Behavioral and neurochemical alterations evoked by p-Chlorophenylalanine application in rats examined in the light-dark crossing test

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Abstract. The aim of the present study is to examine the effects of serotonin synthesis inhibition with p-Chlorophenylalanine (p-CPA) in rats on (1) anxiety behavior examined in the light-dark crossing test and, (2) regional brain concentration of monoamines (NA, DA and 5-HT) and their metabolites (MHPG, DOPAC, HVA and 5-HIAA) as well as GABA in the hypothalamus, amygdala, hippocampus, midbrain central gray matter and the frontal cortex. Treatment of animals with p-CPA produced a significant increase in time out from the illuminated part of the chamber and in time of locomotor activity in the illuminated part of the chamber. HPLC analysis showed a significant reduction of 5-HT and 5-HIAA concentration in all examined brain regions with the exception of the frontal cortex. Additionally, a significant decrease in DA and its metabolites, DOPAC and HVA occurred in the hypothalamus and amygdala. Moreover, we observed a significant decrease in frontal cortex NA concentration after p-CPA administration. The results of our study suggest that administration of p-CPA is effective in reduction of anxiety through depletion of 5-HT accompanied by diminution of catecholamines, especially DA and its metabolites in the main emotional brain regions.

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Key words: light-dark crossing test, anxiety, p-CPA, brain monoamines, GABA, HPLC, rat

INTRODUCTION

Anxiety is an emotion which is as a state necessary perceived to avoid threat and ensure survival (Hendrie et al. 1996) but anxiety becomes pathological when it occurs without objective reasons, or when its intensity is excessive.

The neural substrate of anxiety has been searched for within the brains neuronal systems. Therefore, it was particularly appropriate to examine the neuronal mechanism of anxiety in animal behavior models (File 1990, Barrett 1991, Hendrie et al. 1996). The animal behavior models used in psychopharmacological studies to examine the mechanisms of anxiety were divided by Barrett (1991) into two types models, based on conditioned behavior and on unconditioned behavior.

Benzodiazepines were studied extensively in these models (Rodgers 1991, Treit 1991). Experiments from several laboratories revealed that a reduction of serotonin turnover was involved in the anxiety-reducing activity of benzodiazepines (for review, see Wise et al. 1972). Moreover, several studies showed that serotonin depletion after administration of p-Chlorophenylalanine (p-CPA) mimicked the anxiety-reducing effects of benzodiazepines (for review, see Wise et al. 1972, Johnston and File 1986, Graeff 1990, Handley et al. 1993). The effects of p-CPA on the mechanism of anxiety were studied in conditioned behavior models (for review, see Wise et al. 1972, Graeff 1990) as well as in unconditioned behavior models (for review, see Johnston and File 1986, Sanchez 1995). As a rule the administration of p-CPA produced anxiolytic effects in the most used animal behavior models (Johnston and File 1986, Gibson et al. 1994, Lopez-Rubelcava 1996). The effects of p-CPA on the brain serotonin system are produced by inhibition of the rate-limiting enzyme indoleamine biosynthetic pathway, i.e., tryptophan-5-hydroxylase (Green 1989).

Additionally, several studies showed that administration of p-CPA also affects noradrenergic and dopaminergic systems (Reader and Gauthier 1984, Reader et al. 1986, Romaniuk et al. 1989). Unfortunately, the results of these studies are contradictory. Reader et al. (1986) reported NA and DA reductions in neocortex in p-CPA-treated rats. Similar changes were observed in other brain regions, i.e., midbrain, hippocampus, pons, cerebellum and spinal cord (Reader and Gauthier 1984). On the other hand, the data from our laboratory demonstrated elevation of DA and NA concentration after

p-CPA administration in cats (Romaniuk et al. 1989). The level of NA was not affected after p-CPA administration in another study (Eide et al. 1988).

