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Serotonin Turnover Rate in Raphe and Cortex of Mice Infected With Venezuelan Equine Encephalomyelitis Virus

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The turnover of serotonin (5HT) was determined in the raphe area and cortex of mice infected with Pixuna, a strain of intermediate virulence of Venezuelan equine encephalomyelitis virus (VEEV). NMRI-mice, 24 days old, were inoculated intracerebrally (ic) with 300 LD_{50} of the virus. The animals were sacrificed 4, 7, 15, 21, 30, and 60 days postinoculation. 5HT and 5-hydrodryindolacetic acid (5HIAA) in raphe and cortex were determined by high performance liquid chromatography (HPLC) with electrochemical detection. Turnover rate of 5HT was determined by the administration of pargyline, *p*-chlorophenylalanine, and probenecid. The content of 5HT or 5HIAA and 5HT/5HIAA ratios were not significantly different in infected compared with control mice. However, a decrease of 5HT turnover rate, determined after pargyline treatment, was observed in the raphe and not in the cortex of infected mice at 4 and 7 days after the inoculation. The turnover rate/$(5HT)_0$ in raphe is decreased in infected mice with signs of illness, suggesting a lower density of 5HT innervation in this brain area. The administration of *p*-chlorophenylalanine and probenecid showed that the cortex is also affected, but the synthesis is less modified than metabolism or elimination. Cell bodies of 5HT neurons seem to be more susceptible than projections to infection by Pixuna strain of VEEV.

Key words: serotonin, turnover, viral encephalitis

INTRODUCTION

Some strains of Venezuelan equine encephalomyelitis virus (VEEV) are reported to decrease central nervous system (CNS) catecholamine metabolism [Bonilla et al, 1975; Levine et al, 1981; Lima et al, 1983], brain choline acetyltransferase activity [Bonilla et al, 1982], brain GABA content and glutamate decarboxylase activity [Bonilla et al, 1980], and ^{3}H-spiroperidol binding in the brain [Bonilla et al, 1984]. However, other CNS enzymes were unaffected, such as GABA-transaminase, glutamate dehydrogenase, and lactate dehydrogenase [Bonilla et al, 1980]. Herpes simplex virus and several other viruses affect the turnover rate of brain monoamines [Lycke et al, 1969, 1970; Lycke and Ross, 1972]. Some of these reports show differences among the various brain areas analyzed, suggesting selectivity of the virus [Levine et al, 1981; Lima et al, 1983; Bonilla et al, 1984]. Moreover, certain brain viral infections could produce neurological and psychiatric disorders related to monoamine disfunction [Miyasaki et al, 1977; Crow et al, 1979; Koehler and Guth, 1979; Rhodes et al, 1984]. Recently several reports focused on the preference of various CNS viral infections for localized brain areas, such as the limbic system [Delsedime et al, 1984; Damasio and Vanhoese, 1985; Delamonte, 1985], basal ganglia [Fishman et al, 1985], locus coeruleus [Maurizi, 1985a], and raphe area [Maurizi, 1985b]. The mechanism of the viral-neuron interaction needs to be clarified. The purpose of this study is to analyze in a systematic manner the potential differential effect of a strain of VEEV of moderate virulence on the central serotonergic system (neurons and terminals). For this reason we investigated the serotonin (5HT) turnover rate in infected mice using three pharmacological agents, namely, pargyline, *p*-chlorophenylalanine (PCPA), and probenecid.

MATERIALS AND METHODS

Animals

Male NMRI/IVIC mice (20±5 g), 24 days old, from our animal facility were used in all the experiments. The animals were kept in groups of 5 per cage and inspected daily in order to follow the course of the disease as described by Lima et al, 1983. At 24 days of age, the mice remain susceptible to infection by Pixuna strain of VEEV, but have a greater survival rate than younger mice [Walder and Bradish, 1979]. A dose of 300 suckling mice ic LD_{50} of Pixuna strain was inoculated ic using

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solutions of bovine albumin as diluent. Controls were inoculated with the same vehicle. Animals were sacrificed by decapitation at 4, 7, 15, 21, 30, and 60 days after inoculation.

