Pharmacology, Biochemistry and Behavior 129 (2015) 1–6

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Comparison of centrally injected tryptophan-related substances inducing sedation in acute isolation stress-induced neonatal chicks

Junki Yoshida [a,1], Edi Erwan [a,1,2], Vishwajit Sur Chowdhury [b], Yumi Ogino [a], Asako Shigemura [a], D. Michael Denbow [c], Mitsuhiro Furuse [a,*]

[a] Laboratory of Regulation in Metabolism and Behavior, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
[b] Division for Arts and Science, Faculty of Arts and Science, Kyushu University, Fukuoka 819-0395, Japan
[c] Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA 24061-0306, USA

ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form 17 November 2014
Accepted 22 November 2014
Available online 29 November 2014

Keywords:
Acute stress
Neonatal chick
Kynurenine
Serotonin
L- and D-tryptophan

ABSTRACT

In the present study, we first focused on the function of L-tryptophan (TRP) metabolites which are synthesized in different metabolic pathways, namely, the kynurenine (KYN) pathway and serotonin (5-HT) pathway during an acute isolation stress. When L-TRP metabolites were intracerebroventricularly injected on an equimolar basis (100 nmol), 5-HT induced a sedative effect in neonatal chicks. Additionally, plasma corticosterone, dopamine, 5-HT, and its metabolite 5-hydroxyindoleacetic acid concentrations were increased in the diencephalon of the 5-HT treated group compared with other groups. Second, the two doses (400 or 800 nmol) of L- and D-TRP were compared under a corticotrophin-releasing hormone-augmented social isolation stress. When comparing the efficacy between L- and D-TRP against stress behavior, both amino acids had a similar effect and quickly suppressed distress vocalizations. Finally, D-amino acid levels in the diencephalon and telencephalon were measured but D-TRP was not found. These results indicate that L- and D-TRP induce the same effect in attenuating stress but the mode of action of TRP derivatives, namely 5-HT differs during an acute isolation stress in neonatal chick. The absence of D-TRP in the diencephalon further suggests that instead of being an endogenous factor it may play role as a pharmacological factor.

1. Introduction

L-Tryptophan (TRP), one of the 20 amino acids constituting proteins, is a dietary essential amino acid in most animals. L-TRP is metabolized mainly through two pathways in an animal's body including the central nervous system. One is the serotonergic pathway, which is active in platelets and neurons and yields serotonin (5-HT) through 5-hydroxy-TRP. Then, 5-HT can be the precursor of a pineal produced hormone, melatonin. However, the main alternative route for L-TRP metabolism is through the kynurenine (KYN) pathway. It is notable that 95% of L-TRP is catabolized through the KYN pathway within the brain (Robotka et al., 2008).

There are some reports suggesting that L-TRP metabolism may be related to stress (Graeff et al., 1996; Richell et al., 2005). It has been shown that 5-HT is involved in mood, sleep, appetite, cognition, and stress (Markus, 2008; Marston et al., 2011). In addition, 5-HT had a sedative effect when co-injected with corticotropin-releasing hormone (CRH) by intracerecroventricular (i.c.v.) injection, but did not have a hypnotic effect (Zhang et al., 2004). On the other hand, kynurenic acid (KYNA) had both sedative and hypnotic effects under a CRH-augmented stress (Yoshida et al., 2012). KYNA was involved in the mechanism of L-TRP-mediated sedative effects and KYNA functioned via the simultaneous inhibition of the α7 nicotinic acetylcholine (α7nACh) receptor and *N*-methyl-D-aspartate (NMDA) receptor subgroup containing the subunits NR2A and NR2B (Yoshida et al., 2013).

Only L-amino acids are found in animal and plant proteins. However, free L-amino acids may be racemized to their mirror image configuration, D-isomers (Friedman, 2010). Therefore, there have been many reports focusing on D-amino acids in recent years. D-TRP, the mirror image isomer of L-TRP, has a sweet taste like D-phenylalanine (Findley and Friedman, 1973; Friedman and Cuq, 1988; Manita et al., 2006; Maehashi et al, 2007). It was found that when replacement of L-TRP with D-TRP in the diet was performed then it required considerably more D-TRP which was not associated with toxicity or sparing effect as like L- and D-phenylalanine responses (Friedman and Levin, 2012). The relative nutritional efficacy of D-TRP compared to that of L-TRP in mice was strongly dose-dependent (Friedman and Cuq, 1988).

Considerable species variation exists for the nutritive value of D-TRP. On the other hand, D-TRP was well utilized by the growing pigs (Arentson and Zimmerman, 1985). In chicks, the relative potency of

* Corresponding author. Tel.: +81 92 642 2953; fax: +81 92 642 2954.
E-mail address: furuse@brs.kyushu-u.ac.jp (M. Furuse).
[1] These authors have equally contributed to this work.
[2] Present address: Department of Animal Science, Faculty of Agriculture and Animal Science, State Islamic University of Suska Riau, Indonesia.

http://dx.doi.org/10.1016/j.pbb.2014.11.016
0091-3057/

the conversion of D- to L-isomers has been reported to be 20% (Friedman, 2010). In neonatal chicks, i.c.v. injection of DL-TRP had sedative effects under an acute stressful condition (Kurauchi et al., 2007), and L-TRP reduced stress-induced responses in neonatal chicks (Yoshida et al., 2012). However, the effect and presence of D-TRP itself in the brain have not been clarified yet. In addition, previous studies with i.c.v. injection of L-TRP metabolites varied in that some were with or without CRH and the concentrations of L-TRP differed, making it difficult to compare directly the efficacy of L-TRP metabolites in affecting the stress response (Zhang et al., 2004; Kurauchi et al., 2007; Yoshida et al., 2012). This study, therefore, further clarifies the functions of L-TRP metabolites.

