

Acetylcholine turnover rates in rat brain regions during cocaine self-administration

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Abstract

The involvement of cholinergic neurons in the brain processes underlying reinforcement has been recently demonstrated. This experiment assessed the potential role of cholinergic neurons in cocaine reinforcement by measuring the turnover rates of acetylcholine in brain regions of rats self-administering cocaine and in yoked cocaine and yoked vehicle-infused controls. The activity of cholinergic innervations of and/or interneurons in the olfactory tubercle, caudate putamen, diagonal band-pre-optic region, ventral pallidum, lateral and medial hypothalamus, hippocampus, ventral tegmental area and visual cortices reflected by the turnover rates of acetylcholine were significantly altered in rats self-administering

cocaine compared to yoked cocaine infused controls. These changes implicate the involvement of cholinergic neurons with cell bodies in the diagonal band-pre-optic region, the medial septum and several brainstem nuclei and interneurons in the caudate-putamen and ventral pallidum in the processes underlying cocaine self-administration. The identified cholinergic neuronal systems may have a broader role in the brain processes for natural reinforcers (i.e. food, water, etc.) since drugs of abuse are believed to produce reinforcing effects through these systems.

Keywords: acetylcholine; acetylcholine turnover rates; cholinergic; drug reinforcement; neurotransmitter turnover rates. *J. Neurochem.* (2004) **88**, 502–512.

Brain neurotransmitters exist in multiple pools with a functional pool, often only a small portion of the total, which likely fills response demands that are within normal physiological limits, while extraordinary demands result in the utilization of additional more firmly bound pools. This circumstance has provided significant challenges for investigation of the role of specific neurotransmitter systems in behavior that primarily involve utilization of the functional pool. Neurotransmitter turnover rate measures were developed as a mechanism for investigating the role of the functional pool. The goal of the experiment described here was to use radioactive pulse labeling techniques to measure turnover rates of acetylcholine (ACh) to assess the role of cholinergic neurons in the brain processes that underlie cocaine self-administration.

Although significant progress has been made in the last two decades in understanding the biological basis of brain disorders, the basic biological processes underlying substance abuse continue to be elusive. Drugs of abuse are thought to produce their addictive effects by activating brain systems that convey the hedonic substrates for natural stimuli (i.e. food, water, sexual behavior, etc.). The demonstration that electrical brain stimulation of distinct brain regions could serve as a

reinforcer (Olds and Milner 1954) implied that discrete neuronal pathways underlie hedonic processes and data showing abused drugs to lower thresholds for this stimulation (Olds and Travis 1960; Kornetsky *et al.* 1979) supported this hypothesis. ACh releasing neurons have been demonstrated to be involved in the processes that underlie brain stimulation reinforcement (Chapman *et al.* 1997; Yeomans *et al.* 1985, 1993; Kofman and Yeomans 1988, Kofman *et al.* 1990) and M₅ muscarinic cholinergic receptors appear to participate in these processes (Yeomans *et al.* 2000).

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Abbreviations used: ACh, acetylcholine; α_7 , nicotinic cholinergic receptor subtype alpha 7; β_2 , nicotinic cholinergic receptor subtype beta 2; CPP, conditioned place preference; D1, dopamine receptor subtype 1; DA, dopamine; Glu, glutamate; 5-HT, serotonin; k, fractional rate constant; LDTg, lateral dorsal tegmental nucleus; M2, muscarinic cholinergic receptor subtype 2; M5, muscarinic cholinergic receptor subtype 5; NAcc, nucleus accumbens; NE, norepinephrine; PPTg, pedunculo pontine tegmental nucleus; VTA, ventral tegmental area.

There is increasing evidence that ACh neurons have a role in the neuronal systems mediating drug self-administration. Early studies showed systemic cholinergic antagonists to attenuate cocaine self-administration (Glick and Cox 1975; Goldberg and Gonzalez 1976). However, these effects were thought to result from a non-specific behavioral disruption (De la Garza and Johanson 1982). More recent studies found that systemic pretreatment with a muscarinic cholinergic receptor mixed agonist-antagonist selectively decreased the reinforcing efficacy of intravenous cocaine in mice (Rasmussen *et al.* 2000) while pretreatment with a nicotinic receptor antagonist produced similar effects in rats at doses that did not alter food maintained responding (Levin *et al.* 2000). Response-independent acute administration of cocaine increases extracellular fluid levels of ACh in the cortex (Day *et al.* 1997) and the hippocampus (Imperato *et al.* 1996). Collectively, these data support a role for cholinergic neuronal systems in the brain processes that underlie cocaine self-administration and suggest that further characterization of this involvement is warranted. The research described here was initiated to identify the loci of cholinergic neurons and the forebrain cholinergic innervations that participate in cocaine self-administration by measuring the turnover rates of ACh in small brain regions of rats intravenously self-administering cocaine and in controls receiving response independent infusions of cocaine or the vehicle.

Experimental procedures

Animals

Forty-two adult male Fischer strain F-344 90–150 day-old-rats (Harlan, Indianapolis, IN, USA) were used in groups of three littermates with one allowed to intravenously self-administer cocaine and the other two receiving either identical infusions of cocaine or the vehicle on a schedule yoked to the self-administering rat. Littermates from this inbred strain were used to minimize genetic variation that could affect the experimental measures. Each litter was initially housed together in group cages in a temperature controlled environment with unlimited access to food and water on a reversed 12 h light/dark cycle (lights on 17 : 00–05 : 00 h) until experimental procedures were initiated which were conducted during the dark cycle. Experimental procedures were reviewed and approved by the Wake Forest University Health Sciences Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23) revised in 1996.

