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Generation and Characterization of Mice Lacking Gastrin-Releasing Peptide Receptor

Etsuko Wada,*[,1] Kei Watase,* Kazuyuki Yamada,* Hiroo Ogura,† Mariko Yamano,‡ Yuji Inomata,§ Junichi Eguchi,§ Kazutoshi Yamamoto,¶ Mary E. Sunday,‖ Hiroshi Maeno,* Katsuhiko Mikoshiba,** Hiroko Ohki-Hamazaki,*[,2] and Keiji Wada*

**Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187, Japan; †Tsukuba Research Laboratories, Eisai Co., Ltd., Tokodai, Tsukuba-shi, Ibaraki 300-26, Japan; ‡Osaka Prefectural College of Health Sciences, Osaka, Japan; §Mitsubishi Chemical Co., Research and Development Division, Yokohama Research Center, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227, Japan; ¶Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku-ku, Tokyo 169-50, Japan; ‖Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115-6195; and **Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan*

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Gastrin-releasing peptide (GRP) is a mammalian bombesin-like peptide which is widely distributed in the central nervous system as well as in the gastrointestinal tract. GRP binds to its high affinity receptor (GRPR) to elicit a wide spectrum of biological effects on behavior, digestion, and metabolism. To define the in vivo function of GRPR, we generated GRPR null mutant mice by gene targeting. The intracerebroventricular administration of GRP caused hypothermia in wild-type mice, but not in mutant mice. The GRPR deficient mice showed significantly increased locomotor activity during the dark period, and social responses scored by sniffing, mounting, and approaching behaviors against an intruder. Aggressive scores such as fighting and biting were not altered in the mutant mice. These phenotypes were observed in mice generated from two independent ES cell clones and backcrossed to a C57BL/6J background. The GRPR deficient mice should be useful for studying the bombesin system *in vivo*.

Bombesin is a tetradecapeptide originally purified from the skin of the European frog *Bombina bombina* (1). At present, two mammalian bombesin-like peptides, gastrin-releasing peptide (GRP) (2) and neuromedin B (NMB) (3), have been identified and cloned. Mammalian bombesin-like peptides have a wide spectrum of physiological effects, including regulation of exocrine and endocrine secretions, smooth muscle contraction, metabolism, homeostasis, and behavior (4). Bioactivities associated with the bombesin-like peptides are mediated by their receptors. To date, three distinct mammalian bombesin receptors have been identified. They are GRP receptor (GRPR) (5) which shows high affinity to GRP, NMB receptor (NMBR) (6) which binds NMB with high affinity, and bombesin receptor subtype 3 (BRS3) (7) which lacks a natural high affinity ligand. Distinct distributions of bombesin receptor subtypes in the central nervous system (8) as well as peripheral organs (9) suggest that these receptors mediate different biological effects. However, the molecular basis of the heterogeneity in biological responses elicited by different bombesin-like peptide receptors remains elusive.

Recently, a *de novo* balanced X chromosomal translocation (46, X, t(X; 8) (p22.13; q22.1) disrupting the GRPR coding region was identified in a patient with autism and multiple exostoses (10). The GRPR gene is located on human chromosome Xp21.2-p22.3 (11) where genes involved in behavioral disorders including Rett syndrome (12) have been mapped (13). Rett syndrome is characterized by autism, apraxia, seizures and stereotypic hand movements. Taken together these observations suggest that the GRPR gene may play a role in regulating behavior and mentality. To define the *in vivo* function of GRPR, we generated GRPR null mutant mice by gene targeting.

MATERIAL AND METHODS

Construction of GRPR gene targeting vector and ES cell selection. The genomic DNA for the mouse GRPR was cloned from a 129/SV

[1] Corresponding author. Fax: +81-423-46-1745. E-mail: wada_e@ncnaxp.ncnp.go.jp.
[2] Present address: Department of Neurochemistry, Tokyo Institute of Psychiatry, Kamikitazawa, Setagaya-ku, Tokyo 156, Japan.

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mouse genomic library (Stratagene) using rat GRPR cDNA as a probe (6). To disrupt GRPR gene expression, the 5′ end of exon 2 was replaced with the neomycin resistance gene (pMC1 neo poly A, Stratagene). At the 5′ end of the targeting vector, the thymidine kinase gene (pMC1 tk) (kindly provided by Dr. J. -I. Miyazaki) was added to provide a means for negative selection against random integration events. The linearized targeting vector was introduced into the E14 ES cells (a gift from Dr. M. Hooper). After a 7 day incubation, G148- and gancyclovir-resistant ES cell clones were purified. Genomic DNA from these clones was digested with Eco NI, and analyzed by Southern hybridization using a 0.8 Kb 3′ probe (see Fig. 1A, B). To confirm homologous recombination events neo probe and 5′ probe (data not shown) were further used.

