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Effect of the β-Adrenoceptor Agonist Flerobuterol on Serotonin Synthesis in the Rat Brain

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ABSTRACT. The influence of 2- and 14-day treatments with flerobuterol, a preferential β_2-adrenoceptor agonist, on regional serotonin (5-HT) synthesis in the rat brain was studied by autoradiography using α-[^{14}C]methyl-L-tryptophan. Flerobuterol was delivered at a rate of 0.5 mg/kg/day using osmotic pumps implanted s.c. The 2-day flerobuterol treatment significantly increased plasma Trp, both free and total, and decreased plasma Leu and Ile. This resulted in a significant increase in the facilitated transport of Trp. There was a significant increase in the synthesis of 5-HT in the 2-day treatment group in the dorsal and median raphe as well as in all postsynaptic structures, with the exception of the hypothalamus. In contrast, after a 14-day treatment, the enhanced facilitated transport of Trp was no longer present, and the increase in the rate of 5-HT synthesis persisted only in the parietal and occipital cortex and the superior colliculus. These data suggest that flerobuterol, similar to other β-adrenergic agonists, acutely increases 5-HT synthesis, in part, through an elevation of brain Trp availability. BIOCHEM PHARMACOL **59**;6:673–679, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. α-methyl-L-tryptophan; depression; serotonin synthesis; chronic and acute treatments

It was reported that β-adrenoceptor agonists, such as isoprenaline, can increase the brain concentration of L-Tyr and L-Trp, while the plasma concentration of many amino acids is decreased [1]. This suggests that brain uptake, at least of some amino acids, can be controlled, in part, by β-adrenergic receptors. The free fraction of Trp in the plasma, however, was not measured concomitantly in these studies. The increase of brain Trp could have special relevance with respect to the synthesis of 5-HT§ because the enzyme tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of 5-HT, is not saturated by Trp. Flerobuterol, a preferential β_2-adrenoceptor agonist (K_i = 926 and 518 nM for β_1- and β_2-adrenoceptor binding, respectively [2]), exhibits antidepressant activity in animals and was shown to enhance serotonergic neurotransmission using *in vivo* electrophysiological techniques [3, 4]. Furthermore, the β_2-adrenergic agonist clenbuterol is reported to act as an antidepressant in depressed patients [5]. It is believed that enhanced 5-HT neurotransmission in some situations is directly related to the 5-HT concentration, such as during MAO inhibition, and it probably is also dependent on the rate of 5-HT synthesis. Since the major classes of antidepressant treatment, including MAO inhibitors, enhance 5-HT neurotransmission [6], it is thus possible that flerobuterol could act on the 5-HT system and exert its antidepressant-like effect in animals by altering 5-HT synthesis.

The influence of norepinephrine on the brain serotonergic system has been documented in different types of investigations [7–9]. It has been shown that the brain uptake of the LNAA (e.g. Trp, Tyr) can be affected by the β-adrenergic agonists isoprenaline [10], isoproterenol, salbutamol [11], and clenbuterol [12] despite a significant reduction in a majority of the LNAA in the rat plasma. These investigations did not determine the fraction of free Trp in the plasma and, thus, it cannot be concluded that the influence of the Trp free fraction is related to the increase in the brain Trp. The conclusion was that, despite a reduction in the plasma concentration of the majority of the LNAA, their brain concentrations were increased. This might suggest that the transport of LNAA is under the direct control of β-adrenergic receptors. Because of these data, suggesting a direct effect of β-adrenergic agonists, we have measured plasma concentration of the LNAA, and calculated the facilitated Trp flux (ν) separately for total (ν_T) and free (ν_f) plasma Trp [13]. These quantities would reflect facilitated flux of Trp in competition by other LNAA sharing the same carrier.

In the present study, measurements of the regional 5-HT synthesis using a recently developed autoradiographic method were carried out [14–16]. It was deemed important to investigate brain 5-HT synthesis in rats treated with flerobuterol for different lengths of time because it was shown that short- and long-term flerobuterol administra-

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§ *Abbreviations*: 5-HT, serotonin; α-MTrp, α-methyl-L-tryptophan; MAO, monoamine oxidase; and LNAA, large neutral amino acids.