In the present study, we first compared the central effects of L-TRP and its metabolites such as KYN and 5-HT on an equimolar basis under acute isolation-induced stress for 10 min, and analyzed brain monoamines and plasma corticosterone concentrations. Second, we focused on the relationship between L- and D-TRP in the brain and behavior. The effect of i.c.v. injection of L- or D-TRP during CRH-augmented stress in neonatal chicks was examined. Furthermore, we examined the presence of D-TRP in the brain to clarify the possibility that it acts as an endogenous factor to control the behavior.

2. Materials and methods

2.1. Animals

One-day-old male layer chicks (Julia strain; *Gallus gallus domesticus*) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) and housed in a windowless room at a constant temperature of 30 ± 1 °C. Continuous lighting was provided. Food (AX, Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were freely accessible. Chicks were reared in a group (20–25 per cage) until the start of the experiment. On the day of the experiment, chicks (5 days old in Experiments 1 and 3; 5–6 days old in Experiment 2) were assigned to treatment groups. The number of animals used in each group was kept to a minimum while still ensuring adequate statistical power. Experimental procedures followed the guide for animal experiments of the Faculty of Agriculture, the Graduate Course of Kyushu University, as well as the Law (No. 105) and Notification (No. 6) of the Government.

2.2. Preparation of drugs

L- or D-TRP methyl ester hydrochloride, KYN sulfate salt and 5-HT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat CRH was purchased from Peptide Institute, Inc. (Osaka, Japan). Drugs were dissolved in 0.85% saline containing 0.1% Evans Blue.

2.3. Experimental procedure

The i.c.v. injections were made using a microsyringe according to the method of Davis et al. (1979) and Koutoku et al. (2005). The stress and pain associated with this method is minimal, as described elsewhere (Koutoku et al., 2005). The injected volume was 10 μl. In Experiment 1, chicks were i.c.v. administered with either vehicle, L-TRP (100 nmol), KYN (100 nmol) or 5-HT (100 nmol). The dose was decided based on our previous report where 100 nmol KYNA was an effective dose to attenuate stress in chicks (Yoshida et al., 2012). We have previously reported that both isolation stress and CRH can increase plasma corticosterone (Saito et al., 2005) which indicates that both isolation stress and CRH can be considered as similar stressor. However, the response was stronger in CRH treatment than in isolation stress. In Experiment 2, stress was augmented with CRH, and chicks were i.c.v. administered with either CRH (0.01 μg), L-TRP (400 nmol) plus CRH (0.01 μg), L-TRP (800 nmol) plus CRH (0.01 μg), D-TRP (400 nmol) plus CRH (0.01 μg), or D-TRP (800 nmol) plus CRH (0.01 μg). The doses of TRP were decided according to the report of Yoshida et al. (2012) where it was shown that 400 nmol TRP attenuated some stress response but not all (Yoshida et al., 2012). Therefore, two doses of L- or D-TRP (400 and 800 nmol) were decided in this study.

After injection, chicks were immediately placed in an acrylic monitoring cage (40 cm × 30 cm × 20 cm), and behavioral observations were made for 10 min. During this period, chicks were deprived of water and diet. Video cameras were positioned to record the behavior of chicks from three different directions on DVD. Based on the method reported by van Luijtelaar et al. (1987), chick behaviors were classified into four categories: (1) active wakefulness; (2) standing/sitting motionless with eyes opened; (3) standing motionless with eyes closed; and (4) sitting motionless with the head drooped (sleeping posture). The sedative effect was evaluated by categories (1), (2), and (3), whereas the hypnotic effect was evaluated by category (4) (Suenaga et al., 2008). The monitoring systems were placed in a separate room to avoid disturbing the animals. At the end of the experiment, the birds were decapitated following anesthesia with isoflurane (Mylan Inc., Tokyo, Japan). The brains were removed and the location of the Evans Blue dye was confirmed. Data from chicks without dye in the lateral ventricle were excluded from analysis. Chick vocalizations were simultaneously recorded and the number of distress vocalizations was counted using Gretchen software (Excla Inc., Japan).

2.4. Plasma corticosterone

In Experiment 1, blood taken from the jugular vein was collected into heparinized tubes. The blood was centrifuged at 4 °C and 10,000 ×*g* for 4 min, and the plasma was collected and stored at −80 °C until analysis. Plasma corticosterone was determined using a corticosterone enzyme immunoassay kit (Assay Designs Inc., NY, USA).

2.5. Analysis of monoamine in the brain

To investigate the influence of the injected drugs on various neurotransmitters in the brain, the concentrations of monoamines and metabolites including dopamine (DA), epinephrine (E), 5-HT, the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the norepinephrine metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), and the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the diencephalon were analyzed using a previously described method (Tomonaga et al., 2008) with some modifications. The samples were homogenized in ice-cold 0.2 M perchloric acid solution containing 0.01 mM EDTA·2Na and left for deproteinization on ice for 30 min. Then, the tissue homogenates were centrifuged at 20,000 ×*g* for 15 min at 0 °C. Supernatants were adjusted to pH 3 with 1 M sodium acetate and were filtrated through a 0.2-μm filter. The filtrates were analyzed using a high-performance liquid chromatography (HPLC) system (Eicom, Kyoto, Japan) with a 150 × 3.0 mm ODS column (EICOMPAK SC-5ODS, Eicom) and electrochemical detector (ECD-300, Eicom) at an applied potential of +750 mV versus an Ag/AgCl reference analytical electrode. The changes in electric current (nA) were recorded on a computer using an interface system (Power Chrom ver 2.3.2.J; AD Instruments, Tokyo, Japan). The mobile phase, pH 3.5, consisted of 0.1 M sodium acetate, 0.1 M citric acid, 100 mg/ml of 1-ocutan sulfonate, and 5 mg/ml of EDTA·2Na. The retention time and height of the peaks in tissue homogenates were measured and compared to a sample of external calibrating standard solution containing MHPG, DA, 5-HT, and 5-HIAA. Concentrations in the samples were calculated and expressed as pg/mg wet tissue.

