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No Association Between the Protein Tyrosine Phosphatase, Receptor-Type, Z Polypeptide 1 (PTPRZ1) Gene and Schizophrenia in the Japanese Population

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NRG1-ERBB signaling influences the risk for schizophrenia pathology. A recent study has reported that MAGI1, MAGI2, and protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1; located on 7q31.3) gene products regulate the NRG1-ERBB4 signaling pathway, and PTPRZ1 is associated with schizophrenia in a Caucasian population. By applying a gene-based association concept, we analyzed any association between PTPRZ1 tagging SNPs and schizophrenia in the Japanese population (576 schizophrenics and 768 controls). After linkage disequilibrium analysis, 29 single nucleotide polymorphisms (SNPs) were genotyped using a 5′-exonuclease allelic discrimination assay. **We found a significant association** of one tagging SNP in a genotype-wise analysis ($P = 0.007$); however, this might be resulted from type I error due to multiple testing ($P = 0.17$ after SNPSpD correction). No association was observed between schizophrenic patients and controls in either allelic, genotypic, or haplotypic analyses. Our results therefore suggest that PTPRZ1 is unlikely to be related to the development of schizophrenia in the Japanese population.

KEY WORDS: association study; NRG1; ERBB4; linkage disequilibrium; HapMap

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INTRODUCTION

Schizophrenia is a chronic and devastating psychiatric disorder with a global morbidity risk of approximately 1%. While schizophrenia is highly heritable (heritability score of approximately 0.8), the underlying genetics are complex, and the interpretation of genetics data has proven difficult [Freedman 2003; Ross et al. 2006]. The hypothesis, which this disease is a developmental disorder of the nervous system with a late onset of characteristic symptoms, has been gaining acceptance over the past years, and several candidate predisposition genes such as neuregulin-1 (NRG1), disrupted-in-schizophrenia 1 (DISC1), dysbindin (DTNBP1), and glutamate decarboxylase 67 (GAD1) have been reported [Lewis and Levitt, 2002; Rapoport et al., 2005; Harrison, 2007].

Among these genes, NRG1 is regarded as one of the most promising susceptibility genes for schizophrenia [Stefansson et al., 2002; Li et al., 2006; Munafo et al., 2006]. NRG1-ERBB signaling may contribute to the pathogenesis of schizophrenia by affecting neuronal migration, cortical connectivity, neurotransmitter receptor expression (NMDA and $GABA_A$), oligodendrocyte development, and myelination [Norton et al., 2006; Li et al., 2007; Woo et al., 2007]. Any associated alterations of NRG1-ERBB signaling would thus support a neurodevelopmental and a glutamate/GABA hypothesis of schizophrenia causation [Corfas et al., 2004].

Recently, MAGI proteins were identified as substrates for the ERBB4 gene product by both a yeast two-hybrid analysis and a kinase assay [Montgomery et al., 2004]. Likewise, MAGI proteins are dephosphorylated by receptor protein tyrosine phosphatase beta (RPTPb) [Fukada et al., 2005], and thus notably, RPTPb may also regulate the NRG1-ERBB4 signaling [Buxbaum et al., 2007].

RPTPb is the PTPRZ1 (protein tyrosine phosphatase, receptor type, Z polypeptide 1) gene product, and RPTPb protein is highly expressed during embryogenesis as a transmembrane protein, primarily in the central nervous system [Levy et al., 1993]. Tyrosine phosphorylation and dephosphorylation play a key role in the signaling of cell growth and differentiation, and the PTPRZ1 gene product is believed to play a role in the recovery and survival of oligodendrocytes in demyelinating disease [Harroch et al., 2002].

In the aforementioned NRG1-ERBB4 study, a case-control association study between PTPRZ1 and schizophrenia has been carried out, and PTPRZ1 has been demonstrated to be associated with schizophrenia in a United Kingdom case-control cohort [Buxbaum et al., 2007]. However, while PTPRZ1 is considered to be one of the plausible candidate genes for schizophrenia, replication of this positive association is required in order to demonstrate that PTPRZ1 is a true susceptibility gene for this disease.

It is widely accepted that there are certain limitations in replicating a genetic association study using the same or a smaller number of single nucleotide polymorphisms (SNPs) as the original investigation. One major limitation is due to differences in allele frequency or variations of the linkage disequilibrium (LD) structure (population dependence) among each ethnicity. To overcome this limitation, a gene-based approach, rather than a SNP-based or haplotype-based approach, is currently recommended [Neale and Sham, 2004]. In such studies, it is important to include both the gene as well as the gene-flanking regions when testing for any associations, and it is also important to select genetic variants which adequately reflect the LD background by the standardized disequilibrium coefficient (D′) and squared correlation coefficient (r^2) in the targeted population (e.g., tagging SNPs). By applying this gene-based association approach, we sought to determine the association, if any, between PTPRZ1 tagging SNPs and schizophrenia in the Japanese population.

MATERIALS AND METHODS

Subjects

The cohorts used in this study consisted of 576 patients with schizophrenia (341 males and 235 females, mean age ± SD = 50.1 ± 15.1 years) and 768 healthy control subjects (365 males and 403 females, mean age ± SD = 40.1 ± 15.6 years). All subjects were unrelated to each other and were of Japanese ethnicity. The patients were all diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria for schizophrenia, with consensus reached among at least two experienced psychiatrists on the basis of unstructured interviews as well as a review of the subjects' medical records. All healthy control subjects were also psychiatrically screened with brief diagnostic unstructured interviews. Subjects who had current or past contact with psychiatric services were excluded. After describing the study to each subject, written informed consent was obtained. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine.

Tagging SNP Selection

To analyze genetic association, we implemented a gene-based approach. This method implies the inclusion of both the gene region and the gene-flanking regions in the association study [Neale and Sham, 2004]. The PTPRZ1 gene contains 30 exons spanning approximately 188900 base pairs (bp), and three other splicing isoforms have been reported thus far [Garwood et al., 2003; Paul and Lombroso, 2003].

We first consulted the HapMap database (release#22; phase2, April 2007, population: Japanese in Tokyo, minor allele frequency (MAF): more than 0.05). All SNPs in the entire gene region covering all isoforms, as well as the 5,000 bp upstream 5′ flanking region and the 5,000 bp downstream 3′ flanking region, were listed.

Then we defined 28 tagging SNPs (Table I) with the criterion of an $r^2 > 0.8$ in 'pair-wise tagging only' mode using the 'Tagger' program, implemented by Haploview software version 4.0 (http://www.broad.mit.edu/mpg/haploview/index.php) [Barrett et al., 2005; de Bakker et al., 2005], considering two points in particular. First, we mandatorily included marginal and significant SNPs reported in previous study (Table I: rs6466808, rs10278079, rs1196471, rs2693657, rs1147502, rs1147497, rs1147489, and rs1206381; indicated by asterisk) as tagging SNPs, except for rs1196513 and rs13241278 [Buxbaum et al., 2007]. Because rs1196513 and rs13241278 were not listed in the HapMap database, we could not analyze the LD patterns of these SNPs. Second, due to unavailability of the reliable genotyping method for rs1206384, we genotyped rs1860721 (No.8) instead, whose r^2 value with rs1206384 was 1 (HapMap data). There were three validated exonic SNPs (MAF > 0.05) and some SNPs in the 3′ or 5′ flanking of the gene, and all these SNPs were covered by the selected tagging SNPs.

Of two other significant SNPs reported by Buxbaum et al. (rs1196513 and rs13241278), rs13241278 was included, but rs1196513 was not included in our analysis because this SNP was not validated and its frequency was not reported in the dbSNP database (NCBI). Overall, 29 SNPs were examined.

SNP Genotyping

Venous blood was drawn from each subject and genomic DNA was extracted from whole blood according to standard procedures. Genotyping of all tagging SNPs was carried out using a TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems Japan Ltd., Tokyo, Japan). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Details regarding reagents or reaction conditions are available upon request.

Statistical Analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) and single-marker allelic association were evaluated using Haploview. Analysis of linkage disequilibrium between markers (r^2 and D′) was also performed using Haploview. Genotypic association was tested by the chi-squared test or by Fisher's exact test. Genotypic association of SNPs that deviated from HWE was analyzed using the Cochran-Armitage trend test for a multiplicative model of inheritance [Balding, 2006]. For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria using Haploview software. Haplotypic analyses were performed with Unphased software version 2.403 [Dudbridge, 2003], which performs log-likelihood ratio tests under a log-linear model for global P values. Rare haplotypes found in less than 3% of both case and control subjects were excluded from the association analysis to provide greater sensitivity and accuracy when the effect was seen in common haplotypes, and the expectation-maximization algorithm was then employed. rs13241278 was excluded from haplotypic analysis because this SNP was not selected by Tagger. The significance level for all statistical tests was $P < 0.05$. Power calculations were performed using the genetic statistical package Genetic Power Calculator [Purcell et al., 2003] (http://pngu.mgh.harvard.edu/~purcell/gpc/). The number of effective independent SNPs assayed was estimated by the spectral decomposition method of Nyholt using SNPSpD software. This software is able to reflect the correlation of markers (LD) on corrected P values to control for inflation of the type I error rate [Nyholt, 2004].

RESULTS

The genotype and allele frequencies of each SNP from schizophrenic patients and healthy control subjects are summarized in Table I. The observed genotype frequencies of two tagging SNPs deviated from HWE (rs1206477 and rs13241278). The LD relationships between markers are provided in Table II. The LD patterns observed in our controls were nearly identical to those of the JPT HapMap samples, but obviously different from those of the CEU HapMap samples. Only rs1196511 showed a significant association with schizophrenics in a genotype-wise analysis ($P = 0.007$). However, this

TABLE I. Association Analysis of Twenty-Nine SNPs of *PTPRZ1*

No.	SNP ID		M/m	N		CON			SCZ			MAF		Genotype *P*-value	Corrected *P*-value	Allele *P*-value[d]	LD block[d]	GRR[e]
				CON	SCZ	M/M	M/m	m/m	M/M	M/m	m/m	CON	SCZ					
1	rs13437930	Intron1	G/C	762	566	559	179	24	399	150	17	0.15	0.16	0.45[a]		0.34	/	1.34
2	rs2402593	Intron1	A/G	762	564	400	303	59	272	239	53	0.28	0.31	0.25[a]		0.10	I	1.27
3	rs1916885	Intron1	T/C	762	565	326	337	99	255	240	70	0.35	0.34	0.69[a]		0.43	I	1.26
4	rs3757548	Intron1	T/C	758	566	664	91	3	499	65	2	0.06	0.06	0.93[b]		0.75	I	1.50
5	rs1206477	Intron1	C/G	761	566	361	307	93	266	242	58	0.32	0.32	0.68[c]		0.68	I	1.26
6	rs6974265	Intron1	A/G	761	566	578	171	12	410	150	6	0.13	0.14	0.19[b]		0.26	I	1.36
7	rs1011692	Intron1	T/C	761	564	443	272	46	318	220	26	0.24	0.24	0.31[a]		0.91	I	1.29
8	rs1860721	Intron2	C/T	761	566	582	164	15	445	118	3	0.13	0.11	0.07[b]		0.16	II	1.36
9	rs4731044	Intron2	C/G	762	563	323	327	112	238	256	69	0.36	0.35	0.39[a]		0.54	II	1.26
10	rs6466808*	Intron2	G/A	759	565	356	323	80	255	238	72	0.32	0.34	0.45[a]		0.28	II	1.26
11	rs1196482	Intron2	C/T	761	566	495	237	29	356	193	17	0.19	0.20	0.42[a]		0.67	II	1.31
12	rs10278079*	Intron2	A/G	762	566	392	295	75	268	235	63	0.29	0.32	0.32[a]		0.14	II	1.27
13	rs1196511	Intron2	G/A	761	565	191	358	212	142	306	117	0.51	0.48	0.01[a]	0.17[f]	0.07	II	1.25
14	rs3817483	Intron6	T/C	762	566	592	158	12	443	111	12	0.12	0.12	0.69[a]		0.99	II	1.37
15	rs10225212	Intron8	G/T	761	566	521	213	27	363	175	28	0.18	0.20	0.18[a]		0.06	/	1.32
16	rs1196471*	Intron8	T/C	762	564	316	349	97	230	261	73	0.36	0.36	0.97[a]		0.81	III	1.26
17	rs2693657*	Intron9	G/A	762	566	316	350	96	230	265	71	0.36	0.36	0.95[a]		0.84	III	1.26
18	rs1196474	Intron10	G/A	761	566	379	304	78	273	236	57	0.30	0.31	0.81[a]		0.70	III	1.37
19	rs17144005	Intron11	A/G	762	565	667	91	4	508	55	2	0.07	0.05	0.38[b]		0.17	III	1.50
20	rs1147502*	Intron13	T/G	762	565	330	337	95	249	254	62	0.35	0.34	0.71[a]		0.54	III	1.26
21	rs12670616	Intron13	T/G	762	565	697	63	2	524	42	0	0.04	0.04	0.53[b]		0.32	III	1.61
22	rs1147497*	Intron14	T/G	761	565	313	357	91	232	265	68	0.35	0.36	1.00[a]		0.97	III	1.26
23	rs1147492	Intron18	C/T	762	563	263	364	135	183	272	108	0.42	0.43	0.67[a]		0.37	III	1.25
24	rs1147491	Intron18	A/G	762	565	457	257	48	341	203	21	0.23	0.22	0.10[a]		0.37	III	1.29
25	rs1918031	Intron18	G/A	761	566	532	203	26	408	146	12	0.17	0.15	0.33[a]		0.23	III	1.32
26	rs1147489*	Intron19	A/G	761	566	506	236	19	372	174	20	0.18	0.19	0.54[a]		0.55	III	1.32
27	rs1147488	Intron22	A/G	762	566	571	174	17	436	122	8	0.14	0.12	0.46[b]		0.27	III	1.35
28	rs1206381*	Intron27	A/C	761	566	322	348	91	249	253	64	0.35	0.34	0.82[a]		0.53	III	1.26
	rs13241278*	Intron8	C/T	759	565	385	275	99	280	223	62	0.31	0.31	0.81[c]		0.80		1.26