Much evidence suggests that the brain noradrenergic system participates especially in anxiety response (Stephens et al. 1986, Charney et al. 1990, Norman et al. 1990, Zagrodzka et al. 1994, Strzelczuk and Romaniuk 1995, Krotewicz and Romaniuk 1995, 1998). Several studies have reported that different kinds of stressors produce elevation of NA concentration in the emotional brain regions, especifically in the hypothalamus (Finlay et al. 1995, Krotewicz and Romaniuk 1995). Depletion of NA by administration of N-2-chloroethyl-N-ethyl-2-bromobenzylamine (DSP-4) results in the inhibition of negative emotions (Zagrodzka et al. 1994, Krotewicz and Romaniuk 1998). Furthermore, pharmacological studies have demonstrated that dopaminergic mechanisms also play an important role in emotional behavior (Alonso et al. 1994, Rodgers et al. 1996, Bartoszyk 1998).

The present study is aimed at: (1) getting further data on whether anxiety reduction depends only on blocking 5-HT synthesis, which results in decreasing the functional activity of serotonergic system, or whether it results from the interaction of 5-HT with catecholaminergic and GABA-ergic systems, and (2) demonstrating how the lowering of brain serotonin synthesis affects these interactions in the key "emotional" structures of the brain, i.e., hypothalamus, amygdala, hippocampus and midbrain central gray matter.

METHODS

Subjects

The experiments were performed on twenty male outbred Wistar rats weighing 280 - 320 g, housed in groups, three per cage. Animals were maintained on a 12 L : 12 D cycle (light on at 0800) in the temperature controlled room ($22 \pm 2°C$) with food and water available continuously. The rats were purchased from the licensed animal breeding of the Institute of Occupational Medicine in Łódź.

Drugs

p-CPA ((±)-p-Chlorophenylalanine, Sigma) was dissolved immediately before use in physiological saline with 3-5 drops of arabic gum. The rats (Group 2) were

given IP p-CPA for a total 450 mg/kg, according to the schedule: 150 mg/kg/12 h in a volume of 2 ml/kg, the last dose 36 h before the experiment. A control group (Group 1) was treated with the solvent in the same way in a volume of 2 ml/kg.

Behavioral procedure

LIGHT-DARK CROSSING TEST (LDC TEST)

The Light-Dark Crossing Test is a modified version of the Light-Dark Transition Test described by Crawley (1981). The experimental chamber (80 x 40 x 40 cm) was divided into two parts (40 x 40 x 40 cm each) separated in the middle by a black wall in which there was a hole (8 cm in diameter) through which the rat could freely pass from one part to the other. Each part of the chamber could be illuminated alternately by two halogen bulbs (jointly 2,000 lx) placed on the end wall of each part of the chamber. 50 cm above the floor. In this manner it was a convertible shuttle box where the illuminated part was aversive (stressful) and a dark was safe (non stressful).

Each animal was individually habituated to the experimantal conditions in two consecutive sessions. In such session rats were introduced into the experimental chamber for 2 min in the absence of any external stimuli. Then, the basic experiment was performed. All rats were tested 3 times: first - before any treatment, second - 36 h after vehicle IP injection, and third - 36 h after p-CPA IP injection. One experimental session consisted of 5 trials. The time duration of the aversive light stimulus was 60 s, and intervals between trials were irregular from 60 to 120 s. The following behavior variables were measured: time out of illuminated part, i.e., time elapsing from the moment of switching on the aversive light stimulus to the moment of the rats passing to the dark part of the chamber; the time of the rat was motionless in the illuminated part, and time of locomotor activity in the illuminated part.

Recording system

Each session was video-recorded and video-computer analysis of all behavioral events was conducted by the Etho-Vision program, Version 1.90 (Noldus Information Technology, Wageningen). The registering equipment and experimenter were in a separate room adjoining the test-chamber room.

The behavioral sessions took place between 0900 and 1200 h.

Biochemical analysis

The concentrations of NA, DA, 5-HT, MHPG, DOPAC, HVA, 5-HIAA and GABA were determinated in the selected brain regions using high-performance liquid chromatography with electrochemical detection (HPLC-ED).

Sample preparation

Four hours after the last LDC Test all rats were killed by decapitation, their brains quickly removed and kept frozen at -70°C. Next day selected brain regions, i.e., hypothalamus (HPT), midbrain central gray matter (MID), amygdala (AMY), hippocampus (HIP) and the frontal cortex (CTX) were dissected according to the stereotaxic atlas of Paxinos and Watson (1982), placed into Eppendorf tubes and weighed. Afterwards brain tissue was homogenized with an ultrasonic cell disruptor (Vibracell 72434, Bioblock, Illkrich-Cedex) in 150 µl 0.1 M perchloric acid containing 0.4 mM sodium metabisulphite. The homogenates were then centrifuged at 10,000 xg for 25 min at 4°C and the supernatants were filtered through a 0.22 µm filter (Sigma) and then 5 µl of filtrates were injected into the HPLC system.

