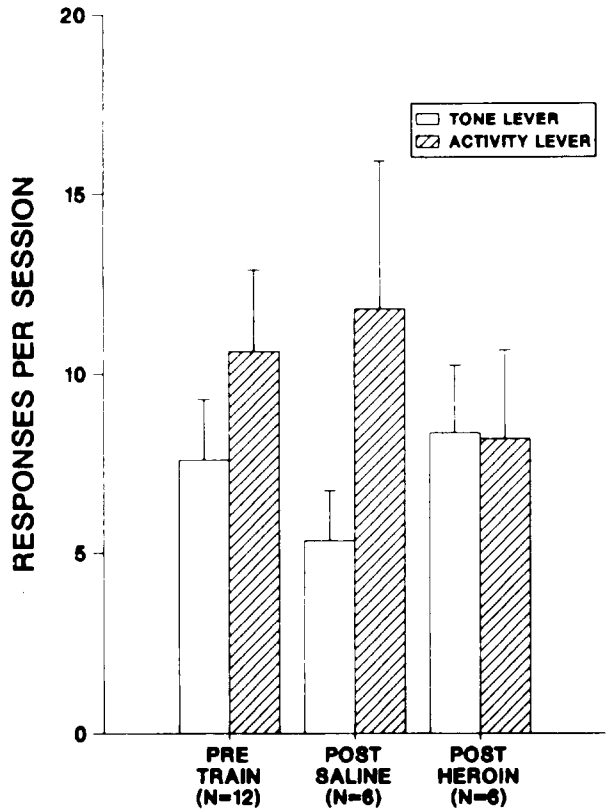

FIG. 3. Mean (±SE) lever response rates of rats before (PRE-TRAIN) and after response-independent pairing of tone to heroin (n=6) or tone to saline infusions (n=6). Response rate on lever producing infusion-paired tone was not statistically different from rate observed on noneffective activity lever before or after training.

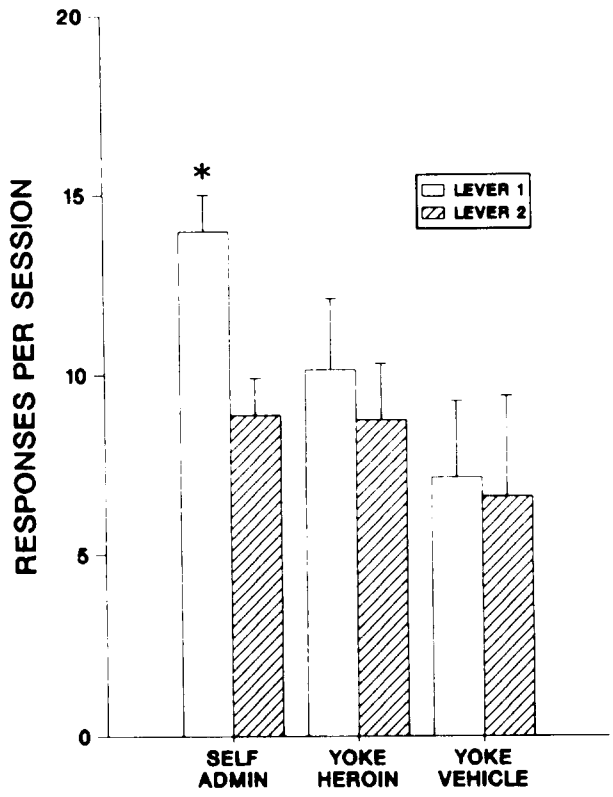

FIG. 4. Mean response rate of rats used in OCTO experiment during the three training sessions prior to sacrifice. Lever 1 of the SELF-ADMIN animals controlled tone-infusion presentations in the three conditions, and response rate on this lever was significantly greater than the rate on noneffective Lever 2 (*$p<0.01$). Responding on both noneffective levers by the yoked-infusion animals was similar to the response rate on the activity lever by SELF-ADMIN rats.

