Acute Effects of L-Tryptophan on Brain Extracellular 5-HT and 5-HIAA Levels in Chronic Experimental Portal-Systemic Encephalopathy

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Portal-systemic encephalopathy (PSE) is associated with increased brain turnover of serotonin (5-hydroxytryptamine; 5-HT). Despite this metabolic increase, neuronal release of 5-HT is unaltered in neocortex of portacaval shunted (PCS) rats. In the present study, frontal neocortical extracellular 5-HT and 5-hydroxyindole-3-acetic acid (5-HIAA) levels were determined in PCS rats and sham-operated controls prior to, as well as, after acute challenge with L-tryptophan (L-TRP; a bolus dose of 280 mg/kg i.p. followed by 5 consecutive hourly doses of 50 mg/kg). Neither basal 5-HT nor 5-HIAA extracellular levels were significantly altered in PCS rats compared to controls. L-TRP administration resulted in unaltered extracellular 5-HT but elevated 5-HIAA levels in PCS and sham rats. These findings do not suggest that changes in brain neuronal 5-HT release play any major functional role in the pathogenesis of chronic PSE. The present data also emphasize the importance of distinguishing between brain 5-HT metabolism and brain 5-HT release.

INTRODUCTION

Despite many years of research efforts, the more precise pathogenesis of portal-systemic encephalopathy (PSE) is still unknown. One hypothesis suggested to explain the development of PSE concerns a perturbation of the metabolic handling of the aromatic amino acid L-tryptophan (L-TRP) in the brain (for overview, see e.g. Bengtsson, 1992). In essence, increases in plasma, brain tissue and brain extracellular fluid levels of L-TRP have been observed in experimental PSE (see e.g. Fischer *et al.*, 1974; Mans *et al.*, 1984; Bengtsson *et al.*, 1991; Bergqvist *et al.*, 1995, 1996). Furthermore, a profound increase in

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brain turnover of the L-TRP-derived neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) has been demonstrated in rats subjected to a permanent portacaval shunt (PCS) (for overview, see e.g. Bengtsson *et al.*, 1989). The PCS rat is a reproducible and the most commonly used non-toxin induced *in vivo* model of PSE (Lee and Fisher, 1961; Bengtsson *et al.*, 1988). To this end, in many hypotheses concerning the significance of disturbances in the brain 5-HT systems in the development of PSE as well as other neuropsychiatric disorders, it has been axiomatically assumed that an altered 5-HT metabolism in the brain also is accompanied by a change in neuronal 5-HT release and thereby in 5-HT function (for overview, see e.g Willner, 1985). In a recent study we showed, however, that increased brain 5-HT turnover in experimental PSE is not associated with increased neuronal release of 5-HT as assessed by *in vivo* microdialysis in the neocortex of PCS rats (Bergqvist *et al.*, 1995). At present, therefore, the functional relevance of a metabolic pertubation in the brain 5-HT systems in PSE is questioned. These findings may have a conceptual bearing also on other neuropsychiatric conditions involving a possible brain L-TRP/5-HT pathology, such as the core psychiatric affective disorders and schizophrenia (e.g. Coppen *et al.*, 1972; Joyce *et al.*, 1993).

In the present study, the possible role of a disturbance in the brain 5-HT systems in the development of PSE was further investigated. *In vivo* microdialysis techniques were applied for monitoring of the frontal neocortical extracellular levels of 5-HT and its major metabolite 5-hydroxyindole-3-acetic acid (5-HIAA). This study focused primarily on acute effects of L-TRP administration on extracellular 5-HT and 5-HIAA levels. In order to evaluate both immediate effects exerted by L-TRP as well as during a short-term steady-state situation with regard to brain L-TRP levels, a bolus dose followed by smaller iterative systemic doses of L-TRP were administered to chronic PCS and sham-operated rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200 g; Möllegaard Breeding & Research Centre A/S, Skensved, Denmark) were used. Animals had free access to laboratory chow containing 18% crude protein (Altromin No 1324; Chr. Peterson A/S, Ringsted, Denmark) and tap water. Rats were housed in groups of three animals in macrolone cages with sawdust bedding under climate controlled conditions for regular in-door temperature and humidity. The rats were kept in a 12:12 h light:dark cycle synchronous with day-light (lights on a.m. 06.00 h). The study was approved by the Animal Ethics Committee at Lund University, Lund, Sweden.

