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Toxic effects of putrescine in rat brain: Polyamines can be involved in the action of excitotoxins

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Summary. After treatment with putrescine (PUT) 200 mg/kg, i.p., male rats displayed a behavioural pattern that included wet dog shakes and motor incoordination. The concentration of PUT in the brain paralleled the severity of clinical signs. Histological examination showed the presence of perivascular edema and moderate spongiosis. These biochemical and histological features were present 2 h after treatment. At 24 h PUT levels in frontal cortex decreased but the histological status of brain tissue remained. Pretreatment with hyperosmolal glycerol did not modify the effect of PUT on the brain content of polyamine or the histological condition at 2 h. These results support a neurotoxic role for putrescine. Such effects were similar to those of kainic acid at convulsant doses, suggesting a role for putrescine in the action of this excitotoxin.

Keywords: Amino acids – Polyamines – Rat brain – Edema – Behavior

Introduction

Many evidences support the involvement of excitatory amino acids (EAA) in degenerative or post-injury processes, Meldrum (1990). The involvement of polyamines in the regulation of the N-methyl-D-aspartate (NMDA) receptor has been recently proven, for a review see Williams (1991). The relationship between EAA and polyamines has stimulated the study of its role in the neurological damage, opening new pharmacological perspectives.

Putrescine (PUT) is a diamine metabolically related to ornithine in animals. PUT is the first product in the synthetic pathway of polyamines (Tabor, 1984). Recent literature shows a growing interest in these substances. Different roles have been proposed for polyamines in relation to cell differentiation and proliferation (Seiler, 1990), as well as in studies of the response of nervous tissue to different kind of injuries (Dienel, 1984; Paschen, 1988; Martínez, 1991; de Vera, 1991).

Polyamines have also been involved, as protective agents, in the nervous cell response after damage (Gilad, 1983; Gilad, 1991). From a molecular point of view, polyamines are ionic products with a key role in the correct function of nucleic acids (Tabor, 1984; Seiler, 1990). They also play an important role in the synaptic events mediated by excitatory aminoacids (Ramson, 1988; Koenig, 1990; Williams, 1991; McGurk, 1990). Work from this (Martínez, 1991; de Vera, 1991), and other laboratories (Reed, 1990) support a role for polyamines -and specially PUT- in the mechanism of neurotoxicity of excitotoxins. Several studies have shown an increased PUT concentration in the brain, in parallel with tissue injury, produced after ischemia (Paschen, 1988), or by kainic acid (de Vera, 1991). However, whether putrescine is a mediator of cell damage or just a biochemical byproduct is not known. Therefore, the main objective of this work is the characterization of the neurotoxic action of PUT. The action of this polyamine is of special interest given the increase of brain PUT found in post-convulsant kainic acid-treated animals (de Vera, 1991). Given the limited transport of polyamines across the blood-brain barrier (Shin, 1985), the pretreatment with glycerol was also evaluated.

Material and methods

Chemicals

Putrescine (PUT), 1, 6-diaminohexane (1, 6-DAH) (free base), and dansyl chloride were obtained from Sigma (St. Louis, Mo). Glycerol was obtained from Carlo Erba (Spain). Other reagents and solvents-HPLC grade- were purchased from different commercial sources.

Animals

Fifty male IFFA-CREDO rats weighing 380–450 g were used. Animals were received from the supplier and maintained for 7 days in the animal room under light- and temperature-controlled conditions. All rats were randomly assigned to the different treatment groups. Rats had free access to standard chow pellets and water before and during the experiments.

Treatments

Rats received saline (i.p.) or PUT (200 mg/kg, i.p.) 30 min after saline (5 ml/kg, per os) or glycerol (90%, 5 ml/kg, per os) according to the following groups: **G/P** Glycerol + Putrescine ($N = 14$), **S/P** Saline + Putrescine ($N = 15$), **G/S** Glycerol + Saline ($N = 11$). Also, a group of rats was administered with saline (i.p.) **S** ($N = 10$). Volume injected was 1 ml/kg.

