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Potential Non-Serotonergic Basis of Hyperphagia Elicited by Intraventricular p-Chlorophenylalanine[1]

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COSCINA, D. V., J. DANIEL AND J. J. WARSH. *Potential non-serotonergic basis of hyperphagia elicited by intraventricular p-chlorophenylalanine.* PHARMAC. BIOCHEM. BEHAV. 9(6) 791–797, 1978.—Previous research has demonstrated that intraventricular injections of 2, 3 or 4 mg p-chlorophenylalanine (pCPA) methylester-HCl can produce dose-dependent hyperphagia in rats. Since the transient time-course of this effect corresponded roughly to large (up to 78%) depletions of endogenous forebrain serotonin (5-hydroxytryptamine or 5HT), these data support previous suggestions of some inhibitory role for brain 5HT neurons in feeding. The dose-response nature of this hyperphagia was replicated here using 2, 3 and 4 mg doses of pCPA as before. However, control infusions of 2, 3 and 4 mg of pCPA's parent amino acid, phenylalanine (PA) in methylester-HCl form, produced dose-dependent hyperphagia of equivalent magnitude and duration. In addition, no association was observed between the occurrence of hyperphagia elicited by either compound and depletion of forebrain 5HT. A number of pharmacological and neural factors potentially operative in explaining these findings are discussed. Because it is possible to dissociate central pCPA-induced hyperphagia from forebrain 5HT loss, our data question the specificity of this method in elaborating hypothesized roles of this brain monoamine in the control of feeding.

Hyperphagia p-Chlorophenylalanine Weight gain Phenylalanine Food intake Intraventricular

CONSIDERABLE indirect evidence has accumulated which suggests that impairment of brain serotonin (5-hydroxytryptamine or 5HT) metabolism can produce overeating (hyperphagia) (for review see [4, 7, 19, 20]). In agreement with this possibility is recent work documenting transient hyperphagia in rats following ventricular infusions of p-chlorophenylalanine (pCPA) [6,21]. Since the magnitude of enhanced feeding reported was dose-dependent (2–4 mg injected) and its time-course was associated with substantial depletion of endogenous forebrain 5HT (up to 78%), these data support an inhibitory role for brain 5HT neurons in food intake.

Notwithstanding the apparent elegance of this work and the parsimonious conclusions it implies, there are additional data which question the generality of these findings. For example, chronic depletion of brain 5HT by midbrain raphe lesions does not elicit overeating [11, 14, 25]. Indeed, such raphe injury can block overeating elicited by medial hypothalamic injury [9,14] which itself can deplete brain 5HT [10,14]. It has been suggested [29,30] that raphe lesions alone are ineffective in producing overeating because of concurrent damage to brain norepinephrine (NE) systems. This general argument is counter-intuitive in light of numerous reports that brain NE depletion itself has been associated with overeating [1, 2, 7, 8, 10, 12, 13, 14, 16, 17, 18, 22]. Additional data mediating against a simple inhibitory role for brain 5HT neurons in feeding include: (1) the inability of several groups (e.g., [5, 8, 21]) to elicit overeating in rats chronically depleted of brain 5HT by central injections of the indole neurotoxin 5,7-dihydroxytryptamine (5,7-DHT); (2) the lack of correspondence in known time-course of brain 5HT depletion following central 5,7-DHT (within days) and the occurrence of overeating (30 days later) reported by one group [29,30]; (3) the inability of central pCPA or 5,7-DHT injections to attenuate the anorexia elicited by the 5HT releaser, fenfluramine [21]; (4) the inability of increasing brain 5HT by systemic tryptophan treatment to suppress deprivation-induced feeding [33].

The contradictory data cited above made us consider the possibility that central injections of pCPA might induce hyperphagia [6,21] by actions not necessarily related to brain 5HT depletion. The following experiments were designed to test this possibility.