Intraabdominal Surgery

A permanent surgical end-to-side PCS (n=12) was created under ether anesthesia with a clean but non-sterile technique according to Lee and Fisher (1961) with minor modifications (Bengtsson *et al.*, 1985; Bergqvist *et al*, 1996). Sham-operated control rats (n=11) underwent a similar surgery procedure but without construction of a portacaval anastomosis.

PCS and sham rats were allowed to recuperate for 5-6 weeks after surgery before the microdialysis experiments were performed. Rats were weighed at time of the PCS-procedure, thereafter weekly and at termination of the experiments.

In vivo Microdialysis

Microdialysis probes were prepared according to a method described by Kalén et al. (1988), using hemophane tubing membranes (GFE Plus16; OD 0.22 mm and ID 0.20 mm; length 4 mm, 2 mm in a dorso-ventral aspect, 7 kDa molecular cut off; Gambro AB, Lund, Sweden). The probe was implanted into the frontal neocortex (rostral, +3.2 mm; lateral, -1.5 mm; ventral, -2.5 mm, relative to bregma and dura mater) under halothane anesthesia (ISC Chemicals Ltd, Avonmouth, England) one day prior to dialysis. At day of dialysis, rats were placed in microdialysis chambers of the CMA/120 system for freely moving animals (CMA/Microdialysis, Stockholm, Sweden) and the probe inlet was attached to a liquid swivel which was connected to a CMA/100 microinjection pump (CMA/Microdialysis). The probes were perfused at 2.5 µL/min with an isotonic Ringer solution (NaCl 147 mmol/L, KCl 4 mmol/L, $CaCl_2$ 2.4 mmol/L, pH 6.0; Pharmacia AB, Uppsala, Sweden). No samples were collected for an initial stabilization period of 1 h. Following stabilization, samples were collected in 15 min fractions rendering approximately 35-40 µL for each sample. The samples were immediately frozen without addition of any antioxidants by immersion into liquid nitrogen and stored at -70°C until the subsequent neurochemical analyses (see below) were performed.

Experimental Design

After collection of three baseline fractions, brain L-TRP availability was exogenously manipulated by systemic (intraperitoneal) loading of the amino acid. The L-TRP challenge protocol applied in the present study was constructed after scrutinizing previous results obtained in normal rats (Hutson et al., 1985; Sarna et al., 1991) and PCS rats (Bengtsson et al., 1991) subjected to various L-TRP loading procedures. Thus, in the present study, the 3h effects of a bolus L-TRP (Sigma Chemical Co., St. Louis, MO) dose of 280 mg/kg body weight plus the effects of lower doses of L-TRP (50 mg/kg) administered hourly as five consecutive i.p. injections were evaluated in terms of response in affecting extracellular 5-HT and 5-HIAA levels in frontal neocortex. The effect of the exogenous L-TRP loading protocol applied in the present study on brain extracellular L-TRP levels has previously been reported (Bergqvist et al., 1996). In essence, these results revealed that after the initial L-TRP loading sham as well as PCS rats will achieve a parallel about 10-fold increase in the extracellular L-TRP level in neocortex (Bergqvist et al., 1996). Over time, though, this elevated extracellular L-TRP level will linger in the PCS rats while in the sham-operated controls the concentration of L-TRP in the extracellular compartment will show a tendency towards normalization compared to the pre-challenge values (Bergqvist et al., 1996).

In some PCS and sham rats, L-TRP was replaced by an equimolar amount of saline (Sigma Chemical Co.) to account for possible confounding variables such as irritation and osmotic effects by the administration procedure. Thus, four experimental groups designated as sham-saline (n=6), PCS-saline (n=6), sham-L-TRP (n=5), and PCS-L-TRP (n=6) were

investigated.

