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Acute effect of simultaneous administration of tryptophan and ethanol on serotonin metabolites in the locus coeruleus in rats

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Abstract

Using the microdialysis method, we investigated whether the levels of serotonin (5-hydroxytryptamine, 5-HT) and its metabolites, 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTPL), in the locus coeruleus are influenced by tryptophan alone or simultaneous administration of tryptophan and ethanol. Tryptophan (50 mg/kg, i.p.) led to a significant increase in the levels of 5-HIAA, but not 5-HT in the locus coeruleus. However, ethanol (1.25 g/kg) had no effect on the levels of 5-HT and its metabolites. Combined administration of tryptophan and ethanol caused very marked increases in 5-HIAA and 5-HTPL levels in the locus coeruleus. A time lag in the increased 5-HIAA levels between tryptophan alone and tryptophan plus ethanol was observed. Moreover, 5-HIAA levels in the locus coeruleus induced by tryptophan were abolished by microinjection of 5,7-dihydroxytryptamine (150 μg/4 μl) into the dorsal raphe nucleus. Judging from the present results, the serotonergic afferents to the locus coeruleus may originate for about 20–30% from cell bodies located in the dorsal raphe nucleus. Teeth-chattering was significantly detected in the tryptophan plus ethanol-treated rats when compared with the tryptophan-treated rats, but not in the saline-treated controls. These results may suggest that the increased levels of 5-HIAA and 5-HTPL in the locus coeruleus induced by tryptophan are potentiated by ethanol, and that these levels are partly responsible for behavioral activation.

Keywords: Locus coeruleus; Dorsal raphe nucleus; Microdialysis; 5-HT (5-hydroxytryptamine, serotonin); 5-HIAA (5-hydroxyindoleacetic acid); Tryptophan; Ethanol; 5,7-Dihydroxytryptamine

1. Introduction

The locus coeruleus and the raphe nuclei are part of the ascending reticular activating system, which implicates the reticular "core" of the brain stem in processes that arouse and awaken the forebrain (Bear et al., 1996). Serotonergic afferents to the locus coeruleus are derived from the dorsal raphe nucleus (Cedarbaum and Aghajanian, 1978; Morgane and Jacobs, 1979; Kaehler et al., 1999a; Mateo et al., 2000), and serotonergic perikarya are present in the locus coeruleus region (Akaoka and Aston-Jones, 1991; Aston-Jones et al., 1997; Kaehler et al., 1999b). In addition, neuronal 5-HT release in the locus coeruleus is modulated by 5-HT_{1A} receptors located within the dorsal raphe nucleus (Mongeau et al., 1998; Kaehler et al., 1999a; Reneric et al., 2002). Recently, Singewald et al. (1997) demonstrated that the serotonergic system of the locus coeruleus is implicated in behavioral activation, pain, noxious and stress stimuli, and modulation of cardiovascular activity (Bear et al., 1996). However, the neuroanatomical and functional interaction between the locus coeruleus and the dorsal raphe nucleus is still not clarified.

Dietary tryptophan augmentation is known to increase brain 5-HT (5-hydroxytryptamine) synthesis and content (Fernstrom, 1983; Stancampiano et al., 1997; Fadda et al., 2000). Conversely, a deficiency in 5-HT synthesis and metabolism has been implicated in several neuropsychiatric disorders, including alcoholism, aggression (LeMarquand et al., 1994) and depression (Meltzer, 1990). 5-HT is oxidatively deaminated by monoamine oxidase to 5-hydroxyindoleacetaldehyde (5-HIAL). The 5-HIAL formed can be metabolized in two ways: dehydrogenation by aldehyde dehydrogenase to 5-HIAA, or reduced by aldehyde reductase to 5-HTPL (Youdim and Ashkenazi, 1982; Gwaltney-Brant et al., 2000). In mammals, ethanol is metabolized into acetaldehyde by alcohol dehydrogenase, cytochrome P-450 2E1 (CYP2E1) and catalase, and then acetaldehyde is further

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metabolized into acetic acid by aldehyde dehydrogenase and CYP2E1 (Lieber, 1999). The most important enzymes catalyzing the conversion of ethanol to acetaldehyde and acetate are alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Aldehyde dehydrogenase activity is detectable in practically all tissues (Pappas et al., 1997; Karamanakos et al., 2001), and acute ethanol intake inhibits the metabolism of other drugs by competition for biogenetic enzymes in liver or brain (Lieber, 1999).

