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AUTORADIOGRAPHIC EVALUATION OF THE INFLUENCE OF HYPOTHALAMIC 5,7-DIHYDROXYTRYPTAMINE LESION ON BRAIN SEROTONIN SYNTHESIS

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Abstract—The influence of a unilateral stereotaxically induced 5,7-dihydroxytryptamine (5,7-DHT) lesion in the dorsolateral hypothalamus on brain serotonin synthesis was evaluated by an autoradiographic method, using labelled α-methyl-L-tryptophan (α-MTrp). The hypothalamus was selected as the lesion site because it receives well defined and relatively large projections from the raphe nuclei. Data suggest that the unilateral lesion in the dorsolateral hypothalamus had a significant influence (an increase) on the rate of serotonin synthesis in the large majority of ipsilateral brain structures examined. It seems that the effect was the greatest in the hippocampal structures, the thalamus, and the parietal and sensory motor cortices. The average increase in the rate of serotonin synthesis on the lesion side when compared with the contralateral side was between 3% (amygdala) and 52% (dorsal hippocampus; CA_3 layer of hippocampus). Since in the sham-injected rats (same volume of saline) there was no obvious injection-contralateral side asymmetry observed (except for two structures, probably affected by the injection needle, which showed a significant difference), we concluded that the effect observed in the present study was most likely related to the 5,7-DHT-induced lesion on the serotonergic terminals in the hypothalamus. Comparison of the rate of synthesis in the dorsal and medial raphe and the pineal body with the rates reported earlier for these structures led us to conclude that either the 5,7-DHT lesion in the hypothalamus did not influence the rates in these structures in their entirety, or the method used was not sensitive enough to reveal this influence. Data reported here also demonstrate how a highly specific tracer (α-MTrp), in conjunction with a specific and localized lesion, could aid our understanding of the brain serotonergic system.

Key words: serotonin synthesis; α-MTrp method; hypothalamic lesion; 5,7-dihydroxytryptamine

5,7-DHT§ has been used as a specific serotonergic neurotoxin [1], which enters the serotonergic neurons via 5-HT uptake sites. 5,7-DHT in larger doses is also neurotoxic to the noradrenergic neurons, and an uptake inhibitor for the catecholamine uptake system is usually injected before 5,7-DHT [2] to protect noradrenergic neurons. The protection of noradrenergic neurons is of importance when a morphological study is done. However, when a very specific label is used for the measurement of the rate of serotonin synthesis (in the present experiments α-MTrp), the protection of noradrenergic neurons is considered to be unnecessary. Stereotaxically injected 5,7-DHT has been shown to be a very convenient method for producing highly localized denervation of brain serotonergic terminals [3]. Soon after intracerebral injection of 5,7-DHT, destruction of serotonergic terminals is localized. The great advantage of stereotaxical intracerebral injection in comparison with intraventricular injection is that it enables us to evaluate the influence of local axotomy on the rate of serotonin synthesis in remote brain structures that cannot be influenced directly by a neurotoxin. This permits us to elucidate brain serotonin synthesis control.

Our newly developed autoradiographic method for the measurement of the rate of serotonin synthesis was used. The principle of the method is measurement of the tissue trapping of radioactively labelled α-MTrp, most likely as a metabolite. α-MTrp was shown to be a metabolic and functional analog of Trp [4–7], and its metabolite, α-M5-HT, was shown to be a functional analog of serotonin [4, 8, 9]; it can functionally replace 5-HT as a substituted neurotransmitter. The rate of the tracer tissue uptake is converted into the serotonin synthesis rate by multiplying it with the plasma concentration of free Trp and dividing it by the LC; a correction factor

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§ Abbreviations: 5,7-DHT, 5,7-dihydroxytryptamine; α-MTrp, α-[^{14}C]methyl-L-tryptophan; Trp, L-tryptophan; 5-HT, 5-hydroxytryptamine (serotonin); α-M5-HT, α-methyl serotonin; TPH, tryptophan hydroxylase; *LC*, lumped constant; MFB, medial forbrain bundle; VD, volume of distribution; and SED, standard error of the difference.

takes into account that the measurements are done with an analog [5, 10]. This autoradiographic method permits measurement in a large number of brain structures and has good anatomical resolution (≈0.1 mm). Since the *in vivo* activity of TPH is accepted as the rate-limiting step in the synthesis of serotonin, and since the tracer is accumulated as a function of TPH activity [5, 11], the assumption that this method assesses the *in vivo* activity of TPH is reasonable.

Here we report a profound effect (an increase) on the serotonin synthesis rate (probably a very good measure of *in vivo* activity of TPH) of the 5,7-DHT lesion in the dorsolateral hypothalamus, seen throughout the ipsilateral brain. The dorsolateral hypothalamus was selected as the site of the lesion because it receives well-defined projections from the dorsal raphe [12–14]. It has also been shown that the greatest accumulation of radioactivity in the hypothalamus occurs after injection of ^{3}H-labelled amino acids in the raphe nuclei [15]. As a result of these relatively large neuronal projections into the hypothalamus, it was expected that the effect, if present, should be the easiest to measure when the lesion was done in this structure. Evaluation of the left–right differences in many brain structures was also possible, because the simplified method permitted estimation of the synthesis rate in different structures on each side of the brain and in each animal. It was hypothesized that the unilateral lesion most likely produced unilateral changes in the rate of synthesis because of the predominant ipsilateral projections of dorsal and medial raphe [16]. Due to the wide distribution of the serotonergic system throughout the brain, mainly ipsilateral to the raphe nuclei, it was also hypothesized that even a highly localized lesion should have widespread effects on the rate of serotonin synthesis in the ipsilateral brain.

MATERIALS AND METHODS

Materials. α-[^{14}C]Methyl-L-tryptophan (sp. act. = 55 mCi/mmol) was synthesized by us [17] from [^{14}C]-CH_3I supplied by Amersham Canada Ltd. A hydrochloride salt of α-MTrp dissolved in normal saline was used in these experiments. 5,7-DHT was purchased from the Sigma Chemical Co. (St. Louis, MO). Other chemicals used in experiments (e.g. solvents for HPLC) were purchased from a local supplier and were always of the highest purity available. A reverse-phase HPLC column was used for the plasma amino acid analysis.

