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Effects of Cocaine and the Cocaine Analog CFT on Glutamatergic Neurons

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ROBINSON, S. E., J. R. MAHER, K. P. McDOWELL AND P. M. KUNKO. *Effects of cocaine and the cocaine analog CFT on glutamatergic neurons.* PHARMACOL BIOCHEM BEHAV **50**(4) 627–633, 1995.—The effects of cocaine and the cocaine analog methyl-3β-(*p*-fluorophenyl)-1αH,5αH-tropane-2b-carboxylate (CFT) on glutamate turnover rate were studied in the nucleus accumbens, striatum, frontal cortex, and parietal-cingulate cortex of the rat, using neurotransmitter turnover rate as an estimate of the activity of the glutamatergic neurons. Both cocaine [15 or 30 mg/kg, intraperitoneally (IP)] and CFT (2.2 mg/kg, IP) increased glutamate turnover in the nucleus accumbens, although the time course of their actions differed. These effects on glutamate turnover appeared at times after maximal motor activation of the animals had occurred. On the other hand, neither cocaine nor CFT affected glutamate turnover in the frontal cortex, parietal-cingulate cortex, or striatum. Neither cocaine nor CFT affected the content of glutamate or glucose in any brain region studied. Thus, although cocaine and CFT affect glutamatergic neurons in the CNS, these actions are not generalized across the CNS, but are restricted to a specific brain region.

Cocaine CFT Frontal cortex Motor activity Nucleus accumbens Parietal-cingulate cortex Striatum Glutamate WIN 35,428

IT IS WELL established that cocaine affects catecholaminergic and serotonergic neurotransmission. Dopaminergic neurons are important in the production of the behavioral effects of cocaine, with mesocorticolimbic and nigrostriatal projections considered particularly important (17,35,48), whereas serotonergic neurons have been implicated in the seizure-inducing properties of cocaine (34). However, there have been few studies of the effect of cocaine on glutamate, a major excitatory amino acid neurotransmitter in the CNS. This is of particular interest, as there is evidence that stimulation of excitatory amino acid receptors is involved in the expression of the biochemical, physiologic, and behavioral effects of psychostimulants such as cocaine. This evidence includes studies demonstrating that these effects are attenuated by pretreatment with *N*-methyl-D-aspartate (NMDA) or other excitatory amino acid receptor antagonists (19,21,33,40,42).

Because cocaine does not displace glutamate or phencyclidine binding (35), activation of neurons using excitatory amino acids as neurotransmitters is more likely the mechanism of action involved in the effect of cocaine on glutamatergic neurotransmission than is direct receptor stimulation. These studies indirectly suggest that cocaine activates neurons that use excitatory amino acids as neurotransmitters. To determine whether psychostimulants can actually activate glutamatergic neurons, the effects of cocaine and the cocaine analog methyl-3β-(*p*-fluorophenyl)-1αH,5αH-tropane-β-carboxylate [CFT or WIN 35,428 (6)] have been studied on the activity of glutamatergic neurons, as indicated by glutamate turnover. Using this noninvasive technique of assessing neuronal activity, it is possible to estimate simultaneously the activity of these amino acidergic neurons at multiple sites throughout the brain. Infusion of labeled glucose results in the selective labeling of the "large" glutamate pool, which has been associated with neurons (16,44). Furthermore, as this pool turns over rapidly, the use of short infusion times preferentially labels the neuronal pool of glutamate. Cremer and Lucas (9) reported that barbiturate anesthesia reduces the incorporation of isotopic label from glucose into glutamate, but not the incorporation of label from butyrate or acetate, which are believed to label nonneuronal glutamate compartments. Results obtained with the technique of Wood et al. (49) are consistent with findings obtained by the use of standard electrophysiologic and neuro-

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chemical techniques. Muscimol decreases glutamate turnover (49) in the same brain regions, as it was demonstrated electrophysiologically that glutamatergic neurons are inhibited by GABAergic interneurons (10). Baclofen, which has been found to decrease the release of glutamate in cortical slices (20) and brain synaptosomal preparations (22), decreases glutamate turnover (49). Thus, this method of measuring brain regional glutamatergic neuronal activity has been validated.

In the present study, the turnover rate of glutamate was determined in the nucleus accumbens and striatum, as well as in cortical regions, brain areas implicated in the actions of psychomotor stimulants and possessing significant glutamatergic innervation (8,14,15). Amino acid neurotransmitters are the most ubiquitous neurotransmitters in the CNS; it is therefore important to understand whether and how they are affected by cocaine.