Chromatographic and detection conditions

The HPLC system consisted of a quaternary gradient delivery pump Model HP 1050 (Hewlett-Packard), a sample injector Model 7125 (Rheodyne, Berkeley), and an analytical column ODS 2 C18, 4.6 x 250 mm, particle size 5 µm (Hewlett-Packard) protected by guard column Lichnospher 100 RP-18, 4 x 4 mm), particle size 5 µm (Hewlett-Packard). The electrochemical detector model HP 1049 A (Hewlett-Packard) with glassy carbon working electrode was used at a voltage setting of +0.65 V for monoamines and their metabolites, and +0.50 V for GABA, vs. an Ag/AgCl reference electrode. The detector response was plotted and measured using a chromatointegrator (Esoft, Łódź). The concentrations of monoamines and their related metabolites in each sample were calculated from the integrated chromatographic peak area and expressed as ng/g wet tissue. GABA concentrations were calculated in the same way as the monoamines, but expressed in µg/g wet tissue.

Monoamines and their metabolites determination

The mobile phase comprised a 0.15 M sodium dihydrogen phosphate, 0.1 mM EDTA, 0.5 mM sodium octanesulphonic acid, 10 - 12 % methanol (v/v) and 5 mM lithium chloride. The mobile phase was adjusted to pH 3.4 with phosphoric acid, filtered through 0.22 μm filter (Sigma) and degassed with helium. A column temperature of 32°C and a flow rate of 1.4 ml/min were used.

GABA determination

The mobile phase for GABA determination was 0.1 mM sodium acetate buffer with 0.1 mM EDTA and 5 mM lithium chloride in 25 % (v/v) methanol. The mobile phase was adjusted to pH 5.5 with acetic acid, filtered through 0.22 μm filter (Sigma) and degassed with helium. The examined amino acid was eluted with the linear methanol gradient from 25% to 75% in 15 min, 75% in next 2 min and from 75% to 25% in following 6 min (a modified method of Arias et al. 1992). Just before the injection into the HPLC system, GABA was derivatized with o-phtalaldehyde-thiol (OPT-thiol) reagent for 2 min

(Joseph and Marsden 1986). A column temperature of 34°C and a flow rate of 1.3 ml/min were used.

Chemicals

Methanol was purchased from Serva (Heidelberg). Other chemicals for HPLC were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistics

The behavioral results were analyzed by the Friedman test followed by the Wilcoxon test for paired data, and biochemical data by a two-way ANOVA followed by the least significant difference test (LSD test).