The brain was quickly removed and washed in a solution of 0.5 mM of sodium metabisulfite in 0.5 N perchloric acid (PCA) and kept in the dark until dissection. Raphe area and cortex were dissected according to the procedure of Aghajanian and coworkers [1973]. A simple examination of these regions was done in sections stained with hematoxiline-eosine. The raphe and cortex were homogenized in 0.5 ml and 1 ml of 0.5 mM sodium metabisulfite in 0.5 N PCA, respectively. A manual homogenizer was used and an aliquot was reserved for protein determination. In normal and infected mice the protein content of raphe was 4.60 + 0.18 mg/ml, and of cortex 14.97 + 1.91 mg/ml (n = 15). After centrifugation at 15,000 rpm for 10 min the supernatant was removed and kept at −80 °C for 1 to 5 days before the determination of 5HT and 5HIAA. Recovery against time was established by the addition of internal standards. A decay curve done over a period of 3 weeks showed recoveries of 89, 80, and 70% for 5HT, and 95, 90, and 85% for 5HIAA after the first, second, and third week, respectively. Protein content was calculated by the procedure of Lowry and coworkers [1951].

5HT and 5HIAA Determination

5HT and 5HIAA were determined by reversed phase HPLC with electrochemical detection. The liquid chromatograph and detection systems consisted of: a LKB 2150 pump; a LKB 2154 manual sample injector; an Altex Ultrasphere-ODS column (25 cm × 4.6 mm ID, 5 μm average particle) placed at 37°C in a LKB 2155 column oven. The amperometric detector was composed of LKB 2143 electrochemical detector, glassy carbon working and auxiliary electrodes, and the reference electrode formed by Ag/AgCl coated with chlorine ions present in the mobile phase. The potential of the working electrode was set at +0.70V versus the reference electrode. The mobile phase was modified from Anderson and coworkers [1981] and consists of 90% 0.01 M sodium acetate, 10 mM EDTA, and 10 mM sodium chloride, adjusted to pH 4.25 with glacial acetic acid, and 10% methanol. Standards were prepared in 0.5 mM sodium metabisulfite in 0.5 N PCA and kept at −20°C. Dilution of standards were made up daily. The flow rate used was 1 ml/min. Retention times were 9 and 14 min for 5HT and 5HIAA, respectively. The amount of 5HT and 5HIAA present in tissues was calculated from the area under the curve using the LKB 2220 recording integrator of actual samples and samples plus external standards.

Turnover Rate Determination

Time course accumulation of 5HT and disappearance of 5HIAA were determined (in min) in the brain of mice injected with pargyline (Sigma), an inhibitor of monoamine oxidase (MAO), given in doses of 100 mg/kg intraperitoneal (ip) [Tozer et al, 1966; King et al, 1985; Whilton et al, 1985]. Also, the decrease in 5HT contents was measured 24 hr after the administration of PCPA (Sigma), an inhibitor of tryptophan hydroxylase, given in a dose of 300 mg/kg ip [Neckers and Meek, 1976]. Finally, the accumulation of 5HIAA was determined in mice treated with probenecid (Palenzona), an inhibitor of acid transport, given in doses of 200 mg/kg ip 2 hr before sacrifice [Neff et al, 1967; Neckers and Meek, 1976].

Turnover rates represent estimates of the fractions of the indole pool (i.e., initial concentration metabolized per hr). These numbers were calculated from a linear curve plotted as indole concentrations against time after pargyline injection. Linearity of regression and regres-

TABLE I. 5HT/5HIAA Ratio in Raphe and Cortex of Infected Mice*

Days after inoculation	Raphe			Cortex		
	C	INS	IS	C	INS	IS
4	1.36 ± 0.07 (6)	1.51 ± 0.10 (6)	—	1.44 ± 0.66 (6)	1.28 ± 0.25 (6)	—
7	1.04 ± 0.16 (6)	1.12 ± 0.15 (6)	1.25 ± 0.33 (6)	1.74 ± 0.11 (6)	1.08 ± 0.16 (6)	1.09 ± 0.28 (5)
15	1.37 ± 0.46 (3)	1.27 ± 0.25 (3)	—	1.95 ± 0.35 (3)	2.11 ± 0.53 (3)	—
21	1.35 ± 0.21 (4)	1.41 ± 0.20 (4)	—	1.74 ± 0.21 (3)	—	—
30	1.30 ± 0.07 (6)	1.12 ± 0.12 (6)	—	1.48 ± 0.24 (6)	1.12 ± 0.22 (6)	—
60	1.55 ± 0.38 (4)	2.03 ± 0.31 (4)	—	2.19 ± 0.38 (4)	2.19 ± 0.55 (4)	—

*Each value is mean ± SE; C, control; INS, infected with no signs; IS, infected with signs; number of animals in parentheses.

sion coefficients were calculated according to Winer [1971]. A two-tailed t test for significant differences ($P < 0.05$) was used to determine variations between groups.

