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Effects of amines on NMDA receptors

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Abstract

N-methyl-D-aspartic acid （NMDA） receptors are widely distributed in the central nervous system and play critical roles in synaptic plasticity and excitotoxicity. Memantine, one of the amines, is a partial agonist of the NMDA receptor and is used as medicine for treatment of Alzheimer's disease. To find a new channel blocker of the NMDA receptor， the agonist and antagonist activities of amines contained in Japanese sake on the NMDA receptor were investigated. Polyamines are present at millimolar concentrations in cells and play important roles in aging, cell proliferation, gene expression and cellular stress. Various foods and beverages including fruits nuts, sake, wine and cheese contain amines. However, the precise biological functions of amines are still unclear. The effects of 12 amines contained in sake on GluN1/GluN2A and GluN1/GluN2B receptors were examined. Agmatine, tyramine, 2-phenylethylamine and 4 other amines inhibited the activity of both receptors. Putrescine inhibited the activity of only the GluN1/GluN2A receptor. Betaine and trimethylamine N-oxide inhibited the activity of only the GluN1/GluN2B receptor. Isoamylamine acted as an antagonist against the GluN1/GluN2B receptor but act as an activator for the GluN1/GluN2A receptor. The results indicated that amines contained in sake can be used as potential therapeutic agents for neural disorders such as anxiety and dementia.

Keywords: amines, sake, NMDA receptor, Oocyte expression system

1. INTRODUCTION

NMDA receptors are widely distributed in the central nervous system and play critical roles in synaptic plasticity and excitotoxicity. NMDA receptors are assembled from two GluN1 subunits and two GluN2 subunits, of which there are four subtypes (GluN1/GluN2A, GluN1/GluN2B GluN1/GluN2C, and GluN1/GluN2D), and the receptors are activated by simultaneous binding of glycine and glutamate to the GluN1 and GluN2 subunits, respectively[1)]. NMDA receptors are involved in various brain disorders including ischemic stroke, Alzheimer's disease and schizophrenia[2)]. Each of the NMDA receptor subunits consists of an intracellular C-terminal domain and three transmembrane domains that form the ion channel pore and a pair of extracellular domains: the ligand binding domain (LBD) and the N-terminal domain. Upon agonist binding, the two lobes close around the agonist, whereas binding of antagonists stabilizes an open domain structure. Nevertheless, a correlation between the steric requirements of the ligand and the extent of receptor activation still seems to exist.

Phencyclidine, one of the antagonists for the receptor, is known to cause the symptom like schizophrenia[3)]. Memantine, an amine and a partial antagonist of a NMDA receptor, has been used as an anti-dementia medicine for more than 15 years in Europe and was recently approved as an anti-dementia medicine in USA and Japan[4)]. Partial agonists of NMDA receptors such as memantine will have the possibility of becoming agents for treatment of psychological illnesses. Many synthesized agents that have agonist or antagonist activity on the NMDA receptor have been examined

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for the possible use as therapeutic agents[5].

Polyamines (putrescine, spermidine, and spermine) are present at millimolar concentrations in cells and play important roles in aging, cell proliferation, gene expression and cellular stress[6-8]. Various foods and beverages including like fruits, nuts, sake, wine and cheese contain polyamines and monoamines[9-12]. However, the precise biological functions of amines are still unclear.

By analyzing the amines in sake and examining their effects on NMDA receptors, we tried to determine the usefulness of the amines and link them to effective utilization. Sake was fractionated using ion-exchange chromatography and subsequently lyophilized as previously reported [13]. The four fractions obtained mainly consisted of basic amino acids (BA fraction), neutral and acidic amino acids (NA fraction), organic acids (OA fraction), and sugars (S fraction). In the present study, we focused on the BA fraction from sake and identified the ingredients of sake that inhibit the activity of the NMDA receptor. We compared the effects of polyamines and monoamines contained in sake on the channel activity of GluN1/GluN2A-(GluN2A) and GluN1/GluN2B-(GluN2B) subtypes of NMDA receptor using the *Xenopus* oocyte expression system. Identification of the naturally derived and subunit-specific NMDA receptor agonists, antagonists and modulators could be valuable pharmacological tools as well as potential new therapeutic agents.

2. MATERIALS AND METHODS

2.1. Chemicals

Putrescine, spermidine, spermine, ornithine, agmatine, 5-oxoproline, choline, betaine, and trimethylamine N-oxide were purchased from Sigma-Aldrich (St. Louis, MO). Tyramine, 2-phenylethylamine, and isoamylamine were purchased from Wako (Japan).