RESULTS

Behavioral data

The Friedman test indicated a significant differences for time of locomotor activity in the illuminated part of chamber ($\chi^2_2 = 6.200$, $P<0.04$) and for time out of the
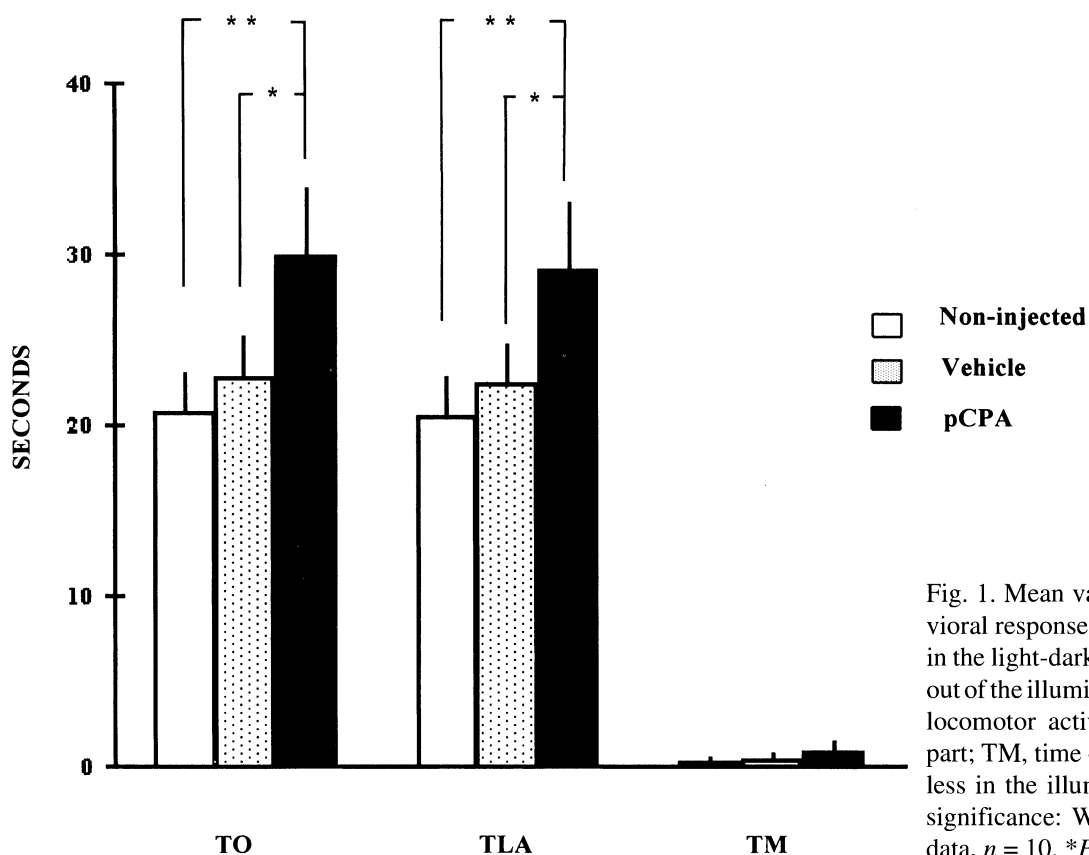

Fig. 1. Mean values (± SEM) of behavioral responses recorded in rats tested in the light-dark crossing test. TO, time out of the illuminated part; TLA, time of locomotor activity in the illuminated part; TM, time of the rat being motionless in the illuminated part. Statistical significance: Wilcoxon test for paired data, $n = 10$, *$P <0.05$, **$P <0.01$.

TABLE I

Regional brain concentrations of monoamines , their metabolites and GABA in p-CPA -treated rats

Group	Brain region	Monoamines and metabolites in ng/g and GABA in µg/g wet tissue							
		NA	DA	5-HT	MHPG	DOPAC	HVA	5-HIAA	GABA
1. Control	HPT	1302.8 ± 177.5	1236.4 ± 286.5	773.3 ± 118.0	40.0 ± 5.9	897.4 ± 177.9	141.5 ± 28.5	536.0 ± 60.9	8.8 ± 1.0
2. p-CPA		1464.5 ± 203.0	883.4 ± 330.4	168.0 ± 34.7	69.8 ± 16.8	428.7 ± 147.1	69.8 ± 23.8	108.5 ± 30.8	9.4 ± 1.2
		NS	$P<0.02$	$P<0.001$	NS	$P<0.005$	$P<0.01$	$P<0.001$	NS
1. Control	MID	891.8 ± 72.3	120.1 ± 14.0	892.2 ± 65.6	45.0 ± 5.1	116.4 ± 16.9	15.5 ± 3.0	814.0 ± 69.9	6.7 ± 1.6
2. p-CPA		650.7 ± 121.8	185.6 ± 43.9	135.7 ± 40.6	48.5 ± 8.9	85.6 ± 23.9	12.3 ± 4.9	134.0 ± 34.0	7.2 ± 1.0
		NS	NS	$P<0.001$	NS	NS	NS	$P<0.001$	NS
1. Control	AMY	720.2 ± 46.2	1629.2 ± 223.4	1053.6 ± 95.1	67.3 ± 16.2	1052.9 ± 135.8	218.7 ± 25.9	616.0 ± 48.4	6.6 ± 0.8
2. p-CPA		759.6 ± 218.1	734.9 ± 290.1	137.0 ± 39.5	119.6 ± 41.9	519.3 ± 119.9	92.0 ± 23.9	164.5 ± 57.1	8.1 ± 1.4
		NS	$P<0.001$	$P<0.001$	NS	$P<0.02$	$P<0.03$	$P<0.001$	NS
1. Control	HIP	540.4 ± 41.4	1551.4 ± 289.4	1059.5 ± 87.8	35.3 ± 3.5	745.2 ± 137.3	188.7 ± 36.6	543.2 ± 38.0	5.0 ± 0.3
2. p-CPA		413.3 ± 93.0	823.7 ± 314.1	169.0 ± 53.2	68.0 ± 25.8	360.4 ± 119.2	155.6 ± 62.7	123.8 ± 33,5	6.9 ± 0.7
		NS	NS	$P<0.001$	NS	$P<0.03$	NS	$P<0.001$	NS
1. Control	CTX	601.0 ± 36.3	162.4 ± 34.0	524.1 ± 65.0	73.6 ± 14.5	157.5 ± 43.2	47.0 ± 10.3	428.6 ± 47.1	6.4 ± 1.3
2. p-CPA		286.4 ± 67.7	87.0 ± 30.3	318.4 ± 69.1	54.4 ± 6.0	74.0 ± 14.0	40.2 ± 9.9	314.5 ± 44.7	6.9 ± 0.7
		$P<0.001$	NS	NS	NS	NS	NS	NS	NS