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tion differentially affect the brain 5-HT system [4]. The α-MTrp method utilizes tracer kinetics and does not require the administration of any other drug to measure the rate of synthesis, thus precluding any influence of auxiliary drugs such as the aromatic amino acid decarboxylase inhibitor NSD-1015, which can, on its own, alter 5-HT synthesis [17].

MATERIALS AND METHODS

Materials

α-[^{14}C]MTrp (sp. act. ≈ 55 mCi/mmol) was synthesized by us, with the method described by Mzengeza *et al.* [18], from [^{14}C]CH_3I purchased from Amersham Canada Ltd. The stereochemical purity was confirmed by HPLC using a chiral column. The chiral and radiochemical purity of the radiopharmaceutical was greater than 98%. Ultrafree-MC filters (Cat. No. UF3LGCOO) with a 10,000 MW cutoff point were purchased from Millipore Canada Ltd. The HPLC solvents were purchased from the Baker Chemical Co.

Experimental Procedures

Male Sprague–Dawley rats weighing about 250 g were treated continuously for 2 or 14 days with saline (controls) or with flerobuterol (0.5 mg/kg/day, s.c.), delivered by osmotic minipump (pump models 2ML1 and 2ML2 for 2- and 14-day infusion, respectively). The minipumps were implanted under chloral hydrate anaesthesia (400 mg/kg, i.p.). This dose of flerobuterol was chosen as the one producing central effects with minimal peripheral effects [3]. After implantation of minipumps, rats were returned to their cages. After either 2 or 14 days of treatment, the rats were anaesthetized with halothane (1 to 1.5%), and arterial and venous catheters were implanted, as part of a normal tracer procedure (see details for animal handling in Ref. 15). Animals were food-deprived for some 20 hr before injection of tracer; however, water was provided *ad lib*. About 2 hr after the rats woke up from anaesthesia, about 50 μCi of α-[^{14}C]MTrp in 2 mL of saline was injected over 2 min using an infusion pump. The arterial plasma samples were taken at increased time intervals for input function determination, and additional samples were collected for the determination of the plasma amino acids (Val, Leu, Ile, Met, Tyr, Phe, Trp) and the plasma concentration of free Trp. The free Trp was determined in the plasma ultrafiltrate [14]. Arterial pCO_2, pO_2, pH, blood pressure, and hematocrit were checked periodically, and in all animals were within laboratory standards. Body temperature, measured through a rectal probe, was maintained at 37° with a heating lamp. To minimize as much as possible any influence of circadian rhythm on the results, the tracer was always injected between noon and 2:00 p.m. Rats were decapitated 60 or 150 min after tracer injection, and brains were extracted, frozen, and cut at about −20° into 30 μm slices in a microtome. The brain slices were contacted to an x-ray sensitive film for 3 weeks along with ^{14}C-standards (tissue equivalent) and developed. Quantification of autoradiograms was done with the aid of a computerized image analyzer (The Image Calculator, Soquelec Ltd.), and tracer concentrations (nCi/g tissue) were determined in thirty structures.

Calculation of the Rate of 5-HT Synthesis

The rates of 5-HT synthesis were calculated by using an approximation method described in details in our previous publications [14, 16, 19]. The method was shown to give results not significantly different from those obtained by the two-time point method described by Nagahiro *et al.* [15]. The method is based on the assumption that the apparent volume of distribution of the precursor pool (V_0; mL/g) is approximately the same in all brain structures, and has a value of 0.45 ± 0.1 mL/g [16, 19]. In the calculations presented here, equality of the V_0 in the control and treatment groups was assumed. Tissue tracer concentrations in each structure and for individual rats were converted into volume of distribution (V_D) by dividing it with the plasma tracer concentration at the end of the experiment [$C_p^*(T)$]. The V_D was then corrected for the contribution of the precursor pool by subtracting 0.45 (V_o; mL/g) and dividing by the exposure time Θ [$\Theta = \int_0^T C_p^*(t)dt/C_p^*(T)$]. The rates of 5-HT synthesis were calculated as per Eq. 1 for each structure and for individual rats, and the average for the group and the standard deviations were calculated from these individual values [16, 19],

$$R = \frac{C_p}{LC} \cdot \frac{V_D - V_0}{\Theta} \tag{1}$$

where C_p (pmol/mL) is the plasma concentration of free Trp and LC is the conversion factor taken to be equal to 0.42 ± 0.07 [20]. LC is a conversion factor that converts the rate of tissue uptake of tracer (α-[^{14}C]MTrp) into the uptake of Trp via 5-HT pathway, which after multiplying by plasma free Trp gives the rate of 5-HT synthesis [20].