METHODS

The activity of glutamatergic neurons was measured by determining the turnover rate of glutamate with the gas chromatographic/mass fragmentographic (GC/MS) method of Wood et al. (49), which uses a constant-rate infusion of [$^{13}C_6$]glucose to label brain regional glutamate. This turnover method uses a single time-point infusion instead of multiple time infusions for each experiment, thereby reducing the number of experimental subjects required. Furthermore, the method of constant-rate infusion leads to efficient labeling of the pools of transmitter so that small samples can be analyzed. This technique allows for simultaneous turnover measurements in multiple discrete brain areas and avoids invasive procedures, which in themselves might alter neuronal activity or amino acid release (46).

All rats were kept on a 12 L : 12 D cycle in a temperature- and humidity-controlled room and given food (Purina Lab Chow, St Louis, MO) and water ad lib. Male Sprague–Dawley rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN) weighing 190–280 g were used in the studies. Rats were injected intraperitoneally (IP) with either 0.9% saline, cocaine HCl, or the cocaine analog, CFT. CFT has been found to exhibit greater potency than cocaine in inhibiting the dopamine transporter, when calculated on a molar basis (David Compton, personal communication); accordingly, the molar dose of CFT chosen was adjusted to take into account this difference in potency. Control rats were treated in the same manner as the drug-injected rats, except that they were injected with 0.9% saline (1 ml/kg). In the time-course studies, control animals were injected with saline at the same times before euthanasia as drug-treated rats. Values for the different treatment time control rats were pooled together in each time-course study, as no differences were detected in any parameters at the various times after injection. Rats were infused for 6 min with uniformly labeled ^{13}C-Dglucose (75 μmol/kg per min) via the tail vein following IP injection of either cocaine, CFT, or physiologic saline, and euthanized by focused microwave irradiation (1.3–1.5 s, 9.5–10 kW; Cober Instruments, Stamford, CN) immediately at the end of the infusion. Gross dissection of the brains was performed on ice and the tissue kept frozen at −70°C until analyzed. The area denoted as "parietal-cingulate" cortex was dissected in such a way as to include portions of cingulate cortex (area 29), temporal cortex (area 41), and parietal cortex (area 2), and the area denoted as "frontal" cortex included the frontal sensorimotor cortex (area 10 and part of area 2), as defined by Krieg (25).

Brain regions were homogenized in 1 N HCl containing the deuterium-labeled internal standards [$^2H_5$]glutamate and [$^2H_2$]glucose. Following centrifugation, aliquots of the supernatant for glutamate or glucose analysis were lyophilized. The HCl-precipitable protein was solubilized in 0.5 N NaOH and protein content measured by the method of Lowry et al. (28); transmitter levels were expressed as nanomoles per milligram of protein. After the fractional rate constant for glutamate utilization (kglu) was determined (49), the turnover rate for glutamate (TRglu) was calculated by multiplying the rate constant by the concentration of the neurotransmitter (49).

An aldononitrile pentacetate derivative of glucose was made according to the procedures of Tserng and Kalhan (43). The freeze-dried extract was reacted with pyridine containing hydroxylamine and then with acetic anhydride. After the addition of methylene chloride, the samples were washed with distilled water. Excess reagents were evaporated under nitrogen before the samples were reconstituted in ethyl acetate. An aliquot was injected into a Hewlett-Packard 5890A gas chromatograph (Sunnyvale, CA) coupled by a capillary direct interface to a Hewlett-Packard 5988A mass spectrometer in the electron impact (EI) mode. A DB-5 bonded-phase (0.25 μm film thickness) fused silica capillary column (30 m × 0.25 mm ID) was maintained at 205° C. In the selected ion monitoring mode (SIM), the ions 328 (endogenous glucose), 330 (glucose internal standard), and 334 (incorporated glucose) were monitored.

To a second freeze-dried aliquot, pentafluoropropionic anhydride (PFPA) and hexafluoroisopropanol (HFIP) were added. After reacting for 1 h, excess reagents were evaporated under nitrogen, and then the samples were reconstituted in ethyl acetate for injection into the gas chromatograph/mass spectrometer, using a column as described earlier, maintained at 150°C. The ions 398 (endogenous glutamate), 403 (glutamate internal standard), and 400 (from labeled glucose incorporated into glutamate) were monitored in the SIM mode.