self-administration chamber operated the infusion pump and tone for the three rats of each group in training, and response rate on this lever was significantly greater than the rate obtained on noneffective Lever 2, $t(7)=4.09$, $p<0.01$. Yoked animals were observed to press both noneffective levers in their chambers at equal rates. All rats in the OCTO experiment received 123±3 tone-paired infusions in the course of training. The response levers were present during the 2 minute OCTO labeling/tone presentation period. Each rat pressed the levers an average of only once during this period and thus there was no difference in response rate among the treatment groups which may have confounded determination of cerebral metabolic activity.

OCTO Data

Autoradiograms obtained from the rats following 2 minutes exposure to the tone and [1-^{14}C]octanoate provided good histological resolution. Autoradiograms obtained from one rat in the yoked-heroin group were not included in the ROD analysis due to insufficient delivery of tracer.

A summary of the results of the image analysis and statistical comparisons are shown in Table 1. ROD was changed in several regions during presentation of a tone previously paired to both passive (yoked) and response-contingent heroin injections. Comparison of yoked-heroin (YH) to yoked saline (YV) animals indicated relative metabolic activity was significantly altered in fifteen of 37 regions as a consequence of pairing the tone to heroin infusions irrespective of behavior (Fig. 5). OCTO labeling density was significantly increased in the medial prefrontal cortex, frontal cortex, olfactory tubercle (level II and III), lateral hypothalamus, ventral thalamus, hippocampus and substantia nigra. Significant reductions in OCTO labeling were found in anterior cingulate cortex, nucleus accumbens, cuadate-putamen (level II and III), diagonal band, medial septum, basolateral amygdala, entorhinal cortex and dorsal raphe.

OCTO labeling density was significantly changed in 19 of 37 regions as a result of pairing the tone to response-contingent heroin infusions (Fig 6) (SA group compared to YH animals). The largest change in ROD was an increase in the vertical limb of the diagonal band. More moderate increases were observed in anterior cingulate cortex, nucleus accumbens, olfactory tubercle, bed nucleus of the stria terminalis, piriform cortex, medial preoptic area, amygdala, entorhinal cortex and auditory cortex. OCTO labeling density was significantly lowered in medial prefrontal cortex, claustrum, caudate-putamen (level II and III), globus pallidus, subthalamus, hippocampus, substantia nigra, dorsal raphe, and central gray.

Changes in cerebral metabolic activity likely related to arousal during the tone presentation are evident in comparison of the SA rats to the YV animals. OCTO labeling density was significantly greater for SA rats in the caudate-putamen