2.6. Analysis of amino acids in the brain

In Experiment 3, L- and D-amino acid contents in the brain were analyzed according to the previously described method (Ohmori et al., 2012). The brains of 5 day old chicks were carefully taken out from the skulls and placed on a cold glass dish kept on dry ice. The telencephalon and diencephalon were dissected from each brain, and stored at

−80 °C until analysis. The telencephalon and diencephalon were identified according to the brain atlas of the chick (Kuenzel and Masson, 1988). The telencephalon collected excluded the diencephalon and mesencephalon. The diencephalon was dissected by cutting between the stria medullaris and posterior commissure. The diencephalon included the hypothalamus and thalamus. The tissues were weighed and homogenized as per Experiment 2. The homogenates were centrifuged at 20,000 ×g for 15 min. Supernatants were adjusted to pH 3 with 1 M sodium acetate and were filtered through a 0.2-μm filter (Millipore, Bedford, MA, USA). Each 20-μl sample of the brain was dissolved with 2 μl of 1 M NaOH and then vortexed. Both the L- and D-isomer of the amino acids were measured by a UPLC (the Acquity™ UPLC system comprised of Waters Binary Solvent Manager, Water Sample Manager and Waters FLR Detector) with an ACCQ-TAG™ ULTRA C18 1.7 μm 2.1 × 100 mm column (Waters Corporation, USA). The excitation and emission wavelengths for fluorescent detection of amino acids were 350 nm and 450 nm, respectively. The system was operated with a flow rate of 0.25 ml/min at 30 °C. The UPLC gradient system (A = 50 mM sodium acetate (pH 5.9), B = methanol) was 10–20% B over 3.2 min, 20% B for 1 min, 20–40% B over 3.6 min, 40% B for 1.2 min, 40–60% B over 3.8 min, 60% B for 1 min, and 60–10% B over 0.01 min. Just before the analysis in UPLC, each sample (10 μl) was transferred to a UPLC tube, and NAC/OPA (20 μl) and a borate buffer (70 μl) were added; then it was left for 2 min in a dark room. The same method was used for the standard solutions. The plasma amino acid concentrations were expressed in nmol/ml, and the amino acid concentrations in the brains were expressed as pmol/mg wet tissue.

2.7. Statistical analysis

In all experiments, data in Experiments 1 and 2 were statistically analyzed by one-way analysis of variance (ANOVA). When significant ($P < 0.05$) effects were detected, the Tukey–Kramer test was used as a post-hoc test. Data were analyzed using StatView Version 5.0 software (SAS Institute, Cary, NC, USA, 1998). Values are presented as means ± S.E.M. All data in each group were first subjected to a Thompson rejection test as described by Kobayashi and Pillai (2013) to eliminate outliers ($P < 0.01$), and the remaining data were used for the analysis among groups.

3. Results

The effect of i.c.v. injection of L-TRP, KYN and 5-HT on distress vocalizations during 10-min isolation-induced stress is shown in Fig. 1. Significant ($F(3, 24) = 3.637$, $P < 0.05$) effects on total distress vocalizations were observed. In the 5-HT treated group, no distress vocalizations were observed. No significant difference was observed in total distress vocalizations when saline group was compared with L-TRP and KYN.

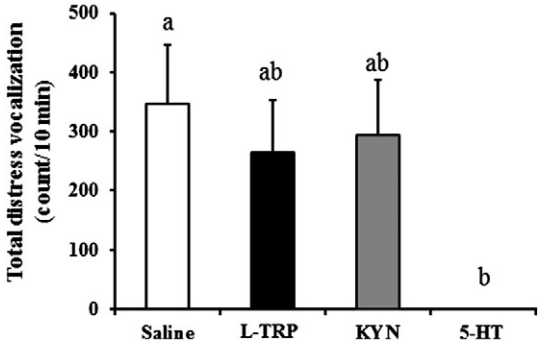

Fig. 1. Effect of i.c.v. injection of L-tryptophan (TRP), kynurenine (KYN) and serotonin (5-HT) on total distress vocalizations during 10 min of isolation in chicks. Results are expressed as means ± S.E.M. The number of chicks used in each group was 7. Groups with different letters are significantly different ($P < 0.05$).

As shown in Table 1, time spent for active wakefulness was significantly reduced ($F(3, 28) = 4.118$, $P < 0.05$) by 5-HT compared to the other groups. In contrast, 5-HT significantly ($F(3, 23) = 9.062$, $P < 0.001$) increased the time spent standing/sitting motionless with eyes open. There were no treatment effects on the time spent in the sleeping posture ($F(3, 23) = 0.947$, $P > 0.05$) and the time spent standing motionless with eyes closed ($F(3, 23) = 1.047$, $P > 0.05$).

The i.c.v. injection of 5-HT significantly ($F(3, 24) = 31.777$, $P < 0.0001$) increased plasma corticosterone concentration compared to other groups (Fig. 2). Table 2 shows the changes in monoamines and their metabolites concentration in the diencephalon due to i.c.v. treatments of TRP related substances. The i.c.v. injection of 5-HT significantly increased 5-HT ($F(3, 24) = 16.405$, $P < 0.0001$), 5-HIAA ($F(3, 24) = 86.349$, $P < 0.0001$), and DA ($F(3, 24) = 31.364$, $P < 0.0001$) levels in the diencephalon compared with other groups. Additionally, KYN significantly ($F(3, 24) = 65.781$, $P < 0.0001$) increased MHPG levels compared to other groups. No significant effects were detected in E ($F(3, 24) = 1.547$, $P > 0.05$), DOPAC ($F(3, 24) = 0.816$, $P > 0.05$) or HVA ($F(3, 24) = 0.943$, $P > 0.05$) by any of the treatments.