Rats were observed during 2 h after PUT for behavioural evaluation, and sacrificed by decapitation at the end of this period or 24 h later. The number of animals included in each time group (2 and 24 h) is shown in Table 1. The behavioural examination during 2 h after treatment was carried out in animals belonging to both time groups.

Brains were removed from the skull and the frontal cortex was dissected from one hemisphere for the analysis of polyamines. The remaining brain was processed for histological studies.

HPLC method

The samples of frontal cortex were homogeneized with 0.4 mol/l cold perchloric acid. The homogenates were centrifuged (10,000 × g, 15 min) and the supernatants were extracted with benzene after dansylation (Desiderio, 1987). 1, 6-DAH was used as internal standard.

Table 1. Rat cortical polyamine levels after 200 mg/kg of putrescine i.p. 2 and 24 h after injection

	Group	N	Putrescine	Spermidine	Spermine
	S	10	0.501 ± 0.066	47.65 ± 6.45	59.97 ± 8.38
2h	G/P	7	2.89 ± 3.83	38.34 ± 3.53[a]	48.84 ± 3.46[a]
	S/P	8	4.99 ± 3.55[a,b]	38.18 ± 6.15[a]	51.61 ± 3.44[a]
	G/S	4	0.419 ± 0.715	35.73 ± 1.31[a]	50.14 ± 3.52[a]
24 h	G/P	6	2.07 ± 1.36[a,c,d]	45.23 ± 6.19	40.92 ± 12.5[a,d]
	S/P	6	1.22 ± 0.340[a,c]	51.59 ± 5.33	60.67 ± 3.72
	G/S	7	0.429 ± 0.054	43.70 ± 7.69	50.56 ± 13.4

P putrescine, *G* glycerol (90%, 5 ml/kg) pretreatment 30 min before P, *S* saline. Results in μg/g, (mean ± S.D.). *N* number of animals
ANOVA Duncan test $p < 0.05$
[a] vs S; [b] vs G/S 2h; [c] vs G/S 24 h; [d] vs S/P 24 h.

A modification of the HPLC method of Desiderio (1987) was used (Martínez, 1991). Briefly, a Spherisorb ODS-1 column was employed. The elution was performed with a gradient consisting of solvent A (1.2 mM Na_2 HPO_4 and 12 mM NaCl) and solvent B (methanol). The flow rate was 1.2 ml/min. Fluorescence detection of dansylated derivatives was performed at 350 nm and 420 nm for excitation and emission respectively.

Histology

Brains selected for histological studies were fixed in Bouin solution for 48 h and gradually dehydrated in alcohol. Then, they were included in paraffin. Slices 5 μm thick were dyed with hematoxylin-eosine according to conventional procedures. Optical microscopic observation was performed on sections corresponding to frontal cortex and hippocampal regions. Histological examination was carried out by a researcher blind to the treatment and the biochemical results.

Results

Behavioural assessment

83% of animals administered with PUT exhibited behavioural alterations beginning 10 min after injection. Most intense changes were observed within the first two hours. The main signs observed were wet dog shakes (WDS), flat body posture, hindlimbs extension and motor incoordination. Two rats that exhibited the most intense alteration, including cyanosis, died 3 and 23 h post-treatment. The clinical status was scored according to the following scale: 0 = no alterations, 1 = WDS and/or slight signs, 2 = WDS and clear postural and coordination deficits that recovered within 2 h, and 3 = WDS and clear postural and coordination deficits not recovering within 2 h.