Motor activity data were collected via Omnitech Digiscan (Columbus, OH) activity monitors enclosed in sound-attenuated darkened chambers. Interruption of infrared beams was registered by a computer, which was programmed (Integrated Lab Animal Monitoring System version 3.5sp) to detect quantity and patterns of beam interruption. The program calculated total activity counts, ambulation counts [(total activity counts)−(stereotypic movements)], number of horizontal movements (recorded each time a pause in ambulatory activity occurs for a period of >1 s), number of stereotypic movements (two or more repeated beam breaks), and number of stereotypic episodes. Rats were injected IP with drug (cocaine 30 mg/kg or CFT, 2.2 mg/kg) or 0.9% saline and immediately placed in the activity chambers. Activity data were collected over 2 h, at 10-min intervals.

Data from the neurochemical experiments were analyzed by one-way analysis of variance (ANOVA), with post hoc comparisons using Fisher's protected least significant difference test (47). Two-way ANOVA with repeated measures was used for the analysis of motor behavior. A level of $p < 0.05$ was accepted as statistically significant.

Cocaine HCl was obtained from the National Institute on Drug Abuse, and CFT was the generous gift of Organix, Inc. (Woburn, MA). Pyridine was purchased from Pierce (Rockford, IL), and hydroxylamine hydrochloride was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). PFPA and HFIP were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetic anhydride was purchased from Supelco, Inc. (Bellefonte, PA). All deuterium and ^{13}C-labeled compounds were purchased from MSD Isotopes (Montreal, Canada).

RESULTS

Time Course of Effect of Cocaine on Brain Regional Glutamate

The effect of cocaine HCl (30 mg/kg, IP) was measured on brain regional glutamatergic neurons 30 min and 2 h after injection. The behavioral effects of cocaine are readily apparent 30 min after injection; thus, neurotransmitter changes observed at that time may be correlated with the behaviors exhibited. The 2-h time point corresponded to the time at which the psychostimulant amphetamine has been demonstrated to maximally release ascorbic acid (37). As ascorbic acid efflux has been suggested to reflect glutamate transport, and hence indirectly indicate glutamate release (3), one could infer that amphetamine may produce a slow-developing increase in glutamate release. If cocaine were to act in a similar fashion, one would expect to find increased glutamate turnover at this later time. As can be seen in Fig. 1, cocaine produced a long-lasting increase in glutamatergic activity in the nucleus accumbens. TRglu was significantly increased in the nucleus accumbens 30 min after injection of cocaine, and remained elevated 2 h after injection [$F(2, 21) = 3.462$, $p < 0.05$]. Glutamate content was not significantly affected in this brain region at either of the times measured [$F(2, 21) = 1.525$, $p > 0.05$]. In the parietal-cingulate cortex, kglu and TRglu were slightly, but not significantly, reduced after cocaine injection [$F(2, 17) = 2.168$ and 1.883, respectively, $p > 0.05$]. Glutamatergic neurons in neither the striatum nor the frontal cortex were significantly affected by cocaine at this dose, as neither the level of glutamate nor its turnover rate was changed following cocaine injection (Fig. 1). In none of the brain regions studied did cocaine affect glucose content or the percent incorporation of ^{13}C label into brain regional glucose (data not shown).

Dose-Response Effect of Cocaine on Brain Regional Glutamate

A dose-response study of the effect of cocaine HCl (5, 15, and 30 mg/kg, IP) on glutamate turnover was performed at 30 min postinjection, as this was the time at which the greatest increase in TRglu was observed in the nucleus accumbens, the main area of interest. There was a significant treatment effect on TRglu in the nucleus accumbens [$F(3, 24) = 3.715$, $p < 0.05$]. TRglu was significantly increased in the nucleus accumbens following IP injection of 15 and 30 mg/kg cocaine HCl, as compared to saline control (Fig. 2). There was no significant effect of 5 mg/kg cocaine HCl on TRglu in this brain region. Glutamate content was not affected 30 min after any of these doses [$F(3, 24) = 2.068$, $p > 0.05$] (Fig. 2). There was no significant effect of any of these doses of cocaine on glutamate content or TRglu in the frontal cortex, parietal-cingulate cortex, or striatum (Fig. 2). Cocaine did not affect glucose content or the percent incorporation of ^{13}C label into glucose in any of the brain regions studied (data not shown).