Animal procedures and 5,7-DHT lesion. The male Sprague–Dawley rats (200–230 g) used in these experiments were housed for at least 3 days in temperature-controlled animal quarters, with lights on from 7.00 a.m. to 7.00 p.m. and food and water were given *ad lib.* A saline solution (250 nL) [3, 18] containing 3 μg of 5,7-DHT (free base) and 0.1 mg/mL of ascorbic acid was injected into the dorsolateral hypothalamus (solution was always freshly prepared). Stereotaxic coordinates of injections were in mm from Bregma: −3.14 post, 1.0 lateral, and 8.1 ventral (below brain surface) [19]. Coordinates were adjusted slightly for the size of the rat. Sham animals were injected stereotaxically with 250 nL of solvent. All intracerebral injections were done under halothane anesthesia in a rat stereotaxic frame. Injection of 250 nL of solution was done over 15 min, and the needle was left in place for at least 10 min after the end of the injection to avoid as much channeling of the neurotoxin as possible. After lesioning and closure of the burr hole with a wax insert, the wound was packed locally with 2% Xylocaine and closed, and rats were returned to their cages. Rats were monitored until they woke up and started to move and take in food and water. Then they were returned to the animal quarters and given food and water *ad lib.* (see above). All procedures used with animals in this research were approved by the Institutional Animal Care Committee and were carried out in accordance with the NIH guidelines.

Since all lesions were done as described by Frankfurt and Azmitia [3] and were shown to be very reproducible in our other experimental protocols done in the laboratory [18,*], the lesions were not evaluated separately to document the extent of neuronal damage in each rat. However, it was clearly visible on all autoradiographic films that the MFB was more pronounced on the lesion side as described before [18]; there was always obvious asymmetry seen in contrast to the sham-injected rats, in which there was symmetry in the MFB. The above observation was considered to be the best indicator of a successful lesioning. In addition, the most recent experiments in which TPH immunostaining was done confirmed the reproducibility of these lesions.*

Animals were given food and water *ad lib.* until the night before the tracer injection (5 days after the lesion was produced) when food was removed. Five days after the induction of the 5,7-DHT lesion or sham injection, and at least 2 hr before the tracer injection, rats were anesthetized with halothane (1.0 to 1.5%), and arterial (for blood sampling) and venous (for tracer injection) catheters were implanted under an operating microscope. After surgery, the lower part of the body was immobilized by a loose-fitting plaster cast, and rats were allowed to awaken. The arterial pH, $PaCO_2$, PaO_2, blood pressure, and hematocrit were checked before and during the experiment. There was no significant difference in any of these parameters compared with those considered normal for the laboratory [5, 11]. There was also no difference in these physiological parameters between control (saline-injected) and lesioned rats. During the experimental procedure, a rectal temperature of approximately 37° was maintained with a heating lamp [20].

Autoradiography. α-MTrp (50 μCi in 1 mL of saline) was injected via venous catheters 5 days after 5,7-DHT lesion (seven rats) or sham injection (five rats) into rats implanted with arterial and venous catheters as described above. Tracer was injected as a constant infusion over 2 min with the aid of an infusion pump. Arterial blood samples (50 μL) were taken at progressively increased time intervals from the beginning of the tracer injection until rats were decapitated [11]. Plasma was separated and

* Ljubić V, Raison S, Weissman D, Hamel E and Diksic M, manuscript in preparation.

deproteinized, and radioactivity was measured by liquid scintillation counting to obtain plasma input function. In addition to these samples (12–15), five samples were taken for the determination of plasma total tryptophan (Trp; two) and free Trp (three), using HPLC [7]. For determination of the plasma-free Trp by HPLC, plasma samples were filtered through a Millipore filter with a cutoff of 10 kDa. Rats were decapitated at 2 hr (two rats), 3 hr (three rats), and 4 hr (two rats) after tracer injection. (This experimental design permitted calculation of the synthesis in two different ways: see later.) Brains were extracted and cut into 30-μm thick slices in a cryostat at −20° and mounted onto microscopic glass slides by drying on a hot plate at 60° [11]. Brain slices were exposed, along with ^{14}C-standards (calibrated in nCi/g of the brain tissue equivalent), to an X-ray film for 3 weeks. Images were digitized with the aid of a personal computer-based image analyzer (The Image Calculator, Soquelec Ltd., Montreal). Usually a third-order polynomial was utilized as a calibration curve for conversion of the optical density into the tissue tracer concentration. Tissue concentration of tracer was measured separately in some twenty-five brain structures on the lesion and contralateral sides.

Calculation of the rate of serotonin synthesis. Since the rationale and basic data on which the method is based have been published previously [5, 20–22], this information will not be repeated here. We will give only a very brief summary of the methods used. Tracer tissue concentrations measured by the image analyzer were converted into VD (mL/g) by dividing them with the plasma tracer concentration at the end of the experiment: $Cp^*(T)$. The plasma input function was used to calculate exposure time (θ) as the ratio of the plasma integral and the plasma tracer concentration at the end of the experiment ($Cp^*(T)$). [$\theta = {}_0\int^T Cp^*(t)\cdot dt/Cp^*(T)$; here $Cp^*(t)$ is the plasma tracer concentration (nCi/g) measured at different times after tracer injection.] The slope (K^*) of the linear relationship between θ and the VD, after an apparent steady-state, was calculated and used in the calculation of the rate of serotonin synthesis (pmol·g^{-1}·min^{-1}) by equation [11, 21]:

$$R = \frac{Cp}{LC}\cdot K^* \qquad (1)$$

where Cp is plasma concentration of free Trp (pmol·g^{-1}·min^{-1}) and LC is the lumped constant ($LC = 0.42 \pm 0.07$) [22]. To permit utilization of paired statistics (left to right comparison in the same rat) in the individual brains, the rates of synthesis were also calculated as:

$$R = \frac{VD - V_0}{\theta\cdot LC}\cdot Cp \qquad (2)$$

where V_0 is the steady-state (or near steady-state) apparent volume of distribution of tracer or the volume of the precursor pool. All other symbols are as above. The rates estimated by Equation 2 are based, in addition to the comparison here, on our previous work. In Equation 2, a V_0 of 0.45 mL/g was used. This value was shown to be an average value for the precursor apparent volume of distribution.* There was excellent agreement between rates calculated by both approaches (see later). The latter approach also was evaluated recently in a set of normal rats,* and was found to give results not significantly different from those obtained by the approach utilizing the estimation of K^* from the time–activity plots.

