Screening of Dipeptides Having Central Functions for Excitation and Sedation

H. Yamane[a], D.M. Denbow[b] and M. Furuse[a,*]

[a]*Laboratory of Advanced Animal and Marine Bioresources, Graduate School of Bioresources and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan;* [b]*Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0306, USA*

Abstract: The naturally-occurring dipeptide carnosine (β-alanyl-L-histidine) and the tripeptide glutathione (L-γglutamyl-L-cysteinylglycine) are found extensively in animal tissues such as brain and skeletal muscle. Central functions for excitation and sedation of them and their derivatives were screened.

Key Words: Dipeptides, carnosine, glutathione, excitation, sedation.

INTRODUCTION

The formation of a dipeptide occurs by either a condensation reaction involving the loss of a water molecule or by the breakdown of a longer peptide containing more than three amino acid residues. In the former case, extensive combinations of dipeptide are estimated. For instance, proteinogenic amino acids, 20 amino acids having the standard genetic code, are found in proteins. In addition, non-proteinogenic amino acids of β-amino acids such as β-alanine can be present as a constituent of dipeptides. Importantly, β-peptides can be made only through biosynthesis, but not breakdown. These amino acids have a chance to place at both the amino and carboxyl terminal.

Carnosine (β-Ala-His) is a naturally-occurring dipeptide that is present in high concentrations in the muscle and brain of mammalian and avian species [1-4], and is synthesized from β-alanine and L-histidine (Fig. **1**). One of the well-known functions of carnosine is its antioxidant activity [2]. On the other hand, reduced glutathione (GSH: L-γglutamyl-L-cysteinylglycine) (Fig. **2**) is also a major protectant in the brain against oxidative stress by interacting directly with reactive oxygen species or by participating in enzyme-catalyzed redox cycling reactions [5]. Recently, we found that both carnosine [6-8] and GSH [9, 10] are involved in stress behavior, the former enhancing and the latter reducing the stress response. In addition, we have screened variations of carnosine derivatives in which amino acids were substituted or exchanged, as well as dipeptides derived from GSH for their effects in the central nervous systems (CNS) with respect to excitation and sedation. The possible role of these dipeptides in neuromedicine is discussed.

FUNCTIONS OF CARNOSINE AND RELATED DIPEPTIDES IN THE CNS

Chicks were injected intracerebroventricular (i.c.v.) with saline (control) or carnosine (0.8, 3.2 and 6.4 μmol) [6]. After injection, the birds were placed in a monitoring cage and video recorded for 15 min. Spontaneous activity was counted with infrared beam sensors and analyzed by software associated with a digital data recording system. The number of vocalizations was also analyzed. Carnosine induced hyperactivity in a dose-dependent fashion. The number of vocalizations also markedly increased in a dose-dependent manner. As described by van Luijtelaar *et al*. [11], four behavioral categories were distinguished: (1) active wakefulness; (2) standing/sitting motionless with eyes open; (3) standing motionless with eyes closed; and (4) sitting motionless with head drooped (sleeping posture). In addition, an abnormal behavior was observed in which birds could not stand and sometimes overturned, but were very active. Carnosine increased the time in active posture compared to the controls in which several postures were observed. Only the highest dose (6.4 μmol) of carnosine caused abnormal behaviors. This behavior observed with the highest dose may be the reason for the rapid decrease in spontaneous activity compared with the 3.2 μmol dose of carnosine.

Hyperactivity and increased vocalization are characteristic behaviors of chicks administered corticotropin releasing factor (CRF) i.c.v. [12], and we previously confirmed that CRF increased plasma corticosterone concentration [13]. Similar to CRF-treated chicks, plasma corticosterone concentration of carnosine-treated chicks dose-dependently increased [6]. Therefore, the results suggest that the action of central carnosine may be related to the central actions of CRF.