5HT/5HIAA ratio was also calculated, as an index of turnover rate. An increase in this ratio reflects reduction of turnover rate [Karoum et al, 1984]. The turnover rate $[5HT]_0$ ratio was calculated as an index of the density of serotonergic innervation [Meek and Loftstrandh, 1976; Neckers and Meek, 1976].

RESULTS

Mice inoculated with Pixuna strain of VEEV did not develop signs of illness before day 6 postinoculation. Between 6 and 8 days after inoculation, signs of encephalomyelitis were observed. These included hypokinesia, lethargy, and paralysis of posterior limbs. This was followed by death. Mortality was about 50% and occurred between 6 and 9 days after inoculation. No mortality occurred in sham-inoculated mice. Groups of animals were designated control (C), infected with no signs (INS), and infected with signs of illness (IS).

Raphe

At 4 days after inoculation, the content of 5HT and 5HIAA in the raphe of infected mice did not change with respect to control group. Also, significant variations were not observed in the 5HT/5HIAA ratio (Table I). By day 7 after inoculation, the concentrations of 5HT in the INS group did not change with respect to control group; there was a 21% decrease in the content of 5HIAA. In contrast, the IS group showed decreases of 21% in 5HT and 30% in 5HIAA content. By day 15, 21, 30, and 60 after inoculation, there were insignificant changes in both the 5HT/5HIAA ratio and 5HT and 5HIAA contents.

Cortex

The concentration of 5HT and 5HIAA did not vary significantly in the cortex of infected animals when the values were compared to those of control groups. However, decreases of 20–30% in either 5HT or 5HIAA were observed in both INS and IS groups. Also in this brain area, the ratio was not significantly affected (Table I). This was expected, since 5HT and 5HIAA decreased simultaneously.

5HT Turnover Rate

After administration of pargyline, 5HT accumulated quickly in the nervous tissue of normal mice. Under the described conditions, 5HT saturation was observed within 30 minutes after the injection of pargyline.

Raphe (Fig. 1). By day 4 after inoculation, linear increase of 5HT content was observed during the first 15 minutes after pargyline administration (Fig. 1A). The content of 5HT at this time was greater than the initial 5HT concentration ($P < 0.05$). However, in the INS group there was a linear increase of 5HT within 15 min after injection of pargyline. This increase was significantly different ($P < 0.05$) from the concentration of this monoamine in the raphe area of the control group (Fig. 1A). The slope of 5HT accumulation in this brain area and the turnover rate were smaller in the INS group compared to those of controls. By day 7 postinoculation, there was a significant accumulation of 5HT only in control mice, 15 min after pargyline administration (Fig. 1B; $P < 0.05$). The content of 5HT in IS mice 15 min after pargyline injection was statistically significantly smaller than in controls. Accumulation of 5HT in INS and IS animals was not statistically significant.

