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GABA depletion and behavioural changes produced by intraventricular putrescine in chicks

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Although polyamines are usually concentrated in tissues with a high rate of biosynthetic activity [1], concentrations higher than those of catecholamines and indoleamines have been found in the brain of different animal species [2]. Their distribution varies in different areas of the brain and spinal cord [3–5] and the neuropharmacological and neurophysiological actions of two polyamines, spermidine and spermine, suggests the possibility that these substances may act as modulators or transmitters in CNS (see ref. 6). In favour of such a role is the finding that polyamines may activate or inhibit acetylcholinesterase [7], although doses of spermidine or spermine which produce central effects are without effect on brain acetylcholine content or on catecholamines, serotonin and GABA content [8]. Putrescine is synthetized by decarboxylation of ornithine and is one precursor of spermidine and spermine (see refs. 9–11). Putrescine also increases GABA synthesis in mammalian brain [12, 13]. Intraventricular and systemic injection of putrescine, spermidine and spermine produces profound behavioural changes in rodents and rabbits, including clonic convulsions and changes in locomotor activity, body temperature and REM and non-REM sleep [14–17]. The present experiments were performed to study in another animal species, i.e. chicks, the acute affects of putrescine given into the III cerebral ventricle on behaviour, electrocortical activity and body temperature. At the time of peak effect, changes in the GABA system both in the diencephalon and brainstem, areas accessible from the third ventricle as shown in rats after intracerebroventricular (IVC) of ^{3}H-putrescine [18] and the effects on spermidine and spermine content were studied. This was to determine whether the acute effects of putrescine were directly or indirectly mediated.

Rhode Island red chicks weighing 240–250 g were used. All operative procedures were performed under halothane anaesthesia. The guide cannula was chronically implanted into the rostral part of the III cerebral ventricle by means of a stereotactic instrument (Horsley–Clark). Cannulae positions were verified histologically at post-mortem. Electrocortical activity was recorded from chronically implanted electrodes on the cortex as previously described [19]. Chicks were tested at least 3 days after operative procedures. Drug injections were made by a 10 μl Hamilton syringe at a rate of 0.5 μl/min. Putrescine was dissolved in distilled H_2O and the pH adjusted to about 7.5 by adding HCl (0.1 N). Control injections of the same volume of vehicle (pH 7.5) lacked effects on behaviour and electrocortical activity. After decapitation the brain hemispheres and diencephalon were quickly dissected out within 30 sec and frozen in liquid nitrogen. GABA was assayed by a Carlo Erba aminoacid analyzer using AMINEX A-5 (0.9 × 13 cm) ion exchange resin as previously described [20].

Glutamate-decarboxylase activity (GAD) was assayed by a slight modification of the method described by Beaven *et al.* [21]. A 15 μl sample of the tissue homogenate (1 : 10) was mixed with 35 μl of reagent inside a 1.5 ml polypropylene Eppendorf vial. The vial was placed inside a 20 ml screw-cap liquid scintillation counting vial. A drop (20 μl) of 30% 2-phenylethylamine in methanol was adsorbed on a square (1 cm^2) of filter paper (Whatman 3MM) placed at the bottom of the counting vial away from the Eppendorf vial. The reagent consists of (a) L-[U-^{14}C]-glutamic acid, 20 nCi; (b) unlabeled L-glutamic acid to bring the concentration of amino acid to 5×10^{-4} M; (c) 1×10^{-5} M pyridoxal-5-phosphate (PLP); (d) 0.1 M sodium phosphate buffer, pH 6.8, and (e) Triton X-100 (final concentration 0.5%). Reaction blanks were prepared by substituting buffer for the sample. The counting vials were tightly capped and incubated at 37° for 30 min; for deproteinization the vials were then placed on ice and uncapped one at a time and 20 μl of 2 N perchloric acid was added to the Eppendorf vials. The counting vials were quickly recapped and reincubated for 30 min. At the end of the second incubation, the Eppendorf vials were removed from the counting vials and discarded. Ten microlitres of liquid scintillation mixture (2.5 g PPO and 150 mg POPOP/1 toluene) was added to each counting vial and radioactivity was measured by a Nuclear Chicago liquid scintillator at room temperature. Preliminary experiments have shown that in these experimental conditions the $^{14}CO_2$ loss was approximately 1.5 per cent. In addition, under our experimental conditions the rate of enzyme reaction was linear as a function of time (up to 30 min) as well as of protein (up to 0.150 mg).