Bondy *et al*. [14] suggested that dietary antioxidants may prevent the age-related decline in cerebral nitric oxide (NO) synthase (NOS) levels. Carnosine has an antioxidant activity and can affect brain function as a dietary supplement [15]. We confirmed that i.c.v. carnosine-induced hyperactivity was prevented by a NOS inhibitor [7]. The non-selective NOS inhibitor N^G-nitro-L-arginine methyl estel HCl (L-NAME) could attenuate carnosine-induced hyperactivity, and the effect was dose-dependent. Because the inactive isomer of the NOS inhibitor, N^G-nitro-D-arginine methyl estel HCl, did not attenuate carnosine-induced hyperactivity, the attenua-

*Address correspondence to this author at the Laboratory of Advanced Animal and Marine Bioresources, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; Tel: +81-92-642-2953; Fax: +81-92-642-2953; E-mail: furuse@brs.kyushu-u.ac.jp

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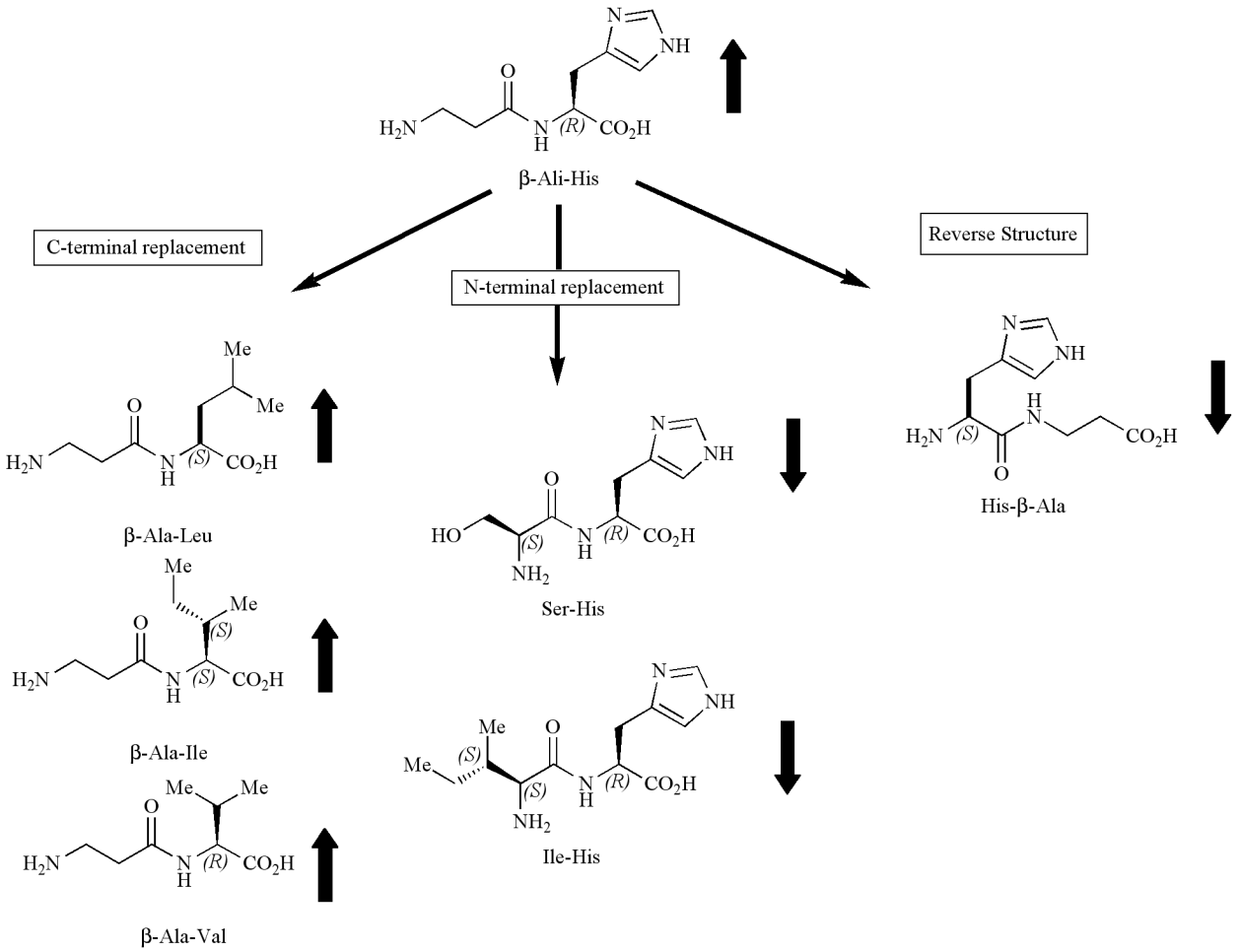

