Serotonin, cortisol, and stress-related psychopathology

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Chapter 2

Low tryptophan diet increases stress-sensitivity, but does not affect habituation in rats

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ABSTRACT

Cerebral dysfunction of serotonin (5-HT) has been associated with stress response and with affective disorders. Stress alone is insufficient to induce depression, since only a minor proportion of subjects that have experienced stressful life events develop depressive episodes.

We investigated whether long-term brain 5-HT depletion induced in rats by a diet with low content of its precursor tryptophan affects stress-responsiveness in rats. Stress-sensitivity was measured through various physiological parameters and by measuring the rats' response to acoustic stimuli. One group of rats was subjected to daily acoustic stimulus sessions for 5 days. Other groups received both immobilization stress and acoustic stimulus sessions daily for either 9 days (chronic experiment) or 1 day (acute experiment).

A low tryptophan diet led to decreases in plasma tryptophan levels, low ratio of tryptophan/large neutral amino acid, whole blood 5-HT, and neuronal 5-HT content in the Dorsal and Median Raphe Nuclei, as well as altered c-fos expression in the brain. Without concomitant immobilization, the diet alone did not affect reactivity and habituation to acoustic stimuli, although plasma corticosterone levels, but not the adrenal weights, were increased on day 5. Low tryptophan and chronic immobilization stress together with the acoustic testing procedure increased adrenal weight, plasma corticosterone levels and reactivity to the acoustic stimuli, but not the rate of habituation to acoustic stimuli.

These results show that cerebral dysfunction of serotonin achieved through a low tryptophan diet, increases the sensitivity of rats to external and stressful stimuli, but does not impair the capacity to adapt to these stimuli. Accordingly, brain-serotonin modulates reactivity to stress, but not stress coping.

INTRODUCTION

There are two major theories on the pathogenesis of affective disorders. The first assumes a disturbed serotonin (5-HT) neurotransmission, while the second supposes a predominant role of stress and stressful life-events in the pathogenesis and patho-physiology of affective disorders. Indeed, abnormalities in the serotonergic system have often been associated with affective disorders such as depression, anxiety, aggression, suicide and poor impulse control (55;78;116;146;147). The second hypothesis is supported by the often reported findings that severe life-events precede the development and relapse of depression (8;9;11). In addition, about 50% of patients suffering from an major depressive episode exhibits a dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis, such as elevated cortisol levels, adrenal hypertrophy and glucocorticoid resistance (28;148-150). Stress alone, however, is not sufficient to induce a depressive episode, as a direct and causal relationship between stress and affective disorders has not yet been established (151). Thus, to induce an affective disorder, additional factors that increase vulnerability to psychopathology are required. One such factor may be concomitant 5-HT dysfunction (144;145).

Cerebral 5-HT function depends on the availability of its precursor L-tryptophan, an essential amino acid. Cerebral tryptophan concentrations and the flux of tryptophan through the blood-brain barrier depend both on its plasma concentration and on the concentration of 6 large neutral amino acids (LNAA's: tyrosine, phenylalanine, leucine, isoleucine valine and methionine) which compete with tryptophan for blood brain barrier transport (49). Accordingly, tryptophan depletion is an effective method to substantially reduce plasma and cerebral tryptophan levels (50) and eventually to reduce brain 5-HT synthesis and consequently, brain 5-HT levels (47;48). Altered plasma tryptophan levels have indeed been associated with psychopathology. Decreased plasma tryptophan levels may, for example, be observed in patients suffering from a depressive episode (58;61;64). Furthermore, reductions in plasma tryptophan levels, which are found in some somatic diseases, have been associated with aggressive behavior and irritability (116;133). Finally, dietary manipulations leading to acute reductions of plasma tryptophan levels may induce mood-lowering effects in patients treated with 5-HT-specific antidepressants (SSRI's) and also in genetically depression-prone and healthy subjects who are at risk for developing a depression (62;64;65).

In animals, 5-HT depletion is also associated with increased stress-reactivity and aggression. Increased reactivity is found in both rats and mice that are fed with a tryptophan-deficient diet (69;70;73;152) and in most studies in which cerebral 5-HT levels are depleted by treatment with p-chlorophenylalanine, a tryptophan hydroxylase inhibitor (71;153-156). Acoustic stimuli are widely used to test reactivity and habituation in rats and the method is very sensitive to stress (118;123;124;127;157). Another advantage of using acoustic stimuli is that the responses are robust across species (126).

Stress-vulnerability may also be assessed by habituation. Habituation refers to a reduced response during repeated presentation of a startling stimulus (132). Previous work has been ambiguous on the role of 5-HT in habituation. Some studies reported decreased habituation after central 5-HT depletion (71-74;74); Other reports indicated that the rate of habituation to acoustic stimuli did not differ between 5-HT depleted and control animals (75;76).

In the present paper, we tested whether chronic low 5-HT-function influences stress-coping as assessed with an unconditional response to acoustic stimuli in rats with or without concomitant exposure to immobilization stress. Depletion of cerebral 5-HT was induced by a low tryptophan diet (47). Stress-sensitivity was measured by plasma corticosterone levels, adrenal weight, body weight and food intake, c-fos expression in a number of brain areas (158;159), and reactivity and habituation to the acoustic stimuli (76;123;125).

The first experiment (where rats were only subjected to acoustic stimuli) demonstrated a mildly increased stress-sensitivity in tryptophan deficient rats. This was manifested as increased corticosterone levels at the time of termination and decreased food intake during the days of testing (118;160). However, this increased stress-sensitivity was reflected neither in increased adrenal weight, nor in the stress-reactivity or habituation to the acoustic stimuli. To evaluate adequately the relationship between tryptophan depletion and stress-sensitivity, we designed a second experiment, in which we augmented the amount of stress by adding a daily 10-minute immobilization period to the protocol. Adding immobilization stress resulted in increased reactivity, as well as increased plasma corticosterone levels and increased adrenal weight, in rats fed with the low tryptophan diet. The present study shows that low brain 5-HT increases stress-reactivity, but that this condition does not alter habituation to unconditioned acoustic stimuli.