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EXPERIMENT 1

Only one laboratory has reported thus far that ventricular pCPA infusion can induce hyperphagia [6,21]. Therefore, one purpose of the first experiment was to replicate the dose-response nature of such overeating. At the same time, we were concerned that this earlier research did not include control infusions of any substance structurally related to pCPA. This seemed important inasmuch as large quantities (2–4 mg) of the drug were required to elicit hyperphagia. Since pCPA is a chlorinated derivative of the essential amino acid phenylalanine (PA), we employed comparable doses of this substance to compare with pCPA.

METHOD

Animals

Thirty-two female albino rats (Wistar strain; High Oaks Ranch, Ontario) were used. Rats weighed 220–280 g at the beginning of experimentation. For the duration of study, all animals were housed separately in hanging metal cages with wire-mesh fronts and floors. The colony room was illuminated 12 hr daily (lights on at 0800 hr) and maintained at 23°C (± 1°C). Sufficient amounts of fresh Teklad Pellet Chow (4% fat) were weighed out daily and made available on cage floors to sustain ad lib food consumption. Food spillage was measured daily by weighing fragments which fell on paper towels placed under the cages. Fresh tap water was always available from bottles fitted with sipper tubes. Bodyweight (BW) and food intake were measured daily for 40 days to the nearest g for all rats using a Mettler triple-beam balance.

Procedure

Injections. Animals were randomly assigned to one of eight groups (n=4 per group): rats receiving 2 mg, 3 mg or 4 mg d,l-pCPA (methyl-ester-HCl form as used earlier [6] dissolved in 20 μl isotonic saline, rats receiving 2 mg, 3 mg or 4 mg d,l-PA methylester-HCl dissolved in 20 μl isotonic saline, rats receiving 20 μl isotonic saline alone, or normal (untreated) rats. Both drugs were obtained from Sigma Chemical Company (St. Louis, MO). Saline (for injection U.S.P.) was obtained from Glaxo-Allenburys (Toronto, Ont.)

With the exception of rats designated for no treatment, all remaining animals were pre-treated with atropine methyl nitrate (0.1 mg IP), anesthetized with sodium pentobarbital (Nembutal; 35 mg/kg IP) and placed in a Kopf stereotaxic instrument. With the incisor bar set 5 mm above the interaural line, infusions of each drug solution or saline vehicle were made into each lateral ventricule (10 μl/side) through a 1-1/2 in., 30 ga hypodermic needle at stereotaxic coordinates 5.8 mm anterior to the interaural line, 1.5 mm lateral to the midline, 3.5 mm ventral to the dura (determined from [28]). Infusions were delivered at a rate of 1 μl/min (± .05 min) by a Sage Instrument Pump (Model 355). At the completion of each hemispheric infusion, the injection needle was left *in situ* for 5 min before withdrawal. Scalp incisions were closed with stainless-steel wound clips and rats were returned to homecages for daily food intake and BW measurements.

Sacrifice and brain tissue preparation. After all rats had returned to normal food intake levels, they were sacrificed by decapitation and brains were removed from the calvaria. After discarding the olfactory bulbs, pineal gland and meninges as well as rinsing away surface blood with cool saline, the forebrain was separated from the hindbrain by a mid-collicular coronal cut. The hindbrain was discarded. The forebrain was further separated into left and right hemispheres by a mid-saggital section. Left hemispheres were stored in 10% Formalin. Right hemispheres were blotted on filter paper, weighed to the nearest 10 mg, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -20°F. The time intervening between sacrifice and freezing of brain tissue was 2–3 min per animal.

Monoamine assays. Right hemispheric 5HT, NE and dopamine (DA) were isolated on a weak cation exchange column [3,15] and assayed fluorometrically [27,31]. Since recoveries of all amines were at least 92%, data reported here were not corrected for recovery loss.

Statistical analyses. All data were analyzed by *t*-tests for independent samples. All probabilities reported represent two-tailed distributions.