To investigate whether the tryptophan-induced release of 5-HT in the locus coeruleus is altered by ethanol, we examined alterations in 5-HT and its metabolites in the locus coeruleus after administration of tryptophan in combination with ethanol to rats. Moreover, behavioral signs during treatment with each drug were also investigated. In addition, we also investigated whether the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT), microinjected into the dorsal raphe nucleus, influences 5-HT release in the locus coeruleus.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (260–330 g, SLC Japan, Hamamatsu, Japan) were purchased and maintained under conditions of 23 ± 1 °C with a 12:12 h light–dark cycle for 1 week prior to surgery. All procedures involving rats were performed using protocols approved by our Institutional Animal Care and Use Committee.

2.2. Drugs

L-Tryptophan, serotonin (5-hydroxytryptamine, 5-HT), 5-hydroxyindoleacetic acid (5-HIAA), *N*-acetyl-serotonin, 5-hydroxytryptophol (5-HTPL) and 5,7-dihydroxytryptamine (5,7-DHT) were purchased from the Sigma (St. Louis, MO). Other reagents were of analytical grade.

2.3. Surgical procedures

As previously described (Hoshi et al., 2000), rats were anesthetized and a CMA/11 guide cannula (Bioanalytical Systems, West Lafayette, IN) was stereotaxically implanted in the locus coeruleus—9.8 mm posterior to the bregma, 1.1 mm lateral, and 6.8 mm below the skull surface. A stainless steel guide cannula (outer diameter 0.35 mm, inner diameter 0.15 mm) with its stylet was stereotaxically inserted until the tip of the cannula was 2 mm above the dorsal raphe nucleus. Coordinates were (in mm) AP 7.8 posterior to bregma; L 0.0; V 5.0 from the skull surface, according to the atlas of Paxinos and Watson (1986). After surgery, rats were given 300,000 units of benzyl penicillin G subcutaneously and were allowed 1 week to recover from implantation. Upon completion of the experimental sequence, each brain was removed and fixed at least 2 days in formalin. The cannula track was verified visually by cutting vertically through the cannula marks on the cortex surface. The brain fragment was frozen and 14 μm sections were cut through the locus coeruleus or the dorsal raphe nucleus using a microtome-cryostat (Ames Lab-Tek, Westmont, IL). The probes were located within the locus coeruleus and the dorsal raphe nucleus area.

2.4. Treatment of animals

Tryptophan was dissolved in 0.25N KOH solution and administered 0.5 ml per 100 g weight. Rats were intraperitoneally given a single dose of tryptophan (50 mg/kg), ethanol (1.25 g/kg) or tryptophan plus ethanol (50 mg/kg + 1.25 g/kg). The control animals received an equivalent volume of saline. To test the effects of the combined treatment on the levels of 5-HIAA in the locus coeruleus, rats were treated with tryptophan (50 mg/kg) 1 h prior to infusion of 5,7-DHT (150 μg in 4 μl) into the dorsal raphe nucleus.

2.5. Measurement of behavioral signs elicited by the drugs

After administration of tryptophan, ethanol and both drugs combined, behavioral signs (teeth-chattering, wet-dog shakes, penis-licking, locomotion, stretching, scratching, salivation, sniffing, rearing and abnormal posturing) were scored for 120 min. For statistical purposes, a simple scoring system (one point for each sign and summation of the 10 signs to give a total score for each rat) was used to compare responses between the control group receiving vehicle and the tryptophan, ethanol alone, or tryptophan plus ethanol groups.

