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Delay of Cancer Anorexia Following Intraventricular Injection of Para-Chlorophenylalanine

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CHANCE, W. T., M. VON MEYENFELDT AND J. E. FISCHER. *Delay of cancer anorexia following the intraventricular injection para-Chlorophenylalanine*. PHARMAC. BIOCHEM. BEHAV. 17(5) 1043–1048, 1982.—Serotonergic mediation of cancer anorexia was investigated in immature female rats following the intraventricular injection of para-chlorophenylalanine (PCPA) or normal saline. Significant anorexia developed 6 days after the induction (IM) of Walker 256 carcinosarcomas in saline-treated rats. Although tumor-bearing rats treated with PCPA ate less than PCPA-injected controls by day 7, their feeding response was significantly greater than that of saline-treated tumor-bearing rats on days 5, 6 and 7. The PCPA treatment had no significant effect on food intake in nontumor-bearing rats. Biochemical analysis revealed significant elevations in plasma free tryptophan, brain tryptophan and brain 5-hydroxyindoleacetic acid in saline-treated tumor-bearing rats. Brain serotonin, 5-hydroxyindoleacetic acid and norepinephrine levels were decreased in PCPA-treated rats. Although these data may provide some support for a serotonergic mediation of cancer anorexia, additional mechanisms are clearly indicated.

Cancer anorexia Food intake Serotonin Tryptophan 5-Hydroxyindoleacetic acid
Para-chlorophenylalanine Norepinephrine Dopamine

ANOREXIA (inappropriately reduced food intake) and the ensuing depletion of host tissue (cachexia) are common features of cancer which often prohibit the aggressive use of therapy [32]. This decrease in food intake may begin when the tumor burden is small and progress to the point of death of the host [22,34]. It has been estimated that two thirds of the cancer fatalities are cachectic at death [24]. Although several variables such as physical obstruction of oral intake, malabsorption of nutrients, nausea and changes in taste thresholds undoubtedly influence caloric intake [9,11], the major abnormality in the anorectic cancer patient appears to be decreased desire for food in the presence of increased demand. Thus, the additional metabolic burden imposed on the host by a tumor is not met by increased appetite and intake. This paradoxical situation suggests that the anorexia induced by the presence of a tumor is central in origin. Hypotheses of the CNS responding to spurious satiety signals elicited by tumors have often been suggested [17, 25, 30, 31]. These postulates, however, have usually been unfocused derivatives of the chaotic metabolic changes initiated by the tumor and delivered to the brain by some unspecified humoral mechanism. If the presence of a tumor induces anorexia by falsely signalling brain mechanisms that a state of satiety exists, experimental investigation of this phenomenon should begin with those mechanisms thought to elicit satiety in normal nontumor-bearing organisms. Since the cancer anorexia-cachexia syndrome can be demonstrated in laboratory animals [20,22], the effect of a tumor burden on these putative satiety mechanisms may be investigated.

Considerable evidence has accumulated to implicate CNS serotonergic (5-HT) neurons as inhibitory in the control of hunger. Thus, direct injection of 5-HT into the rat lateral ventricle elicits satiety [15], as does systemic administration of the 5-HT precursor, tryptophan (Trp) [16]. The anorectic drugs fenfluramine [12] and fluoxetine [13], indirectly increase functional 5-HT activity by increasing 5-HT release or blocking its synaptic reuptake, respectively. Furthermore, systemic injection of the 5-HT receptor stimulant drug, quipazine, decreases food intake in rats [29]. Antagonism of CNS 5-HT systems has been observed to produce the opposite effect and elicit eating. The 5-HT receptor blockers, methysergide and cyproheptadine, abolish the anorectic effect of 5-HT agonists [7,12] as well as increase food intake in rats [2], cats [6] and humans [26]. Intraventricular (IVT) injection of the 5-HT synthesis inhibitor, parachlorophenylalanine (PCPA), has also been reported to elicit hyperphagia in rats [4].