The facilitated Trp flux (ν; nmol/g/min) from blood to brain was calculated by the Michaelis–Menten formula [21],

$$\nu = \frac{C_p \cdot V_{\max}^{Trp}}{K_m^{Trp}\left(1 + \frac{C^{AA}}{K_m^{AA}}\right) + C_p} \tag{2}$$

where C_p (nmol/mL) is the plasma concentration of Trp, and C^{AA} and $K_m{}^{AA}$ are concentrations and the Michaelis–Menten constant, respectively for the competing amino acids. The K_m values used in these calculations were taken from Miller *et al.* [22].

The HPLC system consisted of a reverse phase column, a fluorescent detector (excitation filter, 330 nm; fluorescence filter, 465 nm), and the buffer as elution solvent with post-column derivatization. Standards were prepared from

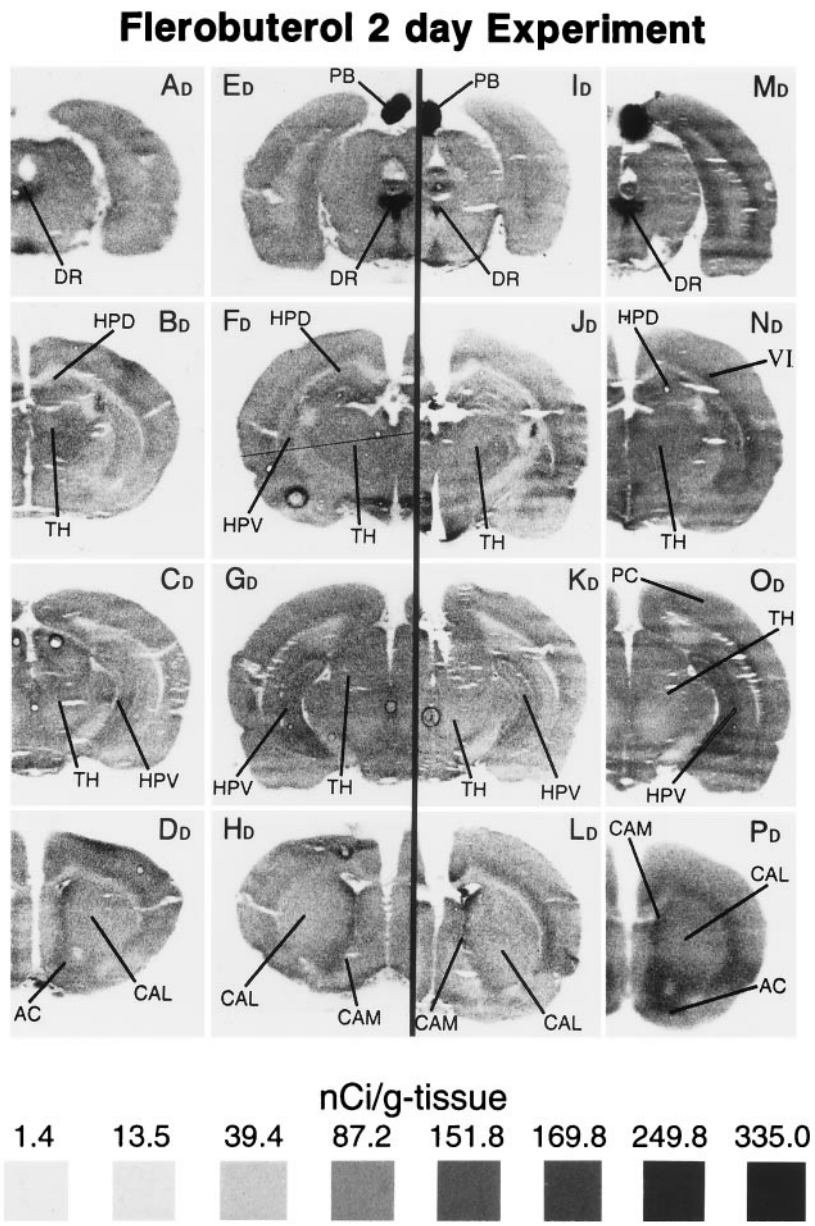

FIG. 1. A set of radiochromatograms obtained in rats treated with flerobuterol (0.5 mg/kg/day delivered by osmotic pump) for 2 days (images I_D to P_D) and respective controls receiving saline without flerobuterol (images A_D to H_D). Images given in A_D to D_D and I_D to L_D are for rats killed 60 min after tracer injection, and those given in E_D to H_D and M_D to P_D are for rats killed 150 min after tracer injection. Some brain structures are identified with letters: dorsal raphe (DR); pineal body (PB); hippocampus-dorsal (HPD); hippocampus-ventral (HPV); thalamus (TH); caudate-lateral (CAL); caudate-medial (CAM); nucleus accumbens (AC); parietal cortex (PC); and layer VI of cortex (VI).

pure chemical and contained about 20 nmol/mL of each LNAA.

RESULTS

There were no significant differences in the physiological parameters or body weight between the groups of control and treated rats. A representative set of autoradiograms is shown in Fig. 1 for 2-day control and treated rats, and in Fig. 2 for 14-day control and treated rats. In general, a large accumulation of the labelled tracer in the brain structures known to have a large concentration of serotonergic cells (e.g. raphe) was readily detectable.

Plasma concentrations of Trp and other amino acids are given in Table 1. There was a decrease in 2-day flerobuterol-treated rats in the plasma concentration of Ile (24%) and Leu (21%) and an increase of total and free plasma Trp (54 and 57%, respectively). In contrast, in the 14-day experiments, there was only a significant increase of the Leu concentration as compared with the respective controls. In the 2-day group, there was also an increase in the facilitated Trp flux, calculated with both free (ν_f) and