GABA-transaminase activity (GABA-T) was assayed in 100 μl of the following reaction mixture containing: GABA 5 μmoles; α-ketoglutarate 5 μmoles; PLP 0.4 μmoles; 0.1 M phosphate buffer, pH 8.4 and homogenate (1 : 10). After 1 hr incubation at 37° the reaction was stopped by adding 0.4 ml of absolute ethanol. Controls were carried out by adding homogenate after ethanol. After centrifugation, the glutamic acid levels in the supernatants were determined by an amino acid analyzer with 3AR/2/A/55 (0.9 × 55 cm) ion exchange resin.

Proteins were determined by the procedure of Lowry *et al.* [22]

For assay of polyamines and putrescine, homogenates from the brain regions studied were immediately added to perchloric acid to make final concentrations of 0.2 M and accurately re-homogenized (3 min at 4°) with a Teflon pestle at 2000 r.p.m. After 3 hr at 4° the samples were centrifuged at 800 *g* for 20 min and the polyamines and putrescine assayed as the dansyl derivatives by mixing one volume of the protein-free supernatant with two volumes of dansyl chloride in acetone (10 mg/ml). $Na_2CO_3 \cdot 10\,H_2O$ was added (80 mg/ml reaction mixture) and the samples were kept overnight in the dark, at room temperature. Standards of commercial polyamines and putrescine in 0.2 M perchloric acid and blank controls (without tissue) were carried throughout the procedure. After completion of the dansylation reaction [23], 10 mg proline in 0.1 ml of water was added to the dansyl chloride excess. The dansylated amines were then extracted with 1 volume of benzene, with accurate mechanical shaking for 30 sec and centrifuged at 2000 *g* for 5 min. The benzene phase was evaporated to dryness *in vacuo*, and each residue was redissolved in 50 μl of benzene. Known aliquots of these samples were applied to Silica-gel thin layer chromatography plates and chromatographed with ethylacetate: cycloexane (2:3) according to Herbst and Dion [24]. The dansyl derivatives of putrescine, spermidine and spermine were visualized on the plate with u.v. light, and the area corresponding to each fluorescent spot was scraped out and the silica gel transferred to a centrifuge tube. Dansyl derivatives were extracted

Table 1. Effect of an intraventricular injection of putrescine (100 μg) on GABA concentration, GAD and GABA-T activity in some areas of chick brain (30 min after injection)*

Brain area	Experimental groups	GABA (μmoles/100 mg protein)	GAD (μmoles CO_2/100 mg protein/hr)	GABA-T (μmoles glutamic acid/100 mg protein/hr)
Diencephalon	Controls	4.70 ± 0.24	17.15 ± 0.40	55.55 ± 0.78
	Putrescine	2.56 ± 0.27†	12.71 ± 0.32†	53.45 ± 1.51
Hemispheres	Controls	7.85 ± 0.24	15.61 ± 0.34	16.08 ± 1.15
	Putrescine	7.31 ± 0.15	14.54 ± 0.69	14.59 ± 1.47

* Results expressed as mean values of 8 single experiments ± S.E.M.
† $P < 0.001$ in comparison with controls.

from the silica gel with a 2 ml of methanol: 25% ammonia (99:1, v/v) mixture. After centrifugation, fluorescence in the supernatant fractions was detected by means of a Turner (mod. 430) spectrofluorometer (activation at 365 nm, emission at 520 nm).