Generation of mutant mice. Four of 258 G148- and gancyclovir-resistant ES cell clones were correctly targeted. Of the four targeted clones, two were used for further blastocyst injection. To produce chimaeric mice, ES cells were injected into C57BL/6J (B6) blastocysts, which were implanted into pseudopregnant jcl:ICR (ICR) female. Chimaeric males were mated to B6 female, to obtain germline transmission of the targeted GRPR disruption allele. Heterozygous female mice (F1) obtained from this cross were mated with B6 males to produce F2 generation including hemizygous male (−/Y), wild-type male (+/Y), and heterozygous female (+/−) mice. In this study, we used mutant and wild-type mice backcrossed to B6 for at least three generations.

Genotype assignments. The genotypes of pups were determined by PCR (Fig. 1C) and Southern hybridization (data not shown) using tail DNA. Primer a and b (see Fig. 1A) were used to detect the 1.8 Kb fragment of the mutant allele or the 0.9 Kb fragment of the wild allele. Primer b and c (see Fig. 1A) were used to detect the 1.1 Kb fragment of the mutant allele. Primer sequences; primer a: 5′-AGA CTT CCC CAG GGA AGA CTT CTT CT-3′, b: 5′-CTT GAC ATG TAT ATT GCC TTC CAC GG-3′, c: 5′-TGA ACA AGA TGG ATT GCA CGC AGG TT-3′. PCR conditions; 94°C for 30 sec, 61°C for 2 min, and 72°C for 3 min for 30 cycles.

Reverse transcription PCR analysis of GRPR gene expression in mouse brain. Reverse transcription PCR (RT-PCR) was carried out on total RNA derived from mouse brain, to determine GRPR gene expression (Fig. 1D). Forward primer on exon 1: 5′-TCA TCT ATG TCA TCC CTG CA-3′ and reverse primer on exon 2: 5′-ATT GTA GGC ACT CTG AAT CA-3′. PCR conditions; 94°C for 30 sec, 61°C for 2 min, and 72°C for 3 min for 30 cycles. As a positive control, specific primers for β actin (14) were used.

Administration of GRP or NMB into murine brain. Microinjection of GRP, NMB or saline into the cerebroventricle of mice was performed as described by Haley and McCormick (15). Five microliters of GRP (0.5 nmol of human GRP, Research Biochemicals International, MA) was introduced into the cerebroventricle of 6 month old mutant (−/Y, n=5) and wild-type (+/Y, n=5) mice. As a control, 5 μl of saline was administered to 6 month old mutant (n=3) and wild-type (n=4) mice. Immediately after the microinjection, mice were kept at 4°C and rectal temperature was recorded for 60 min (thermister thermometer, Nihon Koden, Tokyo). After that mice were returned to room temperature (26°C) and rectal temperature was recorded for another 60 min (Fig. 2). Effects of NMB on body temperature was also studied in the same manner (0.5 nmol of porcine NMB, Research Biochemicals International, MA).

Social interaction test. Behavior of mutant (n=10) and wild-type (n=10) mice (4 month old) against an intruder were observed and monitored by video photography. Mice were housed individually for four weeks and intruders were ICR male mice housed in a group of five mice. Twenty independent intruders were prepared. Total social responses were scored by counting the number of events of sniffing, mounting, and approaching to the intruder's head. The incidence of biting and fighting was scored as total aggressive responses. After transferring an intruder to the resident home cage, social responses and aggressive responses were counted every 10 seconds for 5 min. Total numbers of social behaviors and aggressive behaviors of mutant and wild-type mice were compared (Fig. 3).

Locomotor activity. Spontaneous locomotor activity in the home cage was monitored by a sensor of magnetic field (MK-110 ANIMEX AUTO, Muromachi Kikai, Tokyo). In each experiment, mutant (n=4) and wild-type (n=4) mice (4 to 5 month old) were housed individually under 24-hr dark or 12-hr light/12-hr dark cycles and were allowed to feed freely. After two weeks in complete darkness, when endogenous biological rhythm was observed, spontaneous locomotor activity was compared (Fig. 4).

Behavioral analysis. The Morris water maze task and elevated plus-maze task were performed as described (16, 17). The tracking trace of mice was observed with video-tracking system (BTA-2, Muromachi Kikai, Tokyo). The rota-rod test (KN-75, Natsume Seisakusyo, Tokyo) and prepulse inhibition test (SR-Lab, San Diego Instrument, CA) were performed as described (18, 19). For these behavioral analyses, three to four month old mutant and wild-type mice were used.