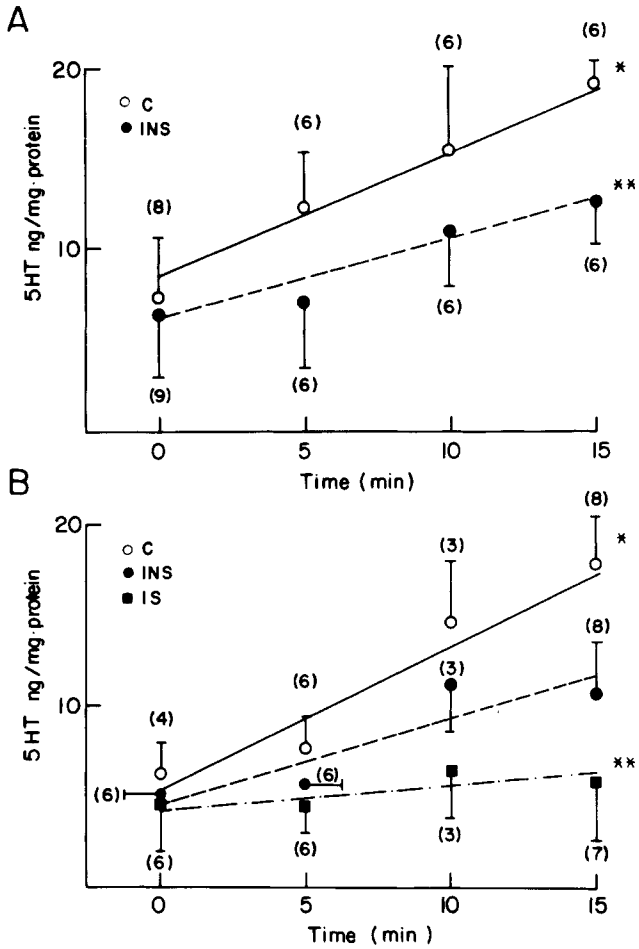

Fig. 1. Levels of 5HT in mice raphe area at various times after pargyline administration (100 mg/kg ip). Groups of mice are: C, control; INS, infected with no signs; IS, infected with signs. **A:** 4 days after the IS inoculation of the virus; r, k (h^{-1}) and turnover rate (ng. mg prot.$^{-1}$ · h^{-1}) are: C: 0.99, 5.44, and 44.24; INS: 0.95, 4.32, and 25.72, respectively. **B:** 7 days after the inoculation, C: 0,97, 8.26, and 47.53; INS: 086, 5.49, and 26.84; IS: 067, 1.08, and 5.16, respectively. $^{*}P < 0.05$ with respect to corresponding 0 time; $^{**}P < 0.05$ with respect to control at 15 min.

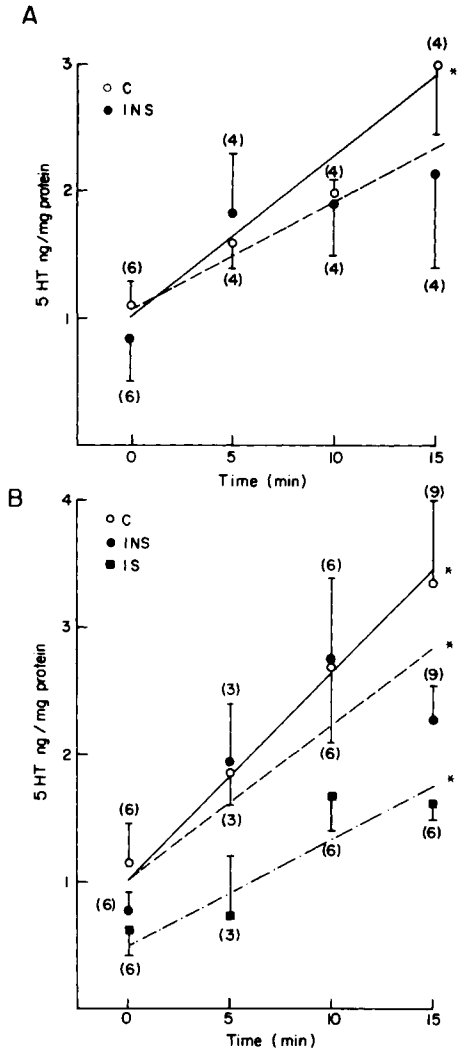

Fig. 2. Levels of 5HT in mice cortex at various times after pargyline administration (100 mg/kg ip). Groups of mice are: C, control; INS, infected with no signs; IS, infected with signs. **A:** 4 days after the inoculation of the virus; 4, k (h^{-1}) and turnover rate (ng.mg. prot.$^{-1}$. h^{-1}) are: C: 0.95, 6.62, and 7.08; INS: 0.89, 4.39, and 4.88, respectively. **B:** 7 days after the inoculation, C: 0.99, 7.89, and 9.08; INS: 0.81, 5.53, and 6.36; IS: 0.90, 8.50, and 4.76, respectively. $^{*}P < 0.05$ with respect to corresponding 0 time.