Statistical comparison. The rates of synthesis on lesion and non-lesion sides were compared by the paired two-tailed t-test. The paired t-test is equivalent to, and gives the same results as, the repeated measure ANOVA. We used the SIGMASTAT (Jandel Scientific, San Rafael, CA) computer code for this analysis. Comparison of the serotonin synthesis rates obtained by the two different methods (Equations 1 and 2) was done using the two-tailed t-test and the one-way ANOVA.

RESULTS

There was no difference (data not shown) in the physiological parameters (PaO_2; $PaCO_2$; blood pH; body weight; body temperature and blood pressure) of rats undergoing injection of saline (sham-injected rats; two in this protocol and three in the protocol from which the results were reported, in part, elsewhere [18]) and those injected with 5,7-DHT in saline (seven rats). There was also no difference between physiological parameters in these groups of animals, when compared with those used in other experiments in the laboratory [5, 11].

A set of representative autoradiograms obtained in rats injected stereotaxically and unilaterally into the dorsolateral hypothalamus with 5,7-DHT (A–D) and saline (E–H; sham-injected rats) is presented in Fig. 1. The entrance of the injection needle produced a cortical increase in the serotonin synthesis rate marked by "N" (Fig. 1C). The asymmetry (left to right comparison) in the MFB was seen clearly in the rats injected with 5,7-DHT (Fig. 1, C and D). There was no asymmetry in the MFB in rats injected with saline (Fig. 1, F and G). An asymmetry in the images of rats injected with toxin could also be noticed in some other structures (Fig. 1). An enlargement of the dorsal raphe image shown in Fig. 1B exemplifies an asymmetry seen in the lateral wings (LV compared with RV) of the dorsal raphe of rats injected with 5,7-DHT. However, the rate of serotonin synthesis was not determined in different parts of the dorsal raphe because of limited resolution of the ^{14}C-images and unclear visualization of lateral wings in all rats. The asymmetry in the lateral wings was never seen in the sham-injected rats (Fig. 1, E–H).

To exemplify the agreement between the serotonin synthesis rates obtained by Equation 1 (open bars) and those by Equation 2 (solid bars), the results (means plus SEM) of these two approaches are shown for comparison in Fig. 2. Comparing the rates of synthesis by the two different methods, on both the lesion and contralateral sides, using the one-way

* Diksic M, Nagahiro S and Grdiša M, The regional rate of serotonin synthesis estimated by the α-methyl-tryptophan method in rat brain from one time method. *J Cereb Blood Flow Metab*, in press.

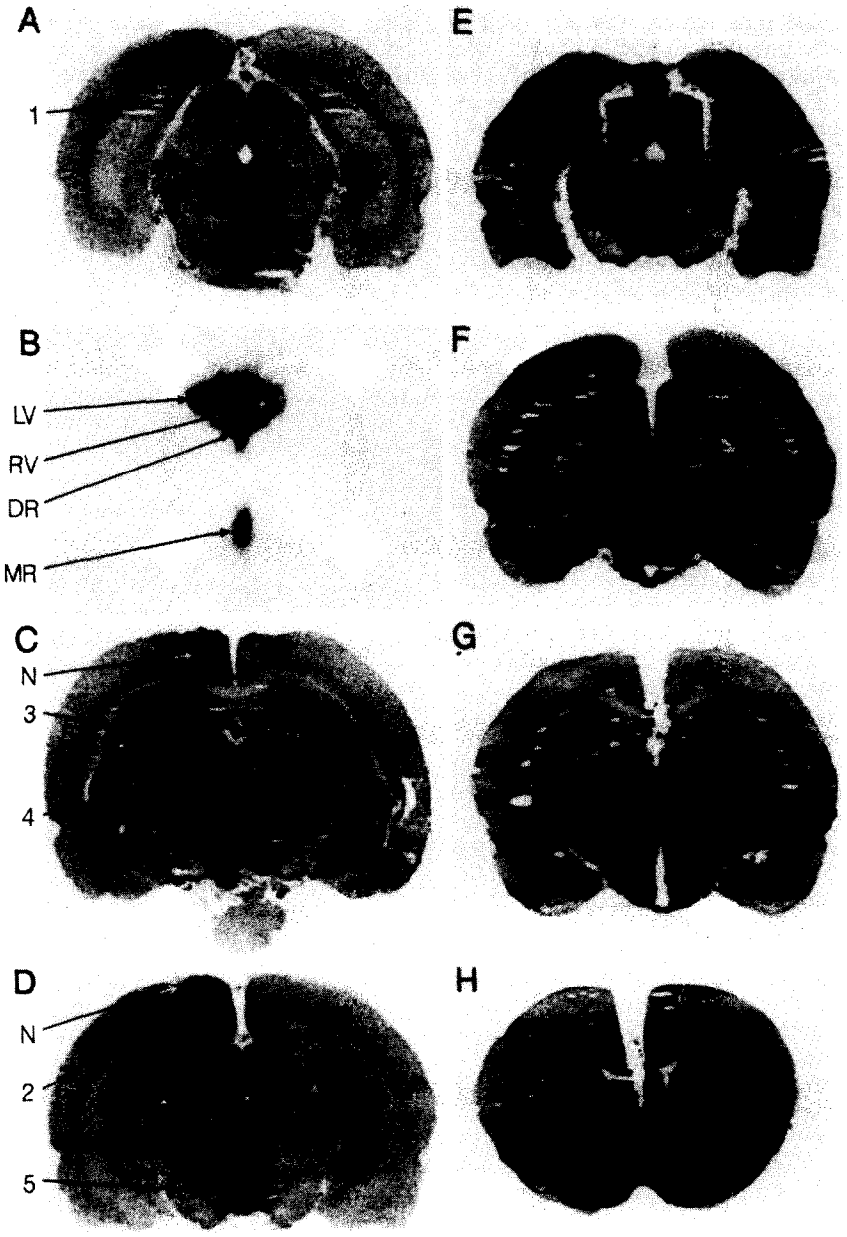

Fig. 1. Representative autoradiograms obtained in rats injected with 5,7-DHT in the dorsolateral hypothalamus (A–D) and those injected with saline (sham-injected; E–H). Asymmetry (left to right) can be noticed in autoradiograms A–D. An enlarged image of the dorsal raphe (Fig. 1B; DR) exemplifies the asymmetry visible especially in the lateral wings (LV and RV), which have more radioactivity ipsilateral to the lesion side (LV is more intense than RV). An increase of radioactivity was also noticed in all images in the vicinity of the brain injured by the entrance of the injection needle (N). A few structures are identified by arrows and numbers: (1) visual cortex; (2) parietal cortex (distal from the lesion); (3) hippocampus; (4) thalamus; and (5) medial forebrain bundle on the lesion side. The asymmetry is probably less pronounced in these images because of the non-linear relation between the tracer concentration and optical density. There was no obvious asymmetry seen in the sham-injected animals. The symmetry could especially be noticed in panel E (dorsal raphe) and panel H (medial part of the caudate).