Initial investigations from this laboratory have suggested that deranged 5-HT metabolism may be associated with cancer anorexia in rats [14]. Thus, rats bearing intramuscular Walker 256 (W 256) tumors exhibited decreased eating 6 days after tumor induction, which increased in severity until the death of the rats (day 10). Biochemical analysis of the brains of tumor-bearing (TB) rats indicated increased levels of Trp and of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA). The absence of these changes in rats that were pair-fed the same amount of food as the TB rats suggests that this increase in 5-HT activity was not merely a

result of malnutrition. In order to more fully investigate the role of brain 5-HT in cancer anorexia, in the present experiments we employed acute IVT injections of the inhibitor of Trp-hydroxylase, PCPA.

METHOD

Subjects

Fifty-nine immature (80–100 g) female Sprague-Dawley rats (Harlan Laboratories, Madison, WI) served as subjects in these experiments. These animals were individually-housed in a temperature and humidity controlled environment under a 12 hr light-dark cycle. Food (Purina rat chow pellets) and water were continuously available. Daily records of food intake, water intake and body weight were obtained to the nearest 0.1 g during the morning hours. Presented values of food consumption are corrected for any spillage and body weight differences of individual animals.

Induction of Tumors

The W 256 tumor was originally obtained from E. G. & G. Mason Research Institute (Worcester, MA). Tumor stock was maintained by harvesting ascites fluid that contained tumor cells 5 days after the IP injection of 1×10^6 viable (by trypan blue exclusion) tumor cells. Tumors were induced in experimental animals by injecting (IM) 5×10^4 (Experiment 1, n=15) to 1×10^5 (Experiment 2, n=15) viable tumor cells into the thigh muscle, with the remaining rats (n=29) receiving control injections (0.1 ml) of normal saline. Our past research has indicated the onset of significant anorexia to be 6 days after these doses of W 256 cells, with spontaneous death occurring by day 10.

Intraventricular Injection of PCPA

Since the systemic administration of PCPA would deplete peripheral stores of 5-HT and perhaps decrease eating by making the animals ill, we chose to acutely inject the drug into the lateral ventricles in a manner analogous to that reported by Breisch *et al.* [4]. To coincide the maximum effect of PCPA with the onset of anorexia, 2 mg of DL-PCPA methyl ester was bilaterally injected under ether anesthesia 2 days after the IM injection of W 256 cells or normal saline. The PCPA was purchased from Sigma Chemical company (St. Louis, MO). Additional groups of TB and control (NTB) rats were also injected (IVT) with an equal volume of normal saline (8 μl, bilaterally) at this time. Each injection was delivered over a 4 min period through a 31 ga hypodermic needle connected to a 50 μl syringe (Hamilton Co., Reno, NV). The hypodermic needle was stereotaxically-positioned at the following coordinates taken from bregma: A. −0.5, L. ±1.5 and V. −3.0 mm below the top of the skull [27]. Following the surgeries, all rats were returned to their home cages, with food and water intake being measured in grams across the next 7 (Experiment 1) or 4 (Experiment 2) days.

Biochemical Analyses

The rats in Experiment 1 were sacrificed by decapitation 9 days after the induction of tumors. In order to assess biochemical changes induced by PCPA at a time point more closely associated with the drug's behavioral effect, all rats in Experiment 2 were sacrificed 6 days after the induction of tumors. Immediately following sacrifice, the brains were removed, dissected into left and right hemispheres and frozen in liquid nitrogen. Blood was collected in heparinized beakers and centrifuged, with the resulting plasma being frozen prior to assay. Plasma free (unbound) and total Trp were assayed fluorometrically as previously described [3], with free Trp being determined in 50 μl of an ultrafiltrate prepared by centrifugation (100 × g, 25 min, 20°C) of 1 ml of plasma (pH=7.4) in a CF 50 Diaflo membrane cone (Amicon Corp., Lexington, MA). In one brain half 5-HT and 5-HIAA were determined fluorometrically [10] following acid-butanol extraction (10 volumes), with 5-HT and 5-HIAA being complexed with o-phthalaldehyde (Sigma Chemical Co.) and read on an Aminco-Bowman fluorometer. Brain Trp was extracted in the same fraction as 5-HT and assayed fluorometrically according to the procedures utilized for plasma Trp.