Cortex (Fig. 2). The 5HT content in the cortex of the control group increased linearly up to 15 min after pargyline administration and became significantly greater than controls without pargyline (Fig. 2A,B). By day 4 after inoculation a linear increase was observed in the INS group, but no significant accumulation was detected. This was perhaps due to population heterogeneity (INS mice + mice that become IS; Fig. 1A). By day 7 after inoculation, there was a linear accumulation of 5HT in the 3 groups under study. This was statistically significant at 15 min after pargyline administration, without statistical differences between the groups (Figs. 2B).

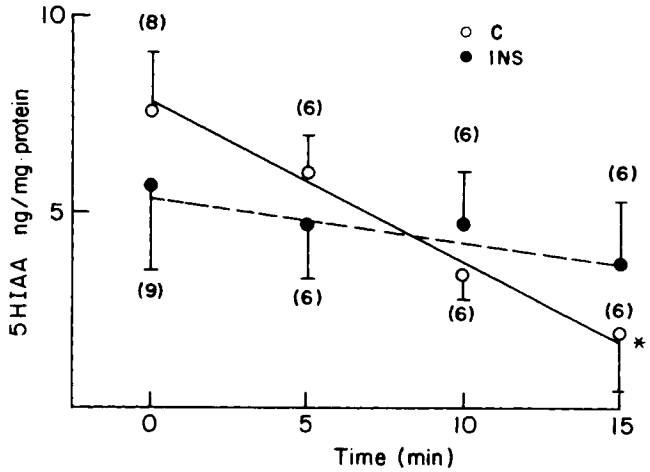

Fig. 3. Levels of 5HIAA in mice raphe area at various times after pargyline administration (100 mg/kg ip) at 4 days after the inoculation of the virus. Groups of mice are: C, control; INS, infected with no signs. r, k (h^{-1}) and turnover rate (ng. mg. prot.$^{h-1}$.h^{-1}) are: C: 0.99, 3.05, and 22.32; INS: 0.97, 1.24, and 6.84, respectively. $^{*}P < 005$ with respect to corresponding 0 time.

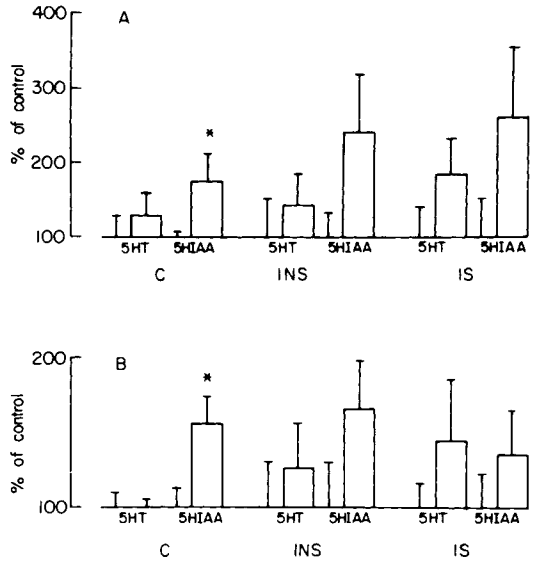

Fig. 4. Effect of probenecid on 5HT and 5HIAA levels in mouse brain. The drug was administered ip in a dose of 200 mg/kg 2 hr before sacrifice. **A:** Raphe area.**B:** Cortex. Groups of mice are: C, control; INS, infected with no signs; IS, infected with signs (n = 4). $^{*}P < 0.05$ with respect to control without probenecid.

The decline of 5HIAA in pargyline-treated mice at day 4 after inoculation is shown in Figure 3. This decline was significantly smaller in infected mice when the values were compared with those of controls.

The decrease of 5HT in the brain of control and infected mice at 7 days postinoculation was determined 24 hours after the ip administration of PCPA. In the control group, PCPA produced 5HT decreases of 35% and 56% in raphe and cortex, respectively (Table II). In

TABLE II. Effect of PCPA on 5HT Concentration in Raphe and Cortex of Infected Mice

	5HT concentration ($ng \cdot mg\ protein^{-1} \cdot h^{-1}$)			
	Raphe		Cortex	
Groups of animals	(−) PCPA	(+) PCPA	(−) PCPA	(+) PCPA
C	6.95 ± 0.32	4.19 ± 0.73*	0.93 ± 0.20	0.32 ± 0.08*
INS	4.78 ± 1.06	7.28 ± 1.24	1.01 ± 0.33	0.78 ± 0.44
IS	4.49 ± 0.89	6.81 ± 1.06	0.91 ± 0.44	0.58 ± 0.11

*Each value is mean ± SE; C, control; INS, infected with no signs; IS, infected with signs. Mice were inoculated ic with Pixuna strain of VEEV (300 LD_{50}). PCPA was given ip in a dose of 300 mg/kg 24 hr before sacrifice. Animals were killed 7 days after inoculation.