MATERIALS & METHODS

The experimental procedures were approved by the Animal Ethics Committee of the University of Groningen. Every reasonable effort was made to minimize the number of animals used and their discomfort.

Experiment 1

Twelve male Wistar rats (Harlan, the Netherlands) weighing 231-276 gram at the start of the experiment, were housed individually in plexiglas cages (45 cm x 28 cm x 20 cm) in a temperature controlled environment (21-23 °C). Animals were kept on a 12h reverse light/dark cycle with lights on from 1900h to 0700h. The experiments were conducted during the dark period. All animals were handled daily to minimize handling stress during the experiment. Food intake and body weights were measured daily. On day –4, standard rat chow was replaced with a synthetic control diet. On day 0, after one week of acclimatization, animals were assigned to one of two treatment groups: a synthetic low-tryptophan diet (Trp^-) or a synthetic control diet (Trp^+). The animals had *ad libitum* access to

water and food. These animals were subjected daily for 5 days to acoustic stimulus sessions starting on day 3. Blood samples were taken on day -1 (baseline levels) and 20 minutes after the acoustic stimulus session on day 1 of the startle protocol. Two hours after the last acoustic stimulus session, animals were sacrificed and an intra-cardiac blood sample was taken to determine plasma corticosterone and tryptophan levels. Thymus and adrenal glands were removed and weighed.

Experiment 2

To adequately evaluate the relationship between tryptophan depletion and stress-sensitivity, we designed a second experiment, in which we augmented the amount of stress. This was done in three ways. 1) A daily 10-minute immobilization session was added to the protocol. Immobilization (10 min/day) was selected as stressor because it is a non-painful manipulation that avoids possible neurochemical and behavioral confounds inherent in stressors that can produce pain. Brief daily exposures to immobilization are sufficient to produce marked behavioral changes and elevation in HPA-axis activity; (118-122). 2) The experiment was extended with a week; 3) Restricted time for food consumption was introduced during testing days (during this time food was still available *ad libitum*). During the weekend, the rats had *ad libitum* access to food.. In addition, histochemical measurements were included to examine changes in a variety of brain nuclei. Furthermore, a single stress group was included to be able to distinguish between the effects of the stress procedures and the effects of the Trp^- diet itself.

In experiment two, 20 animals (10 Trp^- and 10 Trp^+) were used in the chronic stress (CS) experiment (Trp^-CS and Trp^+CS). These animals were subjected to daily immobilization stress and acoustic stimulus sessions for 10 days starting at day three. Ten animals (five Trp^- and five Trp^+) were used in the acute stress experiment (AS). These rats received both procedures only once: the acoustic stimulus session on the day before termination and immobilization stress on the day of termination. Rats were immobilized daily by manually placing them on their backs in a light room for 10 minutes, 3 hours before acoustic stimulus tests were conducted.

Blood samples were drawn on day 6. Rats were terminated two hours after the immobilization stress, when fos levels are maximal (161). The rats were anaesthetized, using isoflurane, and intra-cardial blood was taken for the determination of plasma corticosterone and tryptophan, all the amino acids and whole blood 5-HT. Subsequently, the rats were transcardially perfused for two minutes with 50 ml heparinized saline (0.9%) and for 20 minutes with 300 ml of a 4% paraformaldehyde solution 0.1 M sodium phosphate buffer (pH = 7.4). The brains were removed and post-fixed in the latter solution overnight at 4°C. Thymus and adrenal glands were removed and weighed and reported as promillage of the body weight.

General procedures.

Diet

All diets are designed by Numico (Wageningen, The Netherlands) and manufactured by Research Diet Services B.V. (Wijk bij Duurstede, The Netherlands). The Trp^+ diet contained 0.24 g tryptophan / 100 g diet, the Trp^- diet contained only 0.024 g tryptophan/100 g diet (162). To equalize the total amount of amino acid in the diets, the changes in tryptophan content were counterbalanced by adjusting the amounts of leucine, isoleucine and valine (LNAA's).

Behavioral testing

Individual rats were tested daily at approximately the same time. The behavioral experiments were carried out in a separate room. The animals received acoustic stimulus sessions (163), using a Startle Response Measuring System (TSE GmbH, Bad Homburg, Germany) in a dark, sound-attenuating chamber (320 x 320 x 320 mm). The rats were restrained in a small wired cage (270 x 100 x 125 mm) restricting major movement and exploratory behavior. This cage was placed on a transducer platform, which allows accurate measurements of the animals' motor reactions. Acoustic sound stimuli were generated by means of high-quality high-linearity speakers situated on both sides of the cage. The whole set-up was operated in a sound-attenuating chamber equipped with a ventilation fan. An IBM-compatible computer with TSE Startle Response Software and control interface was used to present stimuli and record data. The rats were subjected to the same conditional program every day; consisting of a five-minute acclimatization period to the startle chamber, and 10 sets of four trials with an inter-stimulus interval of 10 seconds. One set contained successively one trial of nothing, one trial of a stimulus alone (20 ms 80dB 5000 Hz), followed by two trials with a paired stimulus, consisting of a 20 ms 80dB 5000 Hz pulse followed100 ms later a 40 ms 120 dB 5000 Hz pulse. During the acclimatization period and the actual experiment, a constant background noise of 70 dB was present. Only the responses to the first paired stimulus of each set were used and hereafter reported. Habituation is also dependent on the definition of habituation (164). Because the Trp^- animals were expected to react stronger to the stimuli, we chose to measure habituation as percent decrease in motor response. Percent motor responses for day x were determined as (average response day x / average response day 1) * 100. The responses on day 1 were set as 100%. Habituation was measured as percent reduction in motor response to the first conditioned stimulus of each day.