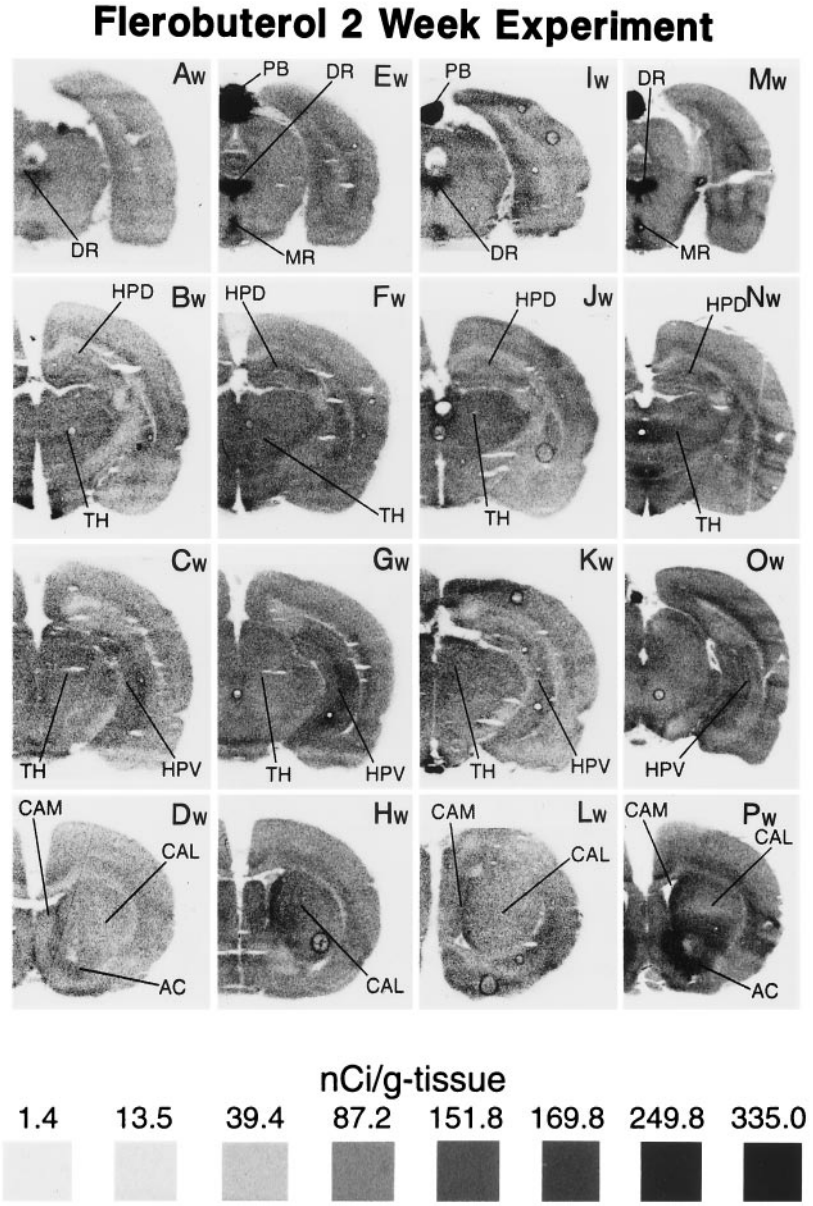

FIG. 2. A set of radiochromatograms obtained in rats treated with flerobuterol (0.5 mg/kg/day delivered by osmotic pump) for 14 days (images I_W to P_W) and the respective controls receiving saline without flerobuterol (images A_W to H_W). Images given in A_W to D_W and I_W to L_W are for rats killed 60 min after tracer injection, and those given in E_W to H_W and M_W to P_W are for rats killed 150 min after tracer injection. Some brain structures are identified with letters: dorsal raphe (DR); median raphe (MR); pineal body (PB); hippocampus-dorsal (HPD); hippocampus-ventral (HPV); thalamus (TH); caudate-lateral (CAL); caudate-medial (CAM); and nucleus accumbens (AC).

total (v_T) Trp. The increase could be related, in part, to a significant increase of plasma Trp and to a decrease in Leu and Ile. Since there is no evidence that plasma Trp is linearly related to the brain 5-HT synthesis rate, it would not be appropriate to normalize brain 5-HT synthesis rates according to plasma Trp. In short, the change in the plasma Trp and some other amino acids should be considered as characteristics of the flerobuterol treatment.

The rates of synthesis in selected discrete brain structures are given in Table 2 for 2-day flerobuterol-treated rats and in Table 3 for 14-day flerobuterol-treated rats. In the 2-day treatment group, a highly significant increase, as compared with the 2-day controls, was observed in the