Surgical techniques

Rats were anesthetized with pentobarbital [50 mg/kg, intraperitoneally (i.p.), Abbott Laboratories, North Chicago, IL, USA] after pretreatment with atropine methyl nitrate (10 mg/kg, i.p., Sigma, St Louis, MO, USA) and Penicillin G procaine [75 000 units, intramuscularly (i.m.), Wyeth Laboratories, Philadelphia, PA, USA] and implanted with venous catheters placed in the right jugular vein using previously described methods (Weeks 1962,

1972; Pickens and Dougherty 1972). The catheter (a small piece of polyvinyl-chloride tubing) was inserted into the right posterior facial vein, guided into the right jugular vein until it terminated just outside the right atrium and anchored to muscle in the area of the vein. The other end of the catheter continued subcutaneously to the back where it exited between the scapulae through a polyethylene shoulder harness. The harness provided a point of attachment for the catheter to a needle tubing leash that passed out the top of the animal chamber. A leak proof swivel (Brown *et al.* 1976) was used to attach the leash to the tubing leading to the infusion pump so that the animals had almost complete freedom of movement. The rats were allowed to recover for four days before the initiation of experimental procedures. The rats were housed in the operant conditioning chambers (Medical Associates, Georgia, VT, USA) that each contained a glass food pellet dispenser tube, a drinking tube providing unlimited access to water, a house light, session light, a stimulus light, tone source and a retractable response lever. Automatically programmed infusions (0.2 mL delivered over 5.6 s) of heparinized saline (1.7 units/mL) were administered at hourly intervals during the 4-day recovery period prior to initiation of daily self-administration sessions. Patency of catheters was evaluated at regular intervals by delivering an intravenous infusion of methohexital (10 mg/kg, Eli Lilly, Indianapolis, IN, USA) and determining the latency for loss of stability or consciousness which occurs within 1–2 s.

Cocaine self-administration

Triads of rats with chronic jugular catheters were exposed to three different treatment conditions, self-administration or simultaneous response independent yoked infusions of either cocaine or vehicle (heparinized-saline) in the operant conditioning chambers in which they were housed. One rat in each triad was trained to intravenously self-administer cocaine (0.33 mg/infusion delivered over 5.6 s in 0.2 mL of heparinized-saline) on a fixed ratio two schedule (two lever presses required for a drug infusion) during daily sessions. Another littermate in each triad received simultaneous identical response independent infusions of cocaine and a third littermate received simultaneous infusions of the vehicle both on a schedule yoked to infusions taken by the self-administering rat. Daily self-administration sessions were initiated at 09 : 00 h with the extension of the retractable lever into the chamber and the illumination of the session light and terminated after 6 h or 60 infusions. Self-administration sessions occurred daily seven days per week with the response requirement initially being one lever press which was rapidly increased to two over the first several sessions. Infusions were paired with the onset of a 20-s tone and light compound stimulus and by retraction of the lever for a 20-s timeout period. To control for the anorectic effects of cocaine, the self-administering rats were provided 24 1-g food pellets (Bioserv, Frenchtown, NJ, USA) each 24-h period (an average amount of food consumed during precocaine unrestricted access). The number of food pellets consumed by the self-administering rat was recorded at 08 : 00 and 17 : 00 h each day and that number of pellets was placed in the tubes located in the chambers of the rats receiving yoked infusions.

Pulse label procedure

After 20 days of stable self-administration, each triad was pulse labeled with a radioactive precursor to ACh 2 h into the

self-administration session. A 55- μ L loop of tubing containing 0.5 mCi of [methyl- 3 H] choline chloride (specific activity 15 Ci/mmol; Amersham, Piscataway, NJ, USA) in 50- μ L of the cocaine solution (self-administration and yoked cocaine infused rats) or saline (yoked vehicle infused rat) was attached below the swivel to the jugular catheters and infused simultaneously with the next self-administered injection to all three rats in the triad. The triads were killed five ($n = 8$ triads) and 10 ($n = 6$ triads) minutes later by immersion in liquid nitrogen. These pulse-label time-points were chosen because they have been demonstrated to be on the log-linear portion of the decay in radioactivity curve for ACh (Nordberg and Sundvall 1977).

Tissue preparation

Animals were killed by total immersion in liquid nitrogen until frozen (5 min). Prior data has shown that the cerebral cortex reaches 0°C within several seconds of immersion in liquid nitrogen so that animals lose consciousness rapidly (Takahashi and Aprison 1964) and brain ACh levels are similar to those obtained with microwave fixation methods (Richter and Shea 1974). The heads were separated and allowed to warm to -20°C and the brains removed. The brains were sectioned in the coronal plane in a cryostat at -18°C into 750 μ m serial sections and dissected into 26 regions. The brain areas of interest (prefrontal, pyriform, motor, somatosensory, anterior cingulate, posterior cingulate, entorhinal-subicular, visual and temporal-auditory cortices, olfactory tubercle, NAcc, caudate nucleus-putamen, diagonal band of Broca, pre-optic region, ventral pallidum-stria terminalis, amygdala, septum, globus pallidus, hippocampus, substantia nigra, ventral tegmental area (VTA), medial hypothalamus, lateral hypothalamus, medial thalamus, lateral thalamus, colliculus and brain stem) were microdissected with the aid of a stereomicroscope and the dissected tissue samples stored at -80°C until analyzed.