The number of WDS exhibited by PUT-treated animals was in the range 1 to 56 (mean value; s.d.: 12.5; 17.6). No differences were observed in the number of WDS between the different experimental groups considered (ANOVA, treatment factor, n.s.) Results of the clinical evaluation after PUT treatment are shown in Fig. 1. In spite of some apparent differences in the groups

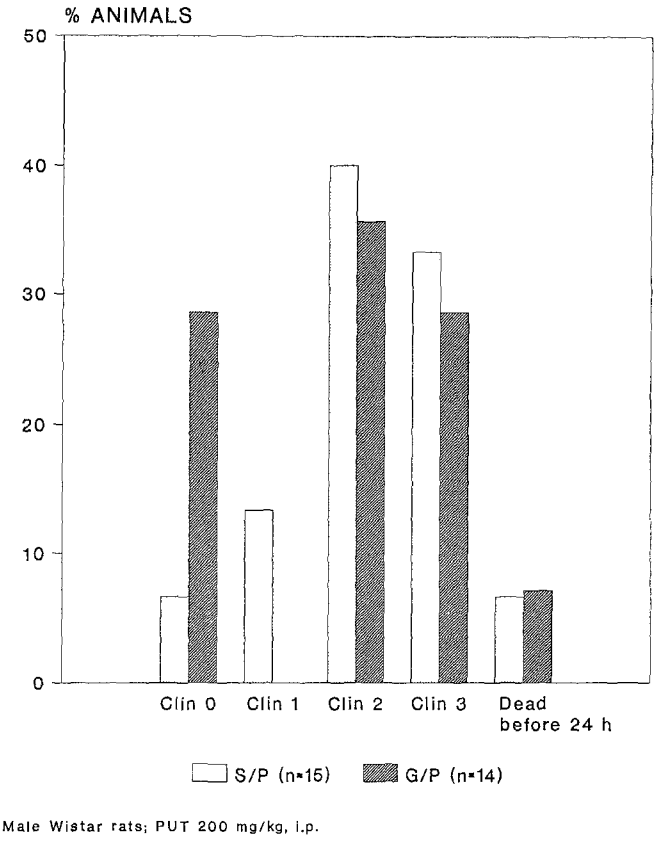

Fig. 1. Behavioural effect of putrescine (200 mg/kg i.p.) administered to rats. Clinical evaluation during 2 h after putrescine: *Clin 0* no behavioural alterations; *Clin 1* wet dog shakes (WDS) and/or slight signs of alteration (see text); *Clin 2* WDS and clear postural and coordination deficits that recovered within 2 h; *Clin 3* WDS and clear postural and coordination deficits not recovered within 2 h. *S/P* saline per os, 30 min before putrescine; *G/P* glycerol (90%, 5 ml/kg) per os, 30 min before putrescine. Ordinates: percentage of animals in each group

scoring 0 and 1, there were no significant differences between the effects of PUT on animals pretreated with glycerol or saline (Chi-square test, n.s.).

Brain polyamines

Frontal cortex concentrations of polyamines in the different groups studied are sumarized in Table 1. The treatment with PUT increased its cortical concentration in a significant manner 2 and 24 h after administration ($p < 0.05$, ANOVA- Duncan test). This effect, however, did not reach statistical significance in the group pretreated with glycerol due to the large variability of data. The difference between the G/P and S/P groups was only significant at 24 h. Groups treated with glycerol and saline showed control values. At 2 h, all groups showed a moderate decrease ($\approx 20\%$) in the concentration of spermidine and spermine respective to the saline group whereas at 24 h only spermine in the G/P group was decreased ($\approx 30\%$). The internal correlation between the concentration of spermidine and spermine in the saline and control groups ($r = 0.608$, $n = 21$;