The microdialysis paradigm comprised in each case of 15 min consecutive aliquot sampling procedure in each of the investigational time-periods. I.e. during the first hour after the L-TRP challenge all collected dialysates were used for neurochemical analyses whereafter only a specified 15 min aliquot was used to represent each of the consecutive 7h following this L-TRP-challenge.

The experiments were terminated by decapitation of the rats, whereafter liver weight was recorded and liver/body weight ratio was calculated and used as a measure for successful chronic patency of the anastomosis and thereby serving as a validation for the PCS function throughout the experimental period.

Neurochemical Assays

Dialysate contents of 5-HT and 5-HIAA were determined by the use of HPLC with electrochemical detection as previously described (Hjorth and Sharp, 1993). The compounds were separated at 30°C on a 2.0 mm x 15 cm column (Ultrasphere ODS 5-µm; Beckman Instruments, Fullerton, CA). Mobile phase consisted of 0.126 M NaH_2PO_4 (pH 4.0) containing 0.85 mM EDTA, 0.01 mM sodium n-octyl sulfate, and 13% (vol/vol) HPLC-grade methanol and was filtered through a cellulose nitrate filter (pore size 0.22 µm; Millipore Ltd., London, U.K.). An aliqout of 20 µL of dialysate was manually injected with a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA). Mobile phase was delivered at 0.2 mL/min through a twin-reciprocating pump (LKB-2150; LKB-Produkter AB, Bromma, Sweden). The indole derivatives were detected by a glassy carbon working electrode set at +0.6 V relative to a Ag/AgCl reference electrode (Waters M460; Millipore AB/Waters Chromatography Division, Sundbyberg, Sweden) and the detection signals were recorded on a Chrom-Jet integrator (Spectra-Physics, San Jose, CA). The detection limit for 5-HT was 0.5 - 1.0 fmol/20 µL.

Statistics

All values are expressed as means ± SEM. A probability of less than 5% (p<0.05) was preset in all statistical comparisons to indicate significant differences. All statistical analyses were performed by the use of the computer software StatView 4.12 for Macintosh (Abacus Concepts, Inc., Berkeley, CA). Statistical comparisons between two independent groups were made by applying a two-tailed Students' t-test for unpaired observations. When frontal neocortical contents of extracellular 5-HT and 5-HIAA following L-TRP or saline administrations were compared between the different experimental groups at a specific time-point during the 7 h follow-up period, a one-factor ANOVA was applied. When significance was reached with the ANOVA, a post-hoc test (the Bonferroni/Dunn's test) was applied to determine which groups differed. When the sham versus PCS effects of L-TRP or saline administrations on frontal neocortical content of extracellular 5-HT and 5-HIAA were evaluated, the area under the curve (AUC) was calculated. The AUC values were then compared between the four experimental groups by subjecting the data to a one-way ANOVA with the Fishers's PLSD test.

RESULTS

All rats survived from intraabdominal surgery until time of dialysis. Administration of L-TRP or saline were not found to affect body or liver weights either within PCS or sham groups. Thus, body weight and liver weight data are presented only as values for the sham versus PCS group. At termination of the experiment, there was a significant difference in body weight between sham and PCS rats (403±7 g, n=11 vs. 259±14 g, n=12; p<0.001). The body weight gain from operation to dialysis in sham (Δ body weight 189±12 g) and in PCS (Δ body weight 62±8 g) showed that both groups had been able to reach a post-surgical anabolic state at time of brain dialysis. In fact, parallel weight-gain curves for sham and PCS rats were present during the week prior to brain dialysis (data not shown). A significant difference in liver to body weight ratio between sham and PCS rats was demonstrated (3.6±0.1%, n=11 vs. 2.3±0.1%, n=12; p<0.001), signifying a relatively lower liver weight of PCS rats which has previously been shown to correlate well with functional liver impairment displayed in this animal model (see e.g. Schröder et al., 1985).