Blood sample analysis

Blood samples of approximately 0.5-0.75 ml were collected from a small tail wound (165) using short general anesthesia with isoflurane. On the last day of the experiments, the rats were anaesthetized using isoflurane, and an intracardial blood sample was taken for determination of plasma corticosterone and tryptophan.

Corticosterone (free and protein-bound) was measured with a locally optimized radioimmunoassay, using ^{3}H-corticosterone as displacement ligand. Plasma tryptophan and 5-HT concentrations were measured by means of HPLC (166). Total amino acid analysis in plasma samples was performed with a BioChrom 20 (Pharmacia, Freiburg, Germany) using post-column reaction with ninhydrin for detection (167) according to manufacturer's protocols with slight modifications. In short, plasma samples were deproteinized by mixing 100 µl of plasma with 100 µl 10 % solution of sulphosalicylic acid in 0.5 M lithium citrate buffer. For amino acid analysis, a standard stepwise elution by 5 lithium citrate buffers was used. The amino acids were detected with ninhydrin reagents through a reaction coil set at 135 ˚C.

Histology

Following an overnight cryoprotection in a 30% sucrose solution, serial 40 µm coronal sections were made with a cryostat microtome and collected in Tris 0.01 M + 0.9% NaCl + 0.01 % Na-azide buffer (TBS). Fos immunostaining was performed essentially following the method of (168), but using TBS instead of 0.02 M potassium phosphate saline buffer. Fos immunostaining was performed on free-floating sections. Sections were rinsed with 0.3% H_2O_2 for 10 min to reduce endogenous peroxidase activity, thoroughly washed with TBS and incubated with the rabbit anti-fos antibody (1:10000; Oncogene Research Products, San Diego, CA, USA) diluted in 0.02 M TBS with 0.1% Triton X-100 and 4% BSA for 72h at 4ºC. The sections were subsequently incubated for 2h with biotinylated goat-anti-rabbit IgG (1:1000 in 0.2 M TBS) and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). After another washing session, the peroxidase reaction was developed with a DAB-nickel solution and 0.3% H_2O_2. Sections were again washed for 15 minutes in buffer and mounted with a gelatin solution and air-dried, dehydrated in graded alcohol and xylol solutions and then coverslipped with DePeX mounting medium (BDH Laboratory supplies, Poole, England).

5-HT immunostaining was performed on free-floating sections. Sections were washed with 0.01 M TBS and subsequently rinsed with 0.3% H_2O_2 for 10 min to reduce endogenous peroxidase activity. Afterwards, all sections were thoroughly washed with TBS and incubated with mouse anti-5-HT antibody (1:100000; L. Leger antibody, France, protocol:(169)) diluted in 0.01 M TBS with 0.4% Triton X-100, 5% NSS and 1% BSA for two hours at 37ºC, followed by 12 h at room temperature and finally 96h at 4ºC. After thorough washing, the sections were subsequently incubated for two hours with biotinylated sheep-anti-mouse IgG (1:200, Amersham Life Science, in pre-incubation mixture) and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). After another thorough washing, the peroxidase reaction was developed with a DAB-nickel solution and 0.3% H_2O_2. Sections were washed for 15 minutes in 0.01 M TBS.

All sections were mounted with a gelatin solution and air-dried, dehydrated in graded alcohol and xylol solutions and then coverslipped with DePeX mounting medium (BDH

Laboratory supplies, Poole, England).

Fos positive cells in the hypothalamic paraventricular nucleus (PVN), prefrontal cortex (PFC), amygdala, nucleus accumbens (NAcc), dorsal raphe nucleus (DRN), and median raphe nucleus (MRN) were quantified using a computerized imaging analysis system. The PVN, PFC and NAcc are part of brain circuits involved in stress behavior and emotional memory (170-172). The DRN was investigated since this region is the origin of serotonergic projections to a.o. the PFC and NAcc.

5-HT positive cells in the DRN and MRN of the two CS rats on a Trp^- and Trp^+ diet, respectively, were quantified. The selected areas were digitized, using a Sony charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at 100 x magnification. Regions of interest were outlined and the positive nuclei were counted in a single focus plane, using a computer-based image analysis system (Leica Imaging System Ltd., Cambridge, UK). Results are reported as number of positive cells/0.1 mm^2. Per rat, four slices were counted and the average was taken as a single observation. No left-right asymmetry of fos immunoreactivity was found and therefore the mean ± S.E.M. was calculated over both sides.

Statistical analysis

Statistical analyses were done using SPSS (version 12.0), with significance determined at $p \leq 0.05$. Data are shown as mean ± S.E.M. Plasma tryptophan concentrations, corticosterone levels, food intake, organ weight (corrected for body weight), c-fos and 5-HT were analyzed using a one-way ANOVA. The behavioral responses on the first day of testing were analyzed with an one-way ANOVA, corrected for body weight. Weight gain and percent motor response were analyzed with a repeated-measurements ANOVA. Correlations were counted using the Pearson correlation coefficient. Greenhouse-Geisser correlations were used when the assumption of sphericity was not met.

RESULTS

Experiment 1

Plasma tryptophan and corticosterone levels (table 1)

Baseline Trp levels did not differ between the experimental groups. Tryptophan levels on day 3 ($F = 131.973$, $P < 0.001$) and at termination ($F = 13.371$, $P = 0.005$) were significantly lower in the Trp^- compared to the Trp^+ rats. No differences are seen in corticosterone levels between the two treatment groups on day 3, but on day 7, plasma levels of corticosterone are significantly higher in Trp^- animals compared to Trp^+ animals ($F = 9.180$, $P = 0.014$).