TABLE 1

REGIONAL RELATIVE OPTICAL DENSITY VALUES (ROD) OBTAINED DURING PRESENTATION OF THE TRAINING TONE

Atlas	Region	Self-Administration (n=5)	Yoked-Heroin (n=4)	Yoked-Vehicle (n=5)
I.	Med. Prefrontal Cx	70.0 ± 1.4*	77.1 ± 1.1†	67.6 ± 1.1
	Frontal Cx	76.0 ± 0.9	77.7 ± 1.0†	73.1 ± 0.8
	Claustrum	81.4 ± 1.1*	88.6 ± 1.1	87.1 ± 0.8‡
II.	Ant. Cingulate Cx	86.2 ± 0.5*	83.5 ± 0.8†	87.9 ± 0.4
	Caudate-Putamen	32.6 ± 0.9*	37.8 ± 2.0†	44.2 ± 1.0‡
	Nucleus Accumbens	37.5 ± 0.4*	32.3 ± 0.9†	36.7 ± 0.4
	Olfactory Tubercle	34.5 ± 0.8	35.5 ± 1.3†	29.2 ± 0.8‡
III.	Ant. Cingulate Cx	89.6 ± 0.4	90.4 ± 0.4	90.9 ± 0.4
	Caudate-Putamen	44.1 ± 0.5*	49.8 ± 0.8†	52.3 ± 0.5‡
	Lateral Septum	38.0 ± 0.9	40.1 ± 1.4	42.3 ± 0.5‡
	Medial Septum	68.9 ± 1.1	67.8 ± 1.3†	78.2 ± 0.9‡
	Olfactory Tubercle	39.8 ± 1.3*	34.7 ± 1.0†	29.5 ± 1.0‡
	Piriform Cx	27.3 ± 1.1*	21.3 ± 1.3	20.5 ± 1.1‡
	Vert. Diagonal Band	71.7 ± 0.8*	62.8 ± 1.0†	68.8 ± 0.8
IV.	Bed N. Stria Term.	48.0 ± 1.1*	41.8 ± 1.0	44.5 ± 0.8
	Caudate-Putamen	54.3 ± 0.4	56.3 ± 0.6	55.6 ± 0.5
	Globus Pallidus	35.4 ± 1.1*	42.1 ± 0.8	38.8 ± 1.2
	Med. Preoptic Area	48.7 ± 0.7*	44.9 ± 1.1	44.7 ± 0.6‡
V.	Lat. Hypothalamus	37.8 ± 0.5	37.1 ± 0.7†	34.4 ± 0.5‡
	Lat. Dorsal Thalamus	94.4 ± 0.6	95.1 ± 0.4	95.1 ± 0.4
	Med. Dorsal Thalamus	79.1 ± 0.9	79.0 ± 1.3	75.6 ± 0.7‡
	Ventral Thalamus	66.7 ± 0.6	68.3 ± 1.2†	64.6 ± 0.8
VI.	Centromedial Amygdala	29.0 ± 0.6	30.2 ± 0.6	29.3 ± 0.8
	Basolateral Amygdala	23.9 ± 0.6*	21.2 ± 0.6†	25.2 ± 0.6
	Post. Cingulate Cx	88.7 ± 0.7	90.8 ± 0.7	90.7 ± 0.5
	Lateral Habenula	88.9 ± 0.7	90.5 ± 0.7	90.7 ± 0.4
	CA Hippocampus	28.2 ± 1.0*	33.1 ± 0.6†	28.6 ± 0.7
	Dentate Gyrus	60.0 ± 1.0	63.1 ± 0.8	59.9 ± 0.8
	Subthalamus	68.8 ± 1.0*	75.8 ± 1.4	72.4 ± 1.1
VII.	Auditory Cx	63.3 ± 0.7*	60.8 ± 1.1	60.0 ± 0.8‡
	Entorhinal Cx	14.3 ± 0.5*	8.3 ± 0.4†	13.7 ± 0.9
	Substantia Nigra	40.8 ± 0.8*	45.6 ± 1.0†	40.1 ± 1.1
	Vent. Tegmental Area	42.7 ± 1.0	46.1 ± 1.1	43.1 ± 1.1
VIII.	Dorsal Raphe	67.8 ± 0.9*	74.2 ± 1.0†	78.7 ± 0.6‡
	Central Grey	48.5 ± 0.5*	52.2 ± 1.3	53.6 ± 0.5‡
	Superior Colliculus	83.0 ± 0.6	83.9 ± 1.0	84.4 ± 0.7
	Inferior Colliculus	85.9 ± 0.3	85.3 ± 0.6	85.7 ± 0.4

Values are means ± SE. Atlas numbers refer to sections described in Fig. 1. Statistical significance ($p<0.01$) of the difference between means as determined with Newman-Keuls test are designated. Regional means for the self-administering animals were compared to the yoked-heroin group (*), the yoked-heroin group means were compared to the yoked-vehicle group (†), and the yoked-vehicle group means were compared to the self-administration group (‡).

(level II and III), lateral and medial septum, and central gray. Significant reductions in labeling density were found in the claustrum, olfactory tubercle (level II and III), piriform and auditory cortex, medial preoptic area, lateral hypothalamus, medial dorsal thalamus, and dorsal raphe (Table 1).