Fig. 3 shows time course changes (A) and total counts (B) in distress vocalization. CRH alone caused vocalizations over the 10 min period, while CRH plus L- or D-TRP strongly inhibited the vocalizations just after i.c.v. injection. Consequently, total vocalizations over the 10 min were significantly ($F(4, 23) = 11.275$, $P < 0.0001$) higher in the CRH alone group than in other groups. Table 3 shows that the time spent in active wakefulness was significantly ($F(4, 22) = 12.354$, $P < 0.0001$) increased by CRH compared to the other groups. In contrast, a significant ($F(4, 22) = 8.405$, $P < 0.0001$) decrease in time spent standing/sitting motionless with eyes open was seen following the i.c.v. injection of CRH. The time spent in sleeping posture was nearly significant ($F(4, 22) = 2.510$, $P = 0.071$), and tended to be longer in the L- or D-TRP coinjected with CRH groups.

It was not possible to separate L- and D-forms of the following amino acids: aspartate (ASP), serine, glutamine, histidine, arginine, alanine (ALA), tyrosine (TYR), methionine, valine, TRP, phenylalanine, isoleucine and leucine. Among them, D-ASP, D-ALA and D-TYR were in detectable amounts (Table 4), but no peaks of D-TRP was confirmed in either the telencephalon or diencephalon. L-TRP was present both in the telencephalon (3536 ± 423 pmol/mg) and diencephalon (2715 ± 583 pmol/mg). Although the levels of D-ALA and D-ASP were lower when compared with their L-isomers in the brain, it was found that D-TYR was higher than L-TYR. Approximately, D-ALA was 24 times lower both in the diencephalon and telencephalon, while D-ASP was 144 times lower in the diencephalon and 105 times lower in the telencephalon. However, D-TYR was approximately 1.3 times higher in both the brain regions.

4. Discussion

Previously, effects of i.c.v. injection (100 nmol) of L-TRP, KYN and KYNA on CRH (0.01 μg)-augmented social isolation-induced behaviors in chicks were investigated (Yoshida et al., 2012). CRH plus KYNA significantly decreased the number of distress vocalizations in comparison with CRH alone. Furthermore, among various behaviors of chicks in response to CRH-augmented social isolation stress, the time spent in active wakefulness was significantly decreased, and in sleeping posture was significantly increased in the KYNA group compared with the CRH alone group. In the present study, the effect of 5-HT was compared to L-TRP and KYN under social isolation stress without CRH-augmentation. Distress vocalizations disappeared following the i.c.v. injection of 5-HT. This result agreed with previous studies (Zhang et al., 2004). For various behaviors, 5-HT decreased active wakefulness, but sleeping posture was almost negligible. These changes were mainly due to increased time spent standing/sitting motionless with eyes open. On the other hand, KYNA significantly increased sleeping posture (Yoshida et al., 2012). These facts imply that 5-HT and KYNA have

Table 1
Effect of i.c.v. injection of L-tryptophan (TRP), kynurenine (KYN) and serotonin (5-HT) on various behavioral categories of chicks in response to social isolation stress for 10 min.

Drugs (nmol)	Saline	TRP (100)	KYN (100)	5-HT (100)
Active wakefulness	312 ± 67^{a}	253 ± 89^{ab}	266 ± 80^{ab}	13 ± 10^{b}
Standing/sitting motionless with eyes open	224 ± 43^{b}	278 ± 74^{b}	276 ± 62^{b}	567 ± 26^{a}
Standing motionless with eyes closed	0 ± 0	0 ± 0	0 ± 0	17 ± 17
Sitting motionless with head drooped (sleeping posture)	64 ± 34	69 ± 36	58 ± 37	3 ± 3
Total	600	600	600	600

Values are means ± S.E.M. in seconds. The number of chicks used in each group was 6–7.
Groups with different letters are significantly different at $P < 0.05$.

different functions in the brain, probably because they act at different receptors. It has been suggested that KYNA functions via the simultaneous inhibition of the α7nACh receptor and NMDA receptor subgroup containing the subunits NR2A and NR2B in chicks (Yoshida et al., 2013) whereas 5-HT acts at several different 5-HT sub-receptors (Bielenica et al., 2013).

