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Aromatic amino acid-leucine dipeptides exhibit anxiolytic-like activity in young mice

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HIGHLIGHTS

- Aromatic amino acid-leucine, FL and WL exhibit potent anxiolytic-like activity.
- FL and WL were orally active, and their retro-sequence dipeptides were inactive.
- Their anxiolytic-like activities were mediated by 5-HT_{1A}, D_1 and $GABA_A$ receptors.

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ABSTRACT

We previously reported that Tyr-Leu (YL) exhibits potent anxiolytic-like activity comparable to diazepam in mice. In the current study, we revealed that aromatic amino acid-Leu, Phe-Leu and Trp-Leu (FL and WL, respectively), exhibited anxiolytic-like activity in the elevated plus-maze and open-field tests. FL and WL were orally active. Retro-sequence peptides of FL and WL were inactive. Similarly to YL, the anxiolytic-like activities of FL and WL were inhibited by WAY100135, SCH-23390 and bicuculline, antagonists of serotonin 5-HT_{1A}, dopamine D_1 and $GABA_A$ receptors, respectively, implying that FL and WL activate a common anxiolytic pathway to that of YL. Taken together, aromatic amino acid-Leu dipeptides such as YL, FL, and WL may exhibit anxiolytic-like activity in a manner dependent on the activation of 5-HT_{1A}, D_1 and $GABA_A$ receptors.

1. Introduction

Recently, we have found that a dipeptide, tyrosyl leucine (Tyr-Leu, YL) had potent anxiolytic-like activity after oral administration (0.3–3 mg/kg), comparable to diazepam, a general anxiolytic pharmaceutical [11]. In the present study, we investigated the anxiolytic-like activity of YL analogs using behavioral tests. We then found that phenylalanyl leucine (Phe-Leu, FL) and tryptophanyl leucine (Trp-Leu, WL) exhibited anxiolytic-like activity; however, their retro-sequence dipeptides were inactive. These results suggest that aromatic amino acid-leucine sequences are important for anxiolytic-like activity. It has been reported that enzymatic digests of proteins sometimes exhibited anxiolytic-like activity after oral administration [8,9,15,16,22,24]. Since YL, FL, and WL sequences are present in the primary structure of many endogenous and exogenous proteins, these findings might contribute to uncover potential functions of proteins-derived peptides after enzymatic digestion.

Next, we investigated the mechanism of the anxiolytic-like activities. Emotional behavior is associated with a number of neurotransmitters, including serotonin, dopamine and γ-amino butyric acid type (GABA) in the central nervous system. It has been reported that serotonin 5-HT_{1A} receptor, dopamine D_1 receptor, and $GABA_A$ receptor play an important key role in anxiolytic-like activity [3,17,19,20]. We then tested whether the anxiolytic-like activity of aromatic amino acid-leucine dipeptide is mediated by activation of these receptors.

2. Materials and methods

2.1. Animals

Male ddY mice (SLC, Shizuoka, Japan), 4 weeks old, were raised in plastic cages in a room with a 12-h light–dark cycle (dark phase: 19:00–7:00), constant temperature (23 ± 1 °C), and constant humidity (50 ± 10%). This ddY strain is a closed colony with a rapid

Abbreviations: EPM, elevated plus maze.

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growth rate and high reproductive potential, and has frequently been used in various biomedical research, including behavioral pharmacological tests, as previously described [10,14,15]. They were housed for more than 3 days for acclimatization to the environment. Animals were fed regular tap water and a commercial solid diet ad libitum. All experiments were approved by Kyoto University Ethics Committee for Animal Research Use. All animals were euthanized by cervical spine dislocation after the experiment.

2.2. Reagents

Tyr-Leu (YL), Phe-Leu (FL), Trp-Leu (WL), Leu-Phe (LF), and Leu-Trp (LW) were purchased from Bachem AG (Bufendorf, Switzerland). WAY100135 dihydrochloride, a serotonin 5-HT_{1A} receptor antagonist; R(+)-SCH-23390 hydrochloride, a dopamine D_1 receptor antagonist; and (−)-bicuculline, a $GABA_A$ receptor antagonist were purchased from Tocris Bioscience (Bristol, UK).

2.3. Elevated plus-maze (EPM) test

Anxiolytic-like behavior was assessed using the EPM test, which was performed as described previously [8,20,23]. Four arms (25 cm long × 5 cm wide) were placed 50 cm above the ground. Two opposite arms were delimited by acrylic vertical walls (15 cm high, closed arms), and two had unprotected edges (open arms). A mouse was placed in the center of the maze facing an open arm and observed for 5 min to measure the cumulative time and frequency of entries into open and closed arms. Arm entry was defined as the entry of four paws into an arm. Open-arm entry time (time spent in open arms) was expressed as a percentage of the total entry time (% of time), and the number of open-arm entries was expressed as a percentage of the number of total entries (% of visit). We confirmed that anxiolytic drug, diazepam, increased in % of time in and visit to the open arms. Each peptide dissolved in saline was administered intraperitoneally (i.p.) or orally (p.o.) 30 min before the test. WAY100135 dihydrochloride (10 mg/kg), R(+)-SCH-23390 hydrochloride (30 μg/kg), or (−)-bicuculline (0.5 mg/kg) were co-administered i.p. with peptide once, 30 min before the test. The dosage of each antagonist was based on previous studies [2,4,7,8,11,18,21]. The total number of visits to open and closed arms, and the cumulative time spent in open and closed arms were measured on a monitor. The data were checked by observers unaware of the experimental groups. The EPM test was started at 10:00 a.m. during the light phase of the light/dark cycle.