In addition to measuring indole activity, norepinephrine (NE) and dopamine (DA) were assayed in Experiment 2 in the remaining brain halves using high performance liquid chromatography with electrochemical detection [19]. Brain halves were homogenized in 5 volumes of 0.4 M $HClO_4$ solution, containing (per 100 ml) 50 mg EDTA, 100 mg $Na_2S_2O_5$ and 10 μg of alpha-methylNE (as an internal standard). After centrifugation (31,000 × g, 15 min, 4°C), shaking (10–15 min) with activated alumina (100 mg) and washing (3 times with 10 ml H_2O), each sample was eluted from the alumina with 1.0 ml of 0.1 N HCl solution (containing 10^{-5} M $Na_2S_2O_5$) and centrifuged (1,000 × g, 10 min). Supernatants (20 μl aliquots) were injected into the HPLC system (Beckman Model 110A pump, Altex reverse phase C-18 column, Palo Alto, CA; Bioanalytical Systems Inc., Model LC-4 electrochemical detector, W. LaFayette, IN), with NE eluting 4.5 min and DA 9.0 min after the injection.

Statistical Evaluation

All data were evaluated employing analysis of variance (ANOVA) techniques, with post hoc comparisons of individual means being made by *t*-tests.

RESULTS

Mean food intake in the first experiment is presented in Fig. 1. As may be observed, the onset of anorexia in TB rats following the IVT injection of PCPA was delayed. Statistical analysis employing a repeated measures ANOVA over days 9 through 13, indicated significant drug, $F(1,26)=11.53$, $p<0.01$, tumor, $F(1,26)=24.78$, $p<0.01$, and trials, $F(4,104)=52.26$, $p<0.01$, effects. The significant ($p<0.01$) interaction effects (trial × drug, trial × tumor and trial × drug × tumor), however, preclude a simple interpretation of the data. Post hoc *t*-tests showed no difference between IVT saline (Sal) injected NTB and PCPA NTB groups on any days. The Sal TB group, however, consumed significantly ($p<0.01$) less food than did the Sal NTB group on days 10, 11, 12 and 13. Although the PCPA TB group did not differ from the PCPA NTB group on days 9 and 10, significant ($p<0.05$) changes were observed on days 11, 12 and 13. In addition, the PCPA TB group ate significantly more on days 9 ($p<0.01$), 10 ($p<0.05$) and 11 ($p<0.05$) than did the Sal TB group. Therefore, it appears that significant anorexia may have been delayed for 2 days (days 10 and 11, as compared to TB controls) following the IVT injection of PCPA. Figure 2 presents the body weight data for the first experiment. A repeated measures ANOVA (days 9–13) indicated no significant effects of drug or tumor treatment. Although all groups lost weight immediately after the surgeries, the significant days effect, $F(4,104)=186.30$, $p<0.01$, suggests re-emergence of daily body weight gain. Mean water intake in

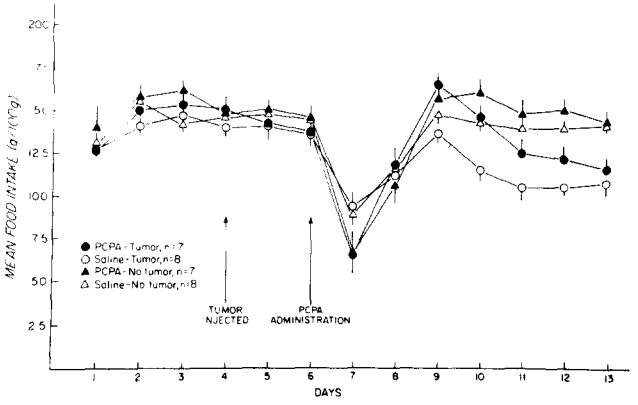

FIG. 1. Mean (±S.E.M.) food intake (g/100 g body weight) by rats following inoculation (IM) with 5×10^4 Walker 256 carcinosarcoma cells or 0.1 ml saline (day 4) and the acute bilateral injection (IVT, 8 μl) of para-chlorophenylalanine methyl ester (PCPA, 2 mg) or saline.