Acetylcholine measurements

The frozen tissue samples were individually pulverized in liquid nitrogen with a stainless steel mortar cooled on dry ice in a cryostat cooled to -20°C. ACh was extracted from pulverized tissue (40–100 mg) with 0.4 mL of ice cold 1 M formic acid/acetone (15 : 85 v/v) and lipids were removed by a heptane/chloroform (8 : 1 v/v) wash. Samples were taken to dryness under dry N₂ and reconstituted with mobile phase for assay using high pressure liquid chromatography with electrochemical detection. Acetylthiocholine chloride was added to the formic acid/acetone extract of each sample as internal standard to correct for recovery. The mobile phase consisted of 20 mM sodium phosphate, pH 8.5 at a flow of 1.0 mL per min. The analytical column used was coupled with an enzyme bonded column to convert ACh to hydrogen peroxide which was oxidized at a platinum electrode held at +0.5 V against Ag/AgCl and detected electrochemically (BAS, Lafayette, IN, USA). The peak was collected directly in a scintillation vial for liquid scintillation counting and the specific radioactivity calculated. Proteins were determined using a previously reported procedure (Lowry *et al.* 1951).

Turnover rate calculation

Turnover rates were determined with previously reported methods (Smith *et al.* 2003) with the assumption that radiolabel was

disappearing from a single open pool (Zilversmit 1960) since there is no acceptable method for determining central nervous system intraneuronal compartmentation *in vivo*. Turnover rates = $k \times$ content where the apparent fractional rate constant (k) was calculated as:

$$k = \ln 2/t_{1/2}$$

and the half life $t_{1/2}$ was extrapolated from a semilogarithmic plot of the specific radioactivities (dpm/pmol) obtained at the two pulse times on the log-linear portion of the decay in the radioactivity curve. The apparent fractional rate constants and turnover rates were determined by comparing each of the eight animals at the 5-min pulse time with all six animals at the 10-min pulse time. A mean of these measures was calculated and used to represent one turnover measure. Thus, for each of the eight animals in each of the three treatment conditions at the short pulse time, up to eight turnover rates were calculated and these values for each of the three treatment conditions were then used to determine significance of differences in turnover rates. This procedure was used to obtain an accurate error estimate that would be representative of both the variation in content as well as radioactivity measurements. The turnover rate was expressed as nmol/mg protein/h and was the product of each rate constant (per h) and each content value (pmol/mg protein). Negative values that resulted on occasion when the specific radioactivity at the short pulse point was lower than the long pulse point were not included in the calculations.

Statistical analysis

ACh content and individual turnover rates calculated as outlined above were then assessed for significant differences by comparing the cocaine self-administering animals with the yoked cocaine infused controls (designated as the 'cocaine self-administration effect') and comparing the cocaine yoked infused controls with the yoked vehicle infused controls (designated as the 'cocaine effect'). The significance of differences were tested for each brain region with a one way ANOVA followed by *post hoc* analysis of differences between means using Bonferroni *t*-tests for multiple comparisons using the yoked cocaine infused group as the control.

Results

Intravenous cocaine self-administration

Animals acquired intravenous cocaine self-administration within the first five sessions with the response requirement raised to the terminal fixed ratio two-value within the first several days and stable baselines and patterns of drug intake were obtained by the tenth session. The number of sessions of exposure prior to pulse labeling was 24.9 ± 3.2 days for all groups (all values presented are means \pm SD unless otherwise specified). The total drug intake for the 14 triads was 50.1 ± 5.4 infusions per session that corresponds to 16.5 ± 1.8 mg per day. The interinfusion intervals did not differ for the two pulse label time groups [5 min (5.4 ± 0.3 min) or 10 min (5.9 ± 0.2 min)] and the total number of infusions did not differ between the groups during

the two pulse label session [5 min (20.6 ± 4.5) and 10 min (24.0 ± 8.5)].

Food intake

Nineteen to 21 1-g food pellets were generally consumed each 24-h period. The average daily intake for the three treatment conditions during the self-administration period were: cocaine self-administration -20.6 ± 1.6 ; yoked cocaine infused -18.8 ± 2.1 ; yoked vehicle infused -19.5 ± 2.1 . Yoking food available to the cocaine self-administering rat resulted in similar daily caloric intake and body weights of the littermates within each triad without restricting the intake of the vehicle infused animals since this group on average consumed 1.5 g less than was available per day.

Acetylcholine content

The content of ACh varied between brain regions in a manner consistent with a neurotransmitter role as previously demonstrated (Racagni *et al.* 1974) (Table 1). The ANOVA

found significant treatment effects in the hippocampus [$F_{2,37} = 5.38$; $p = 0.009$], lateral hypothalamus [$F_{2,37} = 5.81$; $p = 0.006$] and lateral thalamus [$F_{2,37} = 4.33$; $p = 0.02$]. *Post hoc* analysis of the difference between means assessed with Bonferroni *t*-tests for multiple comparisons found three significant decreases in content were seen between the yoked vehicle infused group and the yoked cocaine infused group which include the hippocampus ($p = 0.032$), lateral thalamus ($p = 0.013$) and lateral hypothalamus ($p = 0.009$). One increase in content was seen in the hippocampus between the yoked cocaine infused and cocaine self-administration groups ($p = 0.008$).