2.2. Fractionation of sake

Japanese sake (junmai shu) was fractionated as described previously[13]. Briefly, the pH was adjusted to 6.5 with 1N NaOH, and sake was fractionated into four different fractions (fractions containing basic amino acids, neutral and acidic amino acids, organic acids, and sugars) using the columns IRC-76, IR-120BH, and IRA-96SB (Oregano Co., Tokyo, Japan). The concentrations and compositions of organic acids and amino acids were confirmed by an organic acid analyzer (LC10AD, Shimadzu, Kyoto) and an amino acid analyzer (LC10A, Shim-pack Amino-Na Column, Shimadzu, Kyoto, Japan), respectively. The four fractions derived from sake were lyophilized and dissolved in Milli-Q water to 50-fold concentrations compared to those in the original sake. The obtained concentrate was used for further analysis. For electrophysiological analysis, the 50-fold concentrated fractions were diluted 50 times by Ba^{2+} Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$ and 10 mM HEPES, pH 7.2).

2.3. Analysis of the BA fraction of sake by CE-TOFMS

50-fold concentrated BA fractions from a sake sample were analyzed using CE-TOFMS. For the analysis, 1 µL of the BA fraction, 49 µL of Milli-Q water, and 50 µL of Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) were mixed thoroughly. The mixture was centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 ×*g* for 60 min at 4°C to remove the proteins and macromolecules. Metabolome measurements were carried out at a facility at Human Metabolome Technology Inc. (Tsuruoka, Japan). CE-TOFMS was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The systems were controlled by Agilent G2201AA ChemStation software, version B.03.01, for CE (Agilent Technologies, Waldbronn, Germany).

2.4. Analysis of amines in sake

Amines were derivatized by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) using AccQ・TagTM Ultra and determined by ACQUITY UPLC system and Quattro Premier XE (Waters Corp, MT, USA).

2.5. Preparation of cRNAs and oocytes

Complementary RNAs of mouse NMDA receptors were synthesized as described previously[14) 15]. Stage V and VI oocytes were obtained from anesthetized *Xenopus laevis* as described previously. The oocytes were injected with mouse GluN2A and GluN1a cRNAs in a molar ratio of 1:1 and with (GluN2A:GluN1a), GluN2B and GluN1a cRNAs in a molar ratio of 1:2 (GluN2B:GluN1a). The total amounts of cRNAs

injected were 43 ng and 5 ng per oocyte, respectively. Before recording, oocytes were incubated in a 35 mm culture dish (Falcon) at 19ºC for 18-26 hrs in Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM NaHCO and 7.7 mM Tris-HCl, pH 7.6).

2.6. Electrophysiological recordings

Currents were recorded by the two-electrode voltage-clamp technique using a TEV200 oocyte clamp (Dagan Corp., Minneapolis, MN). Electrodes were filled with 3 M KCl and had resistances of 1-5 M Ω. Oocytes were perfused by a constant stream of Ba^{2+} Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$ and 10 mM HEPES, pH 7.2) at 23-25ºC. The oocyte membrane was voltage-clamped at –70 mV. Under standard assay conditions, currents were evoked by bath perfusion with Ba^{2+} Ringer's solution containing 100 μM L-glutamate and 100 μM glycine for 20 s, followed by a washout with standard Ba^{2+} Ringer's solution. All data were obtained from 2-5 different frogs and normalized with the control current at each time unless otherwise stated. Responses to ligands were normalized as I %= (I/Imax) x100, where I is the peak amplitude of current response and Imax is the maximal current produced by ligands measured in each individual cell. Normalized responses were pooled and graphed as the mean ± SEM from at least three oocytes. The IC_{50} value (inhibitor concentration for half-control response) and Hill coefficient value of each amine for NMDA receptors were calculated according to theoretical curves that were drawn according to the equation $F=1/[1+(G/IC_{50})^n]$, where F is the fractional response, G is the concentration of amines, and n is the Hill coefficient.

2.7. Animal experiments

Nine-week-old male C57BL/6 mice (Charles River Japan, Yokohama, Japan) were maintained under controlled conditions (ambient temperature, 22 ± 2 °C; 12-h light/dark cycle, lights on from 0:00 am to 12:00 pm). The mice had free access to food and water. All of the animals received human care as outlined in the Guide for the Care and Use of Laboratory Animals (Kindai University Animal Care Committee).