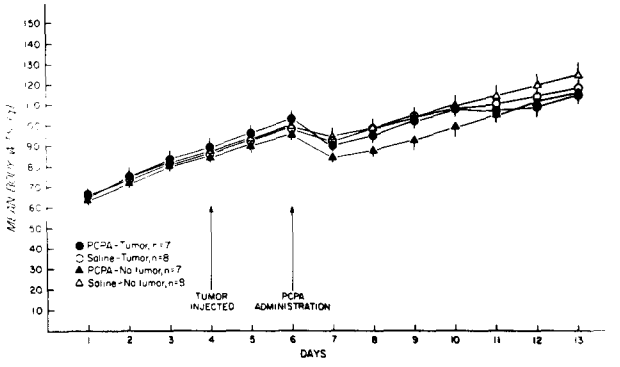

FIG. 2. Mean (±S.E.M.) body weight (g) changes by tumor-bearing and nontumor-bearing rats following the IVT injection of PCPA or saline.

TABLE 1

MEAN 5-HT, 5-HIAA AND TRYPTOPHAN LEVELS OF TUMOR-BEARING AND CONTROL RATS 7 DAYS AFTER THE IVT INJECTION OF PCPA OR SALINE

Treatment	N	Brain 5-HT (ng/g)	Brain 5-HIAA (ng/g)	Brain TRP (μg/g)	Plasma Total TRP (μg/ml)	Plasma Free TRP (μg/ml)
Sal-Sal	8	721 ± 20	521 ± 19	2.6 ± 0.1	15.6 ± 1.0	3.1 ± 0.3
PCPA-Sal	7	629 ± 27†	424 ± 36†	2.7 ± 0.1	13.1 ± 1.6	2.9 ± 0.2
Sal-Tumor	7	700 ± 14	617 ± 18*	3.3 ± 0.1*	9.7 ± 1.0*	4.6 ± 0.2*
PCPA-Tumor	7	655 ± 19	514 ± 25‡	3.0 ± 0.3	8.9 ± 1.3*	4.6 ± 0.4*

*Different from Sal-Sal $p<0.01$.
†Different from Sal-Sal $p<0.05$
‡Different from Sal-Tumor $p<0.01$.

this experiment is presented in Fig. 3. There were no significant overall effects of drug or tumor treatments on water intake. However, the drug × tumor interaction was significant, $F(1,26)=8.57$, $p<0.01$, primarily due to the increased drinking response of the PCPA TB group.

Analysis of the biochemical data for Experiment 1 (Table 1) indicated that although plasma total Trp was decreased, $F(1,25)=17.59$, $p<0.01$, two-way ANOVA), free (unbound) plasma Trp was elevated, $F(1,25)=28.58$, $p<0.01$, in TB rats. Plasma Trp was not affected by the PCPA treatment in either TB or NTB rats. Brain Trp was also elevated in TB rats, $F(1,25)=10.24$, $p<0.01$, as was brain 5-HIAA, $F(1,25)=13.08$, $p<0.01$. The PCPA treatment significantly reduced both brain 5-HT, $F(1,25)=11.49$, $p<0.01$, and 5-HIAA, $F(1,25)=15.21$, $p<0.01$, with post hoc t-tests revealing decreased 5-HT in NTB ($p<0.05$) and decreased 5-HIAA in NTB ($p<0.05$) and TB ($p<0.01$) groups.

In order to obtain biochemical data that more closely corresponded to the onset of the anti-anorectic effect of PCPA, the above experiment was replicated with the rats being sacrificed 4 days after the IVT injections. As may be observed in Fig. 4, the effect of PCPA on food intake was similar to the results of the previous experiment. Statistical

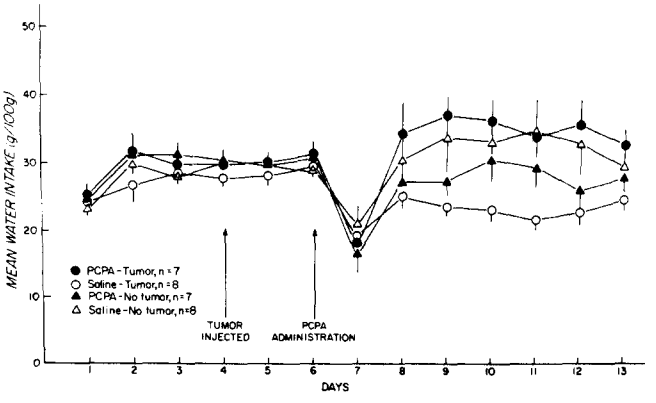

FIG. 3. Mean (±S.E.M.) water intake (g/100 g body weight) by tumor-bearing and nontumor-bearing rats following the IVT injection of PCPA or saline.