ANOVA, showed that there was no significant difference between the two approaches. However, the serotonin synthesis rates were somewhat greater in several structures on the lesion side, but not significantly greater (two-tailed t-test; $P > 0.05$), when estimated from Equation 2 (solid bars; Fig. 2). A similar trend was observed on the contralateral side (actual comparison not shown here), but again

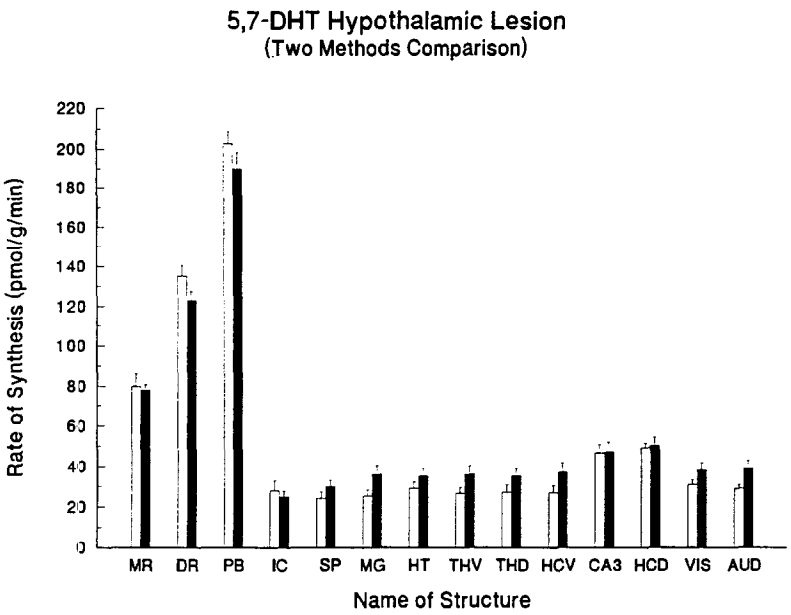

Fig. 2. Graphical comparison of the rate of serotonin synthesis on the 5,7-DHT-lesioned side of rats injected with 3 μg of 5,7-DHT in 250 nL of saline into the dorsolateral hypothalamus. The mean rates were estimated from Equation 1 (open bars) and from Equation 2 (solid bars). The error bars, representing standard error of the mean (+SEM), are shown as vertical lines. The brain structures, identified on the x-axis, are: medial raphe (MR); dorsal raphe (DR); pineal body (PB); inferior colliculus (IC); superior colliculus (SP); medial geniculate body (MG); hypothalamus (HT); thalamus ventral (THV) and dorsal (THD); hippocampus ventral (HCV), CA_3 layer of hippocampus (CA_3); dorsal hippocampus (HCD); cortex visual (VIS), and auditory (AUD). Note that MR, DR, and PB were measured only as one structure, and not actually separated into lesion and contralateral sides.

there was no significant difference ($P > 0.05$). The simplified approach (Equation 2) was of special relevance here where our objective was the comparison of the rate of synthesis in lesion and non-lesion sides of the same rat brain. This way the contralateral side was used as a control.

The percent differences in the rate of serotonin synthesis measured on the injection side compared with that on the contralateral side in the sham-operated rats is shown in Fig. 3 (solid bars). There was a significant increase ($P < 0.05$) in the rate of synthesis only in the dorsal hippocampus and parietal cortex. The rate of serotonin synthesis measured in the brain structures of five sham-injected rats (see above) did not show significant asymmetry, excluding the two structures already mentioned (Fig. 3), permitting us to conclude that the surgery/injection alone did not contribute significantly, in the large majority of structures, to the rate of serotonin synthesis observed in the 5,7-DHT-lesioned rats. In Fig. 3, for comparison, we also plotted the percent SED between the two sides (open bars) in different structures. One could notice that the percent SED was greater than the percent difference between two structures, in all structures except in the parietal cortex and hippocampus dorsal.

To better exemplify the side-to-side difference in the rate of serotonin synthesis and the power of the paired statistics in this analysis, the measurements obtained on different sides in individual rats are shown for several structures in Fig. 4. As seen from Fig. 4, there was generally a higher rate of synthesis on the side ipsilateral to the lesion (striped bars), but since there was quite a bit of variability between different rats, the average values shown in Table 1 might not properly represent the effect of the toxin in individual rats. However, by substantially increasing the number of animals, the difference in the means between the two sides would become significant even if the two-group comparison was done by an ANOVA or t-test.

In Table 1, we only presented the rate of synthesis (given as means ± SEM) obtained by the simplified approach, Equation 2, because only this kind of data was appropriate for paired statistical comparison. The probability shown under the column "P" (Table 1) was calculated from the paired two-tailed t-test comparison. The same probability would be obtained by the repeated measure ANOVA, which is actually an equivalent way of assessing this comparison. We also show the percent increase in the rate of synthesis between the two sides. The percent increase was expressed relative to the rate in the contralateral side. The rate of synthesis in the hypothalamus was estimated in the entire structure because the limited resolution of ^{14}C-images and the small size of different nuclei made it impossible to isolate the part of the nuclei damaged by the needle. We did not find a significant difference between the two sides, and for reasons just mentioned, we excluded the