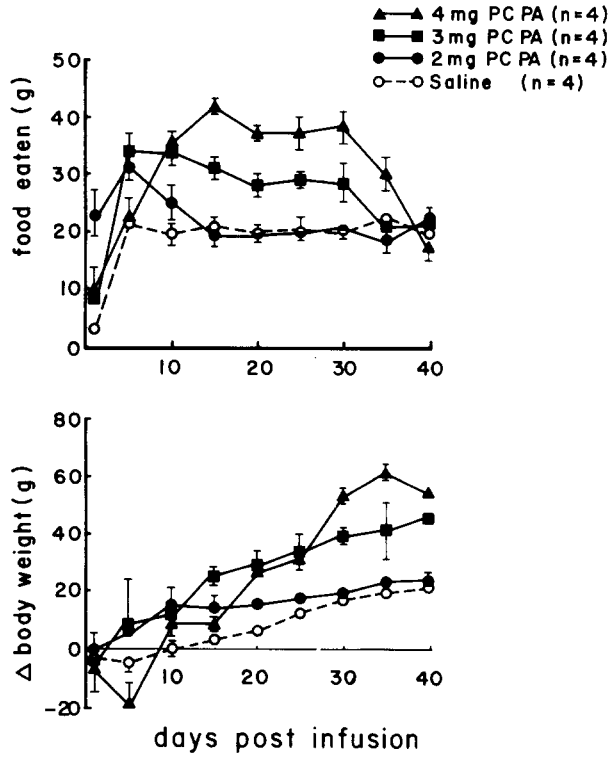

FIG. 1. Effects of 2, 3 or 4 mg of intraventricularly administered pCPA vs isotonic saline on 24-hr food intake (upper graph) and cumulative bodyweight gain (lower graph). Data points represent mean g ± standard error of mean g plotted every 5 days for a total of 40 days. See figure inset for group symbol definition and ns.

RESULTS AND DISCUSSION

Rats receiving saline vehicle infusion did not demonstrate altered food intake or BW gain as compared to untreated controls. Therefore, all comparisons of drug-treated groups were made with saline controls as described in the seminal work on pCPA-induced hyperphagia [6].

As shown in Fig. 1, the lowest dose of pCPA (2 mg) elicited a rapid onset of overeating ($ps<0.05$ compared to saline on Days 1 and 5). This effect subsided by Day 10. Higher doses of pCPA also elicited hyperphagia after an initial delay of several days. However, once overeating began,

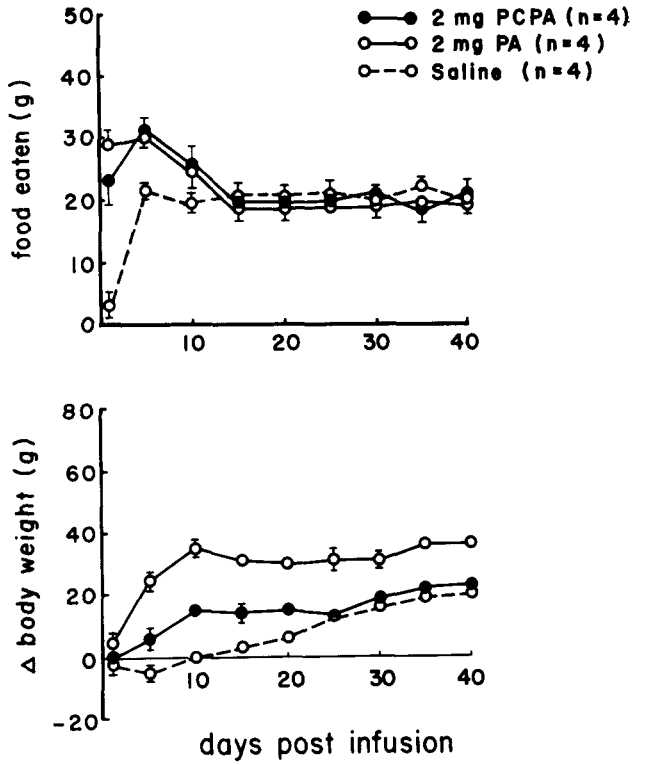

FIG. 2. Comparison of 2 mg pCPA (from Fig. 1) and 2 mg PA in comparison with isotonic saline (from Fig. 1) on food intake and body weight gain as described in Fig. 1.