DISCUSSION

Results obtained from the two behavioral experiments indicate that the behavioral contingency associated with tone-heroin pairings used in training yielded different moti-

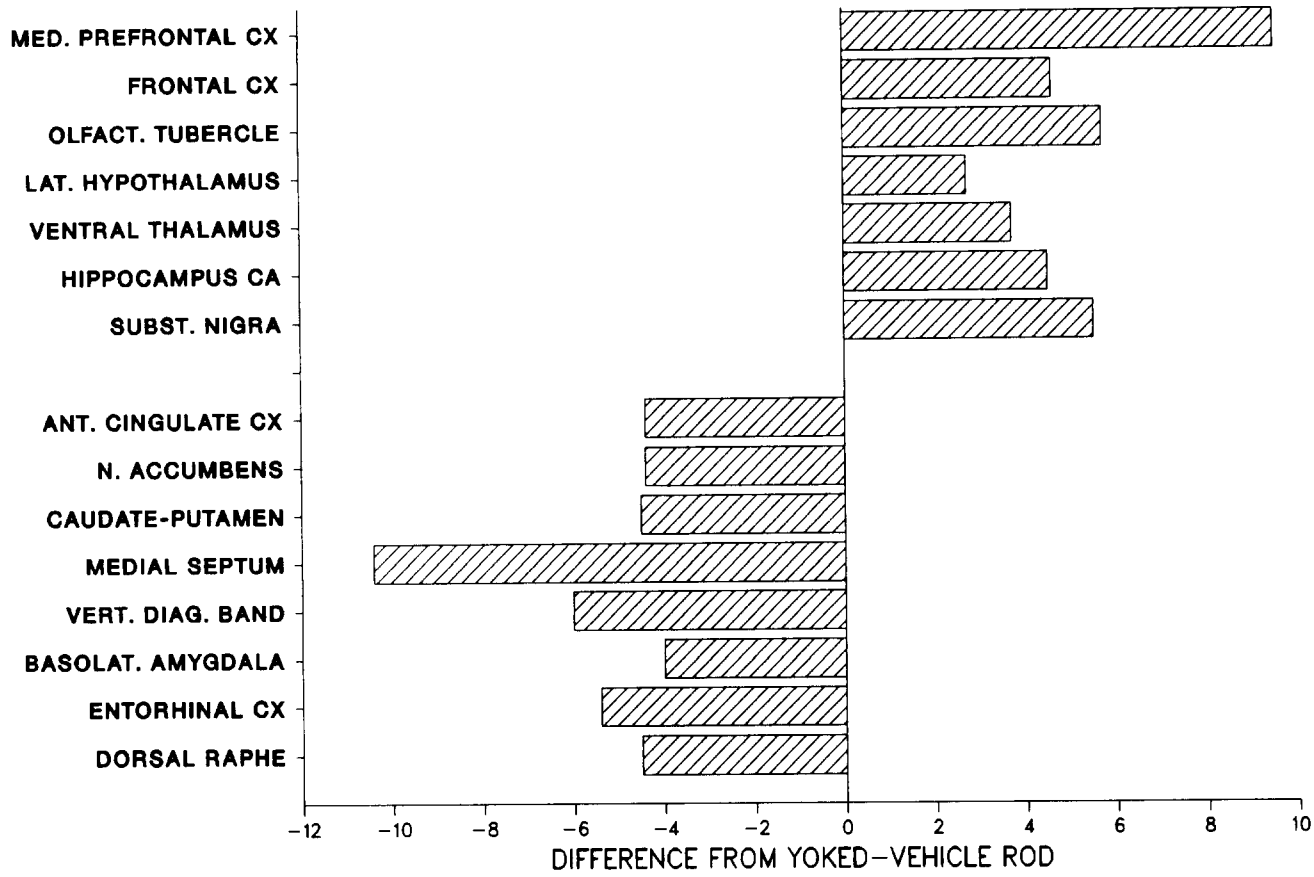

FIG. 5. Regional changes in ROD produced by the tone in rats previously paired to heroin infusions independent of behavior (YH group). Increments in ROD relative to yoked-vehicle animals indicate increases in functional activity.