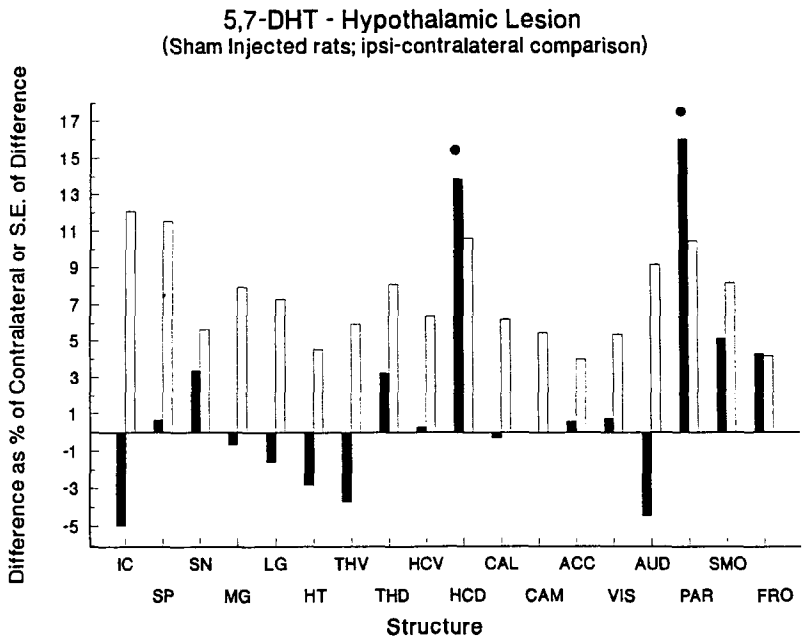

Fig. 3. Difference in the rate of serotonin synthesis, measured in sham-injected animals on the injection side and on the contralateral side, presented as the percentage of the rate on the contralateral side (solid bars) for different brain structures. The open bars represent the percent standard error of the difference (SED) between rates on two sides (shown as open bars in the positive direction; note that this quantity also has an equal negative value), calculated as the square root of the sum of the squares of the SEM rates on each side. Data are presented for the following structures: inf. colliculus (IC); sup. colliculus (SP); substantia nigra (SN); medial geniculate body (MG); lateral geniculate body (LG); hypothalamus (HT); thalamus ventral (THV); thalamus dorsal (THD); hippocampus ventral (HCV); hippocampus dorsal (HCD); caudate lateral (CAL); caudate medial (CAM); accumbens nucleus (ACC); visual cortex (VIS); auditory cortex (AUD); parietal cortex (PAR); sensory motor cortex (SMO); and frontal cortex (FRO). The positive change indicates a larger synthesis rate on the injection side. A significant difference ($P < 0.05$) in the rate of serotonin synthesis, marked by a solid circle, was found in the sham-injected rats.

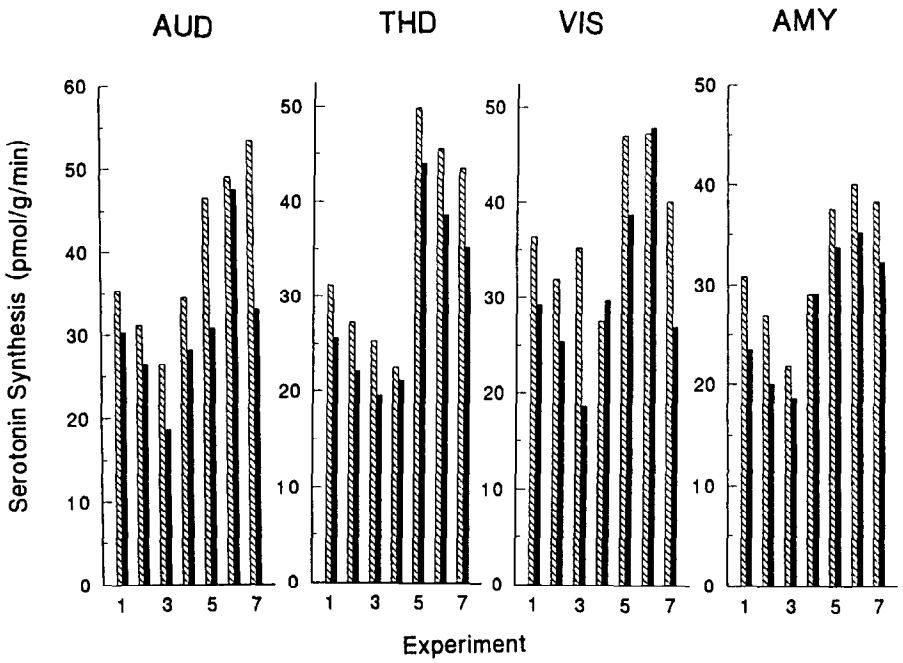

Fig. 4. Comparison between the rate of serotonin synthesis on the 5,7-DHT lesion side and that on the contralateral side, given for several structures [auditory cortex (AUD); thalamus dorsal (THD); visual cortex (VIS); amygdala (AMY)], as measured in individual rats (experiment number: x-axis). The solid and striped bars indicate the contralateral and lesion sides, respectively.

Table 1. Rate of serotonin synthesis (R) measured in rats with 5,7-DHT unilateral stereotaxic lesion in the left dorsolateral hypothalamus

Structure	R (pmol·g^{-1}·min^{-1})*		P† (paired t-test)	Increase as % of contralateral rate
	Lesion side	Contralateral side		
Medial raphe	NA‡	78 ± 3		
Dorsal raphe	NA	124 ± 5		
Pineal body	NA	190 ± 8		
Cortex: Auditory	39 ± 4	31 ± 3	0.014	25.8
Visual	38 ± 3	31 ± 4	0.034	22.6
Parietal	32 ± 3	25 ± 2	0.001	28.0
Sensory motor	36 ± 3	29 ± 3	<0.001	24.1
Frontal	36 ± 2	29 ± 3	0.023	24.1
Hippocampus: CA_3	47 ± 5	31 ± 2	0.004	51.6
Ventral	37 ± 5	34 ± 5	0.048	8.8
Dorsal	50 ± 4	33 ± 3	0.001	51.5
Thalamus: Dorsal	35 ± 4	29 ± 4	<0.001	20.7
Ventral	36 ± 4	32 ± 4	<0.001	12.5
Accumbens	40 ± 3	40 ± 2	NS	0.0
Caudate: Medial	37 ± 5	35 ± 4	0.027	5.7
Lateral	24 ± 3	22 ± 3	NS	9.1
Amygdala	32 ± 3	31 ± 3	0.003	3.2
Medial geniculate body	36 ± 5	31 ± 3	0.037	16.1
Inferior colliculus	25 ± 3	24 ± 2	NS	4.2
Superior colliculus	30 ± 3	28 ± 3	0.007	7.1
Substantia nigra	34 ± 3	31 ± 3	0.013	9.7
Lateral geniculate body	31 ± 3	26 ± 3	0.002	19.2

Rates were calculated from autoradiographic images obtained after injection of 50 μCi of α-[^{14}C] methyl-L-tryptophan.