TABLE 2

MEAN BRAIN 5-HT, 5-HIAA, NE AND DA LEVELS OF TUMOR-BEARING AND CONTROL RATS 4 DAYS AFTER THE IVT INJECTION OF PCPA OR SALINE

Treatment	N	5-HT (ng/g)	5-HIAA (ng/g)	NE (mg/g)	DA (mg/g)
Sal-Sal	7	718 ± 21	535 ± 29	519 ± 12	1000 ± 23
PCPA-Sal	7	568 ± 47*	387 ± 42*	490 ± 11	987 ± 24
Sal-Tumor	7	699 ± 35	597 ± 24	510 ± 7	992 ± 29
PCPA-Tumor	8	597 ± 25*	498 ± 39	485 ± 11	1014 ± 34

*Different from Sal-Sal $p<0.01$.
†Different from Sal-Tumor $p<0.05$.

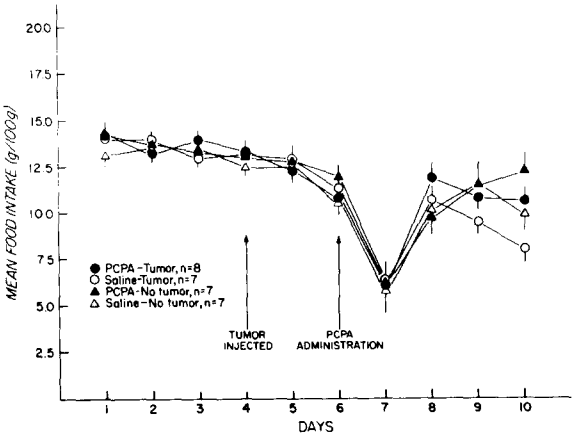

FIG. 4. Mean (±S.E.M.) food consumption (g/100 g body weight) by rats following tumor inoculation (1×10^5 Walker 256 cells) or control injections of saline (0.1 ml, IM) on day 4. PCPA (2 mg, 8 μl) or saline (8 μl) was acutely-injected (IVT) on day 6, with all rats being sacrificed for biochemical analysis on day 10.

analysis of eating across days 8 through 10 (repeated measures ANOVA) revealed a significant overall effect of PCPA, $F(1,25)=6.14$, $p<0.01$, and a significant trials × tumor interaction, $F(2,48)=6.54$, $p<0.01$. Post hoc t-tests indicated the Sal TB rats to be eating less than the NTB group by day 9 ($p<0.05$) and the PCPA TB group to be eating more than the Sal TB group on day 10 ($p<0.01$). Analysis of body weight and water intake data in the second experiment revealed no significant effects of drug or tumor treatments. However, there were significant days effects for both body weight gain, $F(2,50)=13.47$, $p<0.01$, and water intake, $F(2,50)=4.14$, $p<0.05$, with both of these parameters increasing across the days of the ANOVA.

Analysis of the biochemical data in Experiment 2 (Table 2) suggests that PCPA significantly reduced 5-HT, $F(1,25)=14.58$, $p<0.01$, as well as 5-HIAA, $F(1,25)=13.59$, $p<0.01$. Post hoc t-tests indicated decreased 5-HT and 5-HIAA in both PCPA NTB ($p<0.01$) and PCPA TB ($p<0.05$) groups. Although the tumor treatment did not elevate brain 5-HT, overall levels of 5-HIAA were significantly increased in tumor groups, $F(1,25)=6.89$, $p<0.01$. Analysis of brain catecholamine data (Table 2) indicated that the PCPA treatment significantly reduced NE, $F(1,25)=6.85$, $p<0.01$, while not affecting DA. The tumor treatment had no significant effect on brain catecholamines.