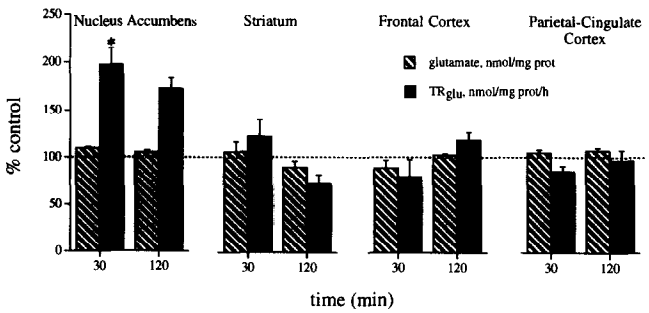

FIG. 1. Effect of cocaine HCl (30 mg/kg, IP) on glutamate neurons in four brain regions at 30 min or 2 h following injection. Glutamate content (hatched bars) and TRglu (solid bars) were measured after IP saline or cocaine HCl injection. The data were calculated as a percentage of the mean saline control response and are expressed as mean + SEM. There are five to 10 subjects per data point. *$p < 0.05$, as compared to control by ANOVA and Fisher's plsd test.

Time Course of Effect of CFT on Brain Regional Glutamate

Methyl-3β-(p-fluorophenyl)-1αH,5αH-tropane-2β-carboxylate (CFT; 2.2 mg/kg, IP), like cocaine, increased glutamate turnover in the nucleus accumbens [$F(3, 20) = 4.898$, $p < 0.05$]. However, as can be seen in Fig. 3, this effect was statistically significant only at the 2-h postinjection time point, with TRglu not significantly affected in the nucleus accumbens 30 and 60 min after injection of CFT. As was the case with cocaine, CFT did not affect the content of glutamate in the nucleus accumbens at any of the times tested [$F(3, 20) = 0.253$, $p > 0.05$]. Also, this dose of CFT (2.2 mg/kg, IP) did not significantly affect glutamatergic neurons in the striatum, frontal cortex, or parietal-cingulate cortex, as neither the level of this neurotransmitter, kglu, nor TRglu was significantly changed following CFT injection (Fig. 3). In none of the brain regions studied did CFT affect glucose content or the percent incorporation of ^{13}C label into glucose (data not shown).

Time Course of Effect of Cocaine and CFT on Motor Activity

The patterns of motor activity were quite different after an injection of cocaine or CFT. Although five different measures of motor behavior were made, only total activity is presented, as the pattern of responses to the various treatments was similar across the measures. There were significant effects of treatment and time, respectively, on total activity [$F(2, 29) = 14.833$, $p < 0.0001$ and $F(11, 319) = 2.064$, $p < 0.05$; number of movements, $F(2, 29) = 18.49$, $p < 0.0001$ and $F(11, 319) = 7.153$, $p < 0.0001$; number of stereotypic episodes, $F(2, 29) = 9.042$, $p < 0.001$ and $F(11, 319) = 2.172$, $p < 0.05$; and number of stereotypic movements, $F(2, 29) = 48.373$, $p < 0.0001$ and $F(11, 319) = 8.889$, $p < 0.0001$]. The only measure in which there was no significant effect of time was ambulatory activity [$F(11, 319) = 1.174$, $p > 0.05$], in which there was a significant treatment effect only [$F(2, 29) = 28.359$, $p < 0.0001$]. In each activity measure, there were significant interactions between treatment and time, indicating that the pattern of the activity response over time differed for the treatments [total activity, $F(22, 319) = 3.816$, $p < 0.0001$; ambulatory activity, $F(22, 319) = 2.701$, $p < 0.0001$; number of movements, $F(22, 319) = 4.353$, $p < 0.0001$; number of stereotypic episodes, $F(22, 319) = 3.733$, $p < 0.0001$; and number of stereotypic movements, $F(22, 319) = 3.29$, $p < 0.0001$]. Whereas the motor effects of cocaine peaked within the first 10 min following injection, the motor activation observed following CFT injection reached a maximum level more slowly and was much more prolonged (Fig. 4). Both drugs increased values in each measure; however, the behavioral effects of CFT were especially pronounced. At almost every time in each measure, the values obtained following CFT injection were significantly higher than those following cocaine injection.