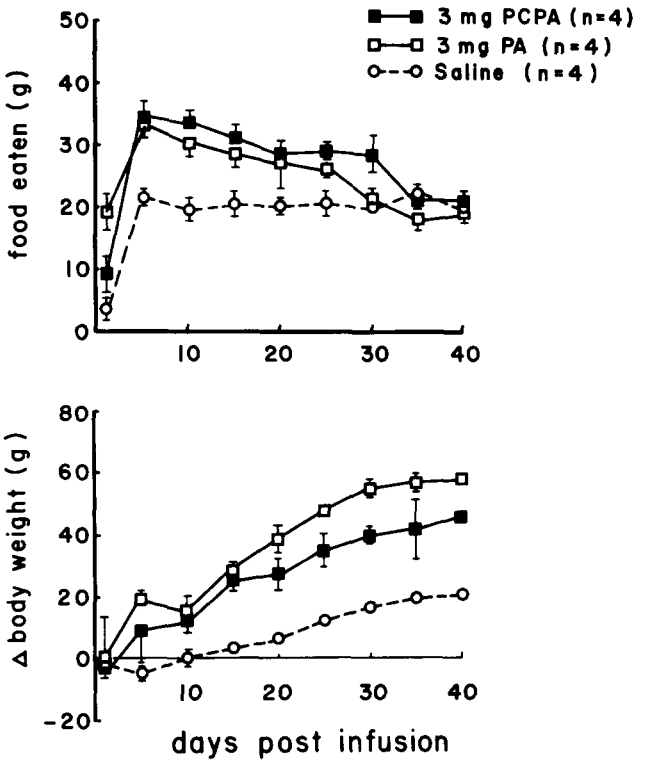

FIG. 3. Comparison of 3 mg pCPA (from Fig. 1) and 3 mg PA in comparison with isotonic saline (from Fig. 1) on food intake and body weight gain as described in Fig. 1.

its magnitude and duration was greater as dose increased ($ps<0.05$ compared to saline Days 5–30 for 3 mg and Days 5–35 for 4 mg; 4 mg effect>3 mg effect Days 15–35). The duration of hyperphagia observed here for 3 and 4 mg doses appears somewhat longer than that reported previously [6]. However, the peak magnitude of overeating per dose appears to agree almost perfectly. BW gain was also observed (Fig. 1) to increase as a function of pCPA dose (4 mg>3 mg>2 mg on Days 30–40). In general, then, we were able to replicate [6] the dose-response capacity of ventricular pCPA to induce hyperphagia and BW gain.

Of particular interest was the additional fact that control infusions of 2 mg (Fig. 2), 3 mg (Fig. 3), and 4 mg (Fig. 4)PA also induced overeating in a dose-response fashion. Indeed, with only a few exceptions (Day 1 for 3 mg dose; Days 1–10, 4 mg dose), there were no significant differences in either the magnitude or duration of hyperphagia produced by pCPA and PA ($ps>0.1$). On the other hand, despite the dose-response equivalence of overeating between these two drug conditions, BW gain appeared greater ($ps<0.05$) for all PA doses studied. The reason for this divergence is unknown, although higher activity levels (not measured here) in pCPA rats [6] may be accountable.

Terminal levels of forebrain 5HT, NE and DA (Table 1) did not vary significantly across all groups studied. This finding, particularly for 5HT, is not necessarily incompatible with the earlier work documenting pCPA-induced hyperphagia [6] inasmuch as brain samples were obtained after overeating had subsided. On the other hand, it is interesting to note that in the earlier work [6] brain 5HT could still be depleted up to 40% from control values after overeating had subsided.