TABLE III. Turnover Rate/$[5HT]_0$ Ratio in Raphe and Cortex of Infected Mice

Brain area	Days after inoculation	Groups of animals C	INS	IS
Raphe	4	6.32 ± 2.43 (8)	4.93 ± 2.56 (9)	—
	7	7.41 ± 1.09 (4)	6.02 ± 2.67 (6)	1.28 ± 0.68* (6)
Cortex	4	6.15 ± 0.92 (4)	6.60 ± 2.06 (4)	—
	7	8.37 ± 2.06 (6)	7.00 ± 1.24 (6)	7.80 ± 2.94 (6)

*Each value is mean ± SE; C, control; INS, infected with no signs; IS, infected with signs; number of animals in parentheses. $P < 0.001$.

infected mice, either INS or IS, the administration of PCPA did not change significantly the content of 5HT in raphe or in cortex.

Probenecid was given to mice 7 days after the inoculation with the virus. This treatment increased 5HT and 5HIAA in raphe and cortex of control and infected mice (Fig. 4A,B). The increase of 5HT and 5HIAA in INS and IS groups was not statistically significant, perhaps because of the greater variations observed within the infected groups.

The turnover rates/$[5HT]_0$ ratio in raphe and cortex are shown in Table III. No variation of this ratio is observed between raphe and cortex of control mice under our experimental conditions. The only statistical significant modification observed within these groups was in the raphe of IS mice, in which there is a decrease of the ratio with respect to control groups (Table III, $p < 0.001$).

Histopathology. Brain of animals 7 days post-inoculation showed various abnormalities. The raphe revealed: 1) in INS mice, edema, moderate gliosis, and mild signs of necrosis in the reticular formation around the raphe; 2) in IS mice, there was disorganization of the tissue in the area, gliosis, and focal necrosis. The cortex showed necrosis in some areas.

DISCUSSION

The effect of viral infection of the CNS on the metabolism of neurotransmitters has been documented in several reports [Lycke et al, 1972; Lima et al, 1983; Bonilla et al, 1984]. Lyck and Ross [1972] showed no change of 5HT levels in the brain of mice infected with herpes simplex virus; however, values of 5HIAA were 10 times greater than those of control mice. Based on these results, the authors suggested that there was an increase in the turnover rate of 5HT in the infected mice.

Studies done in some brain regions of animals infected with various encephalytic viruses have shown high selectivity for certain regions. For instance, Levine and coworkers [1981] demonstrated that the activity of tyrosine hydroxylase decreases in striatum, mesencephalum, and hypthalamus of rats surviving an infection of the Guajira strain of VEEV. Early changes in catecholamine turnover rates were demonstrated in 8 brain areas of mice infected with Pixuna strain of VEEV [Lima et al, 1983]. However, the levels of catecholamines in striatum, mesencephalum, hypothalamus, and olfactory bulb-tuberculum did not return to normal by day 18 after inoculation [Lima et al, 1983]. These areas correspond to the areas in which tyrosine hydroxylase is decreased [Levine et al, 1981]. Moreover, in the Guajira strain, significant decrease in the density of ^3H-spiroperidol binding sites has been reported to occur in striatum, midbrain, and frontal cortex [Bonilla et al, 1984]. The observed decreases in the density of the binding sites do not suggest a generalized biochemical alteration. Moreover, the activity of some brain enzymes, such as GABA transaminase, glutamate dehydrogenase, lactate dehydrogenase, succinate dehydrogenase, and NAD-malate de-

hydrogenase did not differ significantly between control and Guajira VEEV-infected mice [Bonilla et al, 1980]. However, the preference of various viruses such as herpes simplex for the limbic system [Delsedime et al, 1984; Damasio and Vanhoese, 1985], the coronavirus for basal ganglia [Fishman et al, 1985] and certain viruses for locus coeruleus and raphe area [Maurizi, 1985a,b] constitute focal lesions in which changes may be observed. Also, strains, of VEEV of moderate or intermedian virulence were used to reduce the damage to the nervous tissue [Lima et al, 1983]. In these studies, the course of the infection may be followed easily and the results are uniform throughout the experiment, especially when a defined dose of the virus is used [Walder and Bradish, 1979]. The advantage of this type of subclinical infection of the CNS in neurochemical studies has been documented [Lima et al, 1983].