2.6. Sampling for microdialysis and analysis of 5-HT and its metabolites

The microdialysis probes (CMA/11, 2 mm tip) and guide cannula were purchased from Bioanalytical System (BAS, Tokyo, Japan) and used within 3 months. The dialysis membrane tip of the probe had an outer diameter of 240 μm and inner diameter of 210 μm, a dead volume of 1 μl, and a molecular weight cut-off of 20,000 Da. The in vitro recovery of 5-HT, 5-HIAA, *N*-acetylserotonin and 5-HTPL was determined by immersing the probes in Ringer's solution containing 100 μM each of 5-HT, 5-HIAA, *N*-acetylserotonin and 5-HTPL at room temperature. Probes were dialyzed with Ringer's solution, and dialysate samples were analyzed by high-performance liquid chromatography (HPLC; BAS LC-4C Detector) with electrochemical detection using a Waters Spherisorb Column (150 × 4.6 mm; 5 μm ODS2) with electrochemical detection. The mobile phase consisted of 75 mM NaH2PO4, 1 mM sodium octylsulfate, 0.05 mM EDTA-2Na, 6% methanol and 6% acetonitrile. The pH was adjusted to 3.0 with concentrated phosphoric acid; the solution was degassed and pumped at a flow rate of 1 ml/min. The system was calibrated using an external standard and the retention time for 5-HT, 5-HIAA, *N*-acetylserotonin

and 5-HTPL was approximately 8.9, 7.2, 9.7 and 7.7 min, respectively. The sensitivity of the assay and the detection limits for those compounds was 0.03, 0.02, 0.03 and 0.01 ng/30 μl samples, respectively. The recovery of 5-HIAA and 5-HTPL was 9.6 ± 0.4% and 9.6 ± 0.7% of the external concentration, respectively. Peaks of 5-HT and *N*-acetylserotonin were not detected. Due to the variability of probe recovery, the extracellular fluid levels of 5-HIAA and 5-HTPL were individually calculated for each animal.

2.7. General experimental procedures

One week following stereotaxic surgery and the day before the beginning of the experiment, a freshly calibrated microdialysis probe was placed into the locus coeruleus guide cannula. The probe was then perfused with filtered Ringer's solution at a low rate (0.2 μl/min) overnight. On the morning of the following day, the flow rate was increased to 2 μl/min. Collection of consecutive 15-min samples for determination of basal values was begun after 2–3 h of equilibration. Following collection of three consecutive stable (not more than 20% of intersample variation) basal samples, a single i.p. injection of tryptophan (50 mg/kg), ethanol (1.25 g/kg) or tryptophan plus ethanol was given, and consecutive 15-min samples were collected for 2 h. Similarly, 5,7-DHT was injected in a volume of 4 μl over a period of 2 min into the dorsal raphe nucleus.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) and the Newman–Keul's test were used. Calculated values of $P < 0.05$ were considered statistically significant. Data are expressed as percentage change from basal values. Mean values ± S.E.M. are reported.

3. Results

3.1. Effects of tryptophan alone and in combination with ethanol on the levels of 5-HIAA and 5-HTPL in the locus coeruleus