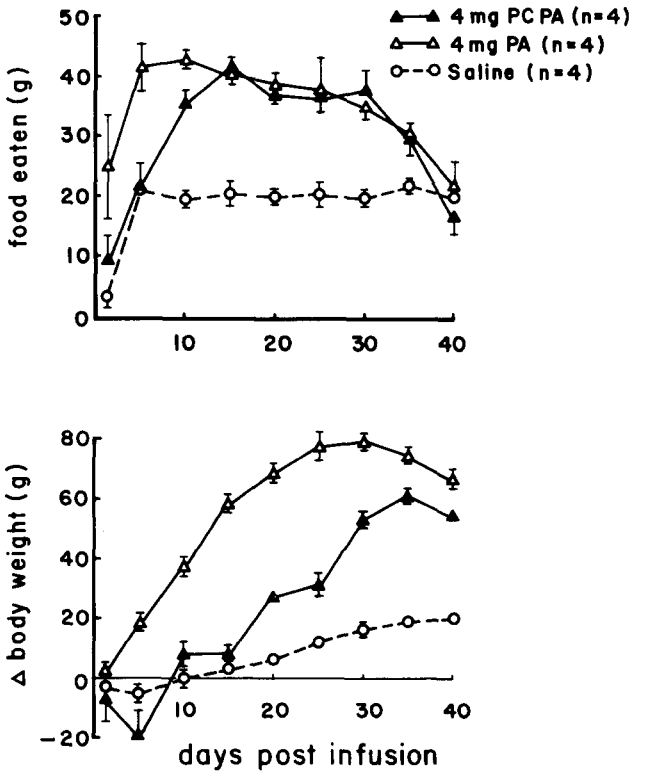

FIG. 4. Comparison of 4 mg pCPA (from Fig. 1) and 4 mg PA in comparison with isotonic saline (from Fig. 1) on food intake and body weight gain as described in Fig. 1.

TABLE 1

TERMINAL LEVELS* OF FOREBRAIN SEROTONIN (5HT), NOREPINEPHRINE (NE) AND DOPAMINE (DA) FOR RATS INJECTED INTRAVENTRICULARLY WITH pCPA, PA OR ISOTONIC SALINE

Group	N	5HT (ng/g)	NE (ng/g)	DA (ng/g)
pCPA				
(4 mg)	4	449 ± 15	312 ± 7	1227 ± 85
(3 mg)	3	475 ± 63	304 ± 9	1318 ± 69
(2 mg)	3	504 ± 40	291 ± 12	1131 ± 31
PA				
(4 mg)	3	414 ± 8	289 ± 0.7	1349 ± 13
(3 mg)	4	430 ± 47	254 ± 27	1199 ± 49
(2 mg)	4	462 ± 23	280 ± 17	998 ± 39
Saline	3	453 ± 10	316 ± 17	1199 ± 178

*All values are expressed as mean ± standard error of mean. None of the values for drug-treated rats vary significantly from saline controls.

In summary, the results of Experiment 1 confirm that ventricular injections of pCPA can indeed elicit overeating and BW gain in a dose-dependent manner. However, comparable injections of pCPA's parent amino acid, PA, produced equivalent dose-dependent hyperphagia and BW gain. Neither drug treatment appeared to produce long-term effects on forebrain levels of endogenous 5HT, NE or DA.

EXPERIMENT 2

Maximal hyperphagia induced by intraventricular pCPA has been associated with substantial (up to 78%) depletion of forebrain 5HT. The purpose of the second experiment was to confirm this observation and to simultaneously determine forebrain monoamine status at this time in other rats overeating after ventricular PA infusion.

METHOD

Animals

Ten rats of the same strain and sex as described in Experiment 1 were used. Animals were housed and maintained as described in Experiment 1.