Because of the multiple regulatory mechanisms of monoamine metabolism in the CNS, the concentration of 5HT may not change even in neuronal disfunction. And even if changes occur, they may not accurately reflect neuronal activity [Neff and Tozer, 1968; King et al, 1985]. Thus, measuring 5HT turnover rate is necessary and should be estimated by various pharmacological approaches. Analysis of the accumulation of 5HT or the disappearance of 5HIAA after inhibition of MAO by pargyline has been widely reported [Tozer et al, 1966; Neckers and Meek, 1976; King et al, 1985]. The accumulation of 5HT or the decline of 5HIAA need not correspond exactly (Figs. 1A,3). This is probably the result of the existance of multiple pools of 5HT [Lane and Aprison, 1978]. Thus other methods such as the administration of inhibitors that do not completely block enzyme activity or administration of probenecid, which effect could differ in different brain regions, generate pharmacological and analytical difficulties [Neckers and Meek, 1976].

In the present report we show that the content of 5HT and 5HIAA are not significantly modified by the infection of the CNS. However, the disfunction is observed only when specific pharmacological agents are given as demonstrated by the decrease in the turnover rate of 5HT in the raphe (neurons) and to a smaller degree in the cortex (terminals) of infected mice. The fact that significant variations of turnover rate are observed in INS as well as IS mice suggests that signs of the illness could be dissociated from the biochemical damage of the brain produced by the virus.

By administering pargyline, a significant decrease in the turnover rate of 5HT in raphe at 4 and 7 days after inoculation was observed (Fig. 1). This decrease was not observed in the cortex by this method (Fig. 1). It is well known that receptors of 5HT in cell bodies terminals are different [Baumgarten and Schlossberger, 1984]. Thus the neuron-virus interaction may be more effective in the cell than in their projections.

The administration of PCPA showed differences in 5HT turnover in the cortex of infected mice (Table III). This observation supports the fact that more than one method should be used for measuring turnover of monoamines [Neckers and Meek, 1976]. However, probenecid treatment, known to be useful in the determination of monoamine turnover in the brain [Tozer et al, 1966], presents some difficulties as shown by Neckers and Meek [1976] and by the results of this study (Fig. 4). It appears that the elimination of 5HIAA in infected mice presents a high degree of variation that does not allow comparison with controls despite the fact that an increase was observed in virus-inoculated groups (Fig. 4). In contrast, blocking MAO may be used to determine turnover rates. The latter is a good index of neuronal activity in different groups of animals. Whereas other methods may be used to analyze all the steps of neurotransmitter turnover, the differences obtained with pargyline in infected mice may be due to a better regulation of synthesis, but not degradation or elimination of metabolities in the cortex (Figs. 2, 3, Table II). Further, the turnover rate/$[5HT]_0$ ratio could be used as an index of the density of serotonergic innervation [Meek and Lofstrandh, 1976]. This ratio has been reported to be greater in cell bodies than terminals, although there is disagreement in the magnitude of this difference [Neckers and Meek, 1976]. The decrease in the function of 5HT neurons in the INS group does not seem to occur because of a decrease in the innervation (Table III). The turnover rate/$[5HT]_0$ rate is smaller in the raphe of IS mice and is significantly different from the control of INS groups (Table III, $p < 0.001$). These results suggest a decrease in the density of 5HT innervation in raphe of IS and not in INS mice. This does not occur in the cortex of the same group (Table III).

The biological significance of the observed virus-induced changes in the turnover rate of 5HT is unknown. We recognize that 5HT is an important neurotransmitter that participates in several psychological and neurological functions. Thus any alteration in the level or metabolism of 5HT may have a bearing on its physiological functions.

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