Basal Brain Extracellular 5-HT and 5-HIAA Concentrations

Basal frontal neocortical extracellular 5-HT and 5-HIAA concentrations prevailing in the rats 5-6 weeks following the creation of a PCS or sham-operation, respectively, are displayed in Table I. As can be seen in Table 1, no significant changes were observed between sham and PCS rats either in basal extracellular 5-HT or 5-HIAA concentrations.

Table I. Basal frontal neocortical extracellular 5-HT and 5-HIAA concentrations in PCS and sham-operated rats.

	Sham (n = 11)	PCS (n = 12)
5-HT (fmol/ 20 μL)	5.1 ± 0.8	6.2 ± 1.0
5-HIAA (pmol/ 20 μL)	2.1 ± 0.3	2.9 ± 0.5

Values are means ± SEM. No statistical significant changes between sham and PCS were observed (two-tailed Students' t-test for unpaired observations).

Effects of L-TRP loading on Brain Extracellular 5-HT and 5-HIAA Concentrations

No changes in the neocortical 5-HT release were displayed in either sham (Figure 1A) or PCS (Figure 1B) rats resulting from the L-TRP loading procedure per se. By difference, the extracellular 5-HIAA concentrations increased in both sham and PCS rats in response to the L-TRP loading (Figure 2A and B). This rise in brain extracellular contents of 5-HIAA tended to be somewhat more pronounced in the PCS-L-TRP (Figure 2B) than in sham-L-TRP rats (Figure 2A) in the later phase of the overall 7 h follow-up period.
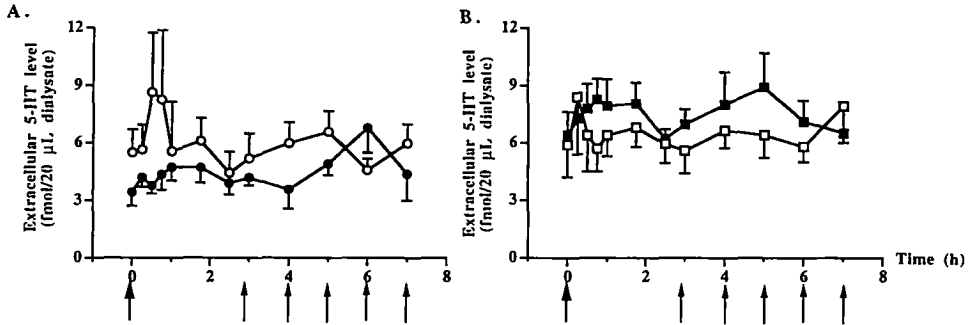
Figure 1. Brain extracellular 5-HT during neocortical dialysis following challenges of saline or L-TRP to (A) sham-operated and (B) PCS rats. The administrations of a single loading dose of L-TRP (280 mg/kg, i.p.) and of five consecutive injections of lower L-TRP doses (50 mg/kg, i.p.) are indicated by a bold arrow and by regular arrows, respectively. *Legend;* sham-saline (○), sham-L-TRP (●), PCS-saline (□) and PCS-L-TRP (■). All values are means ± SEM and expressed as fmoles/ 20 µL dialysate fluid. The number of samples is in all cases 4-6.
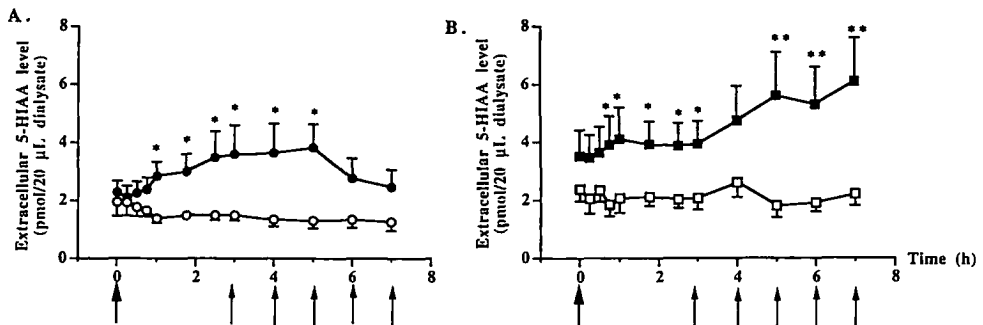