Table 1: Plasma levels corticosterone and tryptophan experiment 1

		Diet	
		Trp+(n=6)	Trp- (n=6)
Corticosterone (nmol/l)	Day 3	812 ± 92	1112 ± 138
	Day 7	241 ± 46	409 ± 34*
Tryptophan (μmol/l)	Day -1	130 ± 4	135 ± 13
	Day 3	118 ± 7	32 ± 3***
	Day 7	111 ± 20	44 ± 4**

Average values of plasma corticosterone and plasma tryptophan levels of animals on a control diet (Trp+) or a low tryptophan diet (Trp-). Day -1: baseline measures; Day 3: immediately after the first acoustic stimulus session; Day 7: two hours after the fifth acoustic stimulus session. Data are presented as mean ± S.E.M. *P≤0.05, **P≤0.01, ***P≤0.001.

Body weight and food intake

Before the switch to the experimental diets, body weight did not differ between the two groups (260 g ±4 gram). After the diet switch the Trp^- animals started to lose weight (interaction effect of day*diet, $F = 72.083$, $P < 0.001$). At the end of the experiments, the Trp^+ animals weighed 286 ± 8 gram, while the Trp^- groups weighed 252 ± 2 gram ($F = 16.420$, $P = 0.002$) No differences in food intake were seen after the switch to the experimental diet (food intake 17 ± 1 gram), but food intake in Trp^- animals was significantly lower than in Trp^+ animals from day three, when the animals were tested daily (Trp^+ 19 ± 1 gram, Trp^- 12 ± 1 gram; $F = 17.508$, $P = 0.002$). In this experiment, a low tryptophan diet did not lead to changes in relative adrenal weight (Trp^+ 0.17 ± 0.005 ‰; Trp^- 0.17 ± 0.15‰) or relative thymus weight (Trp^+ 1.94 ± 0.07 ‰; Trp^- 1.7 ± 0.12‰).

Behavioral testing (figure 1).

Repeated exposure to the acoustic stimuli resulted in a decrease in motor response in all animals, both within one session (short-term habituation; main effect of trial $F = 3.647$, $P = 0.024$) and across experimental days (long-term habituation; main effect of day $F = 10.390$ $P = 0.001$). There was no statistical significant difference between both treatment groups on startle reactivity, nor on the rate of habituation of the animal.

Experiment 1 demonstrated an increased stress-sensitivity in Trp^- rats. This was seen in increased corticosterone levels at the time of termination and decreased food intake during the days of testing (118;160). However, this increased stress-sensitivity was neither reflected in increased adrenal weight, nor in the stress-reactivity or habituation to the acoustic stimuli.

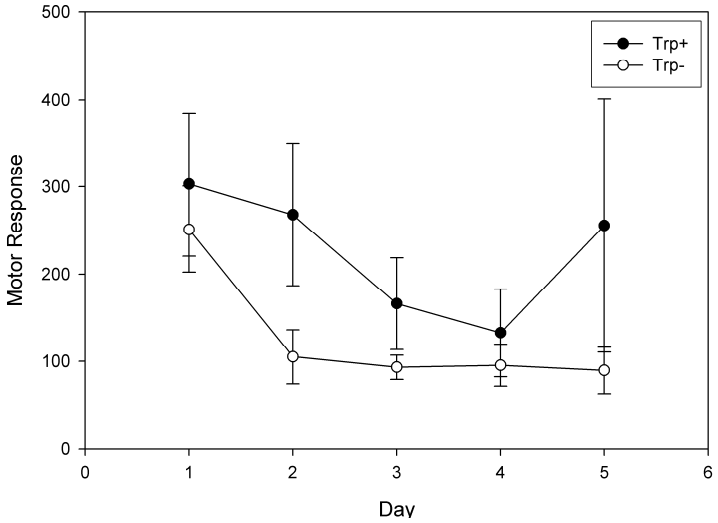

Figure 1: Response to acoustic stimulo experiment 1. Motor response to acoustic stimuli in rats fed with a control diet (Trp^+) or a low tryptophan diet (Trp^-). Results are expressed as average value per day ± S.E.M.

Experiment 2

Tryptophan, serotonin and corticosterone levels (figure 2)

Plasma Tryptophan concentrations were significantly lower in Trp^- rats than in Trp^+ animals, on day 6 (CS F = 326.08, P < 0.001; AS F = 119.43, P < 0.001) and day 14 (CS F = 249.36, P < 0.001; AS: F = 180.91, P < 0.001).

A dietary effect was seen in whole blood 5-HT levels in both the CS and AS experiment on day 6 (CS F=8.463, P = 0.009; AS F = 5.672, P = 0.049) and day 14 (CS F = 6.023, P = 0.023; AS: F = 7.133, P = 0.028). Furthermore, a stress effect was seen in the Trp^+ group on day 6 (F = 7.073, P = 0.020). No significant differences were seen between Trp^-CS and Trp^-AS.

On day six, tryptophan depletion had no effect on baseline plasma corticosterone levels of the AS animals. A dietary effect was also not noted with respect to plasma corticosterone levels in the CS animals. However, a stress effect was observed between Trp^-CS and Trp^-AS (F = 6.647, P= 0.023), and between Trp^+CS and Trp^+AS (F = 9.475, P= 0.009).

On day 14, a significant dietary effect was seen in the CS experiment (F= 19,216, P < 0.001) and in the AS experiment (F= 6,323, P= 0.036), and a stress effect was observed between Trp^-CS and Trp^-AS animals (F =8.668, P= 0.011).

Amino Acids (table 2)

Trp$^-$ diet increased plasma concentration of glycine and arginine. The concentrations of tryptophan, valine, leucine, isoleucine, methionine, asparagine, glutamic acid, and phenylalanine were reduced in these animals. Tryptophan:LNAA ratio is significantly higher in the Trp$^+$ group compared to the Trp$^-$ group ($F= 57.512$, $P< 0.001$).