Acetylcholine turnover rates

The significant changes in ACh turnover rates were generally of four types (Table 2): (i) those that were the result of cocaine and seen in both the self-administering and the yoked cocaine infused groups (designated as the 'cocaine effect'); (ii) those that were the result of the ability to

Table 1 Content of acetylcholine in the brain regions of cocaine self-administering, yoked cocaine and yoked vehicle infused rats

Area	Yoked vehicle (pmol/mg protein)	Yoked cocaine (pmol/mg protein)	Self-administration (pmol/mg protein)
Prefrontal cortex	50.04 ± 3.06	45.49 ± 2.60	47.58 ± 1.41
Olfactory tubercle	158.03 ± 9.33	156.38 ± 7.00	152.20 ± 10.30
Pyriform cortex	102.97 ± 4.73	99.55 ± 3.56	98.72 ± 4.16
Nucleus accumbens	117.07 ± 3.31	115.95 ± 5.66	116.18 ± 7.40
Motor cortex	25.28 ± 1.90	22.84 ± 1.96	20.39 ± 1.39
Somatosensory cortex	33.14 ± 1.63	28.59 ± 1.36	30.49 ± 1.56
Caudate putamen	289.47 ± 11.93	305.32 ± 15.03	299.69 ± 14.75
Anterior cingulate cortex	39.88 ± 2.61	34.04 ± 3.46	35.39 ± 2.05
Septum	114.16 ± 5.44	99.48 ± 8.24	112.83 ± 5.79
Diagonal band	132.75 ± 3.20	123.82 ± 9.65	117.20 ± 10.71
Ventral pallidum	52.95 ± 2.80	47.64 ± 2.77	48.17 ± 2.18
Amygdala	177.44 ± 11.98	149.61 ± 10.31	167.37 ± 11.05
Hippocampus	73.36 ± 2.11	64.82 ± 2.73*	75.08 ± 2.23†
Medial thalamus	96.09 ± 1.70	86.23 ± 5.27	91.97 ± 3.19
Lateral thalamus	65.56 ± 2.05	54.45 ± 3.18*	61.65 ± 2.68
Medial hypothalamus	40.69 ± 2.30	37.06 ± 4.53	38.82 ± 2.05
Lateral hypothalamus	58.24 ± 3.98	43.28 ± 4.03†	44.14 ± 2.22
Posterior cingulate cortex	24.28 ± 1.68	28.88 ± 2.97	27.06 ± 2.57
Entorhinal subicular cortex	87.89 ± 2.76	78.94 ± 3.69	81.77 ± 2.29
Ventral tegmental area	90.85 ± 7.13	73.68 ± 10.30	80.53 ± 6.41
Brain stem	45.60 ± 2.15	41.05 ± 3.16	43.22 ± 3.35
Visual cortex	28.32 ± 1.56	26.23 ± 0.99	27.46 ± 1.49
Temporal, auditory cortex	39.29 ± 1.84	36.43 ± 2.59	38.90 ± 1.52
Colliculus	35.45 ± 2.95	29.22 ± 2.89	36.42 ± 3.28

Data are mean ± SEM for $N = 14$ per treatment condition. The significance of differences were tested with a one way ANOVA followed by *post hoc* analysis of differences between means using Bonferroni *t*-tests for multiple comparisons with the yoked cocaine group as the control. The yoked infused vehicle group was compared with the yoked infused cocaine group to identify drug effects (symbols denoting significant differences located to the right of the yoked cocaine group values) and the yoked infused cocaine group was compared with the self-administration group to identify self-administration effects (symbols denoting significant differences located to the right of the yoked cocaine group values). Significances of differences between means were: * $p < 0.05$ and † $p < 0.01$.

Table 2 Classification of changes in neurotransmitter turnover rates

	Yoked cocaine significantly different from yoked vehicle	Self-administration significantly different from yoked cocaine	Significant changes of both comparisons in the same direction	Significant changes of both comparisons in the opposite direction
Cocaine effect	xx			
Self-administration effect		xx		
Self-administration enhancement effect	xx	xx	xx	
Reversal effect	xx	xx		xx

Area	<i>F</i> -value	<i>p</i> -value	Comparison	<i>Post hoc</i> analysis
Olfactory tubercle	$F_{2,19} = 7.41$	$p = 0.004$	SA versus YC	$p = 0.046$
Nucleus accumbens*	$F_{2,16} = 9.41$	$p = 0.002$		n.s.
Pyramidal cortex*	$F_{2,19} = 4.79$	$p = 0.021$		n.s.
Caudate putamen	$F_{2,13} = 18.48$	$p < 0.001$	SA versus YC YC versus YS	$p < 0.001$ $p < 0.001$
Diagonal band	$F_{2,18} = 13.70$	$p < 0.001$	SA versus YC	$p < 0.001$
Ventral pallidum	$F_{2,19} = 12.99$	$p < 0.001$	SA versus YC	$p = 0.004$
Hippocampus	$F_{2,19} = 3.54$	$p = 0.049$	SA versus YC	$p = 0.033$
Medial hypothalamus	$F_{2,14} = 8.25$	$p = 0.004$	SA versus YC	$p = 0.006$
Lateral hypothalamus	$F_{2,17} = 41.20$	$p < 0.001$	SA versus YC YC versus YS	$p = 0.003$ $p < 0.001$
Posterior cingulate cortex	$F_{2,20} = 10.92$	$p < 0.001$	YC versus YS	$p = 0.003$
Ventral tegmental area	$F_{2,12} = 6.33$	$p = 0.013$	SA versus YC	$p = 0.008$
Brain stem	$F_{2,16} = 27.36$	$p < 0.001$	YC versus YS	$p < 0.001$
Visual cortex	$F_{2,19} = 5.85$	$p = 0.010$	SA versus YC	$p = 0.006$
Temporal auditory cortex	$F_{2,15} = 29.23$	$p < 0.001$	YC versus YS	$p < 0.001$