The drug used was putrescine (Fluka, Switzerland) and the pH of the solution was adjusted to 7–7.5 with 0.1 N HCl.

The intraventricular injection of putrescine in chicks produced a dose-dependent (from 25 to 150 μg) behavioural stimulation, increase in locomotor activity, vocalization, side to side head jerks and tachypnoea. This symptomatology started after a 5–10 min period of sedation and lasted from 15 min to 2 hr according to the dose. After the highest doses used (100 and 150 μg), intense behavioural stimulation with marked postural changes, semisquatting and wing abduction, continuous vocalization, head–neck rotation, ataxia, tachypnoea (up to 180 min), periodic crisis of circling or escape responses and feather erection were observed for about 2 hr. In addition, frequent myoclonic movements of the limbs and clonic convulsions occurred during the first 2 hr. Electrocortical activity was characterized during this and prior to this symptomatology by high voltage continuous spikes activity (Fig. 1). Body temperature was decreased up to 2° for approximately 1–3 hr according to the dose. The above symptomatology was followed by behavioural sedation for about 90 min, after which there were no apparent behavioural and electrocortical changes.

Putrescine (100 μg) given into the III cerebral ventricle of chicks (N = 8) produced 30 min later, in comparison to controls receiving the same volume of vehicle, a significant depletion of GABA in the diencephalon but not in the cerebral hemispheres. This effect appears to be dependent on the inhibition of GAD, the GABA synthesizing enzyme, since no changes were observed in GABA-T activity (Table 1). Thirty minutes after putrescine administration, i.e. at the peak of the behavioural symptomatology and concomitant to GAD inhibition, putrescine did not affect the spermidine and spermine content of these brain areas but increased the concentration of putrescine by 433 per cent.

The diencephalic spermidine and spermine concentration were 0.362 μmoles/g w.t. ± 0.05 (N = 6) and 0.217 μmoles/g w.t. ± 0.04 (N = 6), respectively, in control chicks and these were only slightly increased (0.435 μmoles/g w.t. ± 0.05 and 0.280 μmoles/g w.t. ± 0.03, respectively) by injection of putrescine.

The present experiments show that, in chicks, putrescine, the precursor of spermidine and spermine, given into the III cerebral ventricle produces marked behavioural and electrocortical changes accompanied by GABA depletion in the diencephalon. The central effects of putrescine in mice have been suggested to be due to the increased formation of spermidine and spermine in the brain and were described to occur 24 hr after the administration [14]. In our experiments using a similar range of doses as in mice, we have found that after a short-lasting period of sedation (5–10 min) there was behavioural excitation, marked postural and electrocortical changes. The latency period does not seem to be due to the time required for putrescine to be metabolized into spermidine and spermine as suggested in mammalian species [14], since the concentrations of these amines were only slightly increased, but to the time required for putrescine to inhibit glutamate-decarboxylase and to significantly lower GABA content in the diencephalon. The mechanisms by which GAD activity is depressed is not known at the present time, nor is it known whether such inhibition is direct or mediated by a putrescine metabolite. Convulsions and electrocortical epileptic discharges occur in several animal species when there is a depletion of brain GABA (see refs. 25 and 26). On the basis of the electrophysiological studies showing that polyamines iontophoretically applied mostly depress neuronal firing of rat and cat brain-stem [27], our experiments