2.8. Elevated plus maze test

The plus maze comprised two pairs of opposing open and closed arms (6 cm × 30 cm) that extended from a central area (9 cm × 9 cm). The maze floor was placed 40 cm above the floor of the room. The open arms included a slightly raised edge, 2 cm high, to reduce the likelihood that animals would fall over the edge. The closed arms were enclosed with three walls with open roofs of 10 cm in height. At the start of the test, each mouse (11-week-old mice) was placed in the central area of the maze facing the open arm. The behavior of each mouse in the plus maze was observed for 10 min. Arm entry was defined as all four paws inside the arm. The number of arm entries and the time spent in the arms were recorded. The percentage of open arm entries and the percentage of time spent in the open arms were defined as measures of anxiety. In this study, we conducted an elevated plus-maze test to investigate the anxiolytic effects of isoamylamine, choline, ornithine, betaine, and 2-phenylethylamine, which are components of Japanese sake that have shown inhibitory effects on NMDA receptors. It has been reported that the concentration at which agmatine showed a significant anxiolytic effect was 40 mg/kg[16], and the sake component to be examined for an anxiolytic effect was measured at 40 mg/kg in this study. Saline, isoamylamine, 2-phenylethylamine, choline, ornithine, and betaine were intraperitoneally (*i.p.*) administered 30 min before the tests as shown in Table 4. All statistical calculations are presented as means and standard error of the mean (SEM) . All reagents were dissolved in sterilized saline. After each trial, the apparatus was carefully cleaned with a wet paper towel (soaked in a mixture of ethanol and water) to remove any residues or odors. All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Kindai University Animal Care Committee KAEN-30-001).

2.9. Statistical analysis

Differences between means were determined using Student's t-test when the F-value was significant. Differences were considered statistically significant at a p-value of 0.05.

3. RESULTS AND DISCUSSION

3.1. Composition of amines in fermented food

As shown in Table 1 (modified from Nishimura 2006)[17], there are various amines contained in fermented foods. Sake contains more than 1 mM of agmatine. Wine, beer and soy sauce contain putresine, tyramine, and spermine. Soy sauce, which is a Japanese traditional sauce made from fermented soy beans, contains a large amount of tyramine.

Table 1 Amines contained in fermented foods

(μM)

Amines	Sake A	Sake B	Red wine	White wine	Beer	Soy sauce
Spermine	—	0.5	—	—	1	10.3
Agmatine	1219	1616	—	—	—	—
Putrescine	7.3	16	54.9	13.6	48.8	557
Tyramine	—	0.4	13.8	7.1	32.8	3032
2-Phenylethylamine	0.5	2.3	11.1	18.6	—	264
Isoamylamine	0.1	0.1	22.4	58.6	—	—

3.2. Effects of the four fractions obtained from sake

The activity of each fraction alone was compared with that of each fraction when Glu100 μM/Gly100 μM was added to each fraction. (Fig1) The results showed that the activity of GluN2B receptors was inhibited by the OA and BA fractions, indicating that these fractions contain substances that inhibit the activity of GluN1/GluN2B receptors. The components of the BA fraction were determined to elucidate the effect of the BA fraction on NMDA receptors.

3.3. Amines contained in the BA fraction of sake

The relative amounts of the components in the BA fraction of sake (48 components) were analyzed. As shown in Table 2, it was found that the BA fraction contains various amines (putrescine, spermidine, 2-phenylethylamine, ornithine, isoamylamine, agmatine, 5-oxoproline, choline, betaine, and trimethylamine *N*-oxide) in addition to basic amino acids. The BA fraction contains particularly high concentrations of agmatine, betaine, and choline. (Table 2)

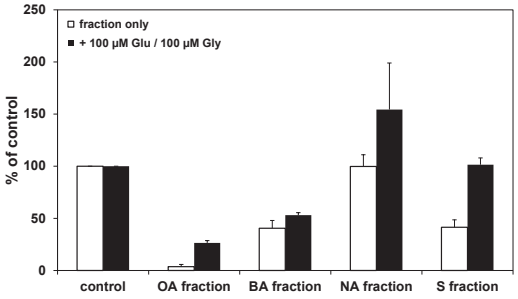

Fig. 1. Effects of fractions of sake on GluN1 / GluN2B receptors

3.4. Activation of NMDA receptors and inhibition of the activity of NMDA receptors by amines in sake

We investigated the effects of 12 amines (putrescine, spermidine, spermine, 2-phenylethylamine, ornithine, isoamylamine, agmatine, tyramine, 5-oxoproline, choline, betaine, and trimethylamine *N*-oxide) contained in sake on GluN2A and GluN2B NMDA receptors expressed in *Xenopus* oocytes. Dose response curves of spermine and isoamylamine are shown in Fig. 2. The IC_{50} values and Hill coefficients for amines on GluN2A