As shown in Fig. 1, administration of tryptophan alone led to a significant increase in 5-HIAA levels, which peaked 60 min after tryptophan administration and slowly returned near to the control level. These levels increased to 130–170% (111–145 nM) of the basal values during 30–120 min sampling, as compared to the control receiving normal saline. However, no significant changes were noted in 5-HIAA levels following a single acute i.p. injection of ethanol or vehicle. Moreover, simultaneous administration of tryptophan plus ethanol produced a delayed increase in 5-HIAA in the locus coeruleus, which reached a peak at 90 min. This increase was sustained for about 30 min before decreasing to the basal values. After combined administration, the dose–response curve for 5-HIAA was significantly shifted to the right as shown in Fig. 1. At 30 min, 5-HIAA levels were different in the tryptophan alone and the tryptophan plus ethanol groups. Moreover, combined administration also caused a significant increase in 5-HTPL levels in the locus coeruleus, although tryptophan alone had no effect on these levels (Fig. 2). In this study, however, the levels of 5-HT and *N*-acetylserotonin in the locus coeruleus remained unchanged after treatment with tryptophan alone or tryptophan plus ethanol (data not shown). Basal levels of 5-HIAA and 5-HTPL averaged 85 ± 16, 83 ± 7, and 76 ± 9 nM, and 5.6 ± 0.7, 5.6 ± 0.9, and 6.0 ± 0.9 nM, respectively, in the tryptophan, ethanol, and control groups.

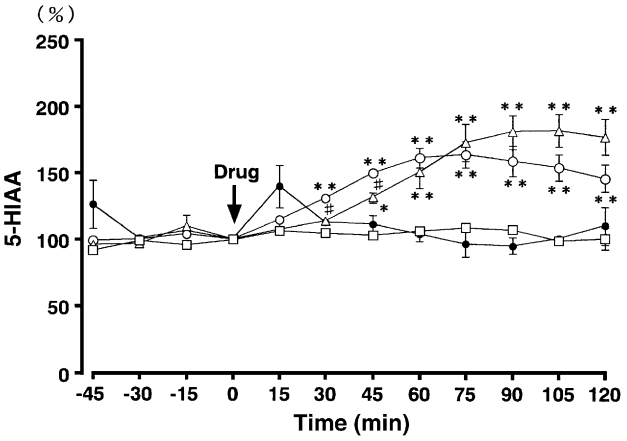

Fig. 1. Extracellular fluid levels of 5-HIAA in the locus coeruleus induced by coadministration of tryptophan and ethanol. Rats were i.p. given a single dose of tryptophan (○, 50 mg/kg; $n = 5$), ethanol (□, 1.25 g/kg; $n = 5$) alone or tryptophan plus ethanol (△, 50 + 1.25 g/kg; $n = 5$), respectively. The control animals received an equivalent volume of saline (●, 50 mg/kg; $n = 4$). Values are expressed as percentage change (mean ± S.E.M.) from basal values. *$P < 0.05$ and **$P < 0.01$, control vs. tryptophan or ethanol plus tryptophan; #$P < 0.05$, tryptophan vs. ethanol plus tryptophan.

3.2. Influence of dorsal raphe nucleus lesion on levels of 5-HIAA in the locus coeruleus

The maximum decrease in 5-HIAA levels in the locus coeruleus elicited by microinjection of 150 μg of 5,7-DHT into the dorsal raphe nucleus was 33–40% (26–32 nM) (Fig. 3); however, saline did not influence the levels of 5-HIAA in the locus coeruleus. The levels of 5-HIAA in the locus coeruleus during 5,7-DHT infusion into the dorsal raphe nucleus 60 min after pretreatment with tryptophan were decreased, and at 30–45 min after 5,7-DHT were approximately 21% lower than those after tryptophan alone (Fig. 3).

3.3. Behavioral signs during tryptophan alone and in combination with ethanol

As shown in Table 1, the composite score for teeth-chattering was significantly higher in the tryptophan plus ethanol-treated rats (42.8 ± 3.8), than in the tryptophan- or