Fig. (1). Dipeptides modified from carnosine (β-Ala-His). The arrows indicate excitation (↑) and sedation (↓), respectively. β-Ala-Leu, β-Ala-Ile, β-Ala-Val and His-β-Ala were named as Excitin-1, Excitin-2, Excitin-3 and rev-carnosine, respectively.

tion of carnosine-induced hyperactivity by L-NAME might be due to the specific inhibition of NOS activity in the brain. Therefore, the results suggested that carnosine-induced hyperactivity may be linked to NO generation *via* NOS in the brain.

NOS isoforms are either constitutively expressed (e.g. neuronal ncNOS, endothelium ecNOS) or inducible (iNOS). Constitutively expressed NO synthases (cNOSs) produce picomolar-nanomolar amounts of NO for short periods in response to receptor stimulation or shear stress. In contrast to

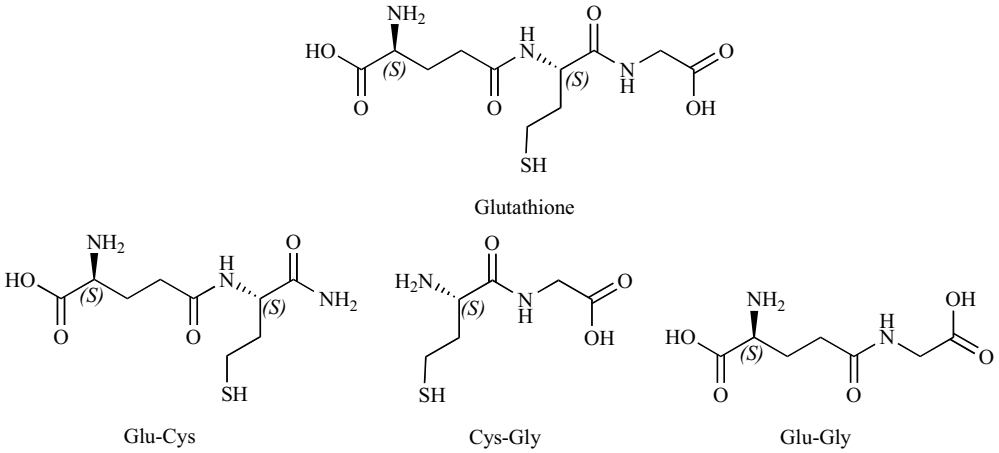

Fig. (2). Theoretically present three types of dipeptides derivable from reduced glutathione.

the ecNOS and ncNOS isoforms, iNOS is expressed following exposure to diverse stimuli such as inflammatory cytokines and lipopolysaccharide, and it generates significantly greater and more sustained amounts of NO when compared to the cNOS [16]. Therefore, carnosine-induced hyperactivity may be linked to cNOS rather than iNOS. To clarify this speculation, we investigated the effect of a selective iNOS inhibitor L-N^6-(1-iminoethyl) lysine HCl (L-NIL) on carnosine-induced hyperactivity. L-NIL did not affect carnosine-induced hyperactivity. Therefore, carnosine-induced hyperactivity may be linked to the stimulation of cNOS rather than iNOS.