Table 2 Major components of the BA fraction of sake

Compound name	Relative Area	Compound name	Relative Area
Gly	2.3E-03	Arg	1.5E+00
Leu	4.1E-03	Lys	8.2E-01
Glu	1.9E-03	Galacturonic acid	6.7E-03
Thr	2.7E-03	Gluconic acid	4.8E-03
Ile	8.5E-03	Ethanolamine	9.9E-01
Val	1.6E-02	Trimethylamine *N*-oxide	1.3E-02
Phe	8.3E-03	Isoamylamine	1.4E-03
5-Oxoproline	4.6E-04	Putrescine	5.4E-03
Gly-Leu	5.4E-03	3-Aminopropane-1,2-diol	1.2E-03
Tyr	1.2E-02	2-Amino-2-methyl-1,3-propanediol	5.7E-03
GABA	1.3E-02	5-Aminovaleric acid	6.8E-02
Succinic acid	8.6E-04	2-Phenylethylamine	5.9E-03
Citramalic acid	2.3E-04	*N*-Acetylputrescine	1.5E-02
Lactic acid	5.0E-03	Agmatine	2.5E-01
3-Hydroxybutyric acid	4.6E-04	Ornithine	1.4E-01
Citric acid	1.0E-03	Spermidine	1.4E-03
Muscimol	1.3E-03	N^6-Methyllysine	1.9E-03
Terephthalic acid	4.7E-04	Mannosamine	8.2E-03
Phthalic acid	1.2E-03	N_ω-Methylarginine	1.3E-03
Betaine	6.6E-01	N^6,N^6,N^6-Trimethyllysine	1.1E-02
Pyridoxine	2.1E-03	ADMA	7.9E-03
Arg-Glu	7.0E-03	Arginine ethyl ester	3.5E-03
Choline	8.8E+00	SDMA	1.2E-03
His	3.4E-01	β-Ala-Lys	5.7E-03

and GluN2B receptors are shown in Table 3. Isomaylamine acted as an antagonist against the GluN2B receptor but acted as an activator for GluN2A receptor (1.6 folds at 30 μM). Spermine acted as an antagonist against the GluN2A receptor, but acted as an activator for the GluN2A receptor (2.0 folds at 300 μM). Spermidine, 2-phenylethylamine, ornithine, agmatine, tyramine, 5-oxoproline, and choline inhibited the activity of both receptors. 2-phenylethylamine especially inhibited the activity of both receptors at low concentrations. IC_{50} values of 2-phenylethylamine for the GluN2A and GluN2B receptors were 1.36 μM and 13.3 μM, respectively. Memantine, an amine and a partial antagonist of the NMDA receptor, has been approved as an anti-dementia medicine. IC_{50} values of memantine for the GluN2A and GluN2B receptors were 0.88 μM and 0.52 μM, respectively[4]. Partial agonists of NMDA receptors such as memantine will have the possibility of becoming therapeutic agents for the psychological illnesses. Askalany et al.[18] reported that the properties of agmatine are similar to the pharmacological profiles of well-characterized NMDA receptor channel blockers such as phencyclidine and ketamine. It was previously reported that agmatine induces anxiolysis in the elevated plus maze task in adult rats[16]. Putrescine inhibited only the activity of the GluN2A receptor. Betaine and trimethylamine N-oxide inhibited only the activity of GluN2B receptor. The effect of spermine was opposite to that of isoamylamine. It was reported that the binding of spermine to a negatively charged amino acid opens the ligand binding site of the GluN2A receptor, but this is not