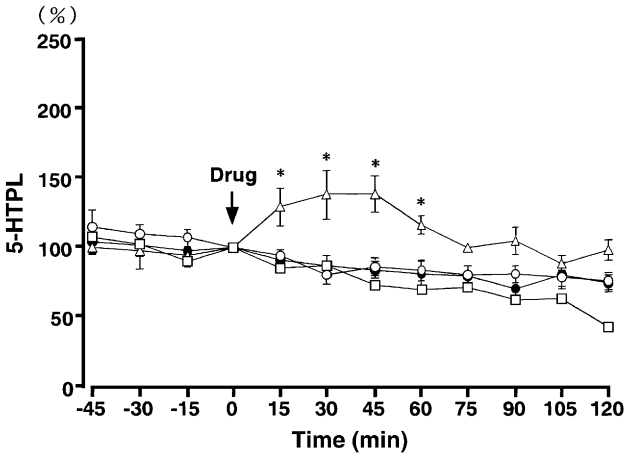

Fig. 2. Extracellular fluid levels of 5-HTPL in the locus coeruleus induced by coadministration of tryptophan and ethanol. Rats were i.p. given a single dose of tryptophan (○, 50 mg/kg; $n=4$), ethanol (□, 1.25 g/kg; $n=4$) or both drugs together (△, 50+1.25 g/kg; $n=4$), respectively. The control animals received an equivalent volume of saline (●, 50 mg/kg; $n=4$). Values are expressed as percentage change (mean ± S.E.M.) from basal values. *$P<0.05$ and **$P<0.01$, control vs. ethanol plus tryptophan; #$P<0.05$, tryptophan vs. ethanol plus tryptophan.

ethanol-treated rats (20.8 ± 6.3 or 18.8 ± 5.6), respectively. Moreover, a significant difference in sniffing was observed between the tryptophan and ethanol groups. However, the composite score for both sniffing and rearing was significantly ($P<0.05$) lower in the tryptophan plus ethanol-treated rats (1.6 ± 0.5 and 1.2 ± 0.6) than in the tryptophan-treated groups (12.6 ± 3.6 and 9.8 ± 3.7). In addition, a significant difference was also noted between the tryptophan and ethanol groups. No significant difference in other behaviors was observed between the tryptophan or ethanol and tryptophan plus ethanol groups. Microinjection of 5,7-DHT into the raphe nucleus did not significantly change behavior in the tryptophan-treated rats compared with the saline-treated controls (data not shown).

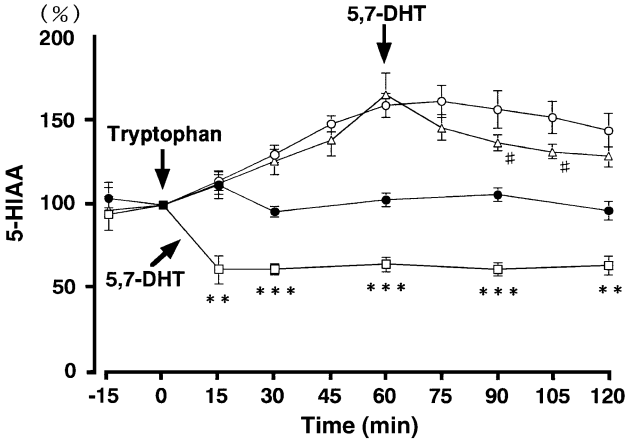

Fig. 3. Influence of dorsal raphe nucleus lesion on levels of 5-HIAA in the locus coeruleus. Rats were i.p. given a single dose of tryptophan (○, 50 mg/kg; $n=5$) in combination with 5,7-DHT (150 μg/4 μl) injected into the dorsal raphe nucleus after pretreatment with tryptophan (△, 50 mg/kg; $n=5$). The control animals received an equivalent volume of saline (●, $n=4$). A single dose of 5,7-DHT (□; $n=4$) into the dorsal raphe nucleus was given over 2 min. Student's t-test was used to establish significant differences in mean values between the control and 5,7-DHT-treated groups or tryptophan alone and combined groups. Values are expressed as percentage change (mean ± S.E.M.) from basal values. **$P<0.01$ and ***$P<0.001$, control vs. 5,7-DHT; #$P<0.05$, tryptophan vs. 5,7-DHT plus tryptophan.