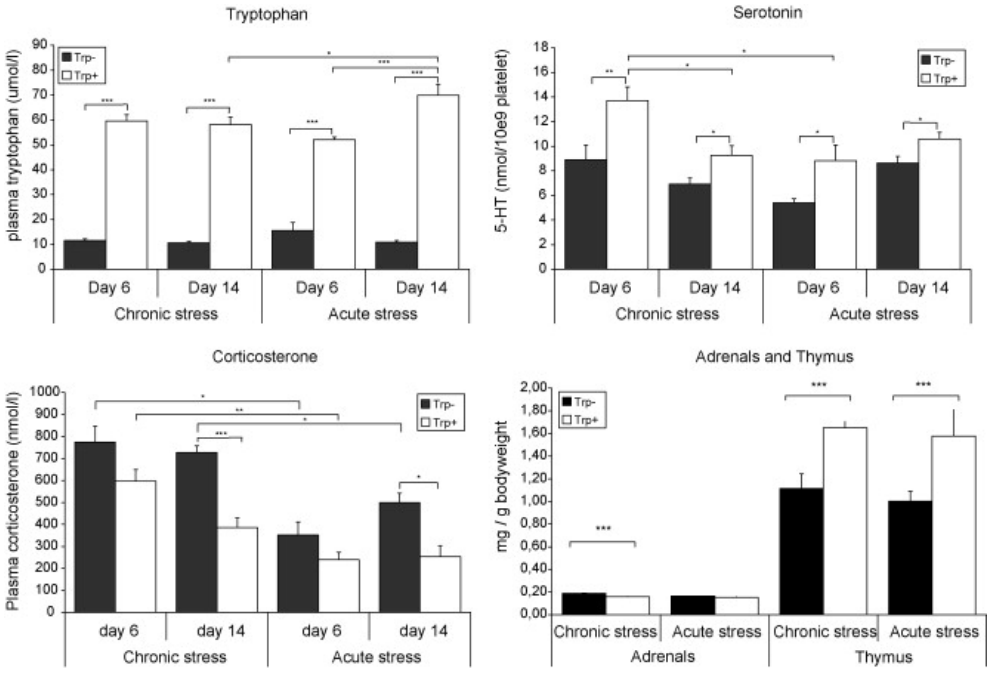

figure 2: Biochemistry and organ weight experiment 2. Plasma levels of tryptophan and corticosterone, whole blood levels of serotonin and adrenal and thymus weight as a permillage of body weight of experiment 2. Groups of male rats received either chronic (9 days) or acute stress (1 day) in the acoustic startle box. All rats were either on a low tryptophan diet (black bars) or on a control diet (white bars). Data are presented as means ± S.E.M. (n=10/group for the chronic stress experiment and 5/group for the acute stress experiment). *P≤ 0.05, **P≤ 0.01, ***P≤0.001

Table 2: Diet

		Diet composition gram / 100 gram		Plasma levels (mmol/l)	
		Trp+	Trp-	Trp+ (n=10)	Trp- (n=10)
Amino acids	Alanine	0.587	0.587	863 ± 32	801 ± 42
	Arginine	0.688	0.688	80 ± 3	125 ± 8***
	Asparagine	0.835	0.835	164 ± 2	121 ± 12**
	Aspartic acid	0.591	0.591	19 ± 2	16 ± 1
	Cystine	0.451	0.451		
	Glutamine	1.589	1.589	852 ± 42	737 ± 32
	Glutamic acid	2.444	2.444	115 ± 9	79 ± 4**
	Glycine	0.344	0.344	257 ± 10	376 ± 9***
	Histidine	0.552	0.552	80 ± 4	95 ± 5
	Isoleucine	0.982	1.034	165 ± 10	113 ± 13*
	Leucine	1.809	1.906	310 ± 20	225 ± 25*
	Lysine	1.758	1.758	529 ± 38	429 ± 51
	Methionine	0.568	0.568	234 ±13	138 ± 14***
	Phenylalanine	0.965	0.965	92 ± 2	76 ± 3***
	Proline	1.723	1.723	785 ± 38	735 ± 45
	Serine	1.137	1.137	431 ± 11	494 ± 29
	Threonine	0.845	0.845	497 ± 12	686 ± 84
	Tryptophan	0.24	0.024	63 ± 3	10 ± 1***
	Tyrosine	1.051	1.051	149 ± 25	117 ± 10
	Valine	1.24	1.307	387 ± 18	280 ± 37*
Carbohydrates	Cornstarch	14.52	14.52		
	Cellulose	5	5		
	Glucose	50	50		
Oil	Soybean oil	5	5		
Minerals	$CaCO_3$	1.24	1.24		
	$NaH_2PO_4.2H_2O$	0.34	0.34		
	$MgCO_3$	0.14	0.14		
	KCl	0.11	0.11		
	KH_2PO_4	1.05	1.05		
Minerals mix		1	1		
Vitamins mix		1.2	1.2		
Total		100	100		

Diet composition of the control diet (Trp+) and low tryptophan diet (Trp-) and plasma levels of the amino acids in rats in experiment 2 at termination. Plasma levels are presented as mean± S.E.M. *P≤0.05, **P≤0.01, ***P≤0.001