N, number; M, major allele; m, minor allele; CON, control; SCZ, schizophrenia; MAF, minor allele frequency; GRR, genotype relative risk ($\alpha = 0.05$, $1 - \beta = 0.8$).
IDs with asterisk (*) represent significant or marginally significant SNPs in Buxbaum's report.
Genotypic *P*-value was calculated by the chi-squared test (a), Fisher's exact test (b), and the Cochran–Armitage trend test(c).
Allelic *P*-value was calculated and LD block (Gabriel's criteria) was defined using Haploview software (d).
GRR was calculated using Genetic power calculator (e).
Corrected *P*-value was calculated by Nyholt's method (f).
Effective number of independent marker loci [Meff]: 24.8646.
Experiment-wide significance threshold required to keep Type I error rate at 5%: 0.002.

significance may be the result of a type I error due to multiple testing. We performed P-value correction by using the SNPSpD program (the effective number of independent marker loci: 24.8646; the experiment-wide significance threshold required to keep type I error rate at 0.05: 0.002: P = 0.17 after SNPSpD correction) [Nyholt, 2004]. No association was observed between the schizophrenic patients and their controls in allelic, genotypic, or haplotypic analyses (Tables I and III). Greater than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk (GRR) was set between 1.25 and 1.61 under a multiplicative model of inheritance assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

DISCUSSION

The 'common disease-common variant' hypothesis postulates that linkage disequilibrium should be detected by the haplotype association test if the risk haplotype is linked to causal variants for disease [Chakravarti, 1999]. Regarding the Japanese population, therefore, the data presented in this article do not provide sufficient evidence for the involvement of the PTPRZ1 gene in conferring susceptibility for schizophrenia.

In this study, we could not replicate a previous report [Buxbaum et al., 2007], which revealed a significant association between PTPRZ1 and schizophrenia in a Caucasian population. The discrepancy between Japanese and Caucasian populations may derive from ethnic differences in the etiology of schizophrenia. Although the sample size used here is smaller than the sample size of the original study, we obtained a statistical power expected to detect any possible association, and so the possibility of a type II error is unlikely. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia [Shifman et al., 2002; Schwab et al., 2003]. In this regard, a prompt gene-based replication study has become feasible as the International HapMap project progresses. In this case, however, PTPRZ1 is a complicated gene with a transcript that is spliced into four distinct isoforms, and the HapMap database focused only one isoform (NM_002851). With such a limitation, other SNPs involved in mRNA splicing, while having attracted great attention in the pathology of schizophrenia [Law et al., 2007], might nonetheless be overlooked. Thus, it might be useful to investigate not only exons but also splice junctions of this gene.

Two additional limitations need to be addressed in order to discuss the present results. First, age-unmatched and male-to-female ratio unmatched cohorts were examined in the present study. The mean age of the controls is younger than that of the patients. This means that a number of these younger controls, though likely not more than eight subjects given a lifetime morbidity risk of 1%, may later develop schizophrenia. This confounding factor might weaken the power of the present study. We also performed exploratory analysis to investigate the effect of age and gender on the disease status. Based on the results of the analysis using logistic regression model, these variables did not seem to be involved in the results of present association study (data not shown). Secondly, another limitation, which must be addressed, is that other candidates related to the NRG1-ERBB signaling would also be in the locus heterogeneity. In this case, causal variants with extremely rare MAFs and allelic heterogeneity should be also considered. Likewise, the combined effect between SNPs on PTPRZ1 and SNPs on the other genes (ERBB4, MAGIs, etc) might actually prove to be a stronger predisposition factor.

Moreover, the definition of phenotypes is vital for a genetic association study. Therefore, endophenotypes (being more

TABLE II. Linkage Disequilibrium Analysis of *PTPRZ1*

No.	SNP ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	rs13437930		0.99	0.97	1.00	0.98	1.00	0.89	1.00	0.65	0.55	0.07	0.57	0.72	0.86	0.58	0.61	0.43	0.43	0.44	0.63	0.49	0.73	0.41	0.17	0.23	0.83	0.02	0.90	0.34
2	rs2402593	0.45		1.00	1.00	0.99	1.00	0.98	0.64	0.80	0.70	0.48	0.58	0.62	0.25	0.63	0.70	0.10	0.10	0.28	0.62	0.52	0.76	0.08	0.11	0.10	0.11	0.41	0.69	0.04
3	rs1916885	0.09	0.21		1.00	1.00	1.00	0.93	0.76	0.89	0.38	0.73	0.32	0.33	0.26	0.90	0.83	0.41	0.41	0.09	0.93	0.90	0.81	0.40	0.89	0.26	0.21	0.57	0.72	0.38
4	rs3757548	0.01	0.03	0.04		1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	0.93	1.00	0.96	1.00	0.89	0.89	1.00	1.00	0.96	1.00	0.88	0.95	1.00	1.00	1.00	0.97	0.88
5	rs1206477	0.08	0.18	0.26	0.14		1.00	0.94	0.77	0.92	0.95	0.94	0.95	0.93	0.90	0.77	0.85	0.64	0.65	0.73	1.00	0.70	0.89	0.65	0.70	0.75	0.73	0.61	0.72	0.65
6	rs6974265	0.03	0.39	0.08	0.01	0.07		1.00	0.18	0.97	0.85	1.00	0.55	0.42	0.35	0.65	0.10	0.17	0.10	0.58	0.51	0.73	0.17	0.03	0.49	0.30	1.00	0.34	0.18
7	rs1011692	0.04	0.12	0.50	0.02	0.13	0.05		1.00	0.89	0.19	0.85	0.12	0.92	0.33	0.90	0.78	0.91	0.91	0.85	0.78	0.89	0.83	0.88	0.90	0.75	0.33	0.65	0.71	0.84
8	rs1860721	0.03	0.02	0.16	0.01	0.04	0.00	0.05		1.00	1.00	1.00	0.95	0.98	1.00	1.00	0.88	0.94	0.94	0.96	1.00	1.00	0.90	0.94	1.00	0.95	0.92	1.00	0.95	0.94
9	rs4731044	0.04	0.14	0.24	0.11	0.71	0.08	0.14	0.08		1.00	1.00	1.00	1.00	0.97	0.94	0.89	0.68	0.68	0.84	1.00	0.83	0.91	0.68	0.75	0.87	0.72	0.67	0.92	0.69
10	rs6466808	0.11	0.40	0.04	0.03	0.20	0.23	0.01	0.07	0.27		1.00	1.00	0.86	0.99	0.87	0.94	0.08	0.08	0.32	0.96	0.73	0.94	0.07	0.34	0.17	0.54	0.90	0.78	0.10
11	rs1196482	0.00	0.02	0.24	0.02	0.10	0.04	0.55	0.04	0.14	0.11		1.00	1.00	1.00	0.98	0.94	0.99	0.99	0.96	0.77	0.98	1.00	0.98	0.96	0.90	0.02	0.80	0.94	0.95
12	rs10278079	0.14	0.31	0.02	0.03	0.18	0.11	0.00	0.06	0.23	0.88	0.10		1.00	0.98	0.97	0.95	0.10	0.11	0.37	0.97	0.88	0.88	0.10	0.44	0.20	0.53	0.89	0.75	0.13
13	rs1196511	0.10	0.16	0.06	0.06	0.39	0.03	0.28	0.13	0.53	0.37	0.25	0.43		0.98	0.98	0.94	0.75	0.75	0.14	1.00	0.90	0.80	0.74	0.60	0.05	0.76	0.73	0.84	0.75
14	rs3817483	0.02	0.02	0.02	0.01	0.05	0.12	0.05	0.02	0.07	0.29	0.03	0.32	0.14		0.97	0.66	0.89	0.89	0.66	0.40	0.94	0.91	0.83	0.96	0.49	0.82	1.00	0.48	0.83
	rs13241278	0.03	0.07	0.20	0.14	0.56	0.03	0.11	0.07	0.71	0.16	0.11	0.18	0.42	0.06		1.00	0.95	0.96	0.89	1.00	0.92	1.00	0.94	0.89	0.86	0.86	0.95	0.93	0.88
15	rs10225212	0.31	0.27	0.08	0.01	0.07	0.01	0.04	0.02	0.09	0.40	0.05	0.47	0.20	0.01	0.10		1.00	1.00	0.91	0.99	0.87	1.00	1.00	0.05	0.41	0.93	1.00	0.58	0.91
16	rs1196471	0.02	0.00	0.17	0.03	0.11	0.01	0.47	0.07	0.14	0.01	0.42	0.01	0.32	0.19	0.23	0.12		1.00	0.97	0.93	0.95	0.97	0.98	0.94	0.95	0.96	0.97	0.98	0.96
17	rs2693657	0.02	0.00	0.17	0.03	0.11	0.01	0.47	0.07	0.15	0.01	0.42	0.01	0.33	0.19	0.23	0.12	1.00		0.97	0.93	0.96	0.97	0.98	0.95	0.95	0.96	0.97	0.98	0.95
18	rs1196474	0.08	0.07	0.01	0.03	0.11	0.00	0.10	0.31	0.17	0.09	0.10	0.13	0.01	0.03	0.16	0.41	0.23	0.23		1.00	1.00	1.00	1.00	0.46	0.98	0.97	1.00	0.98	0.95
19	rs17144005	0.16	0.07	0.03	0.01	0.03	0.00	0.01	0.01	0.04	0.14	0.01	0.16	0.07	0.00	0.03	0.32	0.03	0.03	0.16		1.00	1.00	1.00	0.96	0.98	0.88	1.00	1.00	0.86
20	rs1147502	0.02	0.06	0.23	0.12	0.45	0.02	0.13	0.08	0.64	0.13	0.12	0.17	0.41	0.06	0.73	0.09	0.27	0.27	0.23	0.04		1.00	1.00	0.98	0.97	0.98	1.00	0.97	0.94
21	rs12670616	0.00	0.01	0.06	0.00	0.02	0.00	0.10	0.01	0.02	0.09	0.01	0.09	0.03	0.28	0.02	0.01	0.08	0.08	0.02	0.00	0.02		1.00	1.00	1.00	0.98	0.71	1.00	0.97
22	rs1147497	0.02	0.00	0.16	0.03	0.11	0.01	0.44	0.07	0.14	0.00	0.42	0.01	0.31	0.17	0.22	0.12	0.94	0.95	0.24	0.04	0.29	0.08		0.98	0.99	0.99	1.00	0.97	0.96
23	rs1147492	0.00	0.00	0.31	0.09	0.33	0.00	0.18	0.10	0.45	0.04	0.16	0.06	0.24	0.09	0.51	0.00	0.35	0.35	0.07	0.05	0.71	0.03	0.37		1.00	1.00	1.00	1.00	0.98
24	rs1147491	0.03	0.01	0.04	0.02	0.08	0.01	0.05	0.43	0.13	0.02	0.06	0.03	0.00	0.01	0.10	0.12	0.15	0.15	0.67	0.22	0.15	0.01	0.16	0.22		1.00	1.00	1.00	0.96
25	rs1918031	0.02	0.01	0.02	0.01	0.05	0.07	0.07	0.03	0.06	0.13	0.00	0.14	0.12	0.45	0.07	0.04	0.34	0.34	0.08	0.01	0.10	0.22	0.36	0.14	0.06		0.94	1.00	0.99
26	rs1147489	0.00	0.01	0.13	0.02	0.04	0.03	0.30	0.03	0.06	0.08	0.59	0.07	0.13	0.03	0.09	0.05	0.38	0.38	0.10	0.02	0.12	0.01	0.40	0.16	0.07	0.04		1.00	0.99
27	rs1147488	0.02	0.03	0.15	0.01	0.04	0.00	0.03	0.83	0.08	0.05	0.03	0.04	0.10	0.01	0.06	0.01	0.08	0.08	0.35	0.01	0.08	0.01	0.08	0.11	0.52	0.03	0.04		1.00
28	rs1206381	0.01	0.00	0.15	0.03	0.11	0.01	0.41	0.07	0.14	0.01	0.40	0.01	0.32	0.17	0.19	0.10	0.88	0.88	0.21	0.03	0.25	0.08	0.91	0.36	0.15	0.37	0.40	0.09	