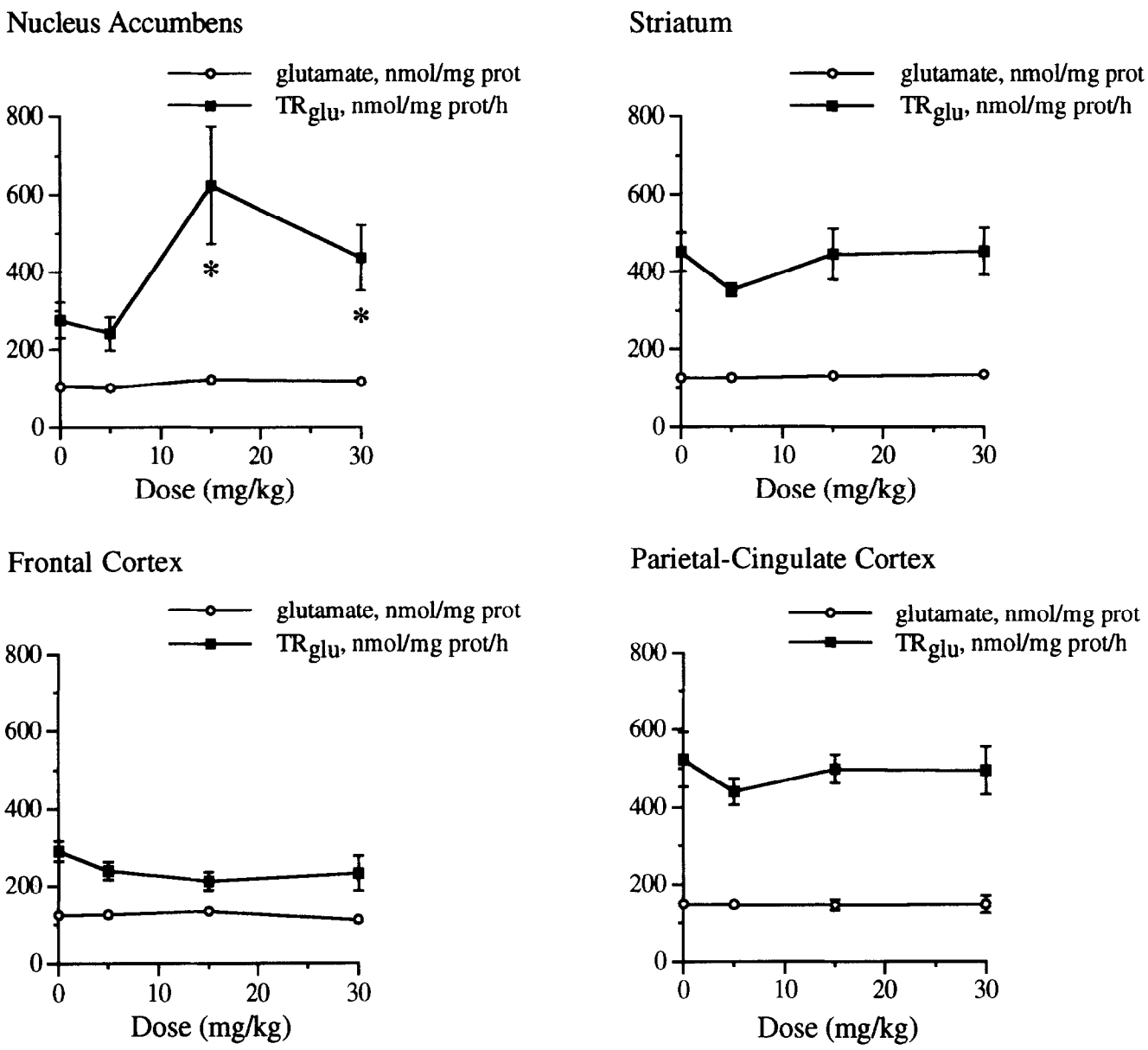

FIG. 2. Dose effect of cocaine HCl on glutamate neurons in four brain regions. Glutamate content (○) and TRglu (■) were measured 30 min after IP saline or cocaine HCl injection (5, 15, or 30 mg/kg). There are six to eight subjects per data point. Data are expressed as mean ± SEM *$p < 0.05$, as compared to saline by ANOVA and Fisher's plsd test.