Table 3 The significant effects of treatment on ACh turnover rates in 14 brain regions

Significant differences of treatment effects upon ACh turnover rates were assessed with a one way ANOVA followed by *post hoc* analysis using Bonferroni *t*-test for multiple comparisons with the yoked cocaine serving as the control. *Although ANOVA found significant group differences, *post hoc* analysis showed no significance differences between yoked cocaine and either of the other groups.

self-administer and seen only in the self-administering group (designated as the 'self-administration effect'); (iii) those seen when the yoked cocaine infused group was significantly different from the yoked vehicle infused group that were significantly enhanced with the opportunity to self-administer (designated as a 'self-administration enhancement effect'); and (iv) those seen when the yoked cocaine infused group was significantly different from the yoked vehicle infused group but were reversed by the ability to self-administer (designated as a 'reversal effect'). ACh turnover rates were significantly affected by treatments in 14 of the 26 brain regions as demonstrated by ANOVA (Table 3). The Bonferroni *post hoc* analysis found 12 of these regions to have significant differences between means while two regions did not (NAcc and pyriform cortex) although a significant *F*-value for treatments was obtained. Three changes in acetylcholine turnover rates were consistent with a cocaine effect (Fig. 1), seven consistent with a self-administration effect (Fig. 2), one consistent with a self-administration

enhancement effect and one consistent with a reversal effect. The cocaine effect included an increase (posterior cingulate cortex) and two decreases (temporal cortex and the brain stem) in ACh turnover. The seven changes in turnover rates consistent with a self-administration effect included three increases (ventral pallidum, hippocampus and medial hypothalamus) and four decreases (olfactory tubercle, diagonal band-pre-optic region, VTA and visual cortex). The change seen in the yoked cocaine infused group that was enhanced even further by the opportunity to self-administer was a decrease in the lateral hypothalamus. The turnover rate change consistent with a reversal effect was seen in the caudate-putamen where it was significantly increased in the yoked cocaine infused group which was reversed in the cocaine self-administration group.

The pharmacological actions of cocaine, as seen in the cocaine yoked-infused group, and the opportunity to self-administer appear to summate in a significant overall treatment effect in several brain regions. These include the

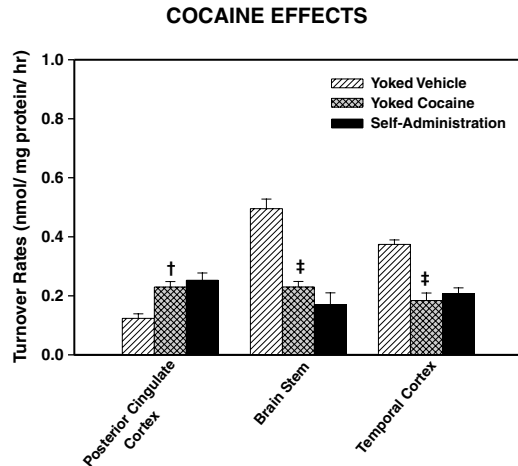


Fig. 1 Significant changes in the turnover rates of acetylcholine in brain regions of intravenous cocaine self-administering, yoked infused cocaine and yoked infused vehicle groups that result from exposure to the drug alone. Significant differences found between the yoked cocaine infused group and the yoked vehicle infused animals were considered to be a 'cocaine effect'. The significances of differences between means were: [†] $p < 0.01$; [‡] $p < 0.001$ for an $n = 14$ (eight at 5-min and six at 10-min pulse times for the three treatment groups).

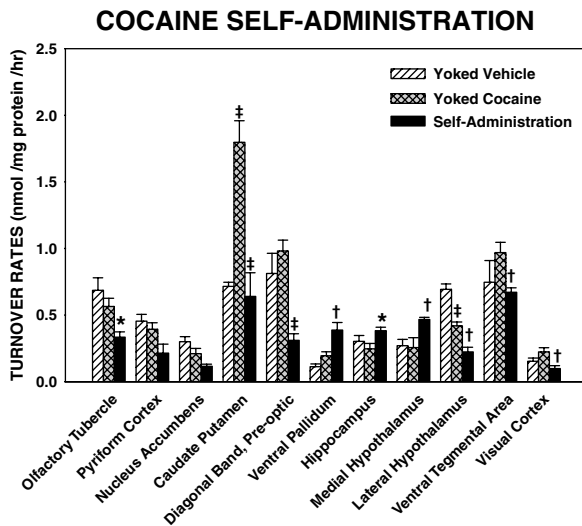


Fig. 2 Significant changes in the turnover rates of acetylcholine in brain regions of intravenous cocaine self-administering, yoked infused cocaine and yoked infused vehicle groups resulting from self-administration. Significant differences found between the self-administering rat and the yoked cocaine infused animals were considered to be a 'self-administration effect'. The significances of differences between means were: * $p < 0.05$; [†] $p < 0.01$; [‡] $p < 0.001$ for an $n = 14$ (eight at the 5-min and six at 10-min pulse times for each of the three treatment groups).