In addition to effects on behavior, the L-TRP metabolites also influence metabolism. 5-HT caused sedative, but not hypnotic, effects as reported previously (Zhang et al., 2004). Plasma corticosterone was increased by 5-HT alone compared with other groups (Fig. 2). This result was also in agreement with a previous study (Zhang et al., 2004). Based on the results of distress vocalizations and time spent for standing/sitting motionless with eyes open, 5-HT greatly suppressed the stress response. However, it was clear that central 5-HT stimulated the hypothalamus–pituitary–adrenal (HPA) axis. In rats, immobilization stress increased the content of 5-HT in the hypothalamic nuclei, which might reflect a role of 5-HT in the regulation of increased pituitary–adrenocortical activity under stress (Culman et al., 1980). Because glucocorticoid is a major mediator of the physiological stress response and impacts many physiological systems to allow the body to react to a stressor (Phillips et al., 2006; Larauche et al., 2012), calm behavioral condition induced by i.c.v. injection of 5-HT may not be calm condition in the brain. The diencephalic areas, as hypothalamus, are rich regions in components of the HPA axis and the 5-HT systems that interact at multiple levels. Some 5-HT receptors may be directly related to the HPA axis under a stressful situation (Goel et al., 2014). Therefore, when 5-HT was injected in chicks then it might have stimulated also corticosterone release. This might be the possible neurobiological reasons why the higher brain concentration of 5-HT increased the corticosterone level. On the other hand, sedation was more common with pooled antidepressants (Vernon et al., 2014). This report supports our current observation in decreased active wakefulness and distress vocalizations. In the present study, i.c.v. injection of 5-HT increased not only 5-HT itself and its metabolite 5-HIAA, but also DA in the diencephalon. It was reported that central serotonergic and dopaminergic systems play an important role in regulating normal and abnormal behaviors (Di Giovanni et al., 2010). According to Bowers et al. (2000), activation of 5-HT_2 receptors stimulates DA release. Thus, the results obtained in the current study suggest that 5-HT stimulates to increase DA content in the diencephalon. Higher 5-HT and DA due to i.c.v. 5-HT stimulate the HPA axis (Locatelli et al., 2010), and release corticosterone. In the present study, the i.c.v. injection of L-TRP did not increase brain 5-HT levels. This result suggests that L-TRP is not quickly metabolized to 5-HT over 10 min. Although we did not measure KYN and KYNA contents after i.c.v. injection of L-TRP, at least synthesis of these metabolites from L-TRP may not be fully completed within 10 min. These ideas support our results that behavioral changes were not observed among control, L-TRP and KYN treatments.

On the other hand, the i.c.v. injection of KYN alone enhanced the MHPG content of the diencephalon. MHPG is a major metabolite of norepinephrine (NE) in the brain and a reliable index of central NE activity (DeMet and Halaris, 1979). Several types of stressors including cold stress (Meek and Neff, 1972; Ceasar et al., 1974) and exhaustive running stress (Stone, 1973) have been correlated with the increase of NE activity in mouse and rat brains, specifically increased MHPG level. While the present results suggest that KYN in the brain is related with stress in neonatal chicks, this metabolite did not activate the HPA axis as evidenced by its lack of effect on plasma corticosterone concentration following i.c.v. injection of KYN. Furthermore, no index for behavioral changes was correlated with enhanced MHPG in the diencephalon.

In a previous study, i.c.v. injection of DL-TRP induced sedative effects during an acute stressful condition in neonatal chicks (Kurauchi et al., 2007). However, it was not clear whether L- or D-TRP was important in attenuating the acute stress response. Under CRH-induced isolation stress, both doses of L- or D-TRP significantly reduced total distress vocalizations and the behavior of active wakefulness compared with the CRH alone group. It has previously been shown that D-TRP is metabolized to D-KYN and KYNA in rats (Ogaya et al., 2010). D-TRP is metabolized to D-KYN by the enzyme, indoleamine 2,3-dioxygenase (Littlejohn et al., 2003) and D-KYN is metabolized to KYNA by the enzyme, D-amino acid oxidase (Fukushima et al., 2009; Ogaya et al., 2010) or kynurenine aminotransferase (Pérez-de la Cruz et al., 2012; Wang et al., 2012). However, D-TRP as well as L-TRP quickly suppressed distress vocalization in the present study (Fig. 3A) and L-TRP was not able to produce

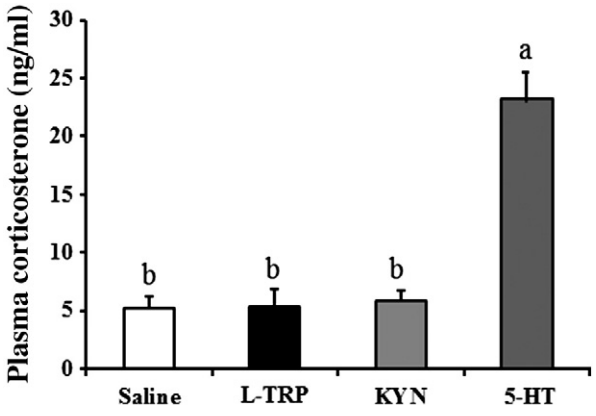

Fig. 2. Effect of i.c.v. injection L-tryptophan (TRP), kynurenine (KYN) and serotonin (5-HT) on plasma corticosterone concentration (ng/ml) in chicks. Results are expressed as means ± S.E.M. The number of chicks used in each group was 5–7. Groups with different letters are significantly different ($P < 0.05$).

Table 2
Effect of i.c.v. injection of L-tryptophan (TRP), kynurenine (KYN) and serotonin (5-HT) on diencephalic monoamines and their metabolites in chicks.

Drugs (nmol)	Saline	TRP (100)	KYN (100)	5-HT (100)
E	312 ± 12	377 ± 15	341 ± 29	331 ± 26
MHPG	51 ± 7^{b}	46 ± 5^{b}	446 ± 47^{a}	67 ± 4^{b}
DA	478 ± 43^{b}	502 ± 79^{b}	453 ± 65^{b}	2959 ± 429^{a}
DOPAC	47 ± 2	47 ± 4	41 ± 3	46 ± 3
HVA	83 ± 5	79 ± 5	86 ± 3	89 ± 5
5-HT	573 ± 31^{b}	558 ± 23^{b}	533 ± 25^{b}	1054 ± 115^{a}
5-HIAA	267 ± 19^{b}	257 ± 14^{b}	263 ± 15^{b}	1792 ± 162^{a}

Vales are expressed as mean ± S.E.M. in pg/mg wet tissue. The number of chicks used in each group was 7.
Groups with different letters are significantly different at $P < 0.05$. E, epinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin; HIAA, 5-hydroxyindoleacetic acid.