Values are mean ± SEM. n = 10 for each group; statistical significance: LSD test.

illuminated part of chamber ($\chi^2_2 = 6.200$, $P<0.04$). No significant change was observed in the time of motionless behavior in the illuminated part of the chamber. The non-parametric Wilcoxon test for paired data showed that 36 h following IP p-CPA injection a significant 44.4 % increase ($P<0.01$) in the time out of the illuminated part of chamber occurred as compared to non-injected rats, and a 31.1% increase ($P<0.05$) as compared to vehicle-injected rats. Also a significant 41.9% increase ($P<0.01$) in the time of locomotor activity in the illuminated part of the chamber occurred as compared to non-injected rats, and a 29.9 % increase ($P<0.05$) as compared to vehicle-injected rats (Fig 1).

Biochemical data

Regional brain concentrations of NA, DA, 5-HT, MHPG, DOPAC, HVA, 5-HIAA and GABA in Group 1 (control) and Group 2 (p-CPA-treated) are presented in Table I.

ANOVA demonstrated statistically significant differences between the groups in the content: of NA ($F_{1,90}$ = 11.885, $P<0.001$), of DA ($F_{1,90}$ = 15.990, $P<0.001$), of DOPAC ($F_{1,90}$ = 19.182, $P<0.001$), of HVA ($F_{1,90}$ = 11.609, $P<0.001$), of 5-HT ($F_{1,90}$ = 125.603, $P<0.001$) and of 5-HIAA ($F_{1,90}$ = 153.589, $P<0.001$). Some further analysis by means of LSD test showed that the level of NA was lower only in CTX ($P<0.001$) in Group 2 vs. Group 1, the DA level was lower in HPT ($P<0.02$) and in AMY ($P<0.001$) in Group 2 vs. Group 1, the level of DOPAC was lower in HPT ($P<0.005$), in AMY ($P<0.02$) and in HIP ($P<0.03$) in Group2 vs. Group 1, the level of HVA was lower in HPT ($P<0.01$) and in AMY ($P<0.03$) in Group 2 vs. Group 1, the level of 5-HT was lower in HPT ($P<0.001$), in MID ($P<0.001$), in AMY ($P<0.001$) and in HIP ($P<0.001$) in Group 2 vs. Group 1, and also the level of 5-HIAA was lower in HPT ($P<0.001$), in MID ($P<0.001$), in AMY ($P<0.001$) and in HIP ($P<0.001$) in Group 2 vs. Group 1. No significant differences occurred between the groups in the contents of MHPG and GABA.

DISCUSSION

The results of our study demonstrate that administration of p-CPA not only decreases 5-HT and 5-HIAA concentration in every emotional brain region examined, i.e., in the HPT, MID, AMY and HIP but also alters the metabolism of dopamine in rats. The neurochemical changes are accompanied by a significant increase in the time out of the illuminated part of chamber and in the time of locomotor activity in the illuminated compartment of the chamber measured in the Light-Dark Crossing Test.