Table 1
Acute effect of simultaneous administration of tryptophan on behavior in rats

Behavioral signs	Control	Tryptophan	25% EtOH	25% EtOH + tryptophan
Teeth-chattering	19.5 ± 6.2	20.8 ± 6.3	18.8 ± 5.6	42.8 ± 3.8[a,b,c]
Wet-dog shakes	1.8 ± 1.0	2.5 ± 2.2	1.8 ± 1.4	1.3 ± 0.6
Penis-licking	3/4	4/5	2/5	3/5
Stretching	0.8 ± 0.5	0.5 ± 0.3	0.4 ± 0.2	0.8 ± 0.8
Locomotion	8.8 ± 3.7	14.8 ± 4.0	10.6 ± 2.6	7.6 ± 4.6
Scratching	8.8 ± 7.4	0.0 ± 0.0	3.3 ± 3.3	6.5 ± 6.2
Sniffing	1.3 ± 1.0	12.6 ± 3.6[d,e]	3.8 ± 1.1	1.6 ± 0.5[c]
Rearing	1.5 ± 0.9	9.8 ± 3.7[e]	1.2 ± 0.5	1.2 ± 0.6[c]
Salivation	2.0 ± 0.4	2.3 ± 0.6	2.8 ± 0.5	1.3 ± 0.8
Abnormal posturing	0.0 ± 0.0	5.6 ± 2.8	2.8 ± 1.9	0.8 ± 0.4

[a] $P<0.05$ control vs. 25% EtOH + tryptophan.
[b] $P<0.01$ 25% EtOH vs. 25% EtOH + tryptophan.
[c] $P<0.05$ tryptophan vs. 25% EtOH + tryptophan.
[d] $P<0.05$ control vs. tryptophan.
[e] $P<0.05$ tryptophan vs. 25% EtOH.

4. Discussion

Our results indicate that the extracellular fluid levels of 5-HIAA in the locus coeruleus were enhanced by tryptophan, and were remarkably increased by simultaneous administration of tryptophan and ethanol. This study provides evidence to indicate that the 5-HIAA level in the locus coeruleus after tryptophan alone or tryptophan plus ethanol is indicative of changes in the activity of serotonergic neurotransmission in locus coeruleus neurons.

Fadda et al. (2000) reported that although a tryptophan-supplemented diet did not significantly change extraneuronal 5-HT content, the significant increase in extracellular 5-HIAA in the frontal cortex nonetheless suggested a stimulation of 5-HT metabolism in serotonergic neurons. In this connection, 5-HIAA, the principal metabolite of 5-HT, reflects central 5-HT turnover (Fernstrom and Wurtman, 1971; Stark and Scheich, 1997; Williams et al., 1999). Moreover, when tryptophan is given to normal rats, there is a much greater accumulation of 5-HIAA than of 5-HT (Curzon et al., 1978).

We noted increases in the extracellular fluid levels of 5-HIAA in the locus coeruleus of rats, which had been i.p. injected with tryptophan alone. In our experiment, increases in 5-HIAA were observed only when the microdialysis probe was properly located in the locus coeruleus; no

increase was seen in adjacent regions. This may indicate that the increased 5-HIAA levels in the locus coeruleus after tryptophan administration are due to an increase in the innervation of locus coeruleus serotonergic neurons (Kaehler et al., 1999a,c). The serotonergic afferents to the locus coeruleus originate for more than 50% from cell bodies located in the dorsal raphe nucleus (Morgane and Jacobs, 1979; Mateo et al., 2000; Reneric et al., 2002). In the present study, however, in spite of the damage to serotonergic neurons caused by dorsal raphe nucleus injection of 5,7-DHT, which destroys presynaptic serotonergic nerve fibers (Kaehler et al., 1999a), 5-HIAA levels in the locus coeruleus decreased by no more than about 35%. In addition, the levels of 5-HIAA in the locus coeruleus during 5,7-DHT infusion into the dorsal raphe nucleus after pretreatment with tryptophan were approximately 21% lower than those after tryptophan pretreatment alone (Fig. 3). This indicates that about 70–80% of the increased 5-HIAA levels in the locus coeruleus seems to be from other distant serotonergic cell groups or be due to activation of locus coeruleus serotonergic neurons (Aston-Jones et al., 1986; Maeda et al., 1991; Luppi et al., 1995).