DISCUSSION

These studies represent the first direct evidence that cocaine and cocaine-like drugs increase glutamate turnover. However, this effect does not represent a direct action of cocaine on all glutamatergic neurons, as the increase in glutamate turnover was restricted to the nucleus accumbens, and did not involve other brain regions with major glutamatergic input, such as the striatum. Thus, it is likely that the cocaine-induced glutamatergic activation observed in the nucleus accumbens results indirectly from an action of the drug on another neurotransmitter system, such as dopaminergic, noradrenergic, or serotonergic neurons, which are known to be affected by cocaine. It is unlikely that the cocaine-induced increases in glutamate turnover merely reflect metabolic activation, as changes in glutamate turnover do not parallel what has been reported in studies of local cerebral glucose utilization, where glucose utilization is increased in the striatum and unaffected in the nucleus accumbens following cocaine (27). The action of CFT on glutamate turnover in the nucleus accumbens was slower to develop than that of cocaine. However, this is consistent with the fact that the effect of cocaine on motor activity peaked earlier than that of CFT.

As excitatory amino acids have been demonstrated to increase extracellular levels of dopamine in the nucleus accumbens (50), cocaine-induced activation of the glutamatergic in-

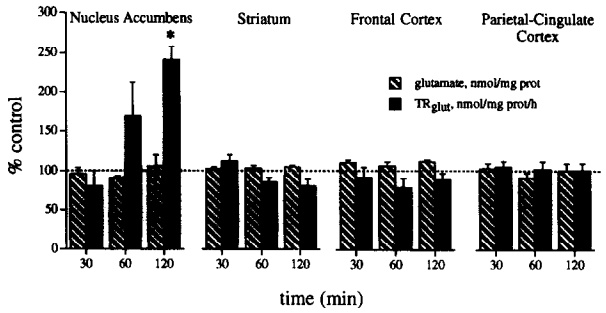

FIG. 3. Effect of CFT (2.2 mg/kg, IP) on glutamate neurons in four brain regions at 30 min, 1 or 2 h following injection. Glutamate content (hatched bars) and TRglu (solid bars) were measured after IP saline or CFT injection. The data were calculated as a percentage of the mean saline control response and are expressed as mean + SEM. There are four to seven subjects per data point. *$p < 0.05$, as compared to saline by ANOVA and Fisher's plsd test.

put to the nucleus accumbens may contribute to that drug's enhancement of dopamine neurotransmission in that brain region. Glutamatergic input to the nucleus accumbens arises in the amygdala, frontal cortex, hippocampus, and thalamus (4,23,39,45). The amygdaloid projection is especially interesting, considering the facts that the amygdala is a very important structure in kindling, glutamatergic neurons have been strongly implicated in that phenomenon as well (5), and repeated injection of cocaine produces kindled seizures (32). The basolateral nucleus of the amygdala, the site of the cell bodies of glutamatergic neurons projecting to the nucleus accumbens (4), is innervated by dopaminergic, noradrenergic, and serotonergic neurons (13), allowing multiple sites through which cocaine could act indirectly upon the glutamatergic neurons. Of course, as there are other sources of glutamatergic input to the nucleus accumbens, other brain regions also may play an important role in the stimulatory action of cocaine on the glutamatergic innervation of the nucleus accumbens.

The present study does not ascertain the physiologic consequences of activation of the glutamatergic innervation of the nucleus accumbens by cocaine; one can only speculate as to what these are. Injection of an excitatory amino acid agonist into the nucleus accumbens induces an increase in locomotor activity (2), whereas cocaine-induced hypermotility is blocked by injection of the AA2-receptor antagonist glutamic acid diethyl ester (GDEE) into the nucleus accumbens (33). Furthermore, the locomotor stimulatory action of intra-accumbens injection of excitatory amino acids can be blocked by dopamine antagonists (12), suggesting a link between these neurotransmitter systems in the production of hypermotility. Thus, it would be expected that cocaine-induced hypermotility is associated with the increase in glutamate turnover observed in the nucleus accumbens. However, the cocaine- and CFT-induced increases in glutamate turnover occur at times at which the animals' activity is beginning to fall toward that of the control animals and, certainly in the case of CFT, are not apparent during the time period in which the animals' activity is increasing. Thus, it is unlikely that psychostimulant-induced hypermotility is directly coupled to an increased activity of glutamatergic projections to the nucleus accumbens. Indeed, the increased activity of the glutamatergic innervation of the nucleus accumbens may reflect an inhibitory response to psychostimulant-induced hypermotility. On the other hand, binding of cocaine and CFT to the (presumed) dopaminergic transporter in the striatum (41) exactly parallels the pattern of locomotor activity observed in the present study, indicating that the action of these drugs on dopamine transport in the striatum is more closely correlated to locomotor behavior than is their action on glutamate neurotransmission in the nucleus accumbens. Another possible consequence of the activation of glutamatergic neurons is the phenomenon of "reverse tolerance." The NMDA antagonist MK-801 has been reported to block reverse tolerance to the locomotor, stereotypic, and convulsive actions of cocaine (21). One could speculate as well that activation of glutamatergic neurons projecting to the nucleus accumbens may contribute to the production of reverse tolerance.