vational properties for the tone. Given an equal number of pairings, a tone associated with response-contingent heroin prolonged extinction of the self-administration response (Experiment 1), while a tone paired to response-independent heroin failed to support lever-pressing behavior above operant rates (Experiment 2). These measures of the ability of a previously neutral stimulus to increase resistance to extinction and support operant behavior are commonly accepted criteria of secondary reinforcing properties [21,30]. Similar criteria have been used to determine the reinforcing capacity of stimuli paired to morphine [5, 7, 32, 38, 41] and other self-administered drugs [6,7]. It should be noted that, contrary to the conclusions of Experiment 2, previous studies have demonstrated the ability of tones paired to behaviorally-noncontingent morphine to act as secondary reinforcers [5, 7, 41]. This discrepancy can likely be explained, however, by the different training parameters used. These studies paired a tone to morphine infusion at a much higher rate (50 pairings in 100 minutes), while 25 tone-heroin pairings occurred in 300 minutes for each training session in this study. Our results suggest that this slower pairing rate, which occurs in rats self-administering the same dose of heroin (Experiment 1), is not sufficient to establish secondary reinforcement properties, and support the conclusion that the tone possessed different motivational properties for the rats trained under the conditions used in the OCTO experiment.

Perhaps the most striking outcome of the tracer study is the numerous changes in relative OCTO labeling density obtained during presentation of the tone. Assuming such alterations reflect differences in functional activity [42], the tone was observed to produce reliable modifications of neuronal activity within several regions as a result of previous association with heroin infusion. Furthermore, different patterns of activity changes were obtained when heroin-tone pairings were made contingent upon behavior. The number and diversity of changes observed is surprising considering that these animals were not infused with heroin during the two-minute tone presentation/OCTO incorporation period, and that all animals appeared equally active prior to sacrifice. Although the production of these alterations by motor or drug-induced effects is thus unlikely, the possiblity exists that they partially represent cerebral functional events associated with extinction, that is, failure of heroin effects to appear. Such effects were not tested by the behavioral design employed, and it is assumed that the alterations in functional activity obtained reflect the differential training history of the stimuli present during OCTO incorporation.

Functional activity changes related to effects produced by simple pairing of the tone to heroin were identified by comparison of regional RODs in yoked-heroin rats to those from yoked-vehicle animals. The affected regions are presented in Fig. 5 as the difference in mean regional ROD between these training groups. An increment in ROD indicates an increase in OCTO labeling density in yoked-heroin rats, and is inter-