Simultaneous administration of tryptophan and ethanol produced a delayed increase in 5-HIAA, which reached a peak level at 90 min in the locus coeruleus, and the dose–response curve for 5-HIAA after combined administration was significantly shifted to the right (Fig. 1). That is, there was a time lag in the levels of 5-HIAA after tryptophan alone and after tryptophan plus ethanol. This may imply that acute ethanol inhibits the metabolism of tryptophan by competing for shared enzymes.

The oxidative metabolite of ethanol, acetaldehyde, may block the metabolism of 5-HIAL to 5-HIAA by competing with it for brain aldehyde dehydrogenase, which may lead to an elevation of 5-HIAL in the brain. Thus, concurrent administration of tryptophan and ethanol may increase not only the locus coeruleus level of 5-HTPL itself but also that of 5-HIAL (Fukumori et al., 1981). Beck et al. (1980) also reported that the 5-HTPL level in the cerebrospinal fluid of alcoholic inpatients is increased. In fact, in our study, 5-HTPL levels in the locus coeruleus were significantly elevated by concurrent administration of tryptophan and ethanol (Fig. 2). However, in this study, a significant increase in 5-HIAA level induced by concurrent administration was also observed. This may be explained by the fact that an increased 5-HIAA level affects not only aldehyde dehydrogenase but also CYP2E1, which is capable of metabolizing 5-HIAL to 5-HIAA (Lieber, 1999). Thus, the stimulation of 5-HT metabolism in locus coeruleus serotonergic neurons resulted from the simultaneous administration of tryptophan and ethanol. Therefore, change in 5-HT metabolites in the locus coeruleus may play an important role in mediating the changes in behavioral activity (Beck et al., 1980; Fukumori et al., 1981).

Neurotransmission in locus coeruleus neurons is closely related to behavioral signs (Rasmussen et al., 1990; Aghajanian et al., 1994; Hoshi et al., 1997). In our experiment, the elevation of 5-HIAA and 5-HTPL in the locus coeruleus after tryptophan plus ethanol resulted in an increase in teeth-chattering. The levels of both 5-HTPL and 5-HIAA increased markedly 15–30 min after substance administration and were sustained for about 1.0–1.5 h. Conversely, sniffing and rearing scores after tryptophan plus ethanol were lower than those after tryptophan alone. The alterations in neurotransmitter levels and behavioral signs after tryptophan alone or in combination with ethanol showed virtually identical time courses. The spontaneous outflow of endogenous 5-HT in the locus coeruleus originates from neuronal sites (Singewald et al., 1997). Thus, tryptophan stimulation may activate serotonergic fibers which influence 5-HT release in the locus coeruleus (Kaehler et al., 1999b), and the spontaneous release of 5-HT in the locus coeruleus may depend in part on the activity of serotonergic neurons of the dorsal raphe nucleus. In addition, our experimental results may be explained by the finding that alterations in 5-HT turnover rate or serotonergic activity (firing rates) are responsible for behavioral signs. This is supported by clinical evidence showing that several neuropsychiatric disorders such as stress and aggression seen after simultaneous dietary tryptophan augmentation and acute ethanol intake in healthy individuals may be caused by active metabolites of 5-HT (Meltzer, 1990; LeMarquand et al., 1994; Williams et al., 1999; Fadda et al., 2000).

These results suggest that the increased levels of 5-HIAA and 5-HTPL in the locus coeruleus in response to tryptophan are potentiated by ethanol and that these levels are partly responsible for behavioral activation.

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