Values shown above the diagonal are D′ and values shown below are r^2.

TABLE III. Haplotypic Analysis of PTPRZ1

No.	SNP ID	Haplotypic global P-value window size 2	3	4
1	rs13437930			
		0.22		
2	rs2402593		0.38	
		0.25		0.57
3	rs1916885		0.46	
		0.64		0.60
4	rs3757548		0.66	
		0.84		0.77
5	rs1206477		0.74	
		0.54		0.90
6	rs6974265		0.77	
		0.64		0.50
7	rs1011692		0.37	
		0.15		0.45
8	rs1860721		0.31	
		0.21		0.26
9	rs4731044		0.34	
		0.58		0.40
10	rs6466808		0.44	
		0.41		0.35
11	rs1196482		0.28	
		0.21		0.24
12	rs10278079		0.19	
		0.18		0.26
13	rs1196511		0.26	
		0.16		0.17
14	rs3817483		0.21	
		0.13		0.41
15	rs10225212		0.29	
		0.21		0.34
16	rs1196471		0.23	
		0.84		0.44
17	rs2693657		0.96	
		0.92		0.44
18	rs1196474		0.43	
		0.26		0.41
19	rs17144005		0.33	
		0.27		0.26
20	rs1147502		0.27	
		0.51		0.43
21	rs12670616		0.73	
		0.58		0.07
22	rs1147497		0.62	
		0.62		0.64
23	rs1147492		0.62	
		0.57		0.39
24	rs1147491		0.32	
		0.22		0.48
25	rs1918031		0.45	
		0.47		0.54
26	rs1147489		0.39	
		0.50		0.36
27	rs1147488		0.37	
		0.34		
28	rs1206381			

Haplotypic global P-value was calculated using Unphased software.

specific than phenotypes) or symptoms are also thought to be important in this field [Gottesman and Gould, 2003; Craddock et al., 2006; Braff et al., 2007]. We did not take advantage of these analyses in order to test for a genetic association, but they may be useful in elucidating the potential role of PTPRZ1 in schizophrenia. Because several studies have demonstrated that Ptprz-deficient mice suffer hippocampal dysfunction [Niisato et al., 2005; Tamura et al., 2006], additional endophenotypic approaches such as cognitive function assessment, brain imaging and other phenotypes that reveal PTPRZ1 traits would further contribute to our understanding of schizophrenia.

In summary, the findings of the present study suggest that PTPRZ1 is unlikely to be related to the development of schizophrenia in the Japanese population. Further replication studies incorporating supplemental populations should be performed for conclusive results.

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Research report

Effects of (*R*)-(−)-1-(benzofuran-2-yl)-2-propylaminopentane hydrochloride [(−)-BPAP] in animal models of mood disorders

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Abstract

(*R*)-(−)-1-(Benzofuran-2-yl)-2-propylaminopentane hydrochloride [(−)-BPAP] is a highly potent enhancer of impulse propagation-mediated monoamine release and an inhibitor of monoamine uptake. We evaluated the efficacy of (−)-BPAP as a drug for mood disorders by using two animal models. (1) Acute, but not chronic, administration of (−)-BPAP and imipramine significantly attenuated immobility in mice induced by forced swimming. Chronic, but not acute, administration of (−)-BPAP ameliorated the impairment of social interaction (SI) behavior following forced swimming, without affecting locomotor activity. The ameliorating effect of (−)-BPAP on the impairment of SI behavior was suppressed by dopamine receptor antagonists, which suggests that the effect was mediated through the activation of the dopaminergic system. Chronic administration of imipramine tended to attenuate the impairment of SI behavior in stressed mice, but not significantly. (2) In the olfactory bulbectomized (OB) rat, chronic (−)-BPAP treatment significantly ameliorated the impairment of SI behavior, prepulse inhibition, and tone–cue fear learning, without affecting locomotor activity in an open field and circadian activity pattern. Furthermore, (−)-BPAP tended to improve sexual dysfunction in OB rats, but imipramine had no such effect. These findings suggest that (−)-BPAP may be clinically effective in treating mood disorders, including comorbid anxiety and depression that are poorly responsive to imipramine.

Keywords: (*R*)-(−)-1-(Benzofuran-2-yl)-2-propylaminopentane hydrochloride [(−)-BPAP]; Dopaminergic system; Forced swimming; Imipramine; Mood disorder; Olfactory bulbectomy

1. Introduction

Mood disorders such as depression, anxiety, obsessive–compulsive disorder and post-traumatic stress disorder are serious problems in today's society. It is difficult to mimic these disorders in the laboratory because of the wide spectrum of disruption that characterizes mood disorders. However, various animal models have been developed based on the hypothesis that mood disorders are caused by stressors (e.g. the chronic variable stress model) or neuronal deficits (e.g. the olfactory bulbectomy model), and utilized widely for evaluating novel compounds for mood disorders in preclinical settings.

Stress has been implicated in both the development and manifestation of mood disorders such as anxiety and depression [17], highly comorbid disorders [24] that may share common elements of an underlying pathophysiology [5]. Chronic stress can accelerate disease processes, cause neural degeneration, and lead to depression or other mood disorders [34]. It has

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been reported that the chronic variable stressors such as mild uncontrollable foot-shock, cold swim, and changes in housing conditions induce behavioral abnormalities, such as decreases in open field activity and social interaction (SI) time, as a sign of increased anxiety [21,22], which are models of human mood disorders. Many antidepressants prevent stress-induced decreases in open field activity, and increase SI time, which indicate their anxiolytic effects [21,22,30].

On the other hand, bilateral removal of the olfactory bulbs in rats produces a complex constellation of behavioral, neurochemical, neuroendocrinic and neuroimmune alterations. These changes are correlated with those observed in major depression and anxiety [23,41,46]. It has been demonstrated that many of these behavioral changes induced by olfactory bulbectomy are reversed by chronic, but not acute antidepressant treatment, which is similar to that observed in patients with depression. Thus, the olfactory bulbectomized (OB) rat is a useful model for evaluating the effects of agents against mood disorders [23].

The monoamine hypothesis based on the deficiency of monoamines is commonly evoked to explain the pathophysiology of depression. This hypothesis, initially based on noradrenaline (NE) and serotonin (5-hydroxytryptamine, 5-HT) deficiency, is extended to dopamine (DA). Brain DA plays an important role in depression, the action of antidepressants, and anxiety [3,19,20]. Changes in DA metabolism have been demonstrated in the frontal cortex of patients with unipolar depression, by positron emission tomography (PET) [3]. Gendreau et al. [14] have suggested an important role for DA in the mediation of social–emotional reactivity in animal models of anxiety. Furthermore, stressful experiences affect emotional behavior and mesolimbic DA functioning in animals [9]. Levels of trace amines such as tryptamine are altered in patients with depression [6]. Trace amines such as β-phenylethylamine and tryptamine enhance the electrically stimulated catecholamines and 5-HT release from isolated rat brainstem [28]. (*R*)-(−)-1-(Benzofuran-2-yl)-2-propylaminopentane hydrochloride [(−)-BPAP] is a tryptamine-derived synthetic substance and enhances impulse-evoked monoamine release [28,49]. In addition, (−)-BPAP is a reuptake inhibitor for DA, NE and 5-HT [43]. Enhancing release of monoamines may serve therapeutic agents for neurologic diseases (e.g. depression, Alzheimer's disease and Parkinson's disease) in which the reduction of catecholamines and 5-HT is observed. (−)-BPAP is expected to improve the non-motor functional deficits such as depression in patients of Parkinson's disease, that are unresponsive to antiparkinsonian drugs [32]. In fact, (−)-BPAP reverses the hypolocomotion in reserpine-pretreated rats [42,43], and impairment of active avoidance performance in the tetrabenazine-induced depression model in rats. These effects may be due to its antidepressive action [27,28], and may be related to its effects on noradrenergic and serotonergic deficits, in addition to a dopaminergic deficit [1,15].

Since (−)-BPAP has more potent dopaminergic action than typical antidepressants, (−)-BPAP is expected to be a novel agent for the treatment of depression and/or other mood disorders. In the present study, we investigated whether (−)-BPAP improved emotional deficits in mice and OB rats and improving effects of (−)-BPAP were mediated via activation of dopaminergic systems in mice subjected to forced swimming.

2. Materials and methods

2.1. Animals

Male ddY mice (7 weeks old; Nihon SLC, Shizuoka, Japan) and male Sprague–Dawley rats (8 weeks old; Nihon SLC) were housed in wire netting and plastic cages, respectively. They received food (CE2; Clea Japan, Tokyo, Japan) and water *ad libitum*, and were maintained on a 12/12 h light/dark cycle (lights on at 8.00 a.m. for mice, and 9.00 a.m. for rats). All experiments were performed in a blind manner in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the institutional guidelines set out in "Principles of Laboratory Animal Care" (NIH publication No. 85–23, revised 1985).

2.2. Drugs

(*R*)-(−)-1-(Benzofuran-2-yl)-2-propyl-aminopentane hydrochloride [(−)-BPAP] ($C_{16}H_{23}NO$ HCl, molecular mass, 281.827) was synthesized in the Research Institute of FP Pharmaceutical Corporation (Osaka, Japan). (−)-BPAP and imipramine hydrochloride (Sigma, St. Louis, MO, USA) were dissolved in saline. (*R*)-(+)-SCH-23390 hydrochloride (Sigma) and (*S*)-(−)-sulpiride (Sigma) were dissolved in distilled water. The doses of (−)-BPAP, imipramine and SCH-23390 were expressed as those of the salt.

2.3. Experiments on forced-swimming-stressed mice

The experimental protocol is shown in Fig. 1A.