* Values are means ± SEM (N = 7).

† The paired two-tailed t-test probabilities are given. In every animal, a comparison of the lesion to the non-lesion side was made. There were seven rats in the group. Since it was possible to compare affected and non-affected sides in the same animal, a powerful paired t-test was used. NS = not significant.

‡ NA = not applicable. In these structures, only one measurement was done representing an average rate for the entire structure.

rate measured in the hypothalamus from Table 1. From those structures examined in this study, only the nucleus accumbens, the lateral part of the caudate, and the inferior colliculus did not have a significant increase ($P > 0.05$; paired two-tailed t-test) in serotonin synthesis. Although every other structure showed a significant increase ($P < 0.05$; paired two-tailed t-test), there was not a uniform difference between the two sides (5th column in Table 1). The most pronounced influence of the dorsolateral hypothalamic 5,7-DHT lesion was seen in the parietal (remote to the needle entrance; 28%) and sensory motor (24%) cortices (there is about an equal increase in other cortical structures), dorsal hippocampus (52%), CA_3 part of the hippocampus (52%), dorsal (21%) and ventral (13%) thalamus, and lateral geniculate body (19%; Table 1). The comparison of the rates measured in other sham-operated animals [11] indicated that no significant effect on the rate of serotonin synthesis was observed in this study in the medial and dorsal raphe, and in the pineal body.

DISCUSSION

The data presented here suggest that local destruction of serotonergic terminals in the hypothalamus (dorsolateral injection) increased the rate of serotonin synthesis in ipsilateral brain structures far from the place of injection of the neurotoxin and throughout the ipsilateral brain. In general, the structures located rostrally from the lesion had a greater increase. This suggests that there must be, at least in part, a feedback control of the serotonin synthesis, from the hypothalamus terminal area throughout the brain hemisphere ipsilateral to the lesion. The synthesis was increased, in part, by general activation of TPH in the MFB at the level of the hypothalamus and/or in axons projecting from the hypothalamus, as reported before [18]. We have also demonstrated the great utility of the highly specific tracer for the serotonergic system, α-MTrp, for *in vivo* evaluation of this system in the brain. On the basis of the results from the two different approaches to estimating the rate of serotonin synthesis, we were able to conclude that this simplified approach, in which the volume of the precursor pool was estimated from other experiments, gave results that were not significantly different from those in which the rate of serotonin synthesis was estimated from the analysis of time–activity plots.

The hypothalamic lesion, as used in this work, has

been reported to produce a very local axotomy with swelling in spared neurons [3,*] and with increased activity (measured *in vitro*) of TPH in spared neurons [23]. The toxin was injected into the dorsolateral hypothalamus [15, 18], but diffusion into adjacent hypothalamic structures [*] and possibly to the fornix could not be ruled out [18]. Since the dorsal raphe projects more densely to the hypothalamus than the medial raphe does [12, 24], and receives projections from the lateral hypothalamus [25], one would expect more influence of the hypothalamic terminal lesion on the serotonin synthesis in the dorsal raphe. The effect could also be expected in other brain structures predominantly receiving projections from the hypothalamus (e.g. dorsomedial hypothalamus) [13, 26]. The results of Stachowiak *et al.* [23] reporting activation of TPH would support our observation that the rate of serotonin synthesis in the hypothalamus as a whole was not reduced on the lesion side at this early stage, despite probable serotonergic denervation in it [3,*].

The significant asymmetry observed only in the parietal cortex and dorsal hippocampus in the sham-injected rats (Fig. 3) suggests that the needle itself (e.g. surgical trauma; Fig. 1, C and D) influenced the synthesis of serotonin to some extent in the structures through which the needle enters the dorsolateral hypothalamus. It is also obvious from Fig. 1 (arrow with letter N) that the needle itself produced increased synthesis in the cortex proximal to the needle entry, but the effect was not so obvious in the subcortical structures (e.g. dorsal hippocampus, thalamus) through which the needle enters the dorsolateral hypothalamus. Since, in general, there was no significant effect ($P > 0.05$; paired two-tailed *t*-test) of the saline injection (sham treatment) on the rate of synthesis in any other structures (Fig. 3), the asymmetry in the synthesis observed in the 5,7-DHT-injected rats (Fig. 4 and Table 1) should be attributed to the remote effect of the serotonergic terminals destroyed by the toxin itself. Incidentally, it should be noticed that there was also the greatest variation in the synthesis rates in those structures with a large SED (open bars in Fig. 3).

The asymmetry in the lateral wings of the dorsal raphe exemplified in Fig. 1B was not as prominent in all rats, which, in part, could be the result of the limited resolution of ^{14}C-images and/or animal-to-animal variability exemplified in Fig. 4. As a consequence of this uncertainty, we decided for now not to report the actual values for the synthesis in the lateral wings of the dorsal raphe. Experiments are underway with ^{3}H-labelled α-methyl-L-tryptophan to answer this question definitively. At this time, we would not like to assign any biological meaning to this observation. However, it is known that the axons from the dorsal raphe lateral wings to the medial thalamus, caudate-putamen and lateral geniculate body pass through the MFB [16] in the proximity of the 5,7-DHT lesion in experiments reported here. This proximity of the axons in the MFB could be responsible, in part, for a significant increase (Table 1) in the serotonin synthesis rate in the thalamus (13–21%) and lateral geniculate body (19%).

There were a few structures (e.g. superior colliculus, amygdala, substantia nigra; Table 1) that on the average did not show a great difference when the mean values of the two sides were compared, but when analyzed with the paired statistics showed a significant difference. This suggests that the animal-to-animal variability in the serotonin synthesis rate, as exemplified in Fig. 4, was substantial and could mask the influence of toxin on the rate of serotonin synthesis when averages of groups were compared.

The data presented in Table 1 suggest that the hypothalamic 5,7-DHT lesion had a profound influence on the rate of serotonin synthesis in structures far from the site of the toxin injection. It is significant to note that structures positioned rostrally from the lesion showed a greater increase in the serotonin synthesis rate, suggesting the possible influence of toxin on the fibres within the MFB passing in the proximity of the lesion [12]. Generally, the structures showing a greater increase in the serotonin synthesis also receive more projections from the dorsal than the medial raphe (e.g. cortex) [16].