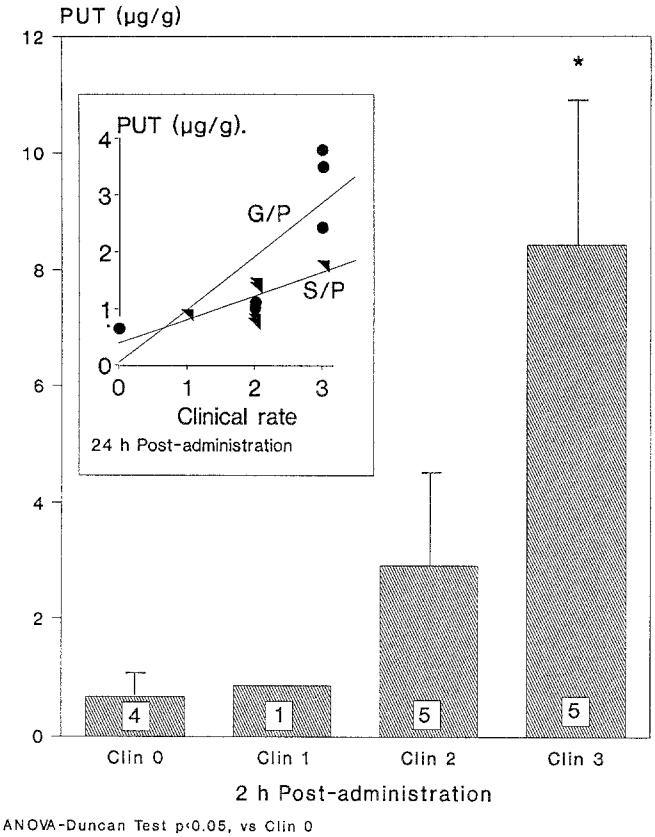

Fig. 2. Concentration of putrescine (μg/g) in frontal cortex of rats treated with 200 mg/kg of putrescine i.p. and sacrificed 2 h after injection according to the clinical status. Individual data at 24 h are plot in the inset. *S/P* saline per os, 30 min before putrescine; *G/P* Glycerol (90%, 5 ml/kg) per os, 30 min before putrescine. Data corresponding to 2 h contain both groups. Number of animals shown within the bars

$p < 0.003$) was also present after the treatment with PUT ($r = 0.525$, $n = 27$; $p < 0.005$).

No correlation was found between polyamine levels and WDS. However cortical PUT levels at 2 and 24 h paralleled the severity of behavioural changes exhibited by the rats (Fig. 2). One-way analysis of variance of PUT levels showed a significant effect of the behavioural status ($p < 0.002$) at 2 and 24 h. Considering both groups together (G/P + S/P), the mean level of PUT in frontal cortex 2 h after treatment with 200 mg/kg PUT can be set as follows: Clin 0 = Clin 1 (PUT ≤ 1 μg/g), Clin 2 (PUT ≤ 3 μg/g) and Clin 3 (PUT ≤ 9 μg/g).

Histology

Analysis of brain sections showed that the main histological signs of damage induced by putrescine 2 h after treatment were the presence of perivascular edema and signs of cellular edema (spongiosis) in both frontal cortex and hippocampus. These effects were more pronounced in the latter. Samples corresponding to rats sacrificed 24 h post-treatment showed the same pattern of