Rodents show natural aversion to illuminated fields. The increase in the time to leave the illuminated part of chamber and the increase in the time of locomotor activity in the illuminated part of chamber indicated reduction of anxiety in rats after p-CPA treatment.

The anxiolytic effect of p-CPA treatment is in agreement with the data obtained in other studies (Crawley 1981, Lopez-Rubelcava 1996). Moreover, the anxiolytic effects of p-CPA were revealed in the most used animal behavior models (Johnston and File 1986, Gibson et al. 1994, Lopez-Rubelcava 1996).

The depletion of 5-HT in the brain of rats following p-CPA administration obtained in our study is also in agreement with the results of a number of other studies (Reader and Gauthier 1984, Ishikava et al. 1986, Reader et al. 1986, Eide et al. 1988, Romaniuk et al. 1989, Lopez-Rubelcava 1996).

On the other hand, changes in 5-HT and 5-HIAA concentration in p-CPA-treated rats were accompanied by the depletion of DA and its metabolites, DOPAC and HVA in the hypothalamus and amygdala. Moreover, we observed a significant decrease in frontal cortex NA concentration after p-CPA administration. These results agree with data obtained in other studies (Reader and Gauthier 1984, Reader et al. 1986). The authors of these studies suggested that a decrease in endogenous 5-HT after p-CPA administration resulted in the loss of negative feedback control between monoamine systems (Reader et al. 1986). It is well known that serotonin and serotonergic agents modulate a release of other neurotransmitters (Feuerstein and Hertting 1986, Decker and McGaugh 1991, Chojnacka-Wójcik 1995). Additionally, it is well established that the locus coeruleus, the main noradrenergic cell group, receives serotonergic projections from the nucleus raphes dorsalis, nucleus centralis posterior and nucleus raphes pontis (for review, see Nieuwenhuys 1985). Moreover, depletion of 5-HT induced by p-CPA or 5,6-dihydroxytryptamine a neurotoxin destroying serotonergic neurons, produced an elevation of tyrosine hydroxylase activity as well as its amount and even mRNA coding for tyrosine hydroxylase in the locus coeruleus (Crespi et al. 1980, Sturtz et al. 1994). As concerns DA, the serotonergic fibers originating from the nucleus raphes dorsalis project to the

substantia nigra, the principal dopaminergic center (Nieuwenhuys 1985). Furthermore, the medial part of the substantia nigra gives rise to axons that pass to the amygdaloid complex (Nieuwenhuys 1985). Our experiment also demonstrated that depletion of 5-HT was accompanied by the depletion of DA and its metabolites in several brain regions, especially in the amygdala. The amygdala is regarded by Graeff et al. (1993) as playing an important role in the neural regulation of fear and defense. In their dual hypothesis Deakin and Graeff (1991) suggested that different 5-HT pathways and receptor subtypes mediate different types of anxiety. According to this view the function of amygdala would be to synthesise the various stimulus inputs from the environment and then signal to the periaqueductal gray matter the degree of threat to the organism. Our results indicating that the neurochemical changes after p-CPA administration were placed in the amygdala may suggested a more complex model of 5-HT regulation of anxiety through an involvement of the DA system.

In summary, the present neurochemical data confirm that p-CPA treatment does not exert an uniform action on monoamine systems of brain (Steinman et al. 1987, Romaniuk et al. 1989). The administration of p-CPA is effective in the reduction of anxiety through depletion of serotonin accompanied by diminution of catecholamines, especially DA and its metabolites, in the main emotional regions of the brain. The effects of p-CPA on catecholamine metabolism may be indirect via interactions among monoamine systems (Reader et al. 1986, Romaniuk et al. 1989). Indeed, the results from several other studies showed that noradrenergic and dopaminergic systems were involved in the control of anxiety (Dorow and Duka 1986, Alonso et al. 1994, Finlay et al. 1995, Rodgers et al. 1996). Furthermore, the effects of p-CPA may be direct via inhibition of transport of amino acids into the brain, e.g., tyrosine and thereby a depletion of brain levels of catecholamines (Green 1989). As has been mentioned, there is much evidence in the literature that catecholamines also play an important role in the mechanism of anxiety, and interact with the 5-HT system in the modulation of anxiety.

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