Figure 2. Brain extracellular 5-HIAA during neocortical dialysis following challenges of saline or L-TRP to (A) sham-operated and (B) PCS rats. The administrations of a single loading dose of L-TRP (280 mg/kg, i.p.) and of five consecutive injections of lower L-TRP doses (50 mg/kg, i.p.) are indicated by a bold arrow and by regular arrows, respectively. *Legend;* sham-saline (○), sham-L-TRP (●), PCS-saline (□), and PCS-L-TRP (■). All values are means ± SEM and expressed as pmoles/ 20 µL dialysate fluid. The number of samples is in all cases 4-6. * denotes significant (p<0.05) differences between sham-saline and sham-L-TRP or between PCS-saline and PCS-L-TRP (ANOVA with Bonferroni/Dunn's post-hoc test).

No differences in overall brain extracellular 5-HT content in the dialysates following L-TRP or saline administrations were observed between any of the four experimental groups (Table II). Although the extracellular 5-HIAA concentrations showed a marked elevation (p<0.05) in general in both sham-L-TRP and PCS-L-TRP rats as compared with sham-saline and PCS-saline, respectively, no differences were observed between sham and PCS rats challenged with L-TRP or between sham and PCS rats administered saline (Table II).

Table II. The effects of L-TRP or saline administrations on frontal neocortical content of extracellular 5-HT and 5-HIAA in PCS and sham-operated rats.

	Sham-saline (n=6)	Sham-L-TRP (n=5)	PCS-saline (n=6)	PCS-L-TRP (n=6)
5-HT (fmol x h / 20 µL)	40.3 ± 9.2	32.9 ± 4.1	45.9 ± 6.6	55.0±6.9
5-HIAA (nmol x h / 20 µL)	10.4 ± 1.2	22.9 ± 5.3†	15.2 ± 2.4	33.3±7.6*

Values are means ± SEM and expressed as area under the curve (AUC). † and * denote significant (p<0.05) differences in AUC's between sham-L-TRP and sham-saline and between PCS-L-TRP and PCS-saline, respectively (one-way ANOVA; Fishers's PLSD test).

DISCUSSION

The present *in vivo* microdialysis study reports two findings concerning a possible role of a disturbance in the brain 5-HT systems in the development of PSE. Firstly, neither basal frontal neocortical extracellular 5-HT nor 5-HIAA levels were altered in PCS rats compared with controls. Secondly, following exogenous administration of a bolus plus repetitive systemic doses of the 5-HT precursor amino acid L-TRP, no changes in brain extracellular 5-HT levels were seen in PCS or sham-operated rats.

The unaltered basal frontal neocortical extracellular 5-HT levels demonstrated in the present study in PCS rats as compared to controls are in accordance with previous results (Bergqvist *et al.*, 1995). The extracellular levels of a neurotransmitter measured by *in vivo* microdialysis are generally considered to represent a synaptic "spillover" to this compartment and thus, indirectly, will reflect neuronal release for this neurotransmitter. Accordingly, it can be assumed that under the current experimental conditions, although an increased brain intraneuronal 5-HT metabolism is prevailing, neuronal 5-HT release and thereby 5-HT function is probably not altered in the brain in conditions of chronic PSE. The previously suggested uncoupling between brain 5-HT metabolism and release in chronic experimental PSE (Bergqvist *et al.*, 1995) is thus supported by the results obtained herein.