NAcc and the pyriiform cortex where a significant F -value was obtained with the ANOVA that is the summation of both of these effects. In these two brain regions although the

differences between the yoked cocaine infused group and the self-administering animals were not significant, there was a trend (pyriiform cortex $p = 0.068$ and NAcc $p = 0.117$). Similar graded effects were seen in the olfactory tubercle, ventral pallidum and lateral hypothalamus. In other regions a clear cocaine only (posterior cingulate and temporal cortices and brain stem) and self-administration only [caudate-putamen, diagonal band-pre-optic region, hippocampus, medial hypothalamus, visual cortex and VTA] effect were seen (Figs 1 and 2).

Discussion

The most important finding from this experiment is the striking differences in the activity of ACh releasing neurons between the passive cocaine infused and the cocaine self-administering rats (referred to as the 'self-administration effect'). Cocaine alone increased the activity of cholinergic innervations of the posterior cingulate cortex and decreased activity of cholinergic innervations of the temporal cortex and brainstem. Although the cocaine response-dependent and -independent animals had identical histories of cocaine exposure, the ability of the self-administering animals to control such exposure with a simple response had substantial neurochemical consequences. This included modulation of the activity of ACh releasing neurons in 11 brain regions [olfactory tubercle, NAcc, caudate-putamen, diagonal band-pre-optic region, ventral pallidum, hippocampus, medial and lateral hypothalamus, VTA, pyriiform and visual cortices].

Neurotransmitter turnover rates – methodological issues

Radioactive pulse labeling turnover rate methodologies have been used sparingly in the last two decades. This probably results partially from the significant effort involved and from the controversy as to what are the acceptable methodologies to be employed. Over the last two decades neurotransmitter content/metabolite content ratios evolved as measures of turnover which are substantially easier to obtain and have been generally accepted as measures of neurotransmitter utilization. There has not been a direct comparison of turnover rates determined with these ratios with those obtained by an alternative method but calculated from the same data base until recently. Neurotransmitter content/metabolite content ratios for dopamine (DA), norepinephrine (NE) and serotonin (5-HT) were compared with pulse labeling measures of turnover from the same data base and found to not be in agreement and be solely the result of changes in the content of the metabolites (Smith *et al.* 2003). In addition, the ratios did not detect changes that have been previously demonstrated and expected. The ACh turnover rates presented here were calculated with the log-linear decay method (Zilversmit 1960) and values for the yoked-vehicle infused group are similar to previously published values for

most of the brain regions obtained with microwave fixation and non-isotopic (Schmidt and Buxbaum 1978; Finberg *et al.* 1979) or with radioisotopic procedures (Brunello *et al.* 1982; Racagni *et al.* 1974, 1976a, 1976b; Wood *et al.* 1978; Zsilla *et al.* 1978; Revuelta *et al.* 1980; Elrod and Buccafusco 1991). In some brain regions the values are four to 10 times higher than those reported here. One potential explanation for these differences is that the lower values in deep brain structures resulted from postmortem decreases with the total freezing procedure versus the rapid inactivation of acetylcholine esterase seen with microwave fixation. However, it is also possible that the method of restraint necessary for microwave fixation or the mode of intravenous administration of precursor produced stress in those studies which may have significantly elevated ACh turnover as previously demonstrated (Finberg *et al.* 1979). Another possible reason for these differences is the method of turnover calculation. The log-linear decay in specific radioactivity of neurotransmitters has been used to calculate turnover rates for a number of neurotransmitters (Goeders and Smith 1993; Smith *et al.* 2003). This procedure has also been used to determine ACh turnover (Smith *et al.* 1984) resulting in values that are in general agreement with non-isotopic procedures that used inhibition of choline uptake and measured the decline in ACh content (Schmidt and Buxbaum 1978; Finberg *et al.* 1979); but not with data calculated with the finite difference or two compartment methods which used the specific activity of choline in the calculation. These two procedures have been used to more accurately define the neuronal compartment which is highly desirable. However, choline is both precursor to, and metabolite of ACh and serves other functions that appear to also include a transmitter role (Papke *et al.* 1996). In addition, there is significant evidence that the choline pool is independent of the ACh pool. Choline specific radioactivity does not correlate with ACh specific radioactivity or with cholinergic neuronal activity while, ACh specific radioactivity does (Atweh and Kuhar 1976). In addition, the postmortem increase in choline is independent of cholinergic neurons since it is still seen in the hippocampus after septal lesions that have removed cholinergic innervations (Freeman and Jenden 1967). Therefore, the use of choline specific activity in turnover rate calculations may not identify the neuronal pool and the methods for ACh turnover rate calculations that use choline may produce values that are the result of the non-precursor roles that choline serves. This type of circumstance resulted in elevated values for GABA turnover when glutamate (Glu) was used as the precursor in the calculations (Bertilsson *et al.* 1972). The log linear decay procedure uses the specific activity of the neurotransmitter itself which avoids this problem. However, in circumstances where the inactivation of a neurotransmitter is not through metabolism, but rather reuptake and reutilization, then turnover rates calculated with the log-linear decay in neurotransmitter specific radioactivity could be affected by

the reuptake process. However, if appropriate control or comparison groups that would not differ in these parameters are used, then relative turnover rates can be valuable in defining cholinergic involvement in brain function. As turnover calculated with neurotransmitter/metabolite ratios are not in agreement with those obtained with the pulse label log-linear decay method calculated from the same data set (Smith *et al.* 2003), then isotopic methods or blockade of synthesis may be the only alternative methods. Isotopic methods are not subject to alterations in the activity of a neuronal system that may occur with blockade of synthesis that can have downstream effects on other systems that may alter turnover estimates. The log-linear decay method used here appears to be subject to fewer potential pitfalls than other isotopic procedures.