Figure **3** shows the mechanism of carnosine for hyperactivity. Carnosine may act to CRF and/or cNOS directly or indirectly. Consequently, hyperactivity was induced in chicks.

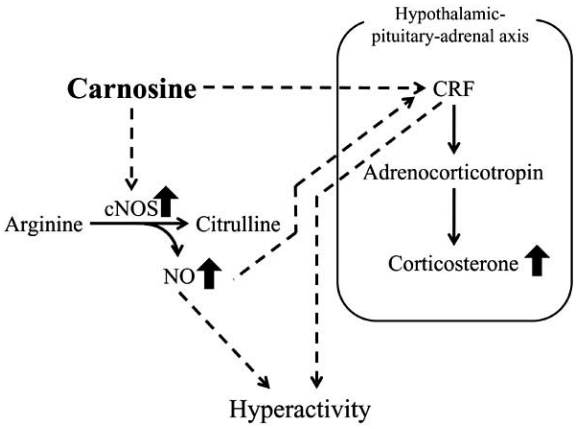

Fig. (3). Speculative schematic model of the action of carnosine. Carnosine may be related to CRF and NO. Carnosine may stimulate CRF release directly or indirectly through NO production.

FUNCTIONS OF β-ALANINE AND CARNOSINE-RELATED DIPEPTIDES

β-Alanine is synthesized in the liver as the final metabolite of uracil and thymin degradation [17]. It was reported that β-alanine decreased the toxic effects of β-amyloid in rat brain endothelial cells [18] or reduced bacterial lipopolysaccharide-induced hepatotoxicity in rats [19]. Although β-alanine is a non-proteinogenic amino acid, it is especially rich in the brain and breast muscle of chickens as a component of carnosine or anserine, or as a free amino acid [8]. Therefore it is reasonable to speculate that β-alanine may have other functions. Accordingly, we focused on its role as a neurotransmitter and investigated the effect of dipeptides having β-alanine at the amino terminus. Conventionally, the discovery of novel dipeptides has been done through the process of isolation and purification, but we designed and manufactured dipeptides related to carnosine, and screened these dipeptides with special reference to their effects on behavior.

Accoding to Tsuneyoshi *et al*. [20], carnosine and β-Ala-Leu (Fig. **1**) significantly enhanced both total spontaneous activity and total distress vocalizations, while β-Ala-Gly has no effect. Since branched chain amino acids (BCAAs), i.e., L-leucine, L-isoleucine and L-valine, have a similar structure, it appeared possible that β-alanyl dipeptides having BCAAs at the carboxyl terminus might have a similar function. Thus, the effect of β-Ala-Leu, β-Ala-Ile and β-Ala-Val (Fig. **1**) were investigated. The i.c.v. injection of β-Ala-Ile had a similar effect on hyperactivity and distress vocalizations as observed with carnosine and β-Ala-Leu. In contrast, the effect of β-Ala-Val was somewhat weaker than that of β-Ala-Leu and β-Ala-Ile. At present, although we could not clarify the difference in efficacies among the three β-alanyl-BCAAs, the low molecular weight of L-valine may be associated with its weak activity. The sedative and hypnotic postures such as standing motionless with eyes closed and sleeping posture were rarely observed with these dipeptides [20]. It was concluded that one of the central functions of β-alanyl-BCAAs is induction of hyperactivity. Thus, we have proposed that β-Ala-Leu, β-Ala-Ile and β-Ala-Val be named Excitin-1, Excitin-2 and Excitin-3, respectively [20].