Content of monoamine in murine brain. The brain was removed from five month old mutant (n=5) and wild-type (n=5) mice. Tissue contents of monoamine in the brain was measured by the high performance liquid chromatography (HPLC) according to Warnhoff (20).

Histological analysis of murine tissues and skeletons. Immunohistochemical staining for tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) (CHEMICON International, Inc., CA) and 5-hydroxytryptamine (5-HT, INCSTAR, MN) was performed using brain sections from 4 month old mice as described (21). For skeletal analysis, 3 month old mutant and wild-type mice were eviscerated and stained with alizarin red S and alcian blue 8GS as described (22). Other tissues were fixed in 4% paraformaldehyde and sections were stained with hematoxylin and eosin, for histopathological analyses.

Blood and physiological analysis. For insulin and glucose measurement, blood was collected from tail of mutant and wild-type mice (9 month old) after 24-hr fast. Plasma levels of the two molecules were measured by using glucose oxides method (Merck) and ELISA kit (Morinaga, Yokohama), respectively. To detect plasma levels of gastrin, amylase and thyroid stimulating hormone (TSH), blood was collected by cardiac puncture from 4 month old mice, and GASTRIN RIA KIT II (Dinabot, Tokyo), Merck auto amylase kit (Merck) and rat TSH assay system (Amersham) were used, respectively. Serum growth hormone (GH) level was measured as described (23) using blood collected from tail of two to three month old mice.

Blood pressure and heart rate of mice (three to four month old) were measured using a blood pressure monitor (MK-1030, Muromachi Kikai, Tokyo). Tail flick test was performed by using TAIL FLICK UNIT 7360 (UGO BASILE, Varese, Italy).

RESULT

Generation of GRPR mutant mouse. The GRPR gene on the X chromosome was disrupted in E14 embryonic stem (ES) cells (Fig. 1A, B). Chimeras obtained from two cell lines transmitted the mutation to heterozygous F1 female mice. Their progeny were generated by backcrossing heterozygous female mice to male C57BL/6J (B6) mice at every generation (Fig. 1C). Mutant and wild-type male offspring were born almost at the same ratio. GRPR deficient mice were viable and fertile. Gross and routine histological analyses of GRPR deficient mice did not show any obvious abnormalities in the brain, lung, and gastrointestinal tract including esophagus, stomach, duodenum, and colon.