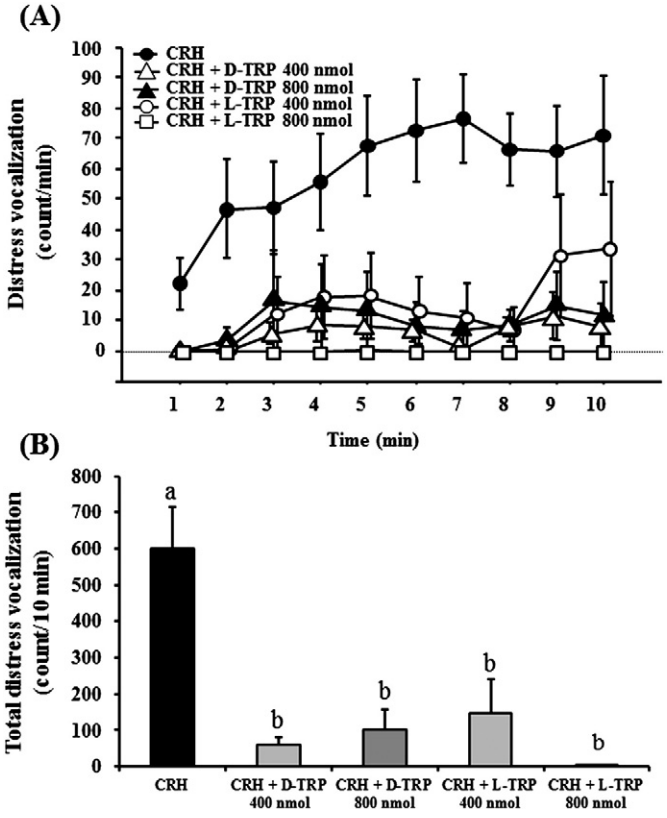

Fig. 3. Effect of i.c.v. injection of CRH or CRH co-injected with L- or D-tryptophan (TRP) on time course (A) and total distress (B) vocalizations during 10 min of isolation in chicks. Results are expressed as means ± S.E.M. The number of chicks used in each group was 4–7. CRH = corticotropin-releasing hormone.

5-HT 10 min after i.c.v. injection of L-TRP (Table 2). It is possible that both L- and D-TRP directly activated some receptors before producing metabolites. In fact, both the L- and D-form of amino acids affected stress behavior, but their mechanism of action appears to be different. We have already examined the sedative effect of several D-amino acids under an acute stressful condition in neonatal chicks. For instance, D-ASP has been shown to cause a sedative effect (Erwan et al., 2012). In this case, D-ASP may function as an endogenous sedative factor in the brain. L-ASP could induce sedative and hypnotic effects for stress behaviors through the NMDA receptor, but the attenuation of stress behaviors by D-ASP might be via simultaneous involvement of other receptors besides the NMDA receptor (Erwan et al., 2014). Furthermore, L- and D-proline similarly attenuated the stress response, but they worked through different mechanisms: L-proline and D-proline induce sedative and hypnotic effects through NMDA and glycine receptors, respectively (Hamasu et al., 2010). In the present study, the mechanism by which L- and D-TRP quickly induced a sedative effect is not clear, but future research should clarify this relationship.

Table 4
Concentration of L- and D-isomers of amino acids which were both detected simultaneously in the brain[a].

	Diencephalon	Telencephalon
L-Alanine	779 ± 76	1283 ± 144
D-Alanine	33 ± 3	54 ± 7
L-Aspartic acid	2442 ± 238	4813 ± 613
D-Aspartic acid	17 ± 2	46 ± 6
L-Tyrosine	154 ± 15	214 ± 40
D-Tyrosine	195 ± 18	292 ± 46

The number of samples used for analysis ranged from 6 to 8.
[a] Values (pmol/mg wet tissue) are means ± S.E.M.

We hypothesized that the L-TRP could be racemized to D-TRP to strengthen sedative effect during a stressful condition. This possibility was further investigated in Experiment 3. Although D-TRP induced a sedative effect in the present study, it was not detected in the diencephalon or telencephalon. It is suggested that TRP racemase may not occur in the chick brain, and D-TRP may function as a pharmacological factor rather than as an endogenous factor.

Nagata et al. (1994) investigated the distribution of free D-serine in the vertebrate brain and reported that D-serine levels were low in chicks. In the present study, we could not detect D-serine. At the sub-receptor sites, D-serine acts as an endogenous ligand for the NMDA receptor-related glycine site (Kleckner and Dingledine, 1988; Hashimoto and Oka, 1997). Asechi et al. (2006) observed that D-serine did not have sedative and hypnotic effects as observed with L-serine in chicks. Therefore, D-TRP differed from D-serine since i.c.v. injection of D-TRP induced a sedative effect but was not present in the brain. In addition to D-serine, D-ALA is a potential coagonist of the NMDA receptor. In the present study, D-ALA was detected in the brain suggesting that it may be the sole endogenous ligand for the NMDA receptor-related glycine site. Higher D-TYR in the brain previously has been confirmed in mice (Takagi et al., 2013) and Djungarian hamster (*Phodopus sungorus*) and Roborovskii hamster *(Phodopus roborovskii)* (Ikeda et al., 2014), and suggests that TYR racemase is highly activated in the vertebrate brain.

It is concluded that L-TRP itself and its metabolites differentially regulate chick behavior under acute stressful conditions.

Acknowledgments

Part of this project has been supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 23248046).

References

Arentson BE, Zimmerman DR. Nutritive value of D-tryptophan for the growing pig. J Anim Sci 1985;60:474–9.
Asechi M, Tomonaga S, Tachibana T, Han L, Hayamizu K, Denbow DM, et al. Intracerebroventricular injection of L-serine analogs and derivatives induces sedative and

Table 3
Effects of i.c.v. injection of several doses of L- or D-tryptophan (TRP) and CRH on various behavioral categories of chicks exposed to social separation stress for 10 min.