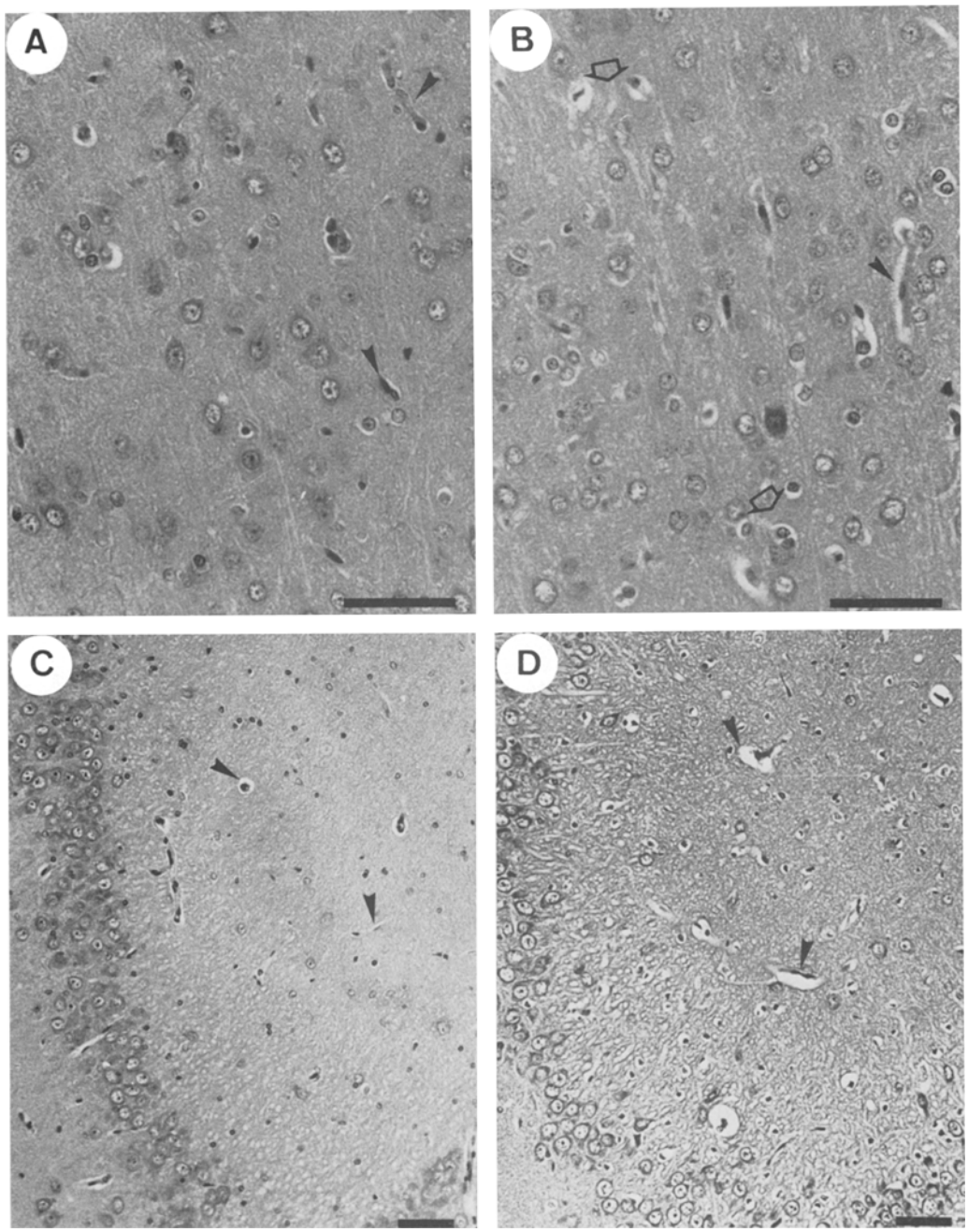

Fig. 3. Rat brain coronal sections from frontal cortex (**A**, **B**) and hippocampus (**C**, **D**). **A** and **C** show the tissue appearance of animals without signs of histological damage (the case selected correspond to a *G/P* group at 2 h). **B** and **D** illustrate the histological damage reported (sample corresponding to the group *S/P* at 24 h). Arrow heads indicate brain vessels. Animals treated with PUT show perivascular edema and moderated spongiosis (empty arrow). Integrity of vasculature and brain cells can be observed in non damaged animals. Bars correspond to 50 μm

damage (Fig. 3). We have not found a clear relationship between the level of PUT in frontal cortex and the histological alterations. Only those animals of the group S/P at 24 h showed more marked lesions in both areas and higher PUT levels in frontal cortex. Animals who received only glycerol also developed signs of edema at 24 h but not at 2 h.

Discussion

The present results show that the systemic administration of PUT induces a characteristic toxic response. Some signs (WDS) are probably related to an action of this polyamine within the CNS. Other signs -flat body posture or motor incoordination- may perhaps reflect some peripheral actions of PUT.

Our results are in good agreement with previous work (Genedani, 1987), showing a strong capacity of PUT to induce WDS. However, these authors did not report on other signs of toxicity, in spite that they used higher doses of PUT. Differences in the strain of rats used or in the sex of the animals may account for this discrepancy.