2.3.1. Forced swimming and SI test

The mice were given forced swimming twice, and then were examined SI behavior. The forced swimming and SI tests were performed according to many previous reports with minor modification [16,35,45]. Firstly, a mouse was put into the glass cylinder (15 cm diameter) filled to a depth of 16 cm with water at about 20 °C, and forced to swim for 15 min (first swimming) to match the immobility time in each group (grouping) and get the immobility time stable at the second swimming. Seven days later, each mouse was forced to swim again in the same environment for 6 min (second swimming), and immobility time was counted during the last 5 min of the 6 min period. Ten minutes after the second swimming, a naive mouse, which had no exposure to swimming and a stressed mouse, which had been exposed, were simultaneously placed into the SI test box, a plastic box (47.5 cm × 30 cm × 35 cm) with a gray plastic base illuminated indirectly. SI behavior, including running toward, sniffing, grooming, mounting and crawling under the other mouse, was recorded for 10 min after placement of the animals in the apparatus. Each mouse was individually habituated to the SI test box, with 10 min of exploration in the absence of objects on 2 consecutive days before the SI test.

2.3.2. Drug administration

Mice were subcutaneously injected with saline, imipramine (10 mg/kg) and (−)-BPAP (0.3, 1 and 3 mg/kg) once daily, from the day following the first swimming to the day of the second swimming (7 days). At the second swimming period, mice were injected with saline, imipramine (10 mg/kg) and (−)-BPAP (0.3, 1 and 3 mg/kg) 30 min before the swimming. In the experiments with DA receptor antagonists, mice were injected with D_1 receptor antagonist SCH-23390 and D_2 receptor antagonist sulpiride 30 and 60 min before injection of (−)-BPAP, respectively. Mice were divided into three groups according to administration schedule: (1) in the control group, mice pretreated with saline for 6 days were injected with saline at the second swimming; (2) in the acute group, mice pretreated with saline for 6 days were acutely injected with (−)-BPAP or imipramine at the second swimming; and (3) in the chronic group, mice pretreated with (−)-BPAP or imipramine for 6 days were injected with saline at the second swimming.

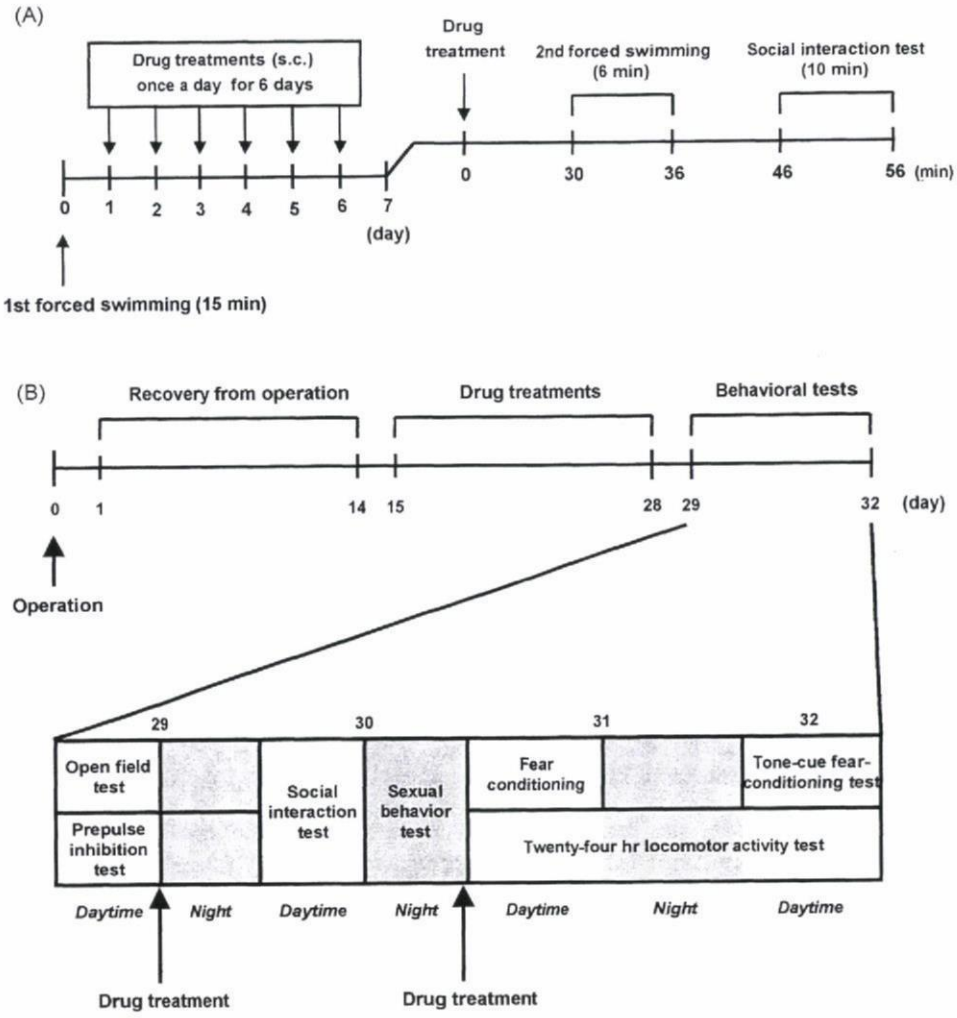

Fig. 1. Experimental protocol in mice (A) and rats (B).

2.3.3. Locomotor activity

The apparatus for locomotor activity consisted of a transparent Plexiglas box (45 cm × 35 cm × 40 cm) with a black plastic base, and were illuminated with 60 W light bulb. Mice were divided into two groups according to experimental schedule. In group A, 7 days after the first swimming, each mouse was individually placed into the test box, and then, locomotor activity was measured for 6 min, the same duration as the second swimming. Mice were injected with drugs 30 min before measurement of locomotor activity. In group B, 7 days after the first swimming, each mouse was forced to the second swimming. Ten minutes after the second swimming, each mouse was individually put into the test box, and then, locomotor activity was measured for 10 min, the same duration as the SI test. Mice were injected with drugs 30 min before the second swimming. Each mouse was individually habituated to the apparatus on 2 consecutive days before the measurement of locomotor activity.

2.4. Experiments on OB rats

The experimental protocol is shown in Fig. 1B.

2.4.1. Surgical procedure of olfactory bulbectomy

Rats were divided into five groups, which were sham-operated (sham); olfactory bulbectomized; imipramine (20 mg/kg)-treated-OB (IMI); (−)-BPAP (0.3 mg/kg)-treated-OB (BP-L); and (−)-BPAP (1 mg/kg)-treated-OB (BP-H). The rats were anesthetized with pentobarbital sodium (50 mg/kg), and fixed on stereotactic instruments (Narishige, Tokyo, Japan). A midline sagittal incision was made to expose the skull overlying the olfactory bulbs. A 4-mm diameter hole was drilled through the skull 6 mm anterior to the bregma. The olfactory bulbs were cut down with a micro-knife, and aspirated by using a pipette tip connected to a water suction pump, with care being taken not to damage the frontal cortex. The cavity of the olfactory bulbs was filled with a hemostatic sponge, the hole in the skull was covered with a piece of gelatin gauze, and the skin was sutured. Sham rats were treated in a similar way, except that the olfactory bulbs were not removed. The animals were allowed to recover for 14 days following the surgery.

2.4.2. Drug administration

The administration of (−)-BPAP (0.3 and 1 mg/kg) and imipramine (20 mg/kg) was started 2 weeks after OB. Rats were subcutaneously injected with drugs daily until 1 day before the 24-h locomotor activity test or fear-conditioning test. After the rats were treated with drugs for 14 days, open field and prepulse inhibition tests were started. The sham and OB rats were subcutaneously injected with saline.

2.4.3. Open field test in OB rats

On the 29th day after the operation, the open field test was performed before drug administration. The open field apparatus, painted gray, consisted of a square arena (60 cm × 60 cm) divided into 15-cm squares by black lines. The wall of the arena was 30 cm high. A 60 W light bulb was positioned at the center, 90 cm above the base of the arena. Each rat was separately put into the center of the open field arena. Starting latency for exploration, number of ambulations, rearing, urination and defecation of each rat were recorded for 3 min. After the test, the rats were allowed to stay in the arena for another 7 min (the rats were

kept in the arena for 10 min in total) as the habituation session for the SI test. After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate the residual odor.

2.4.4. SI test in OB rats

On the 30th day after the operation, SI behavior was observed before drug administration. The same apparatus and testing environment as those of the open field test were used for the SI test, except that the illumination was milder (8 W) than that in the open field test. Rat pairs from the same treatment group housed in different cages were put into two different corners of the open field arena. SI behavior, including running toward, sniffing, grooming, mounting and crawling under the other rat, was recorded for 10 min after placement of the animals into the apparatus. After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate the residual odor.

2.4.5. Sexual behavior test in OB rats

On the night of the 30th day after the operation, sexual behavior was observed before drug administration. The apparatus for the sexual behavior test consisted of a transparent Plexiglas box (45 cm × 27 cm × 39.5 cm) with a black plastic base, illuminated with a red lamp (60 W). Sexual behavior was observed from 10.00 p.m. to 3.00 a.m. in the dark phase of the illumination cycle. A male rat was first placed into a Plexiglas box to be habituated to the environment for 3 min. Then, a sexually receptive female rat which had received subcutaneous injections of 0.14 mg estradiol at 72 and 48 h before the test and 0.7 mg progesterone at 4 h before the test for estrus was introduced. The sexual behavior of the male rats was observed for 30 min. The following parameters of sexual behavior were recorded: starting latency and number of genital sniffing, the starting latency and frequency of grooming female rats, thrusting, and ejaculation. After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate the residual odor.

2.4.6. Tone–cue fear-conditioning test in OB rats

On the next 2 days of the SI test, the tone–cue fear-conditioning test was performed. The apparatus for the test consisted of a transparent Plexiglas box (45 cm × 27 cm × 39.5 cm, the neutral cage) with a black plastic base, and a Perspex box (32 cm × 26 cm × 48 cm, the conditioning cage) with a steel grid floor, which was connected to an electric shock generator (Neuroscience Idea, Osaka, Japan) and was enclosed in an opaque compartment. The neutral cage was illuminated with a red lamp (60 W) and the conditioning box was illuminated with a fluorescent lamp (6 W).

For measuring basal levels of the freezing response (preconditioning phase), rats were placed individually in the neutral cage and the total immobility time for 1 min was recorded, and then placed in the conditioning cage for 2 min. For conditioning (conditioning phase), each rat was placed in the conditioning cage, and then a 60-s tone (75 dB) was presented as a conditioned stimulus. Just before the end of the tone, a 0.5-mA electric foot-shock lasting for 0.5 s was delivered as an unconditioned stimulus through a shock generator. It should be noted that the unconditioned stimulus was not strong enough to produce a stable conditioned response in all of the rats, hence a difference in learning and memory ability among the rats could be observed.

On the following day, the tone–cue fear-conditioning test was carried out. The freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus. The freezing response was defined as follows: all four paws of the rats remained still and the animal was immobilized with fear. After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate the residual odor.

2.4.7. Prepulse inhibition (PPI) test in OB rats

Before drug administration on the 29th day after the operation, the PPI test was performed using two units of the automated SRLab startle system (San Diego Instruments, San Diego, CA, USA). Each unit consisted of a Plexiglas cylinder 9 cm in diameter on a platform, under which a sensitive piezo-electric accelerometer was attached, which measured the whole-body displacement. During the sessions, a rat was kept in the cylinder within a sound-attenuating cabinet, in which a 70 dB white background noise (baseline) was delivered through speakers in the ceiling of the box (29 cm × 28.5 cm × 29.5 cm). Stimuli were similarly delivered and responses in rats were measured using the SRLab software (San Diego Instruments) running on a PC in an adjacent room. Rats were placed in the boxes for a 10-min acclimatization period with white background noise. After this period, rats were exposed to 10 sessions of stimuli that consisted of random combinations of three types of stimuli: a background noise (60 dB); a startle pulse (120 dB) without prepulse; and a startle pulse of 120 ms preceded by a 40-ms prepulse (74 dB). After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate the residual odor.

2.4.8. Twenty-four hour locomotor activity test in OB rats

On the next 2 days of the sexual behavior test, locomotor activity was measured. The rats received the fear-conditioning test were not included in this test. Rats were subcutaneously injected drugs at 4.00 a.m. on the day of the 24-h locomotor activity test, and were individually placed in testing cages (33 cm × 28 cm × 17 cm), above each of which there was a locomotor sensor that was connected to an IBM notebook through a hub, food being supplied. The 24-h locomotor activity test began at 8.00 a.m. in the morning and finished at 10.00 a.m. on the following day, during which the locomotor activity of the rats was recorded by the computer.