As the second step, the amino terminus β-alanine of carnosine was substituted with other amino acids [21]. The i.c.v. injections of Ser-His and Ile-His (Fig. **1**) decreased spontaneous activity and distress vocalizations, while Val-His decreased only spontaneous activity. These results in behavioral changes were completely reverse from those obtained by carnosine. It was interesting that replacement of β-alanine with L-serine or L-isoleucine produced an opposite function. On the other hand, i.c.v. injections of Ala-His, Gly-His and Met-His did not cause any behavioral changes. In conclusion, some combination of amino acids with the carboxyl terminal histidine attenuates stress-induced behavior.

Finally, we investigated carboxyl β-alanine related dipeptides in which the terminal amino acids were replaced with other amino acids [22]. Among the three dipeptides Gly-β-Ala, His-β-Ala and Val-β-Ala having a carboxyl terminus β-alanine, His-β-Ala (Fig. **1**) alone had a sedative and hypnotic effect. This implies that even if the dipeptide has β-alanine, the amino acid at the amino terminal has an effect on function. His-β-Ala is the reverse structure of carnosine, and its effect following i.c.v. injection was opposite to that of carnosine. Thus, we propose to name this dipeptide reverse carnosine (His-β-Ala; rev-carnosine).

FUNCTIONS OF TRIPEPTIDE, GSH, AND RELATED DIPEPTIDES IN THE CNS

GSH is a tripeptide which consists of L-glutamate, L-cysteine and glycine, and oxidized glutathione (GSSG) is a dimer of GSH. Glutathione-related enzymes glyoxalase 1 and glutathione reductase 1 regulates anxiety in mice [23]. Glyoxalase 1 detoxifies dicarbonyl metabolites, and then uses glutathione as a cofactor [24]. Glutathione reductase irreversibly catalyzes the reduction from GSSG to reduced GSH [25]. Greater than 99.5% of tissue "total glutathione" (i.e., GSH and GSSG, in GSH equivalents) is in the form of GSH [26]. Additionally, GSH appears to bind to the *N*-methyl-*D*-aspartate (NMDA) receptor, which has both glutamate and glycine recognition sites, *via* the γglutamyl moiety [27, 28]. To clarify the central function of glutathione as a neurotransmitter in the stress reaction, the effect of i.c.v. injection of GSH and GSSG were investigated using a chick isolation-induced stress model [9]. Both GSH and GSSG dose-dependently decreased distress vocalizations and induced sleep-like behavior in chicks under acute stressful condi-

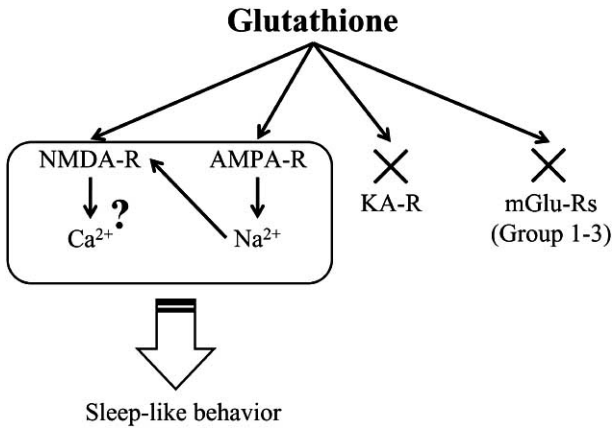

Fig. (4). Speculative schematic model of glutathione signal transduction. Glutathione, particularly GSH may be binding for NMDA and AMPA receptors, but not for kainate receptor (KA-R) and metabotropic glutamate receptors (mGlu-Rs).

tions. However, which glutathione is actually responsible for inducing sleep was unclear since glutathione cycles between GSH and GSSG in which two tripeptides are linked by a disulfide bond. Therefore, the behavior of chicks was monitored following the i.c.v. injection of S-methyl-glutathione (SMG). SMG does not form a disulfide bond due to the methylation of the SH group of the cysteine moiety. SMG had similar effects as observed with GSH and GSSG. In conclusion, glutathione and its derivative have sedative and hypnotic effects, and might be effective in improving psychic stress such as anxiety [9].