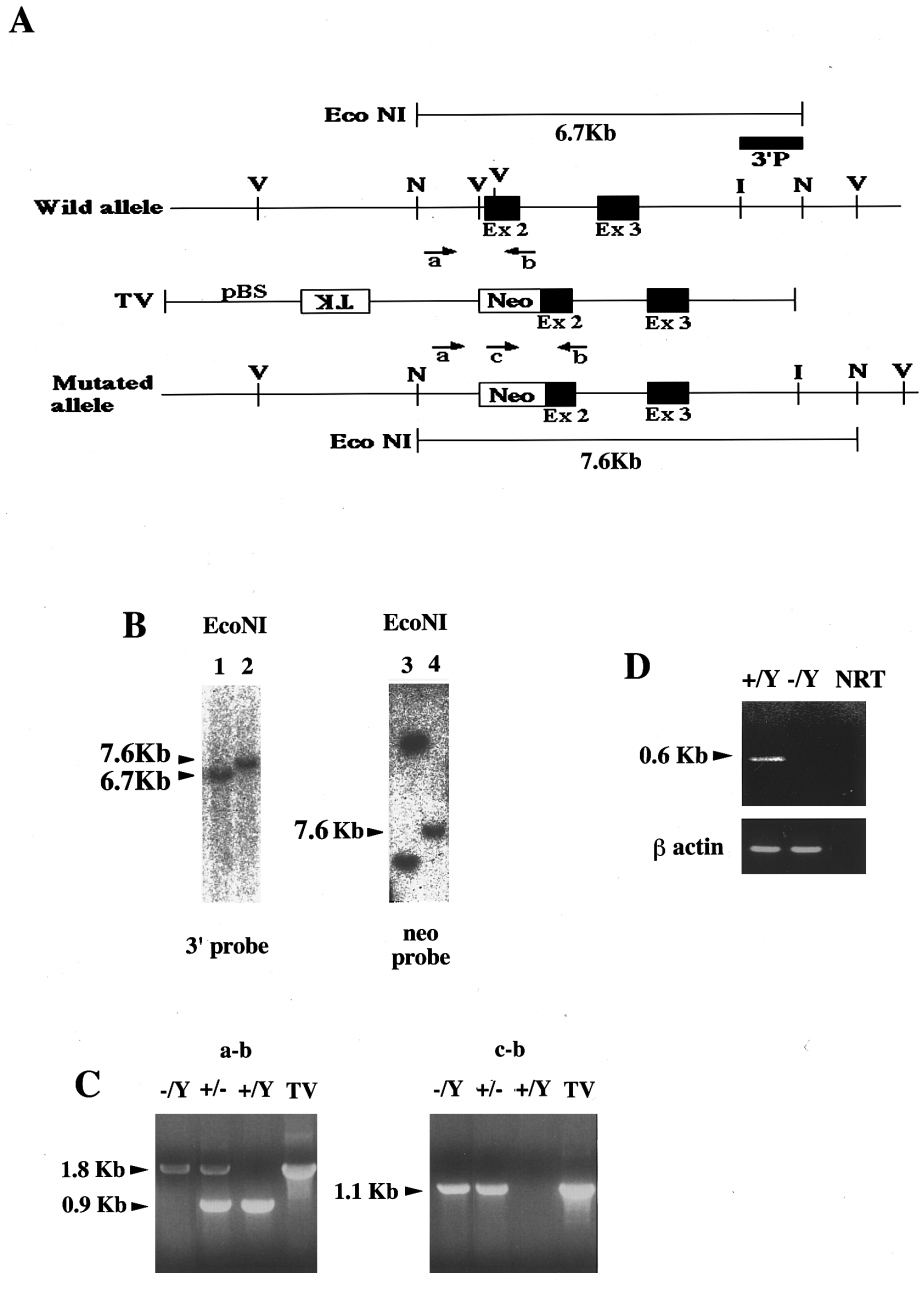

FIG. 1. Generation of GRPR knockout mice by homologous recombination in ES cells. A, Schematic representation of wild-type GRPR allele (top), targeting vector (middle), and the predicted mutated allele (bottom). Restriction sites indicated are Eco RI (I), Eco NI (N), and Eco RV (V). Abbreviations: Ex 2, exon 2; Ex 3, exon 3; Neo, neomycin resistance gene pMC1 neo poly A; pBS, pBluescript SK-vector; TK, thymidine kinase gene pMC1 tk; TV, targeting vector. B, Southern blot analysis of genomic DNA from cloned ES cells transfected with the GRPR targeting vector, showing diagnostic molecular weight changes in Eco NI fragments, from 6.7 Kb (lane 1) to 7.6 Kb (lane 2) with 3′ probe. A homologous recombination event was confirmed with a neo probe by detecting single 7.6 Kb fragment (lane 4) which is not detected in non-homologous recombinant ES cells (lane 3) after Eco NI digestion. C, PCR analysis of tail DNA from a mutant male (−/Y), a heterozygous female (+/−) and a wild-type male (+/Y) with two sets of primers (see A). Targeting vector (TV) was used as a template for a positive control. D, RT-PCR analysis of total brain RNA extracted from wild-type and mutant mice using primers specific for GRPR gene (top) and β actin (bottom). As a negative control, the same reactions were performed without reverse transcriptase (NRT).

Expression of GRPR in the mutant mouse. Absence of GRPR gene expression in mutant mice was demonstrated by RT-PCR (Fig. 1D). PCR product between two primers on exon 1 and exon 2 was detected only in RNA extracted from wild-type mouse brain. No PCR product was detected in RNA extracted from mutant mouse brain.

Functional deficit of GRPR in mutant mice was confirmed by monitoring body temperature after the intra-

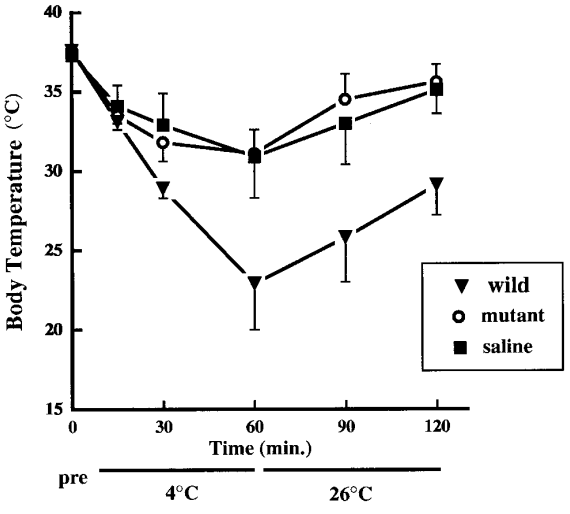

FIG. 2. Loss of functional GRPR in mutant mice as revealed by the intracerebroventricular administration of GRP. After microinjection of GRP (0.5 nmol), rectal temperature (represented by means ± SEM) were measured at 4 °C and room temperature (26°C). As a negative control, saline was administrated into wild-type mice (squares). Only wild-type mice injected with GRP (inverted triangles) showed a significant decrease in body temperature at 4 °C. Mutant mice (open circle) injected with GRP, and wild mice injected with saline showed a slight decrease in rectal temperature.