2.5. Statistical analysis

Results were expressed as means ± S.E. for 7–42 rats. A SAS program (ver. 5.0, SAS Institute, Cary, NC, USA) was used to perform all analyses. Statistical differences among the experimental groups were tested using one-way ANOVA for behavioral tests, two-way ANOVA for analyzing the circadian activity pattern in the 24-h locomotor activity test, and Dunnett's test was employed for multiple comparisons. Differences were considered significant at $P<0.05$.

3. Results

3.1. Effects of (−)-BPAP and imipramine on emotional deficit in forced-swimming-stressed mice

Immobility time in mice treated with acute, but not chronic (−)-BPAP (3 mg/kg) and imipramine (10 mg/kg) was significantly shortened compared to that of the saline group [one-way ANOVA, $F=5.62$, d.f. = 6, $P<0.0001$; Dunnett's test, $P=0.0016$ ((−)-BPAP); one-way ANOVA, $F=10.25$, d.f. = 2, $P=0.0003$; Dunnett's, $P=0.0014$ (imipramine)] (Fig. 2A and B). The locomotor activity in the period of the second swimming was increased in the acute (−)-BPAP (3 mg/kg) group (one-way ANOVA, $F=19.82$, d.f. = 4, $P<0.0001$; Dunnett's, $P<0.0001$) (Fig. 3A), but not in the acute imipramine group (data not shown). Therefore, the decrease in immobility time induced by (−)-BPAP might have been due to the increase in locomotor activity, rather than an antidepressant-like effect.

Mice after the second swimming showed a significant decrease in SI behavior compared to naive (non-stressed) mice [one-way ANOVA, $F=4.92$, d.f. = 7, $P<0.0001$; Dunnett's, $P=0.0027$ (Fig. 4A); one-way ANOVA, $F=18.74$, d.f. = 3, $P<0.0001$; Dunnett's, $P<0.0001$ (Fig. 4B)] (Fig. 4A and B). Acute administration of (−)-BPAP and imipramine treatment significantly decreased SI behavior compared to that with saline [one-way ANOVA, $F=4.92$, d.f. = 7, $P<0.0001$; Dunnett's, $P=0.0268$ ((−)-BPAP); one-way ANOVA, $F=18.74$, d.f. = 3, $P<0.0001$; Dunnett's, $P=0.0008$ (imipramine)] (Fig. 4A and B). However, chronic administration of (−)-BPAP (1 and

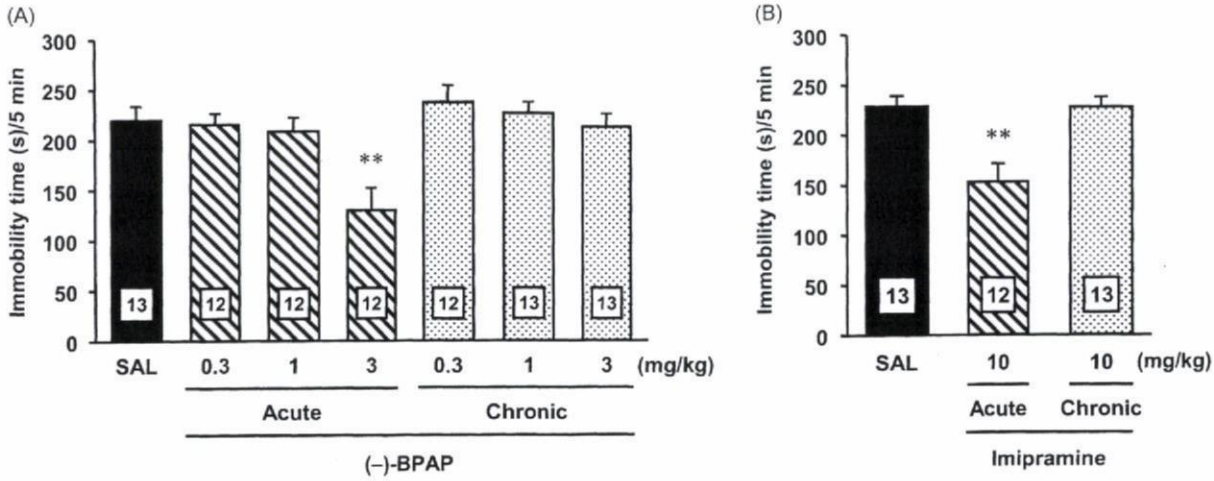

Fig. 2. Effects of (−)-BPAP and imipramine on immobility in mice exposed to forced swimming. Mice were injected subcutaneously with (−)-BPAP, imipramine or saline once daily from the day following the first swimming to the day of the second swimming (7 days). At the second swimming, mice were injected with (−)-BPAP, imipramine or saline 30 min before swimming. A and B show the result of treatment with (−)-BPAP (A) and imipramine (B). SAL, saline. Values represent means ± S.E.. The number of mice used in each group is shown in the column. $^{**}P<0.01$ vs. saline-treated mice.

3 mg/kg), but not of imipramine, attenuated the impairment of SI behavior in saline-treated stressed mice [one-way ANOVA, $F=4.92$, d.f. = 7, $P<0.0001$; Dunnett's, $P=0.0163$ (1 mg/kg), $P=0.0051$ (3 mg/kg)] (Fig. 4A and B).

The locomotor activity at the time of the SI test was increased in the acute (−)-BPAP (3 mg/kg) group compared to the saline-treated stressed group (one-way ANOVA, $F=6.25$, d.f. = 4, $P<0.0001$; Dunnett's, $P=0.0396$), whereas the locomotor activity in chronic group was not significantly changed compared to saline-treated stressed mice (Fig. 3B). These results indicated that (−)-BPAP ameliorated forced-swimming-induced deficiency in SI behavior.

3.2. Antagonistic effects of SCH-23390 and sulpiride on the improving effect of chronic (−)-BPAP on SI behavior in stressed mice

To determine whether the improving effects of chronic administration of (−)-BPAP on the impairment of SI behavior induced by swimming are mediated via DA receptors, we examined the effect of SCH-23390, a D_1 antagonist, and sulpiride, a D_2 antagonist, on the effect of (−)-BPAP in stressed mice. We preliminarily confirmed that administration of SCH-23390 (0.02 mg/kg) or sulpiride (3 mg/kg) alone had no effect on the impairment of SI behavior in stressed mice. The

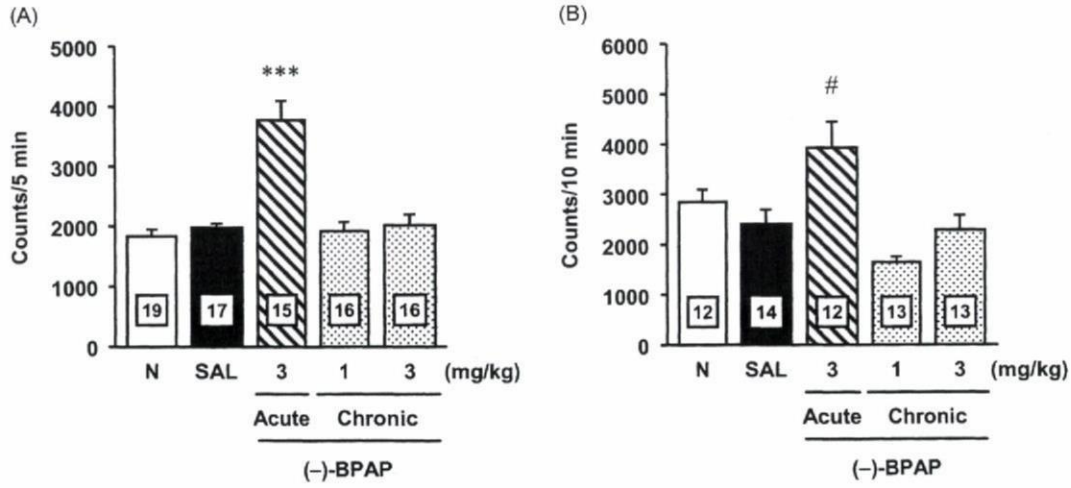

Fig. 3. Effects of (−)-BPAP on locomotor activity in mice exposed to forced swimming. Mice were injected subcutaneously with (−)-BPAP or saline once daily from the day following the first swimming to the day of the second swimming (7 days). At the second swimming, mice were injected with (−)-BPAP or saline 30 min before the swimming. Panels A and B show the locomotor activity at the second swimming period (A) and the SI test period (B). N, naive; SAL, saline. Values represent means ± S.E.. The number of mice used in each group is shown in the column. $^{***}P<0.001$ vs. saline-treated mice. $^{\#}P<0.05$ vs. saline-treated-stressed mice.

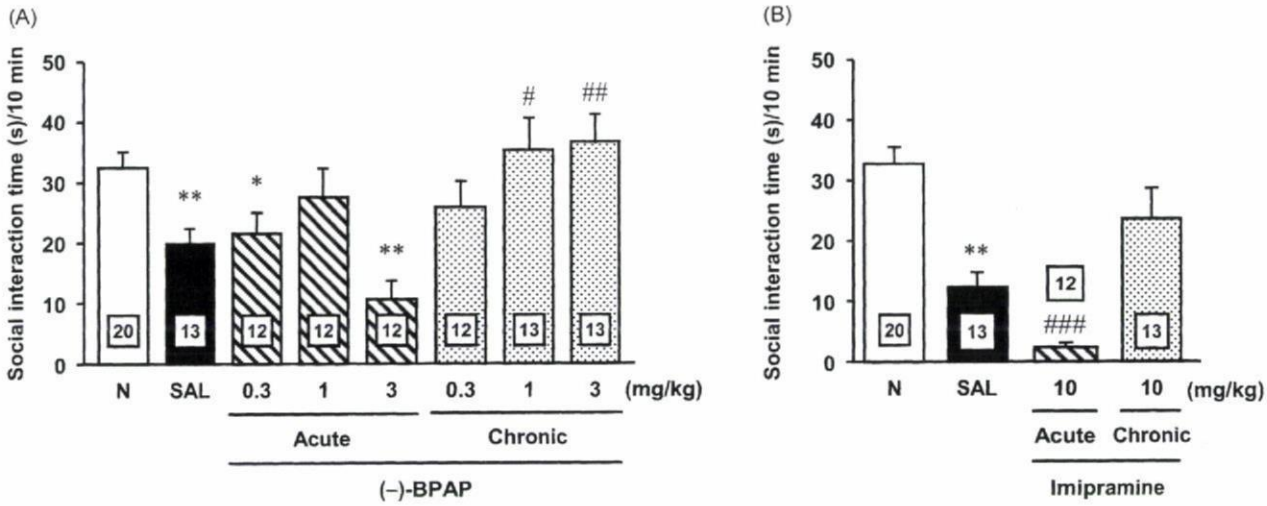

Fig. 4. Effects of (−)-BPAP (A) and imipramine (B) on SI behavior in mice exposed to forced swimming. Mice were injected subcutaneously with (−)-BPAP, imipramine or saline once daily from the day following the first swimming to the day of the second swimming (7 days). At the second swimming, mice were injected with (−)-BPAP, imipramine or saline 30 min before swimming. N, naive; SAL, saline. Values represent means ± S.E.. The number of mice used in each group is shown in the column. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. naive mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. saline-treated-stressed mice.

improving effects of chronic (−)-BPAP in stressed mice were significantly antagonized by chronic co-administration of SCH-23390 (0.02 mg/kg) or sulpiride (3 mg/kg) [one-way ANOVA, $F=13.80$, d.f. = 4, $P<0.0001$; Dunnett's, $P=0.0131$ (SCH-23390), $P=0.0115$ (sulpiride)] (Fig. 5). This result indicated that enhancement of the dopaminergic system was involved in the effect of (−)-BPAP on stressor-induced behavioral deficits.