2.4. Open-field test

The open-field test was performed as previously described with slight modification [1]. The apparatus consisted of a circular arena of 60 cm diameter and wall height of 50 cm. The apparatus was gray with black lines on the bottom, which divided the open-field into 25 parts of similar area by two concentric circles as a series of radii. Each mouse was placed in the center circle and its movement monitored for 5 min. We confirmed that diazepam increased in % of time in and visit to the center circle. The data were checked by observers unaware of the experimental groups. The time spent in the center circle was measured. The open-field test was started at 11:00 a.m.

2.5. Receptor binding assay

Receptor binding assays of serotonin 5-HT_{1A}, dopamine D_1 and $GABA_A$ receptors were performed, as previously described. Briefly, 2 nM [^{3}H]-serotonin were incubated with the membrane from rat cortex homogenate in 50 mM Tris–HCl, pH7.4 for 10 min at 37 °C [13]. Specific binding was calculated by subtracting non-specific binding in the presence of excess unlabeled 10 μM serotonin from the total binding.

In the receptor binding assay of dopamine D_1 receptor, recombinant DNA-transformed CHO cells expressing the human D_1 receptor were used [6]. 1.4 nM [^{3}H]-SCH23390 was incubated with the cell lysate in 50 mM Tris–HCl, pH7.4 for 120 min at 37 °C. Specific binding was calculated by subtracting non-specific binding in the presence of excess unlabeled 10 μM (+)-butaclamol from the total binding.

In the assays of benzodiazepines- and GABA-binding sites of $GABA_A$ receptor, 1 nM [^{3}H]-flunitrazepam or 1 nM [^{3}H]-muscimol was incubated with the membrane from brain homogenate in 50 mM phosphate buffer, pH7.4 for 60 min at 25 °C or 50 mM Tris–HCl, pH7.4 for 10 min at 4 °C, respectively [5,12]. Specific binding was calculated by subtracting non-specific binding in the presence of excess unlabeled 10 μM diazepam or 0.1 μM muscimol from the total binding.

2.6. Statistical analysis

All values are expressed as the means ± SEM. Analysis of variance (ANOVA) followed by Bonferroni's test was used to assess differences among groups. $P < 0.05$ was considered significant.

3. Results

3.1. Aromatic amino acid-leucine dipeptides exhibit anxiolytic-like activities in mice

We investigated whether YL analogs exhibited anxiolytic-like activity in the EPM and open-field tests. In the EPM test, FL and WL (1 mg/kg, i.p.) increased the percentage of time spent in open arms, similarly to YL (Fig. 1A). These dipeptides also increased the percentage of visits to open arms, whereas they did not affect total visits, which indicate locomotor activity (Fig. 1B and C). These results suggest that FL and WL exhibit anxiolytic-like activity in the EPM test. The anxiolytic-like activity of WL at a dose of 0.1 mg/kg was comparable to that of FL at a dose of 1 mg/kg in the EMP test (Fig. 2A and B), implying that WL exhibits more potent anxiolytic-like activity than that of FL. In

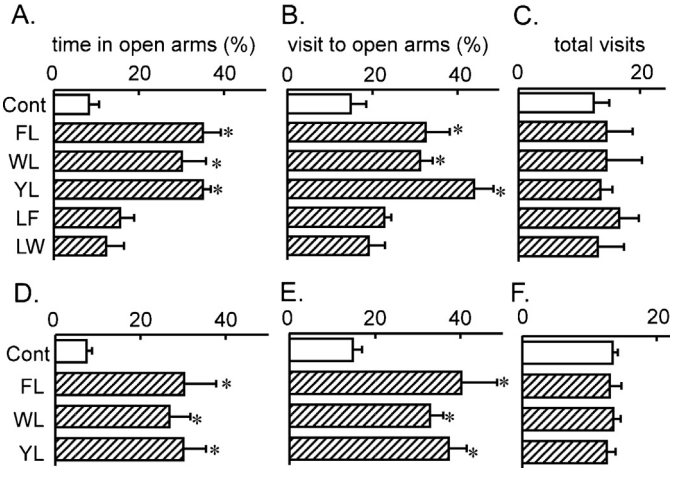

Fig. 1. Aromatic amino acid-leucine dipeptides exhibited anxiolytic-like activity in the elevated plus-maze (EPM) test in mice. (A–C) FL, WL, YL, LF, or LW (1 mg/kg, i.p) was administered to mice 30 min before the EPM test. (D–F) FL, WL or YL (1 mg/kg, p.o.) was administered to mice 30 min before the EPM test. The percentages of time in the open arms (A and D), visits to the open arms (B and E), and total visits to both open and closed arms (C and F) during the test for 5 min were measured. Each value is expressed as the mean ± SEM ($n = 6$–7). * $P < 0.05$, compared with the saline-treated control group.