rate of 5-HT synthesis. In the 14-day experiment, only parietal and occipital cortices and the superior colliculus still showed an increase in 5-HT synthesis, while there was a significant decrease in the mammillary body and the medial part of the caudate nucleus (Table 3). It should be noticed that while 2-day treatments produced decreases in the plasma concentrations of some amino acids with a significant increase in total and free Trp, the 14-day treatment resulted in a significant increase (15-%) in leucine (Table 1) with non-significant changes in other amino acids.

TABLE 1. Plasma concentration of some essential amino acids sharing the same carrier with Trp in 2- and 14-day flerobuterol-treated rats and respective controls

	2 days		14 days	
	Control (N = 10)	Treated (N = 10)	Controls (N = 14)	Treated (N = 13)
	Plasma concentration (nmol/mL)			
Val	364 ± 45	324 ± 67	406 ± 34	424 ± 31
Met	66 ± 7	64 ± 7	66 ± 5	61 ± 6
Lle	167 ± 21	127 ± 30*	97 ± 11	106 ± 15
Leu	337 ± 37	265 ± 35*	254 ± 21	291 ± 19*
Tyr	65 ± 7	66 ± 9	94 ± 8	94 ± 9
Phe	104 ± 15	99 ± 14	99 ± 8	109 ± 13
Trp (total)	44 ± 10	68 ± 15*	82 ± 10	89 ± 10
Trp (free)	3.7 ± 0.6	5.8 ± 0.9*	6.6 ± 1.5	7.2 ± 1.7
ν_f†	0.098 ± 0.027	0.17 ± 0.04	0.19 ± 0.07	0.19 ± 0.07
ν_T†	1.10 ± 0.37	1.84 ± 0.56*	2.09 ± 0.60	2.12 ± 0.57

Values are means ± SD; N represents the number of rats.
*Indicates a significant difference ($p < 0.05$; ANOVA).
†Represent-facilitated Trp fluxes calculated according to Eq. 2.