3.3. Effects of chronic administration of (−)-BPAP on behavior in OB rats

In the open field test, OB rats showed increases in ambulation and rearing behavior in the first 3 min compared to sham rats (Table 1). Both imipramine and (−)-BPAP failed to decrease the ambulation distance and rearing frequency in OB rats. There was no change among any of the groups in urination and defecation frequencies.

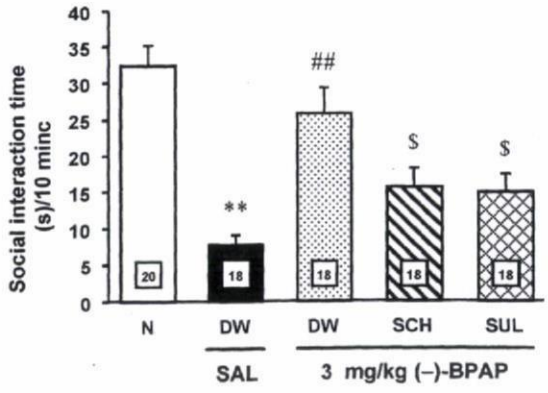

Fig. 5. Antagonistic effects of SCH-23390 and sulpiride on the improving effect of chronic (−)-BPAP on SI behavior in stressed mice. Mice were injected subcutaneously with (−)-BPAP or saline once daily from the day following the first swimming to the day of the second swimming (7 days). At the second swimming, mice were injected with saline 30 min before the swimming. N, naive; SAL, saline; DW, distilled water; SCH, SCH-23390 (0.02 mg/kg); SUL, sulpiride (3 mg/kg). Values represent means ± S.E.. The number of mice used in each group is shown in the column. $^{**}P<0.01$ vs. naive mice. $^{\#\#}P<0.01$ vs. (saline + DW)-treated-stressed mice. $^{\$}P<0.05$ vs. [(−)-BPAP + DW]-treated-stressed mice.

In the sexual behavior test, the starting latency of genital sniffing, grooming, thrusting and ejaculation in OB rats was significantly increased [one-way ANOVA, d.f. = 4, $F=21.18$, $P<0.0001$ (sniffing); $F=8.94$, $P<0.0001$ (grooming); $F=6.42$, $P=0.0001$ (thrusting); $F=4.00$, $P=0.0048$ (ejaculation); Dunnett's, $P<0.0001$ (sniffing), $P<0.0001$ (grooming), $P=0.0024$ (thrusting), $P=0.0215$ (ejaculation)] and the number of these behaviors in OB rats was significantly decreased [one-way ANOVA, d.f. = 4, $F=11.58$, $P<0.0001$ (sniffing); $F=8.88$, $P<0.0001$ (grooming); $F=5.25$, $P=0.0007$ (thrusting); $F=4.98$, $P=0.0011$ (ejaculation); Dunnett's, $P<0.0001$ (sniffing), $P<0.0001$ (grooming), $P=0.0031$ (thrusting), $P=0.0062$ (ejaculation)] compared to those in sham rats (Table 1). The increased starting latency of grooming in OB rats was shortened by the administration of (−)-BPAP (1 mg/kg) [one-way ANOVA, $F=8.94$, d.f. = 4, $P<0.0001$; Dunnett's, $P=0.0122$], but not imipramine. Furthermore, the increased starting latency of thrusting and ejaculation in OB rats tended to decrease with (−)-BPAP (1 mg/kg). In addition, the frequency of thrusting in OB rats tended to increase with (−)-BPAP (1 mg/kg), but not imipramine.

In the SI and PPI tests, OB rats showed significant impairment of SI behavior and PPI compared to sham rats [one-way ANOVA, d.f. = 4, $F=6.59$, $P=0.0003$ (SI), $F=2.84$, $P=0.0296$ (PPI); Dunnett's, $P<0.0001$ (SI), $P=0.0449$ (PPI)] (Fig. 6A and B). The impairment of SI behavior in OB rats was attenuated by chronic administration of imipramine or (−)-BPAP (0.3 and 1 mg/kg) [one-way ANOVA, $F=6.59$, d.f. = 4, $P=0.0003$; Dunnett's, $P=0.0005$ (imipramine), $P=0.0028$ (0.3 mg/kg (−)-BPAP), $P<0.0001$ (1 mg/kg (−)-BPAP)] (Fig. 6A). The impairment of PPI in OB rats was ameliorated by the administration of (−)-BPAP (1 mg/kg) [one-way ANOVA, $F=2.84$,

Table 1
Effects of (−)-BPAP and imipramine on performance in the open field and sexual behavior tests in OB rats

	Sham	OB	IMI	(−)-BPAP	
			20	0.3	1 (mg/kg)
Open field test (n = 23–37)					
SL (s)	12.15 (±1.95)	8.68 (±1.38)	9.07 (±1.38)	7.55 (±1.10)	8.36 (±1.00)
No. of AMB	33.61 (±2.58)	53.54*** (±2.78)	50.44*** (±2.95)	52.43*** (±3.28)	51.51*** (±3.07)
No. of RE	13.22 (±1.22)	21.92*** (±1.46)	19.69** (±1.58)	23.53*** (±1.45)	20.92*** (±1.54)
No. of URI	0.79 (±0.19)	0.84 (±0.12)	0.68 (±0.11)	0.83 (±0.21)	0.76 (±0.11)
No. of DEF	2.54 (±0.35)	2.97 (±0.32)	4.16 (±0.41)	3.00 (±0.31)	3.14 (±0.39)
Sexual behavior test (n = 17–22)					
SL (s)					
SN	19.91 (±5.52)	1591.05*** (±120.35)	1401.37*** (±180.06)	1274.69*** (±182.50)	1264.79*** (±173.78)
GR	300.45 (±115.44)	1393.46*** (±158.11)	1410.49*** (±177.76)	1149.34*** (±182.73)	766.76$^{*,\#}$ (±179.07)
TH	914.41 (±183.51)	1633.06** (±115.07)	1800.00*** (±0.00)	1700.94** (±99.06)	1353.80 (±177.31)
EJ	1189.15 (±152.53)	1671.13* (±89.07)	1800.00** (±0.00)	1727.50** (±72.50)	1382.47 (±166.00)
No.					
SN	45.53 (±11.86)	0.18*** (±0.11)	1.05*** (±0.65)	0.82*** (±0.36)	5.80** (±3.36)
GR	10.32 (±2.09)	1.14*** (±0.55)	2.71* (±1.78)	0.78*** (±0.26)	3.05** (±0.92)
TH	14.36 (±3.66)	1.86** (±1.36)	0.00** (±0.00)	2.56* (±2.56)	6.80 (±2.83)
EJ	1.36 (±0.35)	0.24** (±0.17)	0.00** (±0.00)	0.17** (±0.17)	0.90** (±0.36)

Two weeks after olfactory bulbectomy, rats were treated subcutaneously with (−)-BPAP (0.3 and 1.0 mg/kg), imipramine (10 mg/kg) or saline daily for 16 days, until the day before the 24-h locomotor activity or fear-conditioning test. OB, olfactory bulbectomy; IMI, imipramine; SL, starting latency; AMB, ambulation; RE, rearing; URI, urination; DEF, defecation; SN, sniffing; GR, grooming; TH, thrusting; EJ, ejaculation. Values represent means ± S.E. for 17–37 animals.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. saline-treated-sham rats. $^{\#}P<0.05$ vs. saline-treated-OB rats.

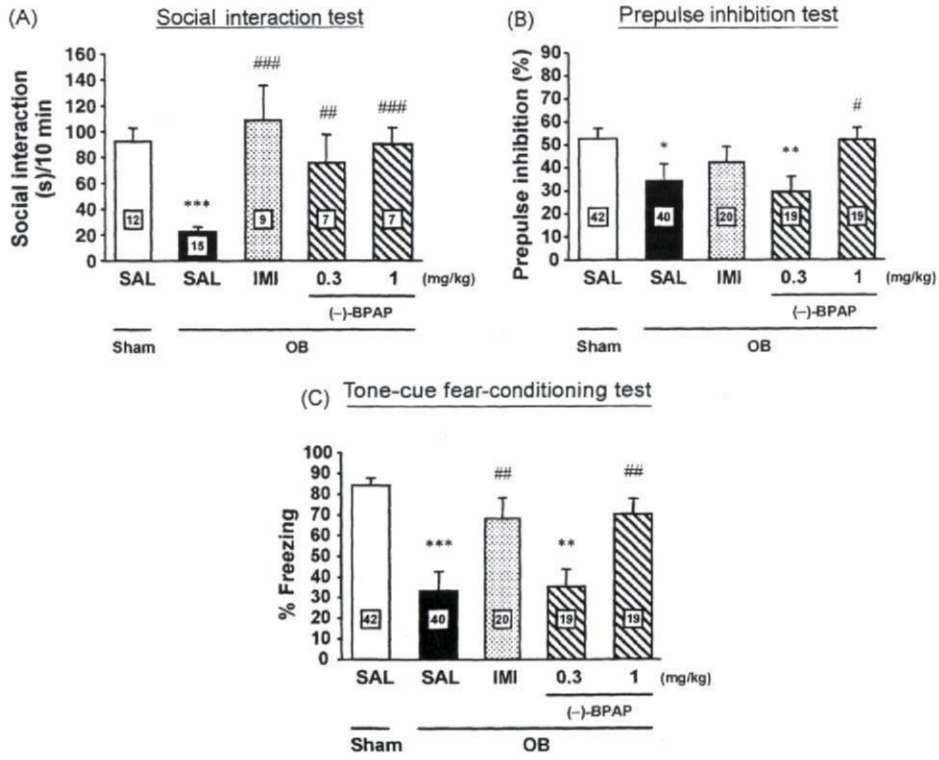

Fig. 6. Effects of (−)-BPAP and imipramine on SI, PPI and tone–cue fear-conditioning tests in OB rats. Two weeks after olfactory bulbectomy, the rats were treated subcutaneously with (−)-BPAP (0.3 and 1 mg/kg), imipramine (10 mg/kg) or saline daily for 16 days, until the day before the 24-h locomotor activity test or fear-conditioning test. Panels A–C show the result of the SI test (A), PPI test (B) and tone–cue fear-conditioning test (C). OB, olfactory bulbectomy; SAL, saline; IMI, imipramine. Values represent means ± S.E.. The number of rats used in each group is shown in the column. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. saline-treated-sham rats. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. saline-treated-OB rats.

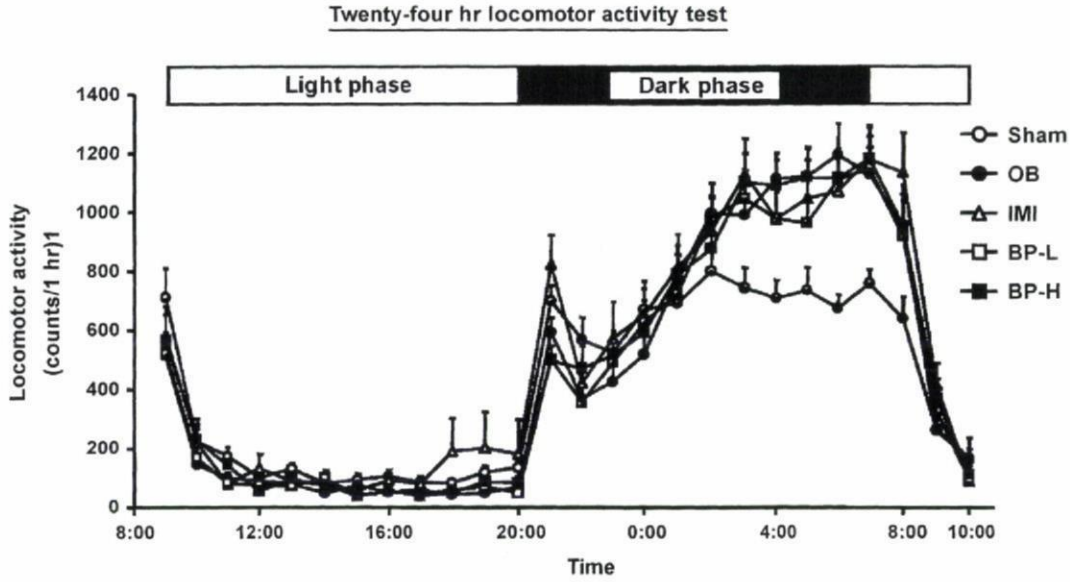

Fig. 7. Effects of (−)-BPAP and imipramine on the 24-h locomotor activity in OB rats. Two weeks after olfactory bulbectomy, rats were treated subcutaneously with (−)-BPAP (0.3 and 1 mg/kg), imipramine (10 mg/kg) or saline daily for 16 days, until the day before the 24-h locomotor activity test. OB, olfactory bulbectomy; IMI, imipramine; BP-L, 0.3 mg/kg (−)-BPAP; BP-H, 1 mg/kg (−)-BPAP. Saline-treated-sham ($n=23$) (○); saline-treated-OB ($n=22$) (●); IMI-treated-OB ($n=19$) (△); (−)-BPAP (0.3 mg/kg)-treated-OB ($n=21$) (□); and (−)-BPAP (1 mg/kg)-treated-OB ($n=23$) (■) groups. Values represent means ± S.E..

d.f. = 4, $P=0.0296$; Dunnett's, $P=0.0452$], but not imipramine (Fig. 6B).