There was a highly significant increase in the rate of serotonin synthesis on the ipsilateral side in the parietal (excluding the entrance of the needle) and sensory motor cortices, and in the dorsal hippocampus and CA_3 layer of the hippocampus. A significant difference observed in the parietal cortex (28%; Table 1), distal from the needle entrance, is probably related, at least in part, to the effect of 5,7-DHT neurotoxicity produced in the ipsilateral hypothalamus; however, a partial influence of the needle cannot be ruled out since the sham injection also resulted in a significant increase (~15%; see above). However, the increase in other cortices, far from the needle entrance, were approximately the same, supporting the notion that the increase in the parietal cortex is real. The increase in the synthesis rate in the cortical structures, which receive rather extensive dorsal raphe projections, could be explained by feedback control and/or by entrance of neurotoxin into fibre in the MFB at the level of the hypothalamus, resulting in activation of TPH by an unknown mechanism. A pronounced increase in the synthesis observed in the CA_3 layer and dorsal hippocampus (above 50%; dorsal hippocampus may have had some influence from the needle) in contrast to the ventral part of the hippocampus (about 9%), points to a possibility that fibres projecting from the raphe nuclei into the former area are located in the MFB proximal to the lesion [12]. In addition to the possibility that axons projecting to the ventral hippocampus in the MFB were not affected by the toxin is the fact that the ventral hippocampus receives more projections from the medial raphe [12, 16].

Clewans and Azmitia [27] also observed increased activity (*in vitro* measurement) of TPH in the brainstem after 5,7-DHT lesion in the cingulum bundle, suggesting a remote influence on TPH activity. The rats used in the present experiments showed an increase in the synthesis rate and presumably also in the *in vivo* activity of TPH in

* Ljubić V, Raison S, Weissman D, Hamel E and Diksic M, manuscript in preparation.

structures far from the lesion. However, it is not easy to find a direct relation between *in vitro* measurement (TPH activity) [23, 27, 28] and *in vivo* assessments of TPH activities (present data) because many *in vivo* factors (e.g. cofactor concentration, Ca^{2+} concentration) play a role in actual *in vivo* brain enzyme activity. Generally, it is accepted that the rate-limiting step in serotonin synthesis is the activity of TPH, which could also be controlled *in vivo*, in some pathological situations, by the concentration of the pteridine cofactor [29].

Our data could, at least in part, be explained by the observations reported by Stachowiak *et al.* [23] and Bendotti *et al.* [28] that the activity of the TPH in the spared neurons is actually increased, compensating for neuronal loss and possibly even overcompensating for it. The remote effect reported by Clewans and Azmitia [27] is also supported by our observation. Overcompensation is suggested from our observation, on the basis of a profound increase in the rate of serotonin synthesis in many ipsilateral brain structures not directly affected by the lesion. The increased activity of TPH in spared and in damaged neurons could increase the synthesis of 5-HT without biochemical control.

Stachowiak *et al.* [23] also reported that 3 days after 5,7-DHT lesion, the TPH K_m for Trp was reduced (meaning increased affinity), and the V_{max} was increased. This would mean that TPH produces more 5-hydroxylated tryptophan and hence more 5-HT. Data presented here also suggest that the neurotoxin used (5,7-DHT) activated TPH (widely spread increase in synthesis) in all serotonergic neurons, not only in those terminating in the hypothalamus, but also in those projecting in the MFB in the vicinity of the hypothalamus into the forebrain areas. A rather extended activation of TPH is supported by a very pronounced asymmetry in a rather large portion of the MFB (present work and [18]), which would be difficult to explain on account of the activation of TPH only in the projections to the hypothalamus, and even less to the dorsolateral hypothalamus. An indication, not yet definite (see above), that the pronounced change in serotonin synthesis was also present in the cell bodies was the asymmetry observed in the dorsolateral wings of the dorsal raphe (Fig. 1B).

The sprouting of the brain serotonergic axons damaged by a neurotoxin has been observed [30] in the 5,7-DHT-lesioned CNS. A limited sprouting (possibly the effect described by Frankfurt and Azmitia [3] as "swollen neurons" or "Bottons" by Björklund *et al.* [30]) between 4 and 6 days was reported in mechanically and electrolytically lesioned CNS. However, in the chemically (dihydroxy-indolamines) lesioned CNS, the sprouting starts at a later date [30]. The increase in the synthesis rate described in this paper could partially be explained by "bottons" [30], which are packed with TPH [23], and in which TPH could also be activated and could synthesize 5-HT without biochemical control; however, sprouting is not likely to occur in the experiments reported here. Since the neurotoxin in the protocol used could not produce axotomy in the structures remote to the lesion (e.g. hippocampal CA_3 layer, frontal cortex), the sprouting ("bottons") in the remote structures could not possibly be a source of increased synthesis.

It is possible that the half-life of TPH protein in the spared neurons was increased, resulting in an increase in the steady-state concentration of TPH. However, note that increased amounts of TPH do not necessarily mean a greater synthesis rate because the enzyme activity is more important for synthesis than enzyme concentration. The increase in the TPH mRNA [28] in neurons spared by 5,7-DHT suggests an increase in *de novo* synthesis of TPH; however, this increase was observed after intraventricular injection of 5,7-DHT. In the experimental protocol of Bendotti *et al.* [28], the toxin was present directly in the dorsal raphe cell bodies (diffusion from ventricle). An indication of an increased synthesis of serotonin after chemical axotomy produced by intraventricular injection of 5,6-dihydroxytryptamine 1–2 weeks after injection was reported by Björklund and Wiklund [31], who found a 30% increase in the serotonin content in the medulla oblongata of the bulbospiral system, the area containing mainly cell bodies spared after intraventricular injection of 5,6-DHT. However, the increase in the serotonin content could also be explained by a reduced catabolism of serotonin.

The data presented confirm the usefulness of this autoradiographic method in assessing *in vivo* activity of TPH. The study of unilateral local brain lesions with labelled α-MTrp should permit us to get a better understanding of the brain serotonergic system and its widespread feedback control of serotonin synthesis. The investigation of the synthesis control should also be possible with some pre-synaptic and post-synaptic serotonergic agonists in an attempt to get a better understanding of the drugs used in the treatment of depression.