Procedure

Animals were randomly assigned to one of three groups: rats receiving 3 mg pCPA (n=3), 3 mg PA (n=3), or saline vehicle (n=4). All drug forms and injection procedures were as described in Experiment 1. Rats were monitored for daily food intake and BW change until stable hyperphagia in both drug groups was observed. Rats were then sacrificed by decapitation, brains removed from the calvaria, dissected and assayed, all as described in Experiment 1.

RESULTS AND DISCUSSION

As observed in Experiment 1, 3 mg of PA intraventricularly elicited reliable ($p<0.01$) overeating compared to saline infusion in the first post-infusion day (see Table 2). pCPA tended to elicit overeating as well but the effect was more variable and not statistically reliable. By Day 3 post-infusion, both pCPA and PA infused rats were eating the same amount of food ($p>0.1$ between groups)

TABLE 2

DAILY FOOD INTAKES* OF RATS INJECTED INTRAVENTRICULARLY WITH 3 MG pCPA, 3 MG PA, OR ISOTONIC SALINE

		Days Postinjection		
Group	N	1	2	3
pCPA	3	9.5 ± 2.0	25.1 ± 5.8	34.5 ± 1.8‡
PA	3	19.5 ± 1.7‡§	29.5 ± 1.9†	33.5 ± 1.1‡
Saline	4	3.8 ± 1.1	18.8 ± 1.9	21.8 ± 1.0

*All values expressed as g mean ± standard error of mean.
†$p<0.05$ compared to saline
‡$p<0.01$ compared to saline
§$p<0.05$ compared to pCPA

TABLE 3

LEVELS* OF FOREBRAIN SEROTONIN (5HT), NOREPINEPHRINE (NE), AND DOPAMINE (DA) FROM RATS TERMINATED DURING HYPERPHAGIA ELICITED BY INTRAVENTRICULAR pCPA (3 mg) or PA (3 mg) AS COMPARED WITH ISOTONIC SALINE

Group	N	5HT (ng/g)	NE (ng/g)	DA (ng/g)
pCPA	3	402 ± 20	286 ± 36	1036 ± 75
PA	3	388 ± 13	273 ± 10	1024 ± 108
Saline	4	429 ± 29	311 ± 23	937 ± 32

*All values expressed as mean ± standard error of mean. None of the values for drug-treated rats vary significantly from saline controls.

which was reliably more ($ps<0.01$) than consumed by saline controls. In fact, the quantities of food consumed by all three groups Day 3 postinfusion were almost exactly the same as the peak response (Day 5) seen in comparable groups in Experiment 1 (see Fig. 3). Accordingly, rats were sacrificed at the end of this period for brain amine assessments. The results of these assays are shown in Table 3. Contrary to earlier work [6,21], we observed no evidence of 5HT (or NE or DA) depletion in either group of rats overeating at the time of sacrifice.

The results of this experiment show that overeating elicited by intraventricular pCPA or its parent amino acid, PA, can occur in the absence of brain 5HT depletion. These observations do not support earlier work [6,21] suggesting that central pCPA induces overeating by depleting brain 5HT.

EXPERIMENT 3

The results of Experiment 2 leave open the possibility that the overeating which occurs after central pCPA or PA infusions are due to pharmacological effects other than those reflected by depletion of brain 5HT (or NE or DA). In this respect, a most parsimonious consideration is that the large amounts of either drug required to elicit such dose-dependent feeding may have produced toxic effects on as yet unspecified brain elements. At least two types of toxicity could have occurred: (1) hyperosmotic stress, producing brain tissue dehydration, and/or (2) methanol-induced toxicity, produced by *in vivo* hydrolysis of the methylester portion of both pCPA and PA. Both possibilities were tested in Experiment 3.

METHOD

Animals

Twelve rats of the same strain and sex as described in Experiment 1 were used. Housing and maintenance conditions were as before.