The lack of changes in extracellular 5-HT levels in the PCS and sham-operated control rats in the immediate period following systemic L-TRP administration is in agreement with observations reported from other authors (De Simoni *et al.*, 1987; Sharp *et al.*, 1992) probably reflecting a generally low potential to alter brain neuronal 5-HT release and function by L-TRP augmentation under normal conditions as well as during chronic PSE. It has previously been shown that brain 5-HT biosynthesis is dependent on the availability of plasma L-TRP (e.g. Fernstrom, 1977). However, the effect of altered L-TRP availability in the brain with regard to neuronal 5-HT release seems to critically depend on the ambient conditions prevailing in the 5-HT systems at the time when L-TRP availability is altered (see e.g. Young and Teff, 1989). For example, Sharp and colleagues have demonstrated that elevation of brain L-TRP availability increases neuronal 5-HT release in hippocampus <u>only</u>

under conditions of an already increased serotonergic neuronal activity (Sharp *et al.*, 1992). Furthermore, it has been shown that exogenous systemic administration of L-TRP induces a much more pronounced increase in brain extracellular 5-HT levels in food-deprived or exercized rats than in *ad lib* fed rats or rats at rest, respectively (Schwartz *et al.*, 1990; Meeusen *et al.*, 1995). It is important, however, to recall that several other previous studies have reported that L-TRP augmentation might increase the neuronal 5-HT release in the brain (Hutson *et al.*, 1985; Carboni *et al.*, 1989; Sarna *et al.*, 1991; Westerink and De Vries, 1991). It should be kept in mind, though, that these various reports, including the present study, utilized different methodology including, for example, varying doses or dose regimens for the L-TRP supplementation.

Although no indications of major changes in the 5-HT release were found in the frontal neocortex of the PCS rats in the present study, the possibility still exists that the 5-HT release may be affected in other regions in the brain in experimental chronic PSE. Further studies therefore have to focus not only on issues such as under which circumstances L-TRP administration might alter brain 5-HT release in normal conditions and in conditions of liver failure but also on possible regional differences in brain 5-HT release in experimental PSE.

Another important issue to take into consideration is the still unresolved issue about the possible existence of two different neuronal pools of 5-HT in the brain, suggesting one small functionally active pool of 5-HT and one larger but functionally inactive, and thus non-releasable, 5-HT pool (Grahame-Smith, 1971, 1973; Shields and Eccleston, 1973; see also Bengtsson *et al.*, 1989). If existing, the two different intraneuronal pools of 5-HT most likely share the same catabolic pathways. An overall 5-HT turnover may therefore not primarily reflect the metabolism of the functionally active compartment but rather mirror the sum of the active and the inactive 5-HT pools in the nerve cell (Grahame-Smith 1971, 1973; Bengtsson *et al.*, 1989). The hypothesis of two different pools of 5-HT may offer some help to explain results obtained previously as well as in the present study. Firstly, the previously reported increased brain tissue 5-HT turnover in PSE (e.g. Bengtsson *et al.*, 1988) has almost entirely been based on neurochemical analysis of brain tissue. Hence, increased brain tissue 5-HIAA levels could be an epiphenomenon because of a major increment in intraneuronal 5-HT metabolism in the non-releasable 5-HT pool but unrelated to the releasable pool and thus to 5-HT function. Secondly, the lack of changes in extracellular 5-HT levels in PCS and control rats following L-TRP administrations, as shown in both the present study as well as in other studies (e.g. Sharp *et al.*, 1992), may indicate that the amount of 5-HT in the releasable pool is sufficient to meet the demand for a normal serotonergic neurotransmission and that the L-TRP-induced increase in 5-HT biosynthesis could be "functionally" buffered, and subsequently metabolized, in the non-releasable compartment. The validity of the hypothesis of two different pools of 5-HT, however, has to be further critically investigated.

In conclusion, the present study showed that after a chronic PCS procedure in the rat neither basal extracellular contents of 5-HT nor 5-HIAA are altered as compared to controls. Following increased brain availability of the precursor amino acid L-TRP, no changes in brain 5-HT release in the PCS and sham groups were seen. The 5-HIAA levels were,

however, increased in both sham and PCS rats following the L-TRP loading procedure indicating an increased intraneuronal 5-HT metabolism following L-TRP loading. These findings do not suggest a major functional significance of an increased neuronal 5-HT release in chronic experimental PSE. The present study also emphasize the importance of distinguishing (just abstract) between brain 5-HT metabolism and brain 5-HT release.

ACKNOWLEDGMENTS

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