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REFERENCES

1. Baumgarten HG, Klemm HP, Sievers J and Schlossberger HG, Dihydroxytryptamine as tool to study the neurobiology of serotonin. *Brain Res Bull* **9**: 131–150, 1982.
2. Gerson S, Baldessarini RJ and Wheeler SC, Biochemical effects of dihydroxylated tryptamines on central indoleamine neurons. *Neuropharmacology* **13**: 987–1004, 1974.
3. Frankfurt M and Azmitia E, The effect of intracerebral injection of 5,7-dihydroxytryptamine and 6-hydroxydopamine on the serotonin immunoreactive cell bodies and fibres in the adult rat hypothalamus. *Brain Res* **261**: 91–99, 1983.
4. Montine TJ and Sourkes TL, Behaviour of α-methyl serotonin in rat brain synaptosomes. *Neurochem Int* **15**: 227–231, 1989.
5. Diksic M, Nagahiro S, Sourkes TL and Yamamoto YL, A new method to measure brain serotonin synthesis *in vivo*: I—Theory and basic data for a biological model. *J Cereb Blood Flow Metab* **10**: 1–12, 1990.
6. Takada A, Grdiša M and Diksic M, Blood–brain

barrier transfer of L-tryptophan and α-methyl-L-tryptophan in Li-treated rats. *Neurochem Int* **21**: 513–519, 1992.
7. Takada A, Grdiša M, Diksic M, Gjedde A and Yamamoto YL, Rapid steady-state analysis of blood–brain transfer of L-Trp in rat, with special reference to the plasma protein binding. *Neurochem Int* **23**: 351–359, 1993.
8. Regunathan S and Sourkes TL, Effect of α-methylserotonin on serotonin receptor-coupled phosphoinositide breakdown in rat cerebral cortex. *Neurochem Int* **17**: 481–486, 1990.
9. Sourkes T and Diksic M, Alpha-methyltryptophan. *Drugs Future* **18**: 799–801, 1993.
10. Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O and Shinohara M, The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized albino rat. *J Neurochem* **28**: 897–916, 1977.
11. Nagahiro S, Takada A, Diksic M, Sourkes TL, Missala K and Yamamoto YL, A new method to measure brain serotonin synthesis *in vivo*: II—A practical autoradiographic method tested in normal and lithium-treated rats. *J Cereb Blood Flow Metab* **10**: 13–21, 1990.
12. Azmitia E and Segal M, An autoradiographic analysis of the differential ascending projections of the dorsal and medial raphe nuclei in the rat. *Brain Res* **196**: 405–415, 1978.
13. Parent A, Descarries L and Beaudet A, Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of [^{3}H]5-hydroxytryptamine. *Neuroscience* **6**: 115–138, 1981.
14. Veening JG, Swanson LW, Cowan WM, Nieuwenhuys R and Greeraed TS, The medial forebrain bundle of the rat. II. An autoradiographic study of the topography and the major descending and ascending components. *J Comp Neurol* **206**: 82–108, 1982.
15. Halaris AE, Jones BE and Moore RY, Axonal transport in serotonin neurons of the midbrain raphe. *Brain Res* **107**: 555–574, 1976.
16. O'Hearn E and Molliver ME, Organization of raphe-cortical projections in rat: A quantitative retrograde study. *Brain Res Bull* **13**: 709–726, 1984.
17. Mzengeza S, Venkatachalam TK, Rajagopal S and Diksic M, Synthesis of enantiomerically pure α-[^{14}C]-methyl-L-tryptophan. *Appl Radiat Isot* **44**: 1167–1172, 1993.
18. Tsuiki K, Takada A, Grdiša M and Diksic M, Effect of hypothalamic 5,7-dihydroxytryptamine lesion on anterograde transport of serotonin as measured with labelled α-methyl serotonin. *Neurochem Int* **24**: 231–239, 1994.
19. Paxinos G and Watson C, *The Rat Brain in Stereotaxic Coordinates*. Academic, Sydney, 1982.
20. Diksic M, Imaging of the serotonergic neuronal system in the brain. *Period Biol* **93**: 525–532, 1991.
21. Diksic M, Nagahiro S, Chaly T, Sourkes TL, Yamamoto YL and Feindel W, The serotonin synthesis rate measured in living dog brain by PET. *J Neurochem* **56**: 155–162, 1991.
22. Vanier M, Tsuiki K, Grdiša M, Worsley K and Diksic M, Determination of the lumped constant for the α-methyl-tryptophan method of estimating the rate of serotonin synthesis. *J Neurochem*, in press.
23. Stachowiak MK, Stricker EM, Jacoby JH and Zigmond MJ, Increased tryptophan hydroxylase activity in serotonergic nerve terminals spared by 5,7-dihydroxytryptamine. *Biochem Pharmacol* **35**: 1241–1248, 1986.
24. Dahlström A and Fuxe K, Evidence for existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol Scand Suppl* **232**: 1–15, 1964.
25. Azmitia E, The serotonin-producing neurons of the midbrain median and dorsal raphe nuclei. In: *Handbook of Psychopharmacology* (Eds. Iverson SD and Snyder SH), Vol. 9, pp. 233–314. Plenum Press, New York, 1978.
26. Beaudet A and Descarries L, Radiographic characterization of serotonin-accumulating nerve cell group in adult rat hypothalamus. *Brain Res* **160**: 231–243, 1979.
27. Clewans CS and Azmitia E, Tryptophan hydroxylase in hippocampus and midbrain following unilateral injection of 5,7-dihydroxytryptamine. *Brain Res* **307**: 125–133, 1984.
28. Bendotti C, Servadio A, Forloni G, Angere HA and Samanin R, Increased tryptophan hydroxylase mRNA in raphe serotonergic neurons spared by 5,7-dihydroxytryptamine. *Mol Brain Res* **8**: 343–348, 1990.
29. Bengtsson F, Bugge M, Johansen KH and Butterworth R, Brain tryptophan hydroxylation in portacaval shunted rat: A hypothesis for the regulation of serotonin turnover *in vivo*. *J Neurochem* **56**: 1069–1074, 1991.
30. Björklund A, Wiklund L and Descarries L, Regeneration and plasticity of central serotonergic neurons: A review. *J Physiol (Paris)* **77**: 247–255, 1981.
31. Björklund A and Wiklund L, Mechanisms of regrowth of the bulbospinal serotonin system following 5,6-dihydroxytryptamine induced axotomy. I. Biochemical correlates. *Brain Res* **191**: 109–127, 1980.