There are three types of dipeptides, i.e., Glu-Cys, Cys-Gly and Glu-Gly that are theoretically present in tissues (Fig. **2**). The combination of Glu-Cys is called γglutamyl-cysteine (γGlu-Cys), which is a GSH precursor. γGlu-Cys synthetase uses glutamate and cysteine as substrates to generate γGlu-Cys. This dipeptide is combined with glycine to synthesize GSH in a reaction catalyzed by GSH synthetase [29]. Cys-Gly is generated from GSH by the γglutamyl transpeptidase (γGT) reaction in the body [30]. The γGT-product Cys-Gly is efficiently utilized in micromolar concentrations as a precursor for neuronal GSH [31], and as a supply of cysteine and glycine for neurons [32]. Glu-Gly is present in the cortex of the rat brain at concentrations of 1.1 ± 0.1 nmol/g wet

Table 1. Effect of Several Substances on Various Behavioral Categories of Chicks

Drugs	Distress Vocalizations	Spontaneous Activity	Active Wakefulness	Sleeping Posture/Sedation
Carnosine	↑	↑	↑	↓
+ L-NAME	−	−	−	−
+ L-NIL	−	−	−	−
β-Ala-Leu	↑	↑	↑	↓
β-Ala-Ile	↑	↑	↑	↓
β-Ala-Val	↑	↑	↑	↓
β-Ala-Gly	−	−	−	−
Ser-His	↓	↓	↓	↑
Ile-His	↓	↓	↓	↑
Val-His	−	↓	−	−
Ala-His	−	−	−	−
Gly-His	−	−	−	−
Met-His	−	−	−	−
Gly-β-Ala	↓	↓	↓	↑
His-β-Ala	↓	↓	↓	↑
Val-β-Ala	↓	↓	↓	↑
GSH	↓	ND	↓	↑
GSSG	↓	ND	↓	↑
SMG	↓	ND	↓	↑
Glu-Cys	↓	−	↓	↑
Cys-Gly	−	↓	−	↑
Glu-Gly	↓	↓	↓	↑

↑: increase. ↓: decrease. −: no change. ND: not determined.

tissues [33]. The combination of Glu-Gly might be synthesized in the metabolism of glutathione *via* the γglutamyl cycle, since a wide variety of amino acids serve as an accepter of the γglutamyl group generated by the degradation of glutathione [34].

Accordingly, all three dipeptides mentioned could possibly bind to the NMDA receptor since they have a glutamate or glycine moiety. Therefore, these peptides may have sedative and hypnotic effects on stress behavior as observed with glutathione.

Yamane *et al*. [10] investigated the effect of i.c.v. injection of three dipeptides, Glu-Cys, Cys-Gly and Glu-Gly, on total distress vocalizations during the 10-min isolation stress. Glu-Cys and Glu-Gly significantly suppressed vocalization, while Cys-Gly had no effect. Glu-Gly and Cys-Gly significantly suppressed spontaneous activity compared to the control. Although a significant difference was not detected, Glu-Cys tended to decrease total spontaneous activity compared to the control. The i.c.v. injections of Glu-Cys and Glu-Gly decreased the time for active wakefulness. No significant effect was found on the time for sleeping posture. When behavioral categories were divided into active wakefulness and sedation (standing/sitting motionless with eyes open, standing motionless with eyes closed and sleeping posture), all three dipeptides caused sedative effects.

Glutamate, NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) induced sedative and hypnosis. However, kainate (KA) and metabotropic glutamate receptors (mGlu-Rs) Group1 agonist had no influence on behavior of chicks [35]. Furthermore, GSH does not bind to mGlu-Rs and has the low affinity to KA receptor [27, 36]. Therefore, it is suggested that the effect of GSH was induced through the interaction between NMDA and AMPA receptors (Fig. **4**).

Finally, the effects of dipeptides and other substances mentioned here on behavioral changes are summarized in Table **1**.

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