Body Weight, food intake and organ weights

Before the exposure to the experimental diets, the animals weighed 267 ± 2 g, with no differences between the groups. Almost immediately after the dietary change, Trp^- animals started to lose weight, while Trp^+ continued to grow (interaction effect of diet*day F= 97.514, P< 0.001) (figure 3). Diet attenuated food intake (day*diet F = 6.268, P < 0.001). Overall, the

mean food intake per day in Trp^- animals was 9.8 ± 0.3 g, while Trp^+ animals ate 16.1 ± 0.3 g per day. The pattern of weight gain on day -4 and day 2 can be explained by the *ad libitum* avalibility of food on these days. In addition, stress augmented the decrease in food intake in the Trp^- group; Trp^-CS animals ate significantly less than Trp^-AS animals (F = 11.357, P = 0.005). The attenuated food intake in Trp^- animals is consistent with previous reports, in which animals fed with a diet lacking only one amino acid, ate 2/3 of the animals on the control diet (Rogers and Lueng 1973). The Trp^- diet resulted in significant decreases in relative thymus weight, both in the CS (Trp^-CS 1.113 ± 0.131, Trp^+CS 1.652 ± 0.054; F= 14.614, P= 0.001) as well as in the AS experiment (Trp^-AS 1.001 ± 0.090, Trp^+AS 1.578 ± 0.232; F= 5.379, P= 0.049). The adrenal glands of the Trp^-CS rats (0.189 ± 0.004) were significantly larger than those of the Trp^+CS animals (0.157 ± 0.004; F= 25.178, P< 0.001); this effect was not seen in the AS experiment (figure 2). Plasma corticosterone was positively correlated with adrenal weight (Pearson correlation 0.725, P< 0.001), and negatively correlated with thymus weight (Pearson correlation -0.468, P= 0.009).

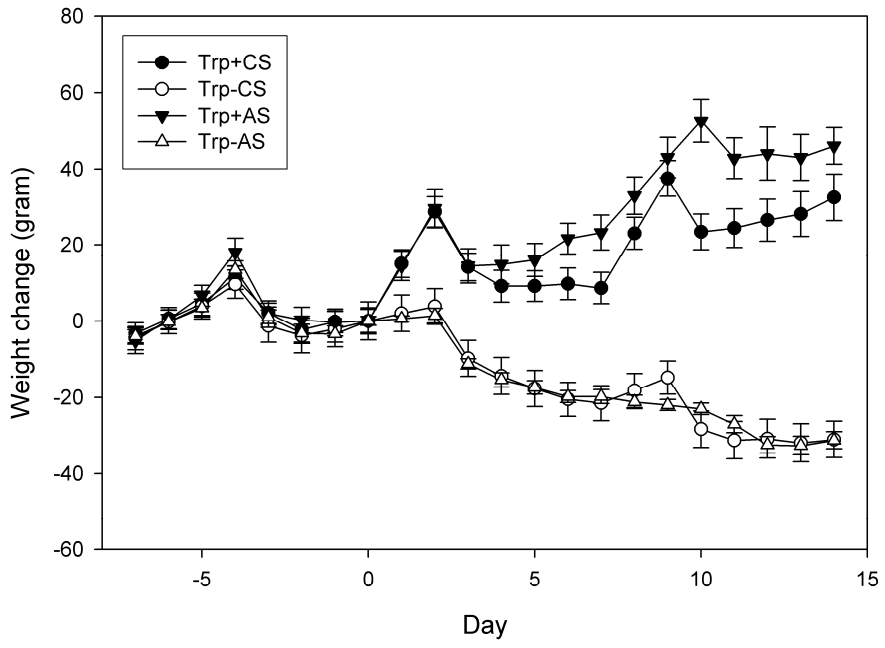

Figure 3: Body weights change during experiment 2 in rats fed with a control diet (Trp^+) and rats on a low tryptophan diet (Trp^-). Day 0: change to experimental diet. CS: chronic stress; AS: acute stress

Behavioral testing (figure 4)

In contrast with the results of experiment 1, a treatment effect was seen in response to the acoustic stimuli in experiment 2. The amplitude of the motor response on the first day of testing was significantly larger in Trp^-CS animals than in Trp^+CS animals ($F = 11.720$, $P = 0.003$), suggesting that Trp^-CS animals were more sensitive to the immobilization period that Trp^+CS animals. Consistent with experiment one, repeated exposure to the acoustic tests decreased motor response in all animals, both within sessions (short-term habituation; main effect of trial; $F= 10.940$, $P < 0.001$) and over the days (long-term habituation; main effect of day, $F= 21.379$, $P< 0.001$). Percent habituation did not differ between Trp^-CS and Trp^+CS animals.

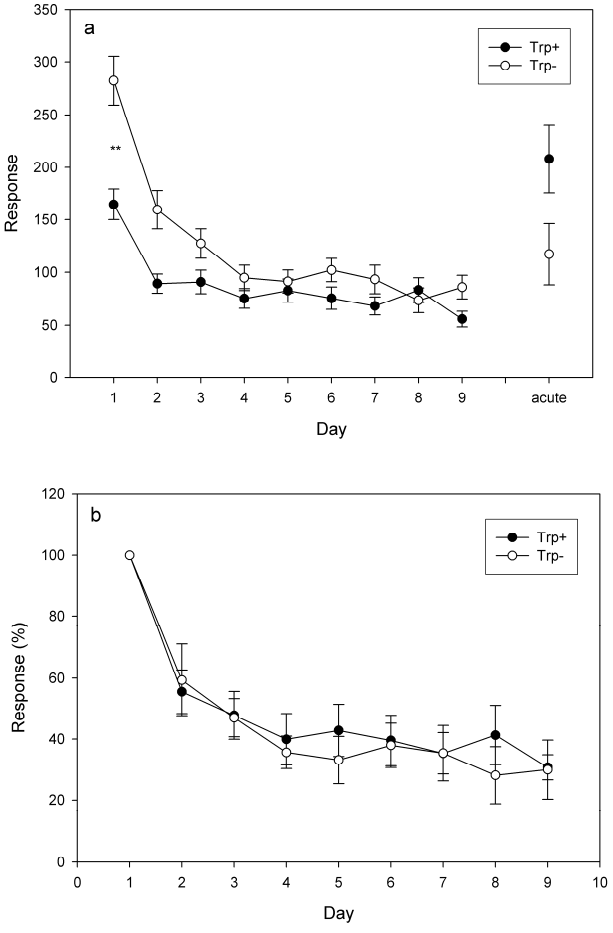

Figure 4: Motor response (a) and percent response compared to day 1 (b) to acoustic stimuli in rats fed with a control diet (Trp^+) or a low tryptophan diet (Trp^-). Day 1 till 9: chronically stressed rats. Acute: rats that were tested only once. Results are expressed as average value per day ± S.E.M. **P < 0.01