DISCUSSION

In the present study, the 2-day flerobuterol treatment resulted in a decrease of some amino acids in plasma, but in a significant increase of plasma Trp (Table 1). This decrease of some amino acids and increase in the free Trp resulted in a significant increase of the facilitated flux of Trp (both ν_f and ν_T). In addition to the facilitated transport of Trp into the brain, there could be a contribution from diffusion of Trp because the diffusion constant in normal rat brain seems to be substantial [23]. This increase in the influx of Trp (both facilitated and diffusion) is responsible, at least in part, for the increases of Trp reported in the brain following treatments with adrenergic agonists [10–12]. It is of interest to note that in 14-day flerobuterol-treated rats there was no significant difference in the plasma free or total Trp between control and treated rats (Table 1). In addition, there was only a significant increase in leucine in the 14-day treated group. However, there was no significant differences in the facilitated transports of either total or free Trp (ν_f and ν_T; Table 1).

In the autoradiographic experiments, a significant increase in 5-HT synthesis was observed in all brain structures except for the hypothalamus in the 2-day treatment group

TABLE 2. Comparison of the rates of 5-HT synthesis in saline-treated (control) rats with that in rats injected for 2 days with flerobuterol at a rate of 0.5 mg/kg/day

Structure	Control	Treated	% Difference from controls*
	5-HT synthesis (pmol/g/min)		
Dorsal raphe	104 ± 12	130 ± 12	+25
Medial raphe	87 ± 12	116 ± 11	+33
Hippocam. ventral	58 ± 6	83 ± 13	+43
Hippocam. dorsal	51 ± 4	72 ± 13	+41
Hypothalamus	55 ± 9	57 ± 11	+9 (NS)
Thalamus	41 ± 4	63 ± 10	+50
Mamillary body	46 ± 7	70 ± 12	+52
Ventr. tegm. area	43 ± 5	58 ± 12	+35
Parietal cortex	39 ± 5	53 ± 9	+36
Temporal cortex	47 ± 7	65 ± 14	+38
Occipital cortex	39 ± 4	56 ± 7	+44
Caudate-medial	51 ± 7	69 ± 13	+35
Caudate-lateral	50 ± 8	65 ± 11	+30
Medial geniculate	54 ± 6	80 ± 13	+48
Superior colliculus	54 ± 4	76 ± 12	+41
Amygdala	42 ± 4	56 ± 13	+33

Values are means ± SD of ten rats.
*All differences were significant ($P < 0.05$; ANOVA) except that in hypothalamus, indicated as NS.

TABLE 3. Comparison of the rates of 5-HT synthesis in saline-treated (control) rats with that in rats injected for 14 days with a continuous infusion of 0.5 mg/kg/day of flerobuterol

Structure	Control (N = 14)	Treated (N = 13)	% Difference from controls*
	5-HT synthesis (pmol/g/min)		
Dorsal raphe	178 ± 16	187 ± 16	NS
Medial raphe	135 ± 13	131 ± 12	NS
Hippocam. ventral	73 ± 7	70 ± 9	NS
Hippocam. dorsal	62 ± 7	56 ± 10	NS
Hypothalamus	74 ± 7	72 ± 7	NS
Thalamus	46 ± 4	42 ± 7	NS
Mamillary body	196 ± 15	172 ± 20	−14†
Ventr. tegm. area	112 ± 11	110 ± 18	NS
Parietal cortex	38 ± 7	46 ± 7	+20†
Temporal cortex	61 ± 7	62 ± 9	NS
Occipital cortex	49 ± 6	54 ± 7	+10†
Caudate-medial	74 ± 12	62 ± 17	−16†
Caudate-lateral	63 ± 14	60 ± 15	NS
Medial geniculate	59 ± 7	62 ± 9	NS
Superior colliculus	58 ± 6	66 ± 10	+13†
Amygdala	67 ± 10	65 ± 13	NS

Values are means ± SD; N represents the number of rats.
*All differences were determined using ANOVA.
†Indicates a significant ($P < 0.05$) difference when compared with the control group.