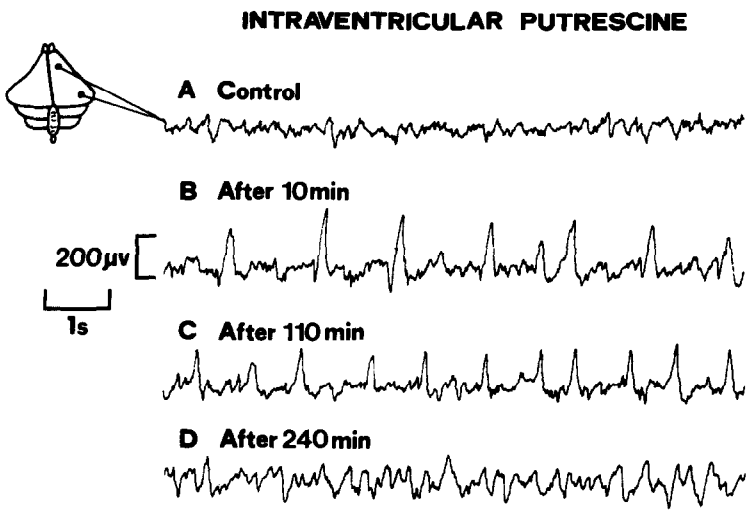

Fig. 1. Effects of a single intraventricular injection of putrescine (150 μg) on electrocortical activity of a chick (240 g) kept at an ambient temperature within thermoneutrality. A: control electrocortical activity. B and C: high voltage spikes in comparison to A, 10 and 110 min from the administration of putrescine. D: Slow-wave, higher amplitude potentials during the behavioural depression (240 min from putrescine) following the first stage of behavioural stimulation.

suggest that amines may inhibit some GABA-ergic neurons, thus producing epileptogenic discharges. Although in mammalian species the enzymatic activities leading to GABA synthesis from putrescine are present [12], the present results indicates that, also if in chicks these are present, the amount of newly synthesized GABA does not counteract GABA depletion due to inhibition of GAD activity.

Despite the fact that in mice and rabbits administration of spermidine and spermine produce marked and long-lasting sedation also in these species the symptomatology culminated after a longer period in tonic–clonic convulsions [14]. The changes obtained in the present experiments in the GABA system in the diencephalon in contrast to the brain hemispheres may reflect the regional distribution of ^{3}H-putrescine in different areas of the rat brain after IVC administration [18]. The effects of putrescine on body temperature in chicks were similar to the hypothermic effects reported to occur in mammalian species [14, 15] and at least in part seem to be due to increased heat loss through tachypnoea.

In conclusion, the profound behavioural changes after intraventricular administration in mammalian and non-mammalian species and the claimed implication of polyamines in the development of aggressivity in mice [28] are in favour of the suggested role for polyamines as modulators or transmitters in CNS [6].

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Effects of depressant drugs and sulfhydryl reagents on the transport of calcium by isolated nerve endings

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One effect of neuronal depolarization is to open the synaptic membrane channels that allow calcium to flow down the electrochemical gradient and enter the nerve terminal [1]. The resultant increase in the concentration of free calcium in the nerve terminal leads to the release of neurotransmitter substances [2]. To terminate the release of neurotransmitter, the excess calcium in the nerve ending must be either sequestered or removed. The mechanisms involved in the removal of calcium are not well understood, but a high affinity, non-mitochondrial, ATP-dependent calcium uptake has been described recently in lysed synaptosomes [3]. This intrasynaptosomal calcium transport system may play a significant role in buffering the intracellular calcium concentration of nerve endings [3]. Since synaptic function is likely to depend upon both the depolarization-dependent influx of calcium and the ATP-dependent sequestration of calcium, we have compared the effects of *in vitro* addition of various drugs on both of these processes in isolated synaptosomes to evaluate the role of calcium transport in the neuropharmacological effects of these drugs. Of particular interest were the observations of increased spontaneous release of neurotransmitters from nerve terminals exposed to ethanol [4–6], barbiturates [7] or sulfhydryl reagents [8–10]. These findings have been taken as evidence to indicate that these agents directly affect the membrane processes responsible for the release of neurotransmitters. However, it is possible that these compounds might also increase the release of neurotransmitters indirectly by inhibiting the intraneuronal sequestration of calcium. In addition, we were interested in the similarities between the ATP-dependent uptake of calcium by brain membranes and by muscle sarcoplasmic reticulum. Accordingly we selected several sulfhydryl reagents and other drugs which are known to inhibit the transport of calcium by sarcoplasmic reticulum and studied their effects on ATP-dependent calcium transport by lysed synaptosomes.