The turnover rate measurements for ACh require short pulse times so that the results represent only the turnover of ACh during the pulse interval. This can be both an advantage and a disadvantage. If a specific treatment of short duration can be identified, then the values can be quite meaningful. However, if duration of the treatment is longer, then the obtained values may not be representative of the total effect. For example, the decrease in ACh content in lateral hypothalamus, lateral thalamus and hippocampus seen in the yoked cocaine group could result from a net increase in turnover of ACh which is not present shortly after the infusion of cocaine when the pulse labeling was completed, if indeed content changes reflected turnover. Turnover rates reflect the activity of the neurons investigated only during the pulse label period. In the present experiment this was directly after a self-administration which may not reflect the overall actions of cocaine which have a longer duration than the pulse label period.

ACh and cocaine self-administration

The turnover rates obtained in this experiment implicate the involvement of discrete cholinergic local circuit neurons and ascending and descending cholinergic afferents from the basal forebrain in the brain processes that underlie cocaine self-administration (Fig. 3). These include cholinergic innervations of the diagonal band-pre-optic region, medial and lateral hypothalamus, hippocampus, ventral pallidum, VTA and pyriform and occipital (visual) cortices and cholinergic local circuit neurons in the caudate-putamen, NAcc, olfactory tubercle and possibly the ventral pallidum. The loci of the changes in ACh utilization suggest that the activity of ACh releasing neurons with cell bodies in several cholinergic nuclei participate, including the diagonal band-pre-optic region, nucleus basalis magnocellularis, medial septum, medial habenular nucleus and the lateral dorsal tegmental (LDTg) and pedunculopontine tegmental (PPTg) brainstem nuclei. The cholinergic cells in the diagonal band-pre-optic nucleus region provide the largest contribution since changes in the turnover rate were seen in efferents to the olfactory

COCAINE SELF-ADMINISTRATION

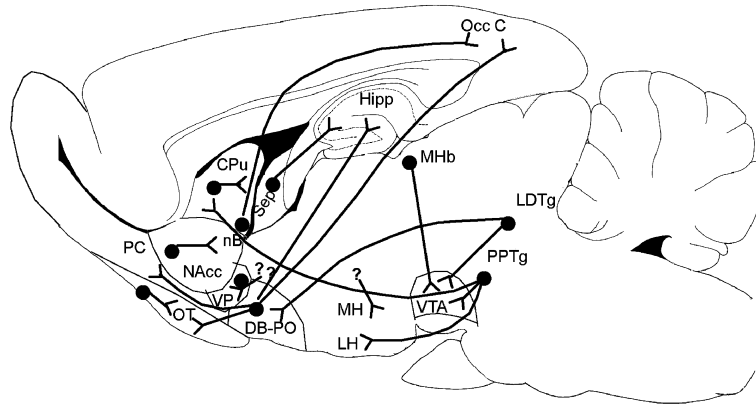


Fig. 3 Neuronal pathways indicated in cocaine self-administration. Abbreviations used: C Pu, caudate nucleus putamen; DB-PO, diagonal band of Broca, pre-optic area; Hipp, hippocampus; LDTg, lateral dorsal tegmental nucleus; LH, lateral hypothalamus; MH, medial hypothalamus; MHb, medial habenula; NAcc, nucleus accumbens;

Occ C, occipital cortex; OT, olfactory tubercle; PC, pyriform cortex; PPTg, pedunculo pontine tegmental nucleus; Sep, septum; VP, ventral pallidum; VTA, ventral tegmental area. Question marks next to symbols indicated unknown origin of the innervations.

tubercle, pyriform cortex as well as the occipital cortex. The LDTg and PPTg cholinergic neurons are also involved since turnover changes were seen in efferents to the VTA, diagonal band-pre-optic region and lateral hypothalamus. In addition, the cholinergic efferents from the medial septum to the hippocampus, from the nucleus basalis magnocellularis to the occipital cortex and from the medial habenular nucleus to the VTA also contribute.