Considering their actions in the nucleus accumbens, it is remarkable that neither cocaine nor CFT affected glutamate turnover in the striatum. The corticostriate projection is one of the most studied glutamatergic projections in the CNS. Dopamine receptors have been identified on glutamatergic corticostriatal terminals (24), and glutamate receptors have been identified on dopaminergic nigrostriatal terminals in this area (36); thus, there is ample potential for interaction between these two neurotransmitter systems. No consistent interaction has been reported between striatal glutamatergic and dopaminergic neurons. Both in vitro and in vivo studies have suggested that, depending on the concentration, excitatory amino acids either stimulate or inhibit the release of striatal dopamine (7,11,18,26,29,37). Berretta et al. (1) reported that both indirect dopaminergic agonists and glutamate agonists induce fos-like protein in the striatum; however, the distribution of neurons affected by these agonists is not identical. The fact that cocaine and CFT do not affect striatal glutamate turnover opposes a possible glutamatergic contribution to the actions of cocaine on striatal dopamine. However, it is possible that the portion of the glutamatergic innervation of the

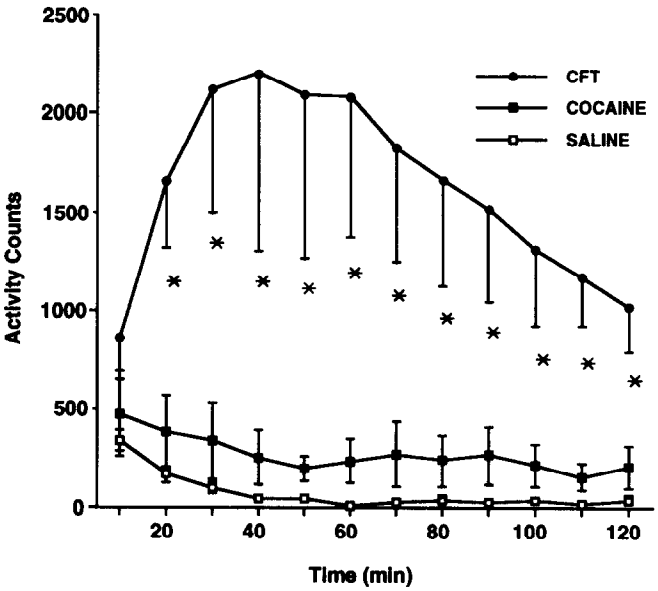

FIG. 4. Effects of saline (□, 16 subjects), cocaine HCl (30 mg/kg, IP, ■, eight subjects) and CFT (2.2 mg/kg, IP, ●, eight subjects) on total motor activity. Data are expressed as mean ± SEM. *$p < 0.05$, as compared to cocaine by ANOVA and Fisher's plsd test.

striatum affected by cocaine is too small to detect with this turnover technique. Furthermore, the possibility exists that the time course of the glutamatergic response to cocaine or CFT in the striatum is very different from that in the nucleus accumbens.

In conclusion, cocaine and CFT affect glutamatergic neurons in the CNS. This action is not generalized, but varies across brain regions. Both cocaine and CFT increase glutamate turnover in the nucleus accumbens, which is consistent with the findings of other investigators who suggested a role for excitatory amino acids in the expression of the biochemical, physiologic, and behavioral effects of psychostimulants. Microdialysis studies currently under way in our laboratory have demonstrated a cocaine-induced increase in extracellular glutamate, lending support to the present study (38). Although cocaine and CFT do not affect glutamate turnover in the frontal cortex, parietal-cingulate cortex, or striatum, an action on other excitatory amino acid neurotransmitters (i.e., aspartate) cannot be ruled out. The mechanism by which cocaine and CFT produce these changes is unknown, but may involve actions of these drugs at dopamine, norepinephrine, or serotonin transporters or even "local anesthetic" (sodium channel-blocking) properties.

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