In the preconditioning phase of the tone–cue fear-conditioning test, mice hardly showed a freezing response. There were no differences in basal levels of freezing response among any of the groups (data not shown). Twenty-four hours after fear conditioning, sham rats showed a marked tone–cue freezing response, whereas OB rats had a lesser freezing response [one-way ANOVA, $F=11.27$, d.f. = 4, $P<0.0001$; Dunnett's, $P<0.0001$] (Fig. 6C). The performance of OB rats was restored by chronic treatment with imipramine or (−)-BPAP (1 mg/kg) [one-way ANOVA, $F=11.27$, d.f. = 4, $P<0.0001$; Dunnett's, $P=0.0077$ (imipramine), $P=0.0015$ (1 mg/kg (−)-BPAP)] (Fig. 6C).

The circadian activity pattern of OB rats was significantly different from that of sham rats [two-way ANOVA, $F=8.64$, d.f. = 4, $P<0.0001$ (group), $F=171.68$, d.f. = 23, $P<0.0001$ (time), $F=1.99$, d.f. = 92, $P<0.0001$ (group × time)] (Fig. 7). The locomotor activity of OB rats in the latter dark phase (2.00–8.00 a.m.) was significantly increased [one-way ANOVA, $F=4.85$, d.f. = 4, $P=0.0013$; Dunnett's, $P=0.0004$] (Fig. 7). The locomotor activity of OB rats in any phase was not affected by the administration of both imipramine and (−)-BPAP.

4. Discussion

The contribution of stress to the induction or exacerbation of mood disorders has been increasingly emphasized [2,17,47]. Preclinical studies have also revealed that stressors induce neurochemical and hormonal alterations that are reminiscent of those observed in patients with depression [2,22,47], and that clinically effective antidepressant drugs are effective against the behavioral alterations promoted by stressful experiences [2,11,33]. Thus, animal models of mood disorders induced by experimental stressors have been used to study alterations of behavior and brain functioning related to human symptoms. We found that SI behavior of mice exposed to forced swimming was impaired compared to that of naive mice. There was not a significant difference in locomotor activity during the SI test between naive and stressed mice exposed to forced swimming. These results support the hypothesis that the impairment of SI behavior in stressed mice indicates an increase in the anxiety level induced by physical stress. It has been reported that physical stress, such as exposure to repeated mild foot-shock, causes an increase in anxiety level [36]. Therefore, the present stressed mouse model may be useful as a stress-based experimental model of mood disorders.

Several studies have demonstrated that stress promotes profound and complex alterations in DA transmission in the mesolimbic and mesocortical systems [9,12,25]. Increasing evidence has suggested that the mesocortical DA system is involved in mood disorders such as depression and social anxiety, which can be induced by stressful experiences [31]. Uncontrollable/unavoidable stressors used to stimulate depressive-like responses in animal models [9] promote inhibition of mesoaccumbens DA [8]. Prolonged exposure to forced swimming promotes a dramatic reduction in DA outflow from the ventral striatum. Behavioral despair (reduced escape attempts) and dopaminergic neuronal dysfunction induced by forced swimming can be counteracted by chronic pretreatment with clinically effective antidepressants [40]. Therefore, drugs which control the dopaminergic system might be available for the treatment of mood disorders.

(−)-BPAP enhances the impulse-evoked release of catecholamines and 5-HT from isolated rat brainstem [26]. (−)-BPAP has been demonstrated to ameliorate motor deficits in

reserpine-treated rats and improves active avoidance behavior in rats with tetrabenazine-induced depression [28]. Therefore, (−)-BPAP is a promising candidate as a treatment for symptoms of depression.

In the present experiments on mice, acute and chronic administration of (−)-BPAP attenuated immobility in the forced swimming test and the impairment of SI behavior that followed forced swimming, respectively. On the other hand, acute administration of (−)-BPAP exacerbated the impairment of SI behavior that followed forced swimming. Mice treated with acute (−)-BPAP showed significant hyperlocomotion compared to those treated with saline during the forced swimming and SI test. The effect of acute administration of (−)-BPAP on immobility in the forced swimming test and SI behavior might be related to enhanced locomotor activity. Since locomotor activity was not affected by chronic administration of (−)-BPAP during the SI test, the effect of (−)-BPAP on impairment of SI behavior might have been due to amelioration of the damage following forced swimming. Furthermore, the ameliorating effect of (−)-BPAP on SI behavior was antagonized by chronic pretreatment with D_1 antagonist SCH-23390 or D_2 antagonist sulpiride. These findings indicate that the dopaminergic system is partly involved in the improving effect of (−)-BPAP. It has been reported that mesolimbic DA transmission appears to play a major role in defensive responses toward aversive stimuli [10,38]. Cabib and Puglisi-Allegra [9] have suggested that stressor-induced behavioral alterations are related to the inhibitory phase of mesolimbic DA response to stressors. Thus, it is possible that (−)-BPAP enhances dopaminergic neuronal transmission in the mesolimbic area, followed by remission of the SI behavioral deficits.

The olfactory bulbs are intimately and extensively connected to a variety of limbic and limbic-related structures and many of the limbic structures and the olfactory bulbs are reciprocally interconnected in a loop pathway that may be involved in the regulation of emotional output [7,37]. The bilateral removal of the olfactory bulbs in rats results in a complex constellation of behavioral alterations, such as an increase in anxiety and aggression, enhancement of exploratory behavior in the open field, reduction of sexual behavior and impairment of spatial learning, and neurochemical, neuroendocrine and neuroimmune alterations. Many of these behavioral deficits in OB rats are correlated with those observed in major depression [23] and depression-related disorders such as anxiety and aggression [41]. In the present study, OB rats produced behavioral abnormalities, such as increased locomotor activity in a novel open field, and impairment of social and sexual behavior 4 weeks after olfactory bulbectomy, which are consistent with those in previous studies [23,46]. OB rats showed impairment of PPI and memory in the tone–cue fear-conditioning test. PPI and tone–cue fear conditioning are associated with the amygdala [13,29]. It has been reported that the amygdala, which is linked to the hypothalamus and frontal cortex, is denervated following olfactory bulbectomy [23]. Thus, dysfunction in these regions may underlie these behavioral deficiencies in OB rats.

In the present study of OB rats, (−)-BPAP ameliorated the behavioral impairments in the SI, sexual behavior, tone–cue fear-conditioning and PPI tests. The mechanisms involved in the effects of (−)-BPAP in OB rats are not clear. However, it has been reported that olfactory bulbectomy induces a decrease in DA turnover in the hypothalamus and DA concentration in the frontal cortex [18,39]. G(olf) protein, a stimulant alpha-subunit of G protein that is coupled to the D_1 receptor, is decreased in OB rats, but is increased by chronic administration of antidepressants [44]. Furthermore, DA receptor agonist rotigotine reverses the hyperactivity of rats subjected to olfactory bulbectomy [4]. In our experiments using mice, (−)-BPAP ameliorated the damage induced by forced swimming that was antagonized by the DA receptor antagonists. Taking all these results together, it is possible that (−)-BPAP ameliorated the behavioral impairments in OB rats through the dopaminergic system.

Finally, chronic administration of imipramine, a typical tricyclic antidepressant, also recovered the impairment of social behavior that followed forced swimming. However, acute administration of imipramine significantly exacerbated the symptoms, which was consistent with a previous study [48]. Imipramine ameliorated impairment of social behavior and tone–cue fear-conditioning learning, but not PPI in OB rats. Therefore, (−)-BPAP might have a wide spectrum of effects against mood disorder, compared to those of imipramine.

In conclusion, (−)-BPAP improved the behavioral deficits induced by forced swimming or olfactory bulbectomy, in part, via the dopaminergic system. However, imipramine failed to improve the behavioral deficit in OB rats. These results suggest that (−)-BPAP may be a new drug for therapy of mood disorders.

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Research report

Synergistic effects of selegiline and donepezil on cognitive impairment induced by amyloid beta (25–35)

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ABSTRACT

Selegiline, an irreversible inhibitor of monoamine oxidase B used in the treatment of Parkinson's disease, has been demonstrated to have a potential cognition-improving effect in patients with Alzheimer's disease (AD) undergoing treatment with an acetylcholinesterase inhibitor donepezil. To confirm such clinical events, we investigated whether co-administration of donepezil with selegiline had a synergistic cognition-improving effect in an animal model of AD. Intracerebroventricular injection of amyloid beta protein fragment 25–35 [$A\beta_{(25-35)}$] induced impairment of learning and memory in a Y-maze, novel object recognition and contextual fear conditioning tests. Either donepezil or selegiline alone improved the cognitive impairments in the Y-maze and conditioned fear learning tasks in $A\beta_{(25-35)}$-injected mice, whereas donepezil, but not selegiline, failed to improve the impairment in a novel object recognition task. Co-administration of donepezil with selegiline, at doses that do not exert efficacy individually, significantly improved the deficits in all three tests, indicating a synergistic cognition-improving effect. These alleviating effects were antagonized by pretreatment with a muscarinic receptor antagonist scopolamine and a dopamine receptor antagonist haloperidol. These results suggest that selegiline potentiates the effect of donepezil on the cognitive impairment, and that the synergistic effect may be mediated through both the cholinergic and dopaminergic systems.

1. Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder in humans, is characterized by the deterioration of cognitive and mental functions, including learning and memory. The formation of extracellular deposits of amyloid beta peptide (Aβ), leading to the formation of neuritic plaques and neurofibrillary tangles in the cortex and hippocampus, is a prominent pathological feature of AD [32]. Aβ, a spontaneously aggregating peptide of 39–43 amino acids, is the primary protein component of senile plaques, the pathological hallmark of AD in the brain [29]. In particular, Aβ fragment 25–35 [$A\beta_{(25-35)}$] seems to be responsible for toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation [37], and protein oxidation in fibroblasts derived from AD patients [4]. In animal experiments, it has been reported that intrahippocampal or intracerebroventricular (i.c.v.) injections of $A\beta_{(25-35)}$ induce histological and biochemical changes, learning deficits [20,21,25] and dysfunction of the cholinergic system, which play an important role in the cognitive deficits associated with aging and neurodegenerative diseases [12,36]. Thus, $A\beta_{(25-35)}$-injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD [12,20].

Cholinergic neurons degenerate in patients with AD and Alzheimer's type senile dementia, and the degree of degeneration correlates well with functional loss in these disorders [26]. Based on a cholinergic hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the cholinomimetic use of acetylcholinesterase inhibitors (AchEIs), acetylcholine precursors and cholinergic receptor agonists. In fact, an AchEI donepezil, has been approved for treatment of cognitive impairment in AD. However, acetylcholine-enhancing drugs can

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compensate for only part of the neuronal dysfunction in AD, and the enhancement of cognition by AchEIs is only transient [13].