Trp^-CS animals showed higher within group variability in response to acoustic startle compared to Trp^+CS animals during almost the entire experiment (day*diet; F= 2.957, P= 0.026), which was most pronounced during the first 5 days of stress (day*diet; F= 4.119, P= 0.013). This variability in response also showed some adaptation, as it decreased over time (main effect of day; F= 10.954, P < 0.001; main effect of trial; F= 6.422, P < 0.001). A positive correlation between body weight and startle response was found in Trp^-CS animals during the first startle session (Pearson: 0.729, P = 0.017), but not on any other day.

Histology (table 3)

Trp^- diet substantially decreased the number of 5-HT stained cells in the DRN (F= 82.062, P< 0.001) and MRN (F= 95.618, P< 0.001).

C-fos expression in PVN cells was significantly reduced in Trp^-CS, compared to Trp^+CS animals (F = 12.975, P<0.001) and Trp^-AS animals (F = 6.564, P=0.011). In the PFC, fos count in Trp^-CS was significantly lower than in Trp^-AS (F = 4.341, P=0.042). In the DRN, Trp^- animals had significantly more fos activation than Trp^+ animals (CS: F= 7.808, P= 0.007 and AS: F = 7.479, P = 0.012). A comparison with 5-HT staining revealed that there was little, if any, co-localization between 5-HT positive cells and c-fos positive cells. Trp^+AS animals showed increased activation in the core of the NAcc, compared to Trp^+CS (F = 8.286, P = 0.006) and Trp^-AS animals (F = 9.948, P=0.004). In the shell of the NAcc, a similar pattern was seen; Trp^+AS had significantly higher fos activation than either Trp^+CS (F = 12.031, P = 0.001) and Trp^-AS (F = 7.048, P = 0.012). Except for the DRN, the results of the fos-study show that following long-term depletion of tryptophan and concomitant low cerebral 5-HT levels, the brain becomes less reactive to the immobilization stress, either given once or repeatedly.

Table 3: Histology

		Control diet		Low tryptophan diet	
Fos positive cells		CS (n=10)	AS (n=5)	CS (n=10)	AS (n=5)
PVN Hypothalamus		133 ± 8	139 ± 7	95 ± 8***	124 ± 9##
Prefrontal cortex		73 ± 8	77 ± 13	74 ± 12	108 ± 12#
Dorsal raphe nucleus		59 ± 8	46 ± 5	106 ± 15**	85 ± 13*
Nucleus accumbens	Core	53 ± 7	86 ± 10##	63 ± 9	49 ± 6**
	Shell	61 ± 5	105 ± 14###	63 ± 5	62 ± 6*
5-HT positive cells		CS		CS	
Dorsal raphe		89 ± 9		8 ± 2***	
Median raphe		113 ± 10		12 ± 3***	

Fos and 5-HT positive cells in brain areas of animals on a low tryptophan diet or a control diet after chronic stress (CS) or acute stress (AS). Data as cells/0.1mm^2 ± S.E.M. Dietary effect: *P≤0.05, **P≤0.01, ***P≤0.001. Stress effect: #P≤0.05, ##P≤0.01, ###P≤0.001

DISCUSSION

The main result of the present study is that a low tryptophan diet increases stress-sensitivity in rats. This is depicted as increased plasma corticosterone levels and adrenal glands, decreased body weight and food intake, in addition to increased responses to acoustic stimuli in Trp^- rats (although mainly when concomitant receiving immobilization sessions). Low tryptophan levels in combination with stress, also enhance c-fos expression in a variety of brain nuclei. However, habituation to acoustic stimuli is not affected by tryptophan depletion. These observations support a modulating function of brain 5-HT on the reactivity to stress but not necessarily on habituation to stressors.

As expected, use of a diet with a low percentage of the amino acid tryptophan, significantly decreased plasma tryptophan, whole blood 5-HT, and neuronal 5-HT content in the dorsal raphe nucleus. Previous studies have shown that a similar low tryptophan diet depletes 5-HT levels in the hippocampus, striatum, and decreases 5-HT release in the frontal cortex and hippocampus of freely moving rats (162;173). In addition to changes in tryptophan and 5-HT levels, Trp^- diet also reduced plasma levels of seven amino acids, including 5 LNAA's, while plasma levels of arginine and glycine were increased. These changes were maximal 50% and often less, as compared to the 6 fold decrease of plasma tryptophan. On the other hand, these changes in plasma amino acid concentration were unrelated to the slight differences in the diet content of some of these amino acids. Although it is shown that tryptophan depletion does not reduce the noradrenaline count in the brain, the dietary tryptophan depletion paradigm may be less specific to study the consequences of reduced brain 5-HT function as has often been suggested (47). To assess the overall consequences of the diet we measured also food intake and bodyweight. The low tryptophan diet caused a decrease in food intake, and consequently weight loss (73). Although (174) reported that attenuated startle response can be due to self-induced food deprivation. It is unlikely that weight loss or decreased food intake affected the present results. There were no correlations between body weight, body weight change, or food intake and behavioral parameters. Thus food intake, and consequently changes in body weight cannot account for the changes in motor reaction to the acoustic stimuli.