Procedure

Rats were randomly assigned to one of three groups: those receiving 1.3×10^{-5} moles of urea (n=4), those receiving 2.8×10^{-6} moles of methanol (n=4), or those receiving saline vehicle (n=4). The quantity of urea (Fisher Scientific, Pittsburgh, PA) injected was calculated to be the molar osmotic equivalent of approximately 3 mg pCPA or PA (Experiments 1 and 2). The quantity of methanol (Baker Chemicals, Phillipsburg, NJ) injected was calculated to be the molar equivalent of potential methanol formed from the complete *in vivo* hydrolysis of the methylester-HCl component of approximately 3 mg of pCPA or PA conjugates as used in Experiments 1 and 2. Both drugs were dissolved in saline and infused in the same volumes as described in Experiment 1. Following all infusions, rats were returned to home cages for 19 days of daily food intake and BW measurements as described in Experiment 1.

RESULTS AND DISCUSSION

As shown in Fig. 5, neither urea nor methanol infusion modified the magnitude or time-course of feeding or BW gain as compared to saline infusion. Since these data were collected, we have additionally tested the possibility that the low pHs (≈3) of pCPA- or PA-methylester-HCl solutions might produce toxic effects associated with overeating. In agreement with previous work [6] which also tested this possibility, there was no evidence of hyperphagia following ventricular infusion of comparably acidified saline. Therefore, the mechanisms by which central pCPA or PA can induce this feeding response remains unknown.

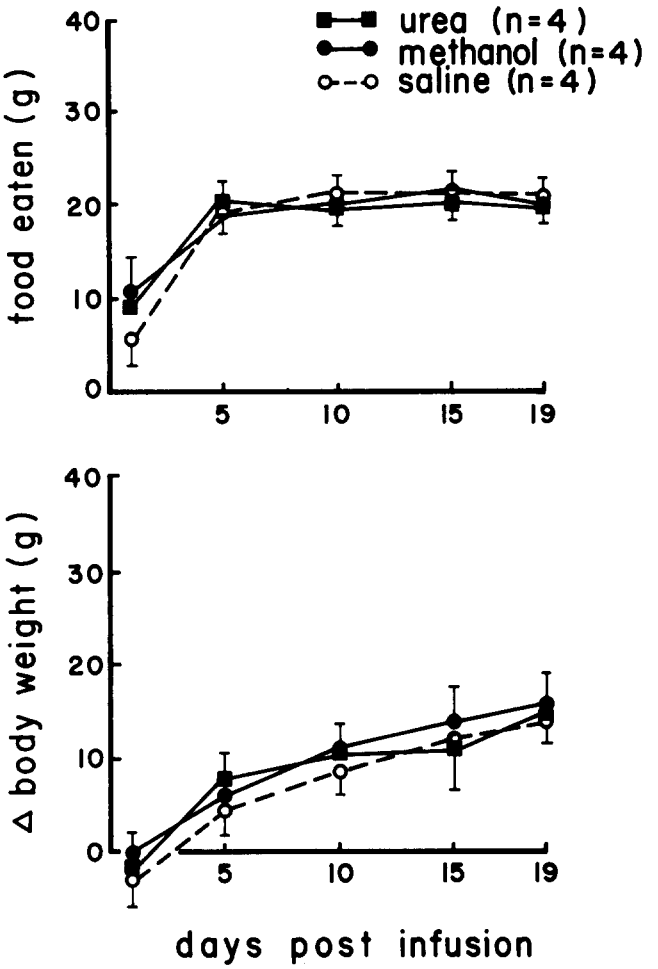

FIG. 5. Effects of intraventricularly administered urea, methanol and isotonic saline on 24-hr food intake (upper graph) and cumulative bodyweight gain (lower graph). Data points represent g parameters as described in Fig. 1 plotted every 5 days for a total of 19 days. See figure inset for group symbol definitions and ns. The quantity of urea injected was calculated to be the approximate molar osmotic equivalent of 3 mg pCPA or PA (see Fig. 3). The quantity of methanol injected was calculated to be the approximate molar equivalent of that potentially formed *in vivo* by complete hydrolysis of methylester-HCls of 3 mg pCPA or PA (see Fig. 3).