CRH (μg)	0.01				
L-TRP (nmol)	0	400	800	0	0
D-TRP (nmol)	0	0	0	400	800
Active wakefulness	448 ± 55[a]	63 ± 24[b]	67 ± 39[b]	133 ± 39[b]	120 ± 62[b]
Standing/sitting motionless with eyes open	152 ± 55[a]	410 ± 40[b]	431 ± 38[b]	430 ± 36[b]	375 ± 22[b]
Standing motionless with eyes closed	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sitting motionless with head drooped (sleeping posture)	0 ± 0	127 ± 57	102 ± 39	37 ± 20	105 ± 58
Total	600	600	600	600	600

Values are mean ± S.E.M. in seconds. The number of chicks used in each group was 4–8. CRH, corticotropin-releasing hormone.

hypnotic effects under an acute stressful condition in neonatal chicks. Behav Brain Res 2006;170:71–7.
Bielenica A, Kozioł AE, Struga M. Binding modes of chain arylpiperazines to 5-HT1a, 5-HT2a and 5-HT7 receptors. Mini Rev Med Chem 2013;13:1516–39.
Bowers BJ, Henry MB, Thielen RJ, McBride WJ. Serotonin 5-HT_2 receptor stimulation of dopamine release in the posterior but not anterior nucleus accumbens of the rat. J Neurochem 2000;75:1625–33.
Ceasar PM, Hague P, Sharman DF, Werdinius B. Studies on the metabolism of catecholamines in the central nervous system of the mouse. Br J Pharmacol 1974;51:187–95.
Culman J, Kvetnanský R, Torda T, Murgas K. Serotonin concentration in individual hypothalamic nuclei of rats exposed to acute immobilization stress. Neuroscience 1980;5:1503–6.
Davis JL, Masuoka DT, Gerbrandt LK, Cherkin A. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiol Behav 1979;22:693–5.
DeMet EM, Halaris AE. Origin and distribution of 3-methoxy-4-hydroxyphenylglycol in body fluids. Biochem Pharmacol 1979;28:3043–50.
Di Giovanni G, Esposito E, Di Matteo V. Role of serotonin in central dopamine dysfunction. CNS Neurosci Ther 2010;16:179–94.
Erwan E, Tomonaga S, Yoshida J, Nagasawa M, Ogino Y, Denbow DM, et al. Central administration of L- and D-aspartate attenuates stress behaviors by social isolation and CRF in neonatal chicks. Amino Acids 2012;43:1969–76.
Erwan E, Chowdhury VS, Nagasawa M, Goda R, Otsuka T, Yasuo S, et al. Central injection of L- and D-aspartate attenuates isolation-induced stress behavior in chicks possibly through different mechanisms. Eur J Pharmacol 2014;736:138–42.
Findley JW, Friedman M. New sweetening agents: N′-formyl-and N′-acetylkynurenine. J Agric Food Chem 1973;21:33–4.
Friedman M. Origin, microbiology, nutrition, and pharmacology of D-amino acids. Chem Biodivers 2010;7:1491–530.
Friedman M, Cuq JL. Chemistry, analysis, nutritional value, and toxicology of tryptophan in food. A review. J Agric Food Chem 1988;36:1079–93.
Friedman M, Levin CE. Nutritional and medicinal aspects of D-amino acids. Amino Acids 2012;42:1553–82.
Fukushima T, Sone Y, Mitsuhashi S, Tomiya M, Toyo'oka T. Alteration of kynurenic acid concentration in rat plasma following optically pure kynurenine administration: a comparative study between enantiomers. Chirality 2009;21:468–72.
Goel N, Innala L, Viau V. Sex differences in serotonin (5-HT) 1A receptor regulation of HPA axis and dorsal raphe responses to acute restraint. Psychoneuroendocrinology 2014;40:232–41.
Graeff FG, Guimarães FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. Pharmacol Biochem Behav 1996;54:129–41.
Hamasu K, Shigemi K, Tsuneyoshi Y, Yamane H, Sato H, Denbow DM, et al. Intracerebroventricular injection of L-proline and D-proline induces sedative and hypnotic effects by different mechanisms under an acute stressful condition in chicks. Amino Acids 2010;38:57–64.
Hashimoto A, Oka T. Free D-aspartate and D-serine in the mammalian brain and periphery. Prog Neurobiol 1997;52:325–53.
Ikeda H, Kawase T, Nagasawa M, Chowdhury VS, Yasuo S, Furuse M. Metabolism of amino acids differs in the brains of Djungarian hamster (*P. sungorus*) and Roborovskii hamster (*P. roborovskii*). , 3. SpringerPlus; 2014. p. 277.
Kleckner NW, Dingledine R. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. Science 1988;241:835–7.
Kobayashi K, Pillai KS. Transformation of data and outliers. A handbook of applied statistics in pharmacology. CRC Press: Taylor & Francis Group; 2013. p. 37–46.
Koutoku T, Takahashi H, Tomonaga S, Oikawa D, Saito S, Tachibana T, et al. Central administration of phosphatidylserine attenuates isolation stress-induced behavior in chicks. Neurochem Int 2005;47:183–9.
Kuenzel WJ, Masson M. A stereotaxic atlas of the brain of chick (*Gallus domesticus*). Baltimore: Johns Hopkins University Press; 1988.
Kurauchi I, Asechi M, Tachibana T, Han L, Hayamizu K, Denbow DM, et al. Intracerebroventricular injection of tryptophan, but not lysine or methionine, induces sedative effect during an acute stressful condition in neonatal chicks. J Appl Anim Res 2007;31:25–8.