The increased reactivity of Trp^- rats in experiment two suggests that 5-HT depletion enhances the aversiveness of stressors. Acoustic stimuli are widely used to assess sensitivity to stress in animals and humans (118). In experiment one, the low tryptophan diet had no effect on motor response to the acoustic stimuli. However, the combination of Trp^- diet and immobilization stress (experiment two) significantly increased the amplitude of the motor response of Trp^-CS animals during the first day of stress. Thus, tryptophan depletion may only affect behavioral responses when the animals receive an additional stressor.

This augmentation effect was described before in medial forebrain lesioned rats, in which 5-HT levels were depleted by 58% (175). In that experiment, only the lesioned rats that also received foot shocks, responded more strongly to auditory stimuli than intact rats.

Habituation to the acoustic stimuli, on the other hand, was not affected by diet in this study, as all animals show both intra-session and inter-session habituation to the acoustic stimuli. Rates of habituation also did not differ between Trp^+ and Trp^- animals. Therefore although behavioral data indicate that the stressor is perceived as more aversive, rats are still able to cope with the stressor.

Also the physiological parameters demonstrated increased stress-sensitivity in Trp^- animals. Plasma corticosterone levels of Trp^- animals were elevated and the adrenal glands of Trp^-CS animals were enlarged. The physiological reaction to stress includes enhanced activation of the HPA-axis and accordingly, increased secretion of glucocorticoids by the adrenals. Prolonged stimulation of the adrenals causes adrenal hypertrophy. Normally, the initial high HPA-axis response fades after repeated exposure to a mild stressor, indicating adaptive capability of the organism (15-18). Therefore, plasma corticocosterone and adrenal weight allow to distinguishing between acute and chronic stress. In our experiment, stressed rats had a lower food intake during days with acoustic stimulus testing than on days without testing. It has been found that male rats eat less during stressful conditions (118;160). Without exposure to additional stress, it has also been shown that rats on a regimen of food restriction, thus growing less, have also elevated corticosterone levels (176). Low food intake alone, however, does not explain the elevated levels of corticosterone, as the increases were less pronounced without stress exposure in the Trp-AS group. Taken together, the data are in line with the idea that tryptophan depleted animals become more sensitive to stress than control rats.

It appeared that the selected brain areas do not respond in a similar way to the diet and to stress. PVN fos immunoreactivity was decreased in chronically but not acute stressed rats on a low tryptophan diet. The reaction to stress is higher at low 5-HT, as illustrated by both the corticosteron levels and the startle response, the lower PVN response can readily be explained by a stronger negative feed-back on HPA-axis activity following the prolonged high levels of glucocorticoids (177). But we did not observe a significant correlation of the PVN-fos counts and circulating corticosterone. So the coupling between PVN activity and circulating glucocorticoids may become dissociated at low 5-HT; such dissociation has also been observed in depression (e.g. Pariante and Miller, 2001). The PVN is required for the maintenance of homeostasis and adaptation to challenges from the internal or external stressors (178). Indeed, we found a correlation between PVN fos count and body weight in CS animals.

There was little, if any, co-localization between 5-HT positive cells and c-fos positive cells. The DRN of the Trp^- animals had significantly more fos activation than that of the Trp^+ animals. These two observations suggest a modulating effect of 5-HT on non-serotonergic

neurons that is not or indirectly mediated by 5-HT-autoreceptors on the serotonergic neurons of the DRN. Increased c-fos expression in the DRN was seen Trp^-CS, as compared to Trp^-AS. One could suggest that when 5-HT modulation is impaired,the response to stress of these non-serotonergic neurons becomes oversensitive. By implication, we suggest that this 5-HT modulation prevents sensitization rather than affecting habituation. Conversely, after chronic stress this diet resulted in decreased fos expression not in the DRN, but in the PFC. The first observation is in line with the idea of an increased sensitivity to stress at low 5-HT levels. The latter also suggests that the immobilization procedure still leads to habituation, irrespective of endogenous 5-HT levels. On the other hand, such a habituating effect of immobilization was not apparent in the normal tryptophan fed rats, possibly because the stress was less dramatically perceived. Either way, these observations illustrate that indeed a stress response and perception of a stressor is susceptible to the cerebral tonus of 5-HT. In contrast, in both core and shell of the NAcc, an increase of c-fos expression in Trp^+AS animals compared to Trp^+CS and Trp^-AS animals was seen. These observations point to the fact that in the NAcc normally functioning 5-HT neurons contribute to respond and cope with immobilization-stress.

Although a limited number of brain nuclei were quantified, highly significant differential effects on c-fos counts were observed in the various brain regions. Our study emphasizes that impaired 5-HT neurotransmission not only modifies the neuronal response and habituation to stress, but may also lead to sensitization in different brain regions. Clear discrepancies between the histological and behavioral observations were noted, however: the neuronal responses to stress persisted and were more augmented in this respect, which is in line with the endocrine data, while behavioral data show effective habituation irrespective of the low 5-HT.

Dysfunction of the 5-HT system has been reported in various types of psychiatric disorders, such as depression, anxiety, aggression and impulse control (58;146;179). Our chronic stress experimental paradigm resembles many of the features seen in e.g. patients suffering from a major depression, such as decreased plasma tryptophan, in addition to enlarged adrenals, increased plasma cortisol levels, weight loss and decreased food intake.

The present results suggest an indirect, rather than a direct and exclusive relationship between 5-HT function and psychopathology. These observations together suggest that impaired cerebral 5-HT function increases the sensitivity to any, mild aversive stimulus. Impaired 5-HT function may then lead to aggravation of the symptoms of an already existing, but sub-clinical psychiatric syndrome, or may facilitate the development of stress-related disorders. Tryptophan supplementation could therefore be considered as a therapeutic or prophylactic treatment of patients.