GENERAL DISCUSSION

It has been well-established that systemic administration of pCPA produces relatively long-term (7–10 days) suppression of both peripheral and brain 5HT [23,24]. While the exact mechanism of this inhibition is not totally clear, it appears to involve the suppression of tryptophan hydroxylase activity, the rate-limiting enzyme in 5HT production [23]. While PA can also suppress 5HT formation by competitive inhibition [23], the time-course of this effect would be expected to be much shorter than suppression of 5HT production by pCPA. However, both pCPA and PA here produced equivalent dose-response effects on the magnitude and time-course of overeating. This fact, along with our inability to detect any depletion of endogenous forebrain 5HT which might correspond to these treatments, shows that such drug-induced hyperphagia can occur in the absence of large changes in brain 5HT as previously reported [6,21]. As such, these data continue to question the general notion of a simple inhibitory role for brain 5HT in feeding (for reviews see [4, 7, 20, 26]).

The present work does not permit us to draw alternative conclusions as to which neural elements may be responsible for the observed hyperphagia. Indeed, it is still possible that 5HT neurons may be involved, either (a) in more restricted brain regions in which small changes in endogenous amine level could not be detected by entire forebrain analyses, or (b) more generally throughout brain, but in some metabolic mode which is not reflected by simple measurements of amine concentration (e.g., metabolite levels, turnover, post-synaptic receptor sensitivity changes). These alternatives remain to be tested.

Instead of or in addition to these possibilities is the chance that other neural elements in the brain are responsible. Most interesting in this respect is a recent report [32] showing that a number of amino acids infused directly into the medial hypothalamus (MH) can elicit chronic overeating and obesity in rats. Additional evidence was provided that several of the effective substances used (l-tryptophan, l-glycine, monosodium glutamate) produced discrete damage to cellular components of this brain region without apparently affecting fibers of passage. The possibility that some of the feeding effects observed here relate to such amino acid ef-

fects in MH [32] is supported by preliminary observations in our laboratory. Infusions of 3 mg monosodium glutamate (MSG) solution (n=4) into the lateral ventricles as described here produced a transient hyperphagia compared to saline infusion (n=4) of somewhat shorter duration (Days 4–15 post-infusion) but of equal magnitude (peak 24-hr eating of 32.6 g vs. 20.3 g for controls) as observed here (Experiments 1 and 2) for the same dose of either pCPA or PA. Indeed, this time-course and magnitude agrees almost perfectly with that reported previously for 3 mg pCPA [6]. Histological analysis of brains from such MSG treated as well as pCPA and PA treated rats here are currently underway to assess potential MH injury as reported previously [32]. Additional support for the possibility that the overeating phenomenon described here is due to amino acid-induced injury to susceptible MH cells derives from additional preliminary work. We have recorded slightly lower (–14%) amounts of protein in hypothalami collected from rats (n=4) overeating 5 days after infusion of 3 mg PA as described here. This decrement was simultaneously associated with lower levels (–14% each) of glutamic acid decarboxylase activity and choline acetyltransferase activity. These enzymes synthesize the formation of gamma-aminobutyric acid and acetylcholine, respectively. To the extent that they serve as markers for the integrity of short (intrinsic) hypothalamic neurons, this evidence is compatible with some general toxic effect of infusing amino acids into brain and altering MH function (producing overeating). The results of these and related studies will be reported in depth elsewhere.

In conclusion, we have several lines of evidence that the hyperphagia attendant upon central injections of pCPA are not necessarily related to brain 5HT depletion. These findings, in conjunction with earlier work demonstrating that depletion of brain 5HT is not a sufficient condition to elicit hyperphagia [5, 8, 9, 11, 14, 21, 25], continue to question the general notion of some simple inhibitory role for brain 5HT neurons in feeding behavior. Of practical consequence, our data imply that this research method has limited value in elaborating functional relationships between this brain monoamine and behavioral states.

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