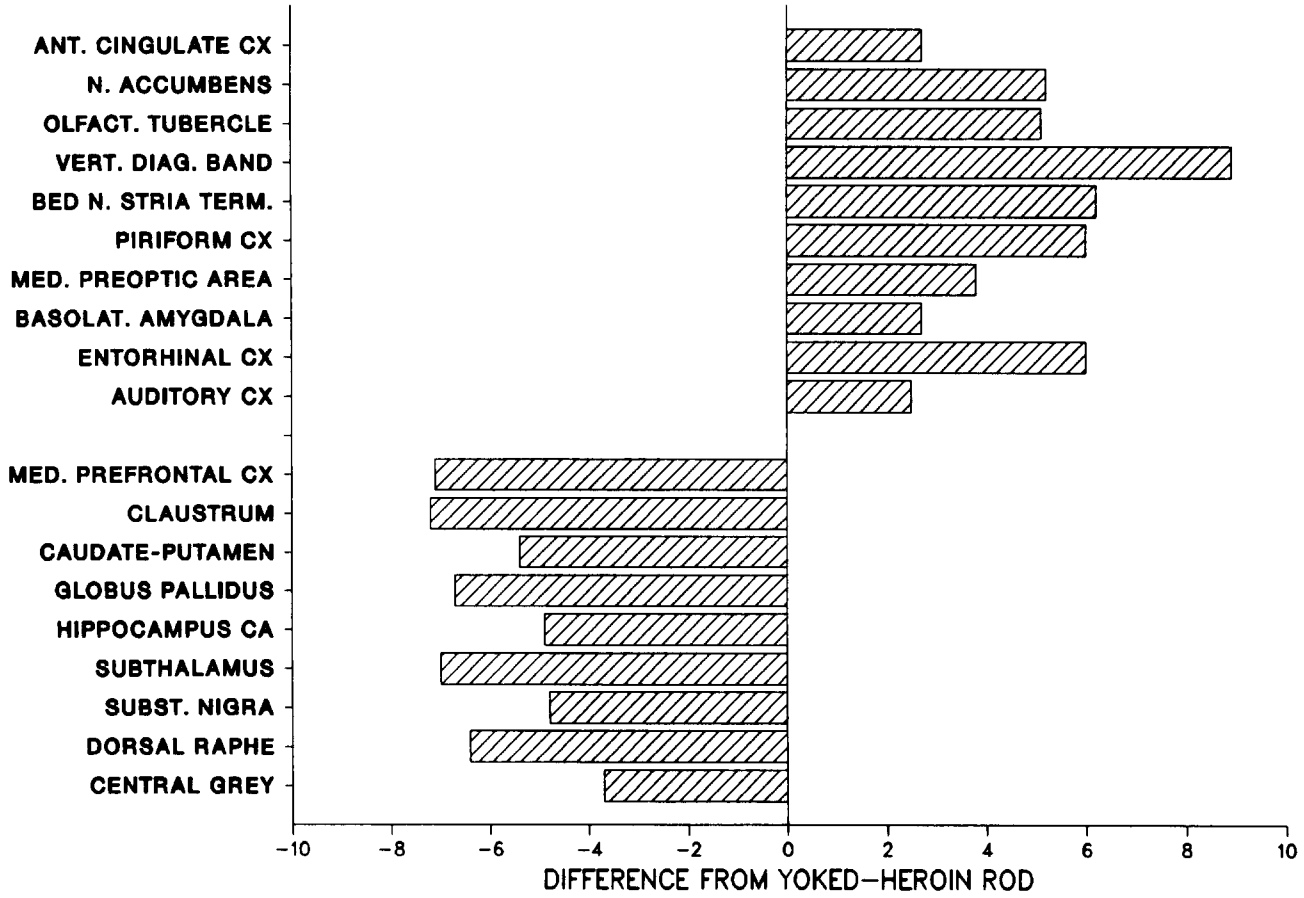

FIG. 6. Regional changes in ROD produced by the tone previously paired to response-dependent heroin infusions (SA group). Increments in ROD relative to YH rats suggest increased functional activity related to the presumed secondary reinforcing property of the tone.

preted as reflecting activation of functional neuronal activity in that region. Conversely, a decrement in ROD denotes activity suppresion. These findings suggest that stimuli passively paired to opiate infusions acquire the ability to alter functional activity in cortex, hypothalamus, thalamus, hippocampus, amygdala, basal ganglia, dorsal raphe and various components of the limbic system. Opiates induce several effects in rats that can be conditioned to neutral stimuli, such as hyperthermia [9], hyperactivity [29] and elevation of serum glucose [23]. Although such effects were not directly measured in this study, the results obtained suggest that cerebral functional events associated with those responses may be detectable with the OCTO method. It is noteworthy that the decrease of neural activity in caudate-putamen observed here is consistent with the observation of Lal *et al.* that a morphine-conditioned stimulus will increase release of dopamine, an inhibitory transmitter, in the striatum [23].