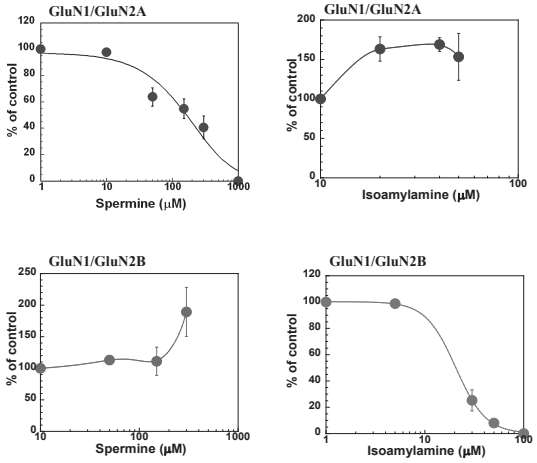

Fig. 2 Effects of spermine and isoamylamine on GluN1/GluN2A and GluN1 /GluN2B receptors

Table 3 Summary of IC_{50} values and Hill coefficients of amines on GluN1/GluN2A and GluN1 /GluN2B receptors

Components	Subtype	IC_{50} (μM)	Hill coefficient
Putrescine	GluN2A	168	1.1
	GluN2B	>1000	n.d.
Spermidine	GluN2A	1795	n.d.
	GluN2B	82.2	n.d.
Spermine	GluN2A	497	2.22
	GluN2B	activation	n.d.
2-Phenylethylamine	GluN2A	1.36	0.46
	GluN2B	13.3	1.65
Ornithine	GluN2A	518	0.31
	GluN2B	379	0.78
Isoamylamine	GluN2A	activation	n.d.
	GluN2B	8.9	2.85
Agmatine	GluN2A	82	1.05
	GluN2B	185	1.76
Tyramine	GluN2A	92.7	0.75
	GluN2B	31.7	0.58
5-Oxoproline	GluN2A	539	0.86
	GluN2B	319	1.2
Choline	GluN2A	857	0.84
	GluN2B	337	0.87
Betaine	GluN2A	No inhibition	n.d.
	GluN2B	346	0.76
Trimethylamine *N*-oxide	GluN2A	No inhibition	n.d.
	GluN2B	7.21	n.d.

applicable to isoamylamine, and further research is therefore needed. By observing differences between the activity of the receptor subtypes, it may be possible to elucidate the mechanism of the action of amines on NMDA receptor channel opening.

3.5. Measurement of anxiolytic effects by an elevated plus-maze test.

Isoamylamine, 2-phenylethylamine and ornithine indicated significant anxiolytic effects by staying longer in open arms at an elevated plus-maze test as shown in Table 4. Choline and betaine showed a trend for anxiolytic effects, while 2-phenylethylamine, isoamylamine and ornithine showed significant anxiolytic effects at concentration of 40 mg/kg. The three components (2-phenylethylamine, isoamylamine, and ornithine) that exhibited significant anxiolytic effects crossed the blood-brain barrier and actually inhibited the activity of NMDA receptors, suggesting that they exerted anxiolytic effects. Memantine, a non-competitive inhibitor of the activity of NMDA receptors, has been reported to show an anxiolytic effect at 100 mg/kg[19] and to improve cognitive impairment at 10 mg/kg[20]. The three components that showed significant anxiolytic effects at lower concentrations than that of memantine, suggesting that they could be applied to anxiolytic drugs.

Amines that act as NMDA receptor agonists, antagonists and modulators could be useful for the

Table 4 Anxiolytic effects indicated by an elevated plus-maze test

i.p. (mg/kg BW)	n	total entry (counts)	% of entry in open arms	% of time in open arms
Ringer	8	28.5 ± 3.8	31.6 ± 3.9	23.0 ± 3.3
2-PEA 40	8	21.1 ± 5.6	62.9 ± 6.6 **	40.2 ± 11

i.p. (mg/kg BW)	n	total entry (counts)	% of entry in open arms	% of time in open arms
Ringer	8	27.1 ± 11	49.2 ± 8.4	54.5 ± 21
IAA 40	8	12.5 ± 8.9 *	69.4 ± 23 *	60.8 ± 40
Choline 40	8	16.0 ± 11	60.7 ± 21	51.8 ± 30

i.p. (mg/kg BW)	n	total entry (counts)	% of entry in open arms	% of time in open arms
Ringer	8	21.9 ± 4.5	40.8 ± 5.8	46.4 ± 7.8
Betaine 40	8	19.3 ± 4.6	44.0 ± 8.3	54.2 ± 10
Ornithine 40	7	20.1 ± 3.6	58.9 ± 8.0	70.7 ± 6.7 *

IAA: Isoamylamine　2-PEA: 2-Phenylethylamine

elucidation of the mechanism and for the development of new therapeutic agents for neural diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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