Larauche M, Mulak A, Taché Y. Stress and visceral pain: from animal models to clinical therapies. Exp Neurol 2012;233:49–67.
Littlejohn TK, Takikawa O, Truscott RJW, Walker MJ. Asp(274) and His(346) are essential for heme binding and catalytic function of human indoleamine 2,3-dioxygenase. J Biol Chem 2003;278:29525–31.
Locatelli V, Bresciani E, Tamiazzo L, Torsello A. Central nervous system-acting drugs influencing hypothalamic–pituitary–adrenal axis function. Endocr Dev 2010;17:108–20.
Maehashi K, Matano M, Kondo A, Yamamoto Y, Udaka S. Riboflavin-binding protein exhibits selective sweet suppression toward protein sweeteners. Chem Senses 2007;32:183–90.
Manita S, Bachmanov AA, Li X, Beauchamp GK, Inoue M. Is glycine "sweet" to mice? Mouse strain differences in perception of glycine taste. Chem Senses 2006;31:785–93.
Markus CR. Dietary amino acids and brain serotonin function; implications for stress-related affective changes. Neuromolecular Med 2008;10:247–58.
Marston OJ, Garfield AS, Heisler LK. Role of central serotonin and melanocortin systems in the control of energy balance. Eur J Pharmacol 2011;660:70–9.
Meek JL, Neff NH. The rate of formation of 3-methoxy-4-hydroxyphenylethyleneglycol sulfate in brain as an estimate of the rate of formation of norepinephrine. J Pharmacol Exp Ther 1972;184:570–5.
Nagata Y, Horiike K, Maeda T. Distribution of free D-serine in vertebrate brains. Brain Res 1994;634:291–5.
Ogaya T, Song Z, Ishii K, Fukushima T. Changes in extracellular kynurenic acid concentrations in rat prefrontal cortex after D-kynurenine infusion: an *in vivo* microdialysis study. Neurochem Res 2010;35:559–63.
Ohmori T, Mutaguchi Y, Yoshikawa S, Doi K, Ohshima T. Amino acid components of lees in salmon fish sauce are tyrosine and phenylalanine. J Biosci Bioeng 2012;112:256–8.
Pérez-de la Cruz V, Amori L, Sathyasaikumar KV, Wang XD, Notarangelo FM, Wu HQ, et al. Enzymatic transamination of D-kynurenine generates kynurenic acid in rat and human brain. J Neurochem 2012;120:1026–35.
Phillips LJ, McGorry PD, Garner B, Thompson KN, Pantelis C, Wood SJ, et al. Stress, the hippocampus and the hypothalamic–pituitary–adrenal axis: implications for the development of psychotic disorders. Aust NZ J Psychiatry 2006;40:725–41.
Richell RA, Deakin JF, Anderson IM. Effect of acute tryptophan depletion on the response to controllable and uncontrollable noise stress. Biol Psychiatry 2005;57:295–300.
Robotka H, Toldi J, Vécsei L. L-Kynurenine: metabolism and mechanism of neuroprotection. Futur Neurol 2008;3:169–88.
Saito S, Tachibana T, Choi YH, Denbow DM, Furuse M. ICV CRF and isolation stress differentially enhance plasma corticosterone concentrations in layer- and meat-type neonatal chicks. Comp Biochem Physiol A Mol Integr Physiol 2005;141:305–9.
Stone EA. Accumulation and metabolism of norepinephrine in rat hypothalamus after exhaustive stress. J Neurochem 1973;21:589–601.
Suenaga R, Tomonaga S, Yamane H, Kurauchi I, Tsuneyoshi Y, Sato H, et al. Intracerebroventricular injection of L-arginine induces sedative and hypnotic effects under an acute stress in neonatal chicks. Amino Acids 2008;35:139–46.
Takagi S, Ikeda H, Kawase T, Nagasawa M, Chowdhury VS, Yasuo S, et al. Orally administered L-tyrosine, but not D-tyrosine, shortly enters into several regions of the mouse brain. J Pet Anim Nutr 2013;16:67–72.
Tomonaga S, Yamane H, Onitsuka E, Yamada S, Sato M, Takahata Y, et al. Carnosine-induced antidepressant-like activity in rats. Pharmacol Biochem Behav 2008;89:627–32.
van Luijtelaar EL, van der Grinten CP, Blokhuis HJ, Coenen AM. Sleep in the domestic hen (*Gallus domesticus*). Physiol Behav 1987;41:409–14.
Vernon JA, Grudnikoff E, Seidman AJ, Frazier TW, Vemulapalli MS, Pareek P, et al. Antidepressants for cognitive impairment in schizophrenia — a systematic review and meta-analysis. Schizophr Res 2014;159:385–94.
Wang XD, Notarangelo FM, Wang JZ, Schwarcz R. Kynurenic acid and 3-hydroxykynurenine production from D-kynurenine in mice. Brain Res 2012;1455:1–9.
Yoshida J, Tomonaga S, Ogino Y, Nagasawa M, Kurata K, Furuse M. Intracerebroventricular injection of kynurenic acid attenuates corticotrophin-releasing hormone-augmented stress responses in neonatal chicks. Neuroscience 2012;220:142–8.
Yoshida J, Shigemura A, Ogino Y, Denbow DM, Furuse M. Two receptors are involved in the central functions of kynurenic acid under an acute stress in neonatal chicks. Neuroscience 2013;248C:194–200.
Zhang R, Tachibana T, Takagi T, Koutoku T, Denbow DM, Furuse M. Serotonin modifies corticotropin-releasing factor-induced behaviors of chicks. Behav Brain Res 2004;151:47–52.