(Table 2). Since there was a similar increase in plasma free Trp, and the plasma free Trp has been related by many investigations to brain 5-HT synthesis [13, 24], the increase in the brain 5-HT synthesis could be attributed, in large part, to the increase in the plasma Trp. It also should be noted (see above) that Trp has a rather large passive diffusion component [23] that also could contribute Trp to the brain Trp pool. It is of interest to note that the increase in the raphe nuclei is similar to the increase in other structures, suggesting that there was no additional influence on 5-HT synthesis mediated via somatodendritic 5-HT_{1A} autoreceptors, which are known to reduce cell body 5-HT synthesis after an increase in the tissue 5-HT levels [17, 25, 26]. In our previous work, the 5-HT releaser/reuptake blocker fenfluramine reduced synthesis in the cell body region of 5-HT neurons [26], while the 5-HT_{1A} agonist buspirone acutely decreased 5-HT synthesis throughout the rat brain [27]. However, one has to consider that fenfluramine also decreases 5-HT neuronal firing as it enhances the extracellular availability of 5-HT [28], which would, in turn, account for the decreased 5-HT synthesis rate. In this respect, the reduction in the firing of dorsal raphe neurons reported by Bouthillier *et al.* [4] in 2-day flerobuterol-treated rats would support results indicating an increase in the 5-HT synthesis in the 2-day treated rats, because by increasing 5-HT synthesis, flerobuterol likely increases the availability of 5-HT. This interpretation finds direct support by the restoration of 5-HT neuronal firing in 2-day flerobuterol-treated rats using the 5-HT_{1A} autoreceptor antagonist spiperone [4]. Our observation that flerobuterol increases the rate of 5-HT synthesis is also in agreement with previous reports that β-adrenoceptor agonists increase 5-HT synthesis in the rat brain [29–31].

In the 14-day flerobuterol treatment, there remained a significant increase in 5-HT synthesis in only three structures (Table 3). Since there was no difference in the plasma Trp between treated and control groups (Table 1), the changes in the 5-HT synthesis observed could be directly related to pharmacological action(s) of flerobuterol. In 14-day flerobuterol-treated rats, Bouthillier *et al.* [4] reported a significantly increased effect of electrical stimulation of the ascending 5-HT in suppressing the firing activity of dorsal hippocampus pyramidal neurons. This was suggested to be the result of a greater amount of 5-HT released for each stimulation-triggered action potential. However, the synthesis measurement in the dorsal hippocampus reported here showed no significant alteration in the 5-HT synthesis rate in the flerobuterol-treated rats when compared with the respective controls. Since Bouthillier *et al.* [4] reported an enhanced 5-HT neurotransmission in the dorsal hippocampus in 14-day flerobuterol-treated rats, the present data suggest that this enhanced neurotransmission is not correlated with 5-HT synthesis unless there is inhibition of MAO with flerobuterol or its metabolites, which has not been shown thus far. Therefore, the exact neurobiological mechanism by which flerobuterol enhances 5-HT neurotransmission in the rat hippocampus following a 2-week treatment remains to be elucidated.

The effect of flerobuterol on 5-HT synthesis observed after 2 days but not 14 days of treatment was likely due to adaptive processes in the 5-HT rather than the β-adrenergic system. Indeed, there is little physiological evidence for adaptation of β_2-adrenoceptors following their long-term activation. Patients with asthma who inhale salbutamol several times a day do not present any clear evidence of attenuated responsiveness, certainly not over a time period similar to that used in the present study.

In summary, short-term flerobuterol treatment has a significant enhancing effect on plasma Trp and some other amino acids competing for the same blood–brain barrier transporter. A 2-day treatment produced a significant increase in 5-HT synthesis throughout the brain, which persisted in only a few structures as the treatment was prolonged to a 14-day period. This adaptive change, and the gradual enhancement of 5-HT neurotransmission observed in a previous electrophysiological study [4], are fully consistent with the delayed onset of action of β_2-adrenoceptor agonists in major depression.

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