The dopaminergic system has been implicated in cognitive processes in a variety of brain regions, including the mesolimbic system, since dopamine modulates transmitter release at cholinergic [11] and glutamatergic [42] synapses in the hippocampus. Previous study has shown that disturbances in the dopaminergic system induce learning and memory impairment [9]. Meanwhile, pathological and clinical evidences reported to date are suggestive of the involvement of the dopaminergic system in dementia. For example, there is a correlation between loss of hippocampal dopamine D_2 receptors and memory impairment in AD [17]. In addition, it has been shown that memory impairment induced by intraperitoneal injection of scopolamine is ameliorated by the injection of dopamine D_2 receptor agonists into the ventral hippocampus [8], suggesting that dopaminergic agents could have therapeutic potential in patients with cholinergic deficits, e.g. those with AD and dementia with Lewy bodies.

Selegiline, a selective monoamine oxidase B inhibitor (MAO-BI), is used worldwide for the treatment of Parkinson's disease [2]. Previous study has shown that selegiline improves episodic memory and learning in patients with AD [35], and spatial memory in aged or a cholinotoxin AF64A-treated rats [18,33]. The increase in dopaminergic activity consequent to the inhibition of monoamine oxidase B activity is often considered to be the neurochemical mechanism involved in the improvement of cognitive performance caused by selegiline in aged rats [3] and individuals affected by Alzheimer's type dementia [35]. Furthermore, selegiline has also been demonstrated to have potential cognition-improving efficacy in AD patients treated with the AchEI donepezil [33].

To confirm such clinical events, the present study was designed to test the hypothesis that co-administration of donepezil with selegiline improves cognitive impairment in an $A\beta_{(25-35)}$-injected animal model of AD, and that the synergistic cognition-improving effects of selegiline and donepezil are mediated via activation of the cholinergic and dopaminergic systems. We attempted to investigate: (1) the effects of single or concurrent administration of selegiline and donepezil on memory impairment induced by $A\beta_{(25-35)}$, and (2) that the cognition-improving effects of selegiline are antagonized by acetylcholine and/or dopamine antagonists in $A\beta_{(25-35)}$-injected mice.

2. Materials and methods

2.1. Animals

Male ICR mice (5-week-old, Nihon SLC, Shizuoka, Japan) were housed in plastic cages, received food (CE2, Clea Japan, Tokyo, Japan) and water *ad libitum*, and were maintained on a 12-h light:12-h dark cycle (lights on at 8.00 a.m.). All experiments were performed in a blind manner and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine (Japan). The procedures involving animals and their care conformed to the institutional guidelines set out in "Principles of Laboratory Animal Care" (NIH publication No. 85–23, revised 1985).

2.2. Drugs and treatment

Selegiline hydrochloride (FP Pharmaceutical, Osaka, Japan), donepezil hydrochloride (Eisai, Ibaraki, Japan), scopolamine hydrobromide (Nacalai Tesque, Kyoto, Japan) and haloperidol (Sigma, St. Louis, MO, USA) were dissolved in saline. The doses of all drugs are expressed as those of the salt. $A\beta_{(25-35)}$ (Bachem, Torrance, CA, USA) was dissolved in distilled water (vehicle) at a concentration of 1 mg/mL (0.9375 mM) and stored at −20 °C.

Mice were intracerebroventricularly injected vehicle or $A\beta_{(25-35)}$ that had undergone incubation for 4 days at 37 °C, a procedure that permits aggregation. Vehicle- and $A\beta_{(25-35)}$-injected mice were subcutaneously administered selegiline, donepezil, scopolamine and haloperidol 30, 30, 45 and 60 min before each behavioral test (Y-maze, novel object recognition and contextual fear conditioning tests), respectively on 3 separate occasions. Three different approaches for drug administration were used as shown in Table 1: (1) selegiline and donepezil by themselves (Fig. 2); (2) co-administration of selegiline and donepezil (Fig. 3) and (3) scopolamine or haloperidol + co-administration of donepezil and selegiline. We preliminarily confirmed that single administrations of donepezil, selegiline, scopolamine and haloperidol, at doses used in this study, had no effect on cognitive function of the vehicle-injected mice in all performed tests.

Table 1
Drugs, doses of drugs and numbers of animals in each experiment

Group No.	N	Treatment			
		Aβ	Drug (mg/kg)		
Experiment 1 (Fig. 2)					
1	27–28	Vehicle	Saline		
2	29	Aβ	Saline		
3	7	Aβ	Donepezil (0.05)		
4	14	Aβ	Donepezil (0.1)		
5	6	Aβ	Selegiline (1.0)		
6	13	Aβ	Selegiline (3.0)		
Group No.	N	Treatment			
		Aβ	Drug 1 (mg/kg)	Drug 2 (mg/kg)	
Experiment 2 (Fig. 3)					
1	37–38	Vehicle	Saline	Saline	
2	36–37	Aβ	Saline	Saline	
3	18	Aβ	Donepezil (0.05)	Saline	
4	16–17	Aβ	Saline	Selegiline (1.0)	
5	18–19	Aβ	Donepezil (0.05)	Selegiline (1.0)	
Group No.	N	Treatment			
		Aβ	Drug 1 (mg/kg)	Drug 2 (mg/kg)	Antagonist (mg/kg)
Experiment 3 (Fig. 4)					
1	10	Vehicle	Saline	Saline	Saline
2	10	Aβ	Saline	Saline	Saline
3	12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Saline
4	11–12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Scopolamine (0.1)
5	10–12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Haloperidol (0.1 or 0.03)

Three different approaches for drug administration were used: (1) selegiline and donepezil separately (Fig. 2); (2) co-administration of selegiline and donepezil (Fig. 3) and (3) scopolamine or haloperidol + co-administration of selegiline and donepezil. Aβ: $A\beta_{(25-35)}$.

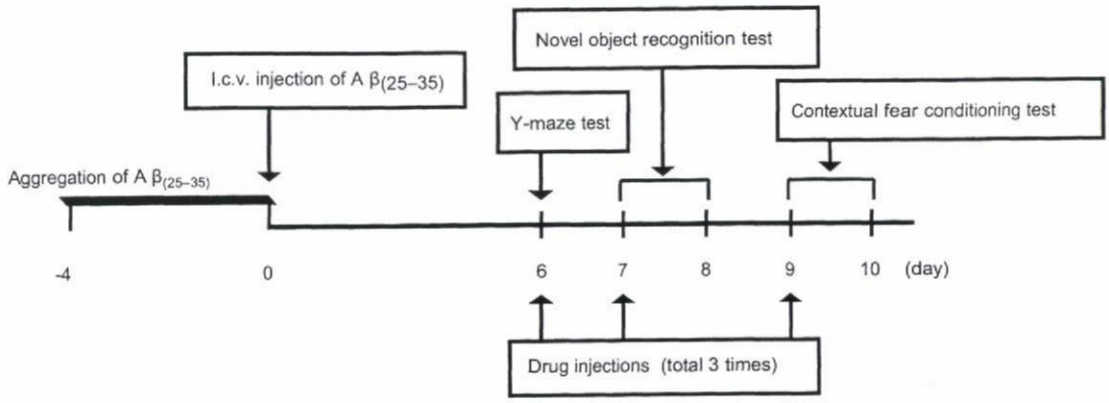

Fig. 1. Behavioral experimental schedule. i.c.v., intracerebroventricular.

2.3. $A\beta_{(25-35)}$-injected mouse model

I.c.v. injections of $A\beta_{(25-35)}$ were performed as described previously [20]. Briefly, a microsyringe with a 28-gauge stainless-steel needle bended an angle of 90° at the point 3 mm far from the tip of the needle was used for i.c.v. injections. Mice were anesthetized lightly with ether to be free from distress and stabilized with nose cone until i.c.v. injection had finished, and the needle was unilaterally inserted by hand 1 mm to the right of the midline point, equidistant from each eye, at an equal distance between the eyes and the ears, and perpendicular to the skull. The skulls of mice were not exposed to perform the injections to save the time and to be free from stress. The i.c.v. injection of $A\beta_{(25-35)}$ (3 nmol/3.2 μL) or vehicle (3.2 μL) was performed slowly over a period of 2 min. Mice exhibited normal behavior within 1 min after injection. We used vehicle as control of $A\beta_{(25-35)}$ in accordance with a previous report [20]. Neither insertion of the needle nor injection of the vehicle had any influence on survival, behavioral responses or cognitive function in consistent with a previous report [20]. The injection site of each mouse was confirmed by injecting Indian ink in preliminary experiments and dissecting the brain after all experiments.

2.4. Behavioral analysis

Previous reports have shown that acute exposure of aged $A\beta_{(25-35)}$ to hippocampal cultures induces apoptosis-mediated neuronal toxicity during 6 days incubation, and cognitive dysfunction in several learning and memory tests in mice [20]. The behavioral tests started on day 6 after $A\beta_{(25-35)}$ injection, and were carried out sequentially according to the experimental schedule shown in Fig. 1. The present study was conducted in a blind manner.

2.5. Spontaneous alternation in Y-maze test

The Y-maze task was carried out on day 6 after $A\beta_{(25-35)}$ injection, as described in previous reports [20]. The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the center of the apparatus, and allowed to move freely through the maze during an 8 min session. The number of arm entries was recorded visually. Alternation was defined as successive entry into the three arms, on overlapping triplet sets. The alternation behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two), multiplied by 100.

2.6. Novel object recognition test

The task was carried out on days 7–8 after $A\beta_{(25-35)}$ injection, according to the method of [15], with a minor modification. The novel object recognition test procedure consisted of three sessions: habituation, training and retention. Each mouse was individually habituated to the box (30 cm × 30 cm × 35 cm high), with 10 min of exploration in the absence of objects on days 5 and 6 after the Y-maze test (habituation session). During the training session on day 7, two novel objects (e.g. object A and B) were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 min. The objects were constructed from a golf ball, wooden column and wall socket, which were different in shape and color but similar in size. The animals were considered to be exploring the object when the head of the animal was facing the object or the animal was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 24 h (day 8) after the training session, in which one of the familiar objects (e.g. object A) used during training was replaced by a novel object (object C). The mouse was then allowed to explore freely for 10 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function [A or B/(B + A) × 100 (%) in the training session, and B or C/(B + C) × 100 (%) in the retention session].

2.7. Contextual fear conditioning test

The contextual fear conditioning task was carried out on days 9–10 after $A\beta_{(25-35)}$ injection, according to a previous report [7], with a minor modification. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in the conditioning cage (25 cm × 31 cm × 11 cm high) for 2 min and the freezing response was continuously measured by experimenter using a stopwatch on day 9. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (85 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (NeuroScience idea, Osaka, Japan). This procedure was repeated four times at 15-s intervals. We excluded the animals that did not represent normal nociceptive response in the conditioning phase from the contextual fear conditioning test. One day after fear conditioning, mice were placed in the conditioning cage, and the freezing response was continuously measured for 2 min (retention session). The freezing response was defined as none of the mouse paws moving.

2.8. Statistical analysis

Results were expressed as means ± S.E.M. for $n = 6$–37. A SAS program (ver. 5.0, SAS Institute, Cary, NC, USA) was used to perform all analyses. Statistical difference among the experimental groups was tested using Kruskal–Wallis analysis for behavioral tests, and Dunnett's test was employed for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of single administration of selegiline or donepezil on $A\beta_{(25-35)}$-induced memory impairment

3.1.1. Y-maze task

$A\beta_{(25-35)}$-injected mice showed significantly reduced spontaneous alternation behavior compared to vehicle-injected mice [Kruskal–Wallis, $H = 36.65$, d.f. = 5, $P < 0.0001$; Dunnett, $P < 0.0001$] (Fig. 2A), indicating impairment of spatial working memory. When $A\beta_{(25-35)}$-injected mice were administered donepezil (0.1 mg/kg) or selegiline (3 mg/kg) alone, the alternation behavior was significantly increased compared to that of vehicle-treated $A\beta_{(25-35)}$-injected mice [Kruskal–Wallis, $H = 36.65$, d.f. = 5, $P < 0.0001$; Dunnett, $P = 0.0211$ (donepezil), $P = 0.0316$ (selegiline)] (Fig. 2A). There was no significant difference in the number of arm entries among any of the groups (Fig. 2B), indicating that all groups