Male Sprague–Dawley rats (200–250 g) (Charles River Breeding Laboratories, Wilmington, MA) were decapitated, and synaptosomes were isolated from whole brain homogenates as described previously [11]. ATP-dependent and potassium-stimulated calcium uptakes were assayed as described by Blaustein *et al.* [3,12]. For both assays, the synaptosomal band was removed from the Ficoll gradient, slowly diluted 5-fold with ice-cold calcium-free Na^+-$5K^+$ (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Tris; pH 7.4) and pelleted at 15,000 *g* for 6 min. For the ATP-dependent uptake, the pellet was then resuspended in hypotonic lysis solution [1.3 mM $MgCl_2$, 2.4 mM NaH_2PO_4 and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), pH 7.4] and incubated at 37° for 3 min to disrupt the synaptosomes. This suspension was placed on ice and diluted with an equal volume of a solution containing 362 mM KCl, 1.95 mM $MgCl_2$, 3.6 mM NaH_2PO_4, 30 mM Hepes, 0.5 mM NaN_3, 0.5 mM dinitrophenol and 5 μg/ml oligomycin, pH 7.4. Aliquots (0.8 ml; 0.4–0.8 mg protein) were added to tubes containing 0.2 ml of various drug solutions or distilled water (control) and incubated at room temperature for 15 min, followed by incubation at 37° for 1 min. At this point, 1 ml of the ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA)-buffered ^{45}Ca solution (100 μM $CaCl_2$, 0.5 μCi/ml $^{45}CaCl_2$, 107 μM EGTA, 145 mM KCl, 1.3 mM $MgCl_2$ and 0 or 2 mM MgATP) was added and incubation was continued for 5 min at 37°. The final concentration of free calcium was calculated to be 2 μM [13]. The uptake was terminated by rapid filtration through GF/C discs which were washed three times with 5 ml of a solution containing 145 mM KCl, 1.2 mM $CaCl_2$ and 1.4 mM $MgCl_2$ The ATP-dependent uptake (ΔATP) was considered to be the difference between the uptake in the absence of ATP (−ATP) and in the presence of ATP (+ATP) (see Table 1). For potassium-stimulated calcium uptake by intact synaptosomes, the synaptosomal pellet was resuspended in Na^+-$5K^+$ (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris and 1.2 mM $CaCl_2$; pH 7.4) to give 1–2 mg/ml protein. Portions (0.5 ml) of this suspension were incubated for 15 min at 37° except for experiments involving ethanol or pentobarbital, which were carried out at 30°. Aliquots of the drug solutions were then added and the incubation was continued for 12 min. Next, 0.5 ml of either a solution of Na^+-$5K^+$ containing ^{45}Ca or a similar solution in which 132 mM KCl was substituted for the NaCl (depolarizing solution) was added. Incubation continued for 1 min and uptake was stopped by the addition of 0.5 ml of an ice-cold EGTA-halting solution (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 30 mM Tris and 30 mM EGTA) plus 5.0 ml of calcium-free Na^+-$5K^+$. Membranes were filtered immediately on GF/C discs and washed twice with 10 ml of calcium-free Na^+-$5K^+$. The ^{45}Ca on the discs was determined by liquid scintillation spectrometry. The depolarization-dependent uptake (ΔK^+) was considered to be the difference between the uptake in a low concentration of potassium ($-K^+$) and in a high concentration of potassium ($+K^+$). Protein concentrations were determined by a modification of the phenol method [14].

The results in Table 1 indicate that depressants such as ethanol (800 mM), pentobarbital (0.5 mM) and acetaldehyde (100 mM) significantly inhibited the ATP-dependent uptake, while chlorpromazine (10^{-5} M), diphenylhydantoin (10^{-4} M) and phencyclidine (10^{-5} M) were without effect. Ethanol and the barbiturates had been shown previously to decrease the ATP-dependent calcium uptake in cardiac sarcoplasmic recticulum vesicles [15–17], while