In the present work, the relationship between the severity of the clinical status of the animals and the concentration of PUT in frontal cortex is remarkable. It is interesting to note that some animals developed vascular and cellular edema in different brain regions of the brain, an effect also described by Genedani (1987). The time course of both histological and behavioural effects shows that they are fastly mediated effects.

A number of similarities can be found between the actions of systemic PUT and those of kainic acid, de Vera (1991): 1) the presence of WDS is similar in time and intensity, 2) the increase of PUT in brain paralleled the severity of the clinical status, 3) the maximum level of PUT attained in frontal cortex is similar: 8.5 μg/g, 2 h after PUT treatment and 10 μg/g, 24 h postconvulsion in kainic acid treated rats, 4) spermidine and spermine levels decreased at 2 h, 5) spongiosis was found in frontal cortex and hippocampus after both treatments but in a different way: the same lesions were found at 2 and 24 h after PUT treatment, whereas the cellular edema exhibited early (2–3 h) after kainic acid treatment became a severe necrosis 24 h after convulsions, 6) a clear relationship between brain PUT levels and brain damage could be established in kainic acid treated animals. This relationship is poor in PUT-treated rats. Therefore, it is likely that the excess of brain PUT observed after KA treatment mediates some of its neurotoxic actions.

The use of a high molarity solution of glycerol to increase the permeability of brain capillaries was selected to avoid other treatments as mannitol that need intravenous administration involving anaesthesia. The animals pre-treated with saline or glycerol and treated with PUT did not show differences on behaviour or cortical PUT levels at 2 h. This may be indicative that glycerol does not modify – at this time – the brain capillary permeability for PUT.

The existing parallelism between brain PUT concentrations and the behavioural status suggests that the individual differences in both variables are due to kinetic factors, among which the crossing of the blood-brain-barrier (BBB) may be crucial. Koenig (1989) has reported that PUT mediates the opening of

the BBB. This effect could be responsible of some of the present results. A fast increase of brain capillary permeability can facilitate the input of circulating PUT and explain the formation of the perivascular edema. The further reaction of the brain tissue could involve modifications in the metabolic regulation of brain polyamines and in the EAA-mediated neurotransmission.

Animals treated only with glycerol showed notable signs of perivascular and cellular edema 24 h post-treatment. According to Koenig (1989), this may indicate that both the treatment with PUT and the treatment with a solution of increased osmolarity increase capillary permeability in brain. In animals treated only with glycerol, brain levels of PUT remain unaltered as expected. Unfortunately, the experimental design does not provide information about the exact time at which glycerol alone induces the formation of edema in the brain.

A recent report (Gilad, 1991) showing that polyamines protect from cell damage after ischemia in gerbils appears to contradict the results – concerning PUT – from this and other reports in the literature (Paschen, 1988; de Vera, 1991). Some experimental differences could be responsible, e.g. use of anaesthesia by Gilad (1991). In addition, polyamines can not be considered as a homogeneous group, since PUT has a different role than spermidine and spermine on the regulation of the NMDA receptor complex (Williams, 1991). The results reported in the present work concerning spermidine and spermine show that both amines had decreased levels 2 h after PUT treatment. Again, this effect was similar to that observed at 3 h post administration of kainic acid (de Vera, 1991). From the present results, it is difficult to support a role for spermidine and spermine in the neurotoxic action of PUT. Spermidine and spermine did not exhibit group differences in relation to the behavioural status or the histological damage. Therefore, it is likely that their slight decreases after PUT treatment do not account for the changes observed.

In conclusion, we have shown that PUT is a toxic agent by itself that induces behavioural and biochemical and histological alterations in the brain of the adult male rat. When comparing these effects with those reported for kainic acid at convulsant doses some analogies can be found. These are more pronounced during the first phase of the action of kainic acid, suggesting that its neurotoxic properties can be partly mediated by putrescine. Nevertheless, the poor relationship between the histological damage and brain PUT concentration is at variance with previous reports. Further work is needed to examine this point.

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