DISCUSSION

In agreement with previous results [14], the data provided by these experiments suggest an association of brain 5-HT with cancer anorexia. Thus, brain levels of the 5-HT precursor, Trp, and the metabolite, 5-HIAA, were significantly elevated in anorectic TB rats in the first experiment. Although levels of 5-HT itself were not changed, the increases in precursor and metabolite levels are suggestive of increased 5-HT turnover. This increase in CNS indole activity is apparently secondary to peripheral changes in circulating levels of Trp. Plasma total Trp was significantly decreased, while the unbound fraction was elevated in TB rats, allowing greater transport of the amino acid into the brain [28]. As previously suggested [14], this increase in plasma free Trp results primarily from a reduction in binding sites due to decreased levels of albumin and displacement of bound Trp by elevated plasma nonesterfied free fatty acids in TB rats. The absence of such changes in pair-fed control animals [14,33] suggests that elevated indole activity was not a result of the anorexia. In addition, we have observed [33] increased brain indole activity just prior to the onset of anorexia (day 5), indicating that the changes in CNS 5-HT metabolism actually precede decreased eating. Although levels of 5-HIAA were not significantly increased in the Sal TB rats in the second experiment on day 10 (day 6 post tumor induction), the trend was clearly in that direction.

Although involvement of brain 5-HT systems in cancer anorexia may also be suggested by the effectiveness of PCPA treatment in delaying the onset of anorexia, the transient nature of this delay suggests additional mechanisms. Thus, levels of 5-HIAA in the PCPA TB group in the first experiment were within normal limits even though these animals were severely anorectic. Therefore, either the effectiveness of PCPA in reversing anorexia is not specific to reduction of 5-HT activity or greater 5-HT changes in specific brain areas are being masked in the whole brain assays. Regional brain assay of indole activity within this model of cancer anorexia has demonstrated elevations of 5-HT in the diencephalon and 5-HIAA in the diencephalon, hippocampus, pons-medulla and cerebral cortex [33]. At present, we are uncertain whether these regional 5-HT and 5-HIAA changes have any functional significance for cancer anorexia.

An additional problem with these PCPA studies is that the

drug was administered acutely during a traumatic surgical procedure. Thus, various factors associated with recovery from the surgeries may have influenced the anorectic response to cancer. These factors include anesthetic effects, blood loss, weight loss, IVT osmotic and volume stresses, any of which could affect subsequent feeding and drinking by the subjects. The Sal TB rats in the second experiment may have become anorectic on day 5 (post tumor transplant) rather than on day 6 as usually observed because of such non-specific factors. Although these same factors may account for some of the variability in the drinking responses, PCPA has been reported to increase water intake [5]. The major change in water intake, however, appears to be in the PCPA TB rats, suggesting that this increase in drinking may at least initially be secondary to their increased feeding response.

Another problem associated with the acute injection of PCPA is the variability of 5-HT depletions. Although Breisch *et al.* [4] reported much greater depletion (75%) in their initial paper, subsequent research by this group [18] has resulted in 5-HT depletions more closely aligned with those in the present report. In this latter paper [18], hyperphagia was observed irrespective of the degree of 5-HT reduction or the specific amino acid methyl ester (PCPA, leucine, tryptophan) being injected into the ventricle. Thus, these data also argue against 5-HT mediation of PCPA-induced hyperphagia.

Although the PCPA treatment was slightly more effective in stimulating feeding in TB rats than in NTB rats, the role of 5-HT reduction in the reversal of cancer anorexia by PCPA reamins to be determined. Since levels of NE were also slightly but significantly reduced by the PCPA treatment in the second experiment, catecholamine mediation of the feeding effect cannot be dismissed. Alternative hypotheses may also be plausible, such as endorphin facilitation of feeding [21] secondary to the stress of the acute injection of high doses of PCPA.

Although these results may offer some support for a role of 5-HT in mediating cancer anorexia, additional research is clearly indicated. A number of alternative hypotheses have been presented to account for the effects of PCPA on feeding. The preponderance of evidence of 5-HT mediation of satiety combined with our reports of increased 5-HT activity in anorectic TB rats makes 5-HT mediation of cancer anorexia a very attractive hypothesis. Nevertheless, additional research employing less traumatic manipulation of 5-HT systems will be required for more complete assessment of this hypothesis.

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