The widespread involvement of ACh in the self-administration of cocaine is not surprising since recent evidence supports such a role. Extracellular fluid levels of ACh were elevated in the NAcc (Consolo *et al.* 1999), cerebral cortex (Day *et al.* 1997) and the hippocampus (Imperato *et al.* 1996) of rats acutely administered response independent cocaine. Cholinergic interneurons in the NAcc may be inhibitory to the effects of cocaine since ablation of these neurons enhanced the locomotor response to cocaine and place preference conditioning (CPP) in mice (Hikida *et al.* 2001). Furthermore, choline acetyltransferase activity was reduced in the NAcc of rats on the last day of cocaine self-administration and after 3 weeks of withdrawal suggesting a down regulation of cholinergic tone in this region (Wilson *et al.* 1994). Although the turnover rates of NAcc ACh did not significantly differ between the yoked cocaine infused and cocaine self-administering groups as assessed with the Bonferonni *post hoc t*-test analysis, the ANOVA demonstrated a significant treatment effect. Figure 2 shows that the turnover is decreased in the yoked cocaine infused group and further decreased in the self-administration group which would be consistent with the inhibitory action mentioned above. In addition, alpha-amino-3-hydroxy-5-methylisoxaz-

ole-4-propionic acid induced lesions of the nucleus basalis magnocellularis (a site containing cell bodies for a major cholinergic system innervating the forebrain) shifted the cocaine dose-intake relationship to the left suggesting an enhancement of reinforcing efficacy (Robledo *et al.* 1998). Moreover, increased extracellular fluid levels of ACh were found in microdialysates of the NAcc from rats during cocaine self-administration (Mark *et al.* 1999a) which could result from previously shown increased tonic stimulatory control of cholinergic activity by D1 dopamine receptors in this structure (Consolo *et al.* 1999). The administration of a muscarinic agonist directly into the NAcc decreased cocaine intake (Mark *et al.* 1999b), while muscarinic blockade with scopolamine did not affect established cocaine self-administration in rats (Ikemoto and Goeders 2000). The attenuation of cocaine self-administration with a muscarinic agonist and absence of an effect with an antagonist is difficult to resolve, but would be consistent with these receptors being quiescent during cocaine self-administration (consistent with the decreased turnover rate), but having an effect if exogenously activated by the administration of the agonist.

Nicotinic cholinergic receptors are involved in these processes as well since systemic pretreatment with the nicotinic antagonist mecamylamine reduced cocaine self-administration at doses that did not alter food maintained responding suggesting an overall excitatory effect on the actions of cocaine (Levin *et al.* 2000). A CPP to cocaine was attenuated in mice lacking the $\beta 2$ nicotinic receptor subunit (Zachariou *et al.* 2001) again suggesting an excitatory role, but intravenous cocaine self-administration occurs at the

same rate as the wild type in this knockout (Epping-Jordan *et al.* 1999). The α_7 receptors in the VTA appears to mediate the enhancing effects of cocaine upon brain stimulation reinforcement since methyllycaconitine attenuated this effect suggesting an excitatory role for this receptor system as well (Panagis *et al.* 2000).

The importance of the hindbrain cholinergic cell nuclei in the LDTg and PPTg in behavior has been recognized for some time. The turnover rates of ACh were decreased in the self-administering rats in the VTA, diagonal band-pre-optic region and lateral hypothalamus which receive input from these brainstem nuclei (Fig. 2). Injection of a nicotinic receptor antagonist into this hindbrain region was reported to increase cocaine self-administration again suggesting an inhibitory role in the actions of cocaine (Corrigall *et al.* 1999). Electrical stimulation of the LDTg resulted in an increase in extracellular DA in the NAcc that could be blocked with intra-LDTg infusions of a M2-selective muscarinic receptor antagonist (Forster and Blaha 2000) suggesting a role for muscarinic receptors in the function of this important brain center. Excitotoxin induced lesions of this brain region antagonized acquisition of a CPP to opioids, psychomotor stimulants and food (Bechara and van der Kooy 1992; Olmstead and Franklin 1993, 1994) and blocked acquisition of intravenous heroin self-administration (Olmstead *et al.* 1998). The LDTg is clearly a behaviorally relevant nucleus that likely includes the cholinergic neurons that originate in this region.

Cholinergic neurons appear to participate in the neuronal circuits activated in intravenous self-administration of other drugs as well. ACh turnover rates were significantly decreased in the pyriform cortex, NAcc and amygdala and increased in the frontal cortex of rats intravenously self-administering morphine (Smith *et al.* 1984). In addition, CPP to morphine is substantially reduced in mice lacking the M₅ muscarinic receptor (Basile *et al.* 2002). The decrease in ACh turnover in the NAcc with both morphine and cocaine self-administration suggests that these neurons could have a broader role in reinforcement processes in general.

Conclusions

The brain mechanisms underlying cocaine self-administration have been shown to involve DA neurons. However, it is clear that a number of other neuronal systems participate in the circuits that are responsible for these complex behaviors. A role of discrete subpopulations of DA, 5-HT, Glu and GABA releasing neurons has recently been demonstrated with neurotransmitter turnover rate methodologies (Smith *et al.* 2003). The striking differences between animals that control the delivery of the drug with those that receive it independent of their own behavior is even more evident when the identified ACh involvement is considered. It is apparent that cholinergic neurons have a

significant role in these processes. Data to date suggest an important role for ACh releasing neurons in the NAcc (Hikida *et al.* 2001; Mark *et al.* 1999a, 1999b) and nucleus basalis magnocellularis (Robledo *et al.* 1998). The turnover rates presented here suggest additional involvement of cholinergic neurons with somata in the diagonal band-pre-optic region, medial septum, caudate-putamen, olfactory tubercle, LDTg, PPTg and ventral pallidum in cocaine self-administration. As cholinergic receptors have been shown to participate in the processes underlying brain stimulation reinforcement (Yeomans *et al.* 2000), it is likely that some of the cholinergic systems identified here represent neuronal systems mediating reinforcement in general, and thus may have broader application to understanding the biological basis of behavior.

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