Changes in functional activity resulting from pairing the tone to response contingent heroin infusions were obtained by comparing rats trained to self-administer heroin to those receiving yoked heroin infusions. Significant regional ROD differences observed between these groups are shown in Fig. 6. The secondary reinforcing capacity of the tone in these animals is inferred from the results of the behavioral studies discussed above. It is assumed that the tone had acquired reinforcing properties in the self-administration rats, and had no detectable motivational significance to the yoked-heroin animals. Thus, these comparisons indicate functional activity changes related to the putative rewarding quality of the tone acquired by association with heroin infusions. The greatest changes in functional activity observed occurred in the nucleus accumbens, vertical diagonal band, bed nucleus of the stria terminalis, claustrum, globus pallidus, subthalamus, and several cortical areas, including medial prefrontal, piriform and entorhinal cortex. Many of these regions have been previously implicated in central processing of opiate reward. Comparison of regional neurotransmitter turnover in rats self-administering morphine to yoked-infusion littermates identified activity in hippocampus, nucleus accumbens, amygdala and frontal-piriform cortex as involved in opiate-seeking behavior [39,40]. The present observations indicate that activity changes in these areas can also become conditioned to stimuli present in the environment of the opiate-seeking animal.

It might be argued that the functional activity changes observed in self administration (SA) rats were not related to reward processes alone, but may also be indicative of arousal effects evoked by the conditioning tone, or reflect learning functions that could also be activated by aversive reinforcers. Unfortunately, the design of the present study does not provide sufficient information to separate arousal effects from those related to reinforcement. However, it is interesting to note that the suppression of activity in caudate-putamen, septum, subthalamus, and globus pallidus

of SA rats is similar to the decrease in metabolic activity of the same regions in rats given arousing electrical stimulation of the midbrain reticular formation [16]. Thus, suppression of activity in these areas is seen after presentation of both rewarding and arousing stimuli, and may represent activation of a common arousal mechanism.

Metabolic function increases in the medial prefrontal and auditory cortices of rats during aversively conditioned auditory training [17,18] while the nucleus accumbens has been shown to increase its metabolic activity during performance of an active avoidance task [20]. These regions were also significantly activated by the heroin-conditioned tone in the SA rats of this study. Therefore, these changes are not likely related to the hedonic value per se of the training stimulus, but may be involved in more general learning processes.

Thus, activity changes observed in the SA group rats most consistent with reward-related functions are to be found in the claustrum, olfactory tubercle, diagonal band, bed nucleus of the stria terminalis, amygdala, preoptic area, piriform cortex, and entorhinal cortex. It is worth noting that metabolic function in most of these areas is suppressed by aversive stimulation [16] and enhanced during presentation of the heroin-conditioned tone in the present study. Electrical stimulation of these areas will produce positive reinforcement effects [33], and increased activity in the diagonal band, bed nucleus and preoptic area is found in rats responding for submaximal rewarding stmulation of the medial forebrain bundle [11]. Since tones paired to rewarding brain stimulation have also been reported to act as secondary reinforcers (see [28] for review), application of the methods of this study to such stimuli appears worthy of investigation.

In summary, regional cerebral functional activity was measured in rats during brief presentation of a tone previously paired with heroin infusions. Different patterns of functional activity responses were observed depending upon whether the tone had been passively paired to heroin, or presented with response-contingent heroin infusions. These latter training conditions were shown to produce secondary reinforcing properties for the tone, thus modifications of brain activity by the tone in these rats is presumed related to these reinforcing effects. Many of the regions affected by the response-contingent tone have been implicated in opiate-seeking behavior, and are activated by rewarding brain stimulation. These results suggest that environmental stimuli paired with primary reinforcers gain the capacity to reinforce behavior by conditioned-activation of reward systems. The OCTO method can be usefully applied to investigations of the role of transient stimuli relevant to behavior.

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