cerebroventricular administration of 0.5 nmol of GRP (Fig. 2). Before the administration of GRP, rectal temperature of wild-type mice was 37.6±0.2°C (SEM, n=5), the same as that of mutant mice (37.6±0.2°C, n=5) at room temperature (26°C). Sixty minutes after the injection at 4°C, the rectal temperature of wild-type mice was 14.7 ± 2.9°C below the baseline temperature prior to administration of GRP. The mutant mice showed a smaller decrease in rectal temperature, which was not significant (6.5±1.5°C below the base temperature). Sixty minutes after the administration of saline at 4°C, rectal temperatures showed a 6.4±2.6°C and 3.2±0.7°C decrease from the baseline temperature in the wild-type and mutant mice, respectively. To compare the potential of GRP as a thermoregulator to NMB, the same dosage of NMB (0.5 nmol) was administrated. Sixty minutes after the administration, mutant and wild-type mice showed a drop in rectal temperature 3.3±0.5°C and 4.1±0.4°C below the baseline temperature, respectively. In addition, receptor autoradiography using ^{125}I-GRP was performed to confirm the lack of functional GRPR. The ^{125}I-GRP binding on the hypothalamic region and cortex where GRPR is abundantly expressed (8) was diminished on brain sections from mutant mice (data not shown).

Social responses of mutant mice in social interaction test. Since autism is characterized by loss of communication skills, we compared GRPR null and wild-type littermates for performance in a social interaction test (Fig. 3). Behavioral responses to introduction of an intruder was observed in both mutant and wild-type control mice of the F4 and F5 generations, which are derived from the two different ES cell lines. Mutant mice showed a significant increase of total social responses ($P<0.002$, student *t*-test), whereas total aggressive responses of mutant mice showed no difference from those of control mice. Among social reactions, each individual sniffing, mounting, and approaching behavior was significantly increased in the mutant mice.

Increased locomotor activity in mutant mice. We did not observe multiple exostoses, seizures and motor discoordination in GRPR deficient mice. Mutant mice did not show any abnormality in the Morris water maze task or an elevated plus-maze task. However, we did observe that locomotor activity is increased in GRPR mutant mice. When movement of mice was monitored under dark/dark cycle, mutant mice showed significantly higher scores in the active period than control mice ($p<0.01$, Student *t*-test, Fig. 4). Increased locomotor activity during the active period also was observed in the 12-hr light/12-hr dark cycle. In the resting period, locomotor activities were similar in mutant and control mice ($p>0.05$, Student *t*-test, Fig. 4). The endogenous circadian rhythm and free-running cycle of mutant mice were not perturbed. The trace of movement of mutant mice was compared with that of wild-type mice. Total path length for the mutant mice was increased but the difference was not significant. The pat-

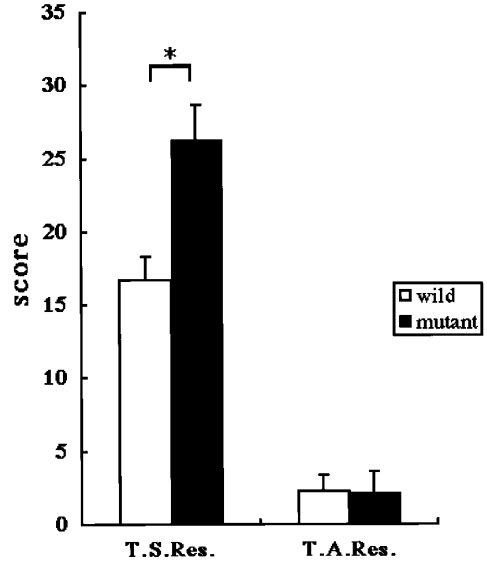

FIG. 3. Increased social responses in mutant mice. The number of total social responses (T. S. Res.) including sniffing, mounting, and approaching behaviors and total aggressive responses (T. A. Res.) including biting and fighting behaviors are represented as mean ± SEM. GRPR mutant mice (closed box) showed a significant increase in total social responses ($p<0.002$, Student's *t*-test). In contrast, there was no significant difference between mutant and wild-type (open box) mice in total aggressive responses ($p > 0.05$). Asterisks indicate a significant difference (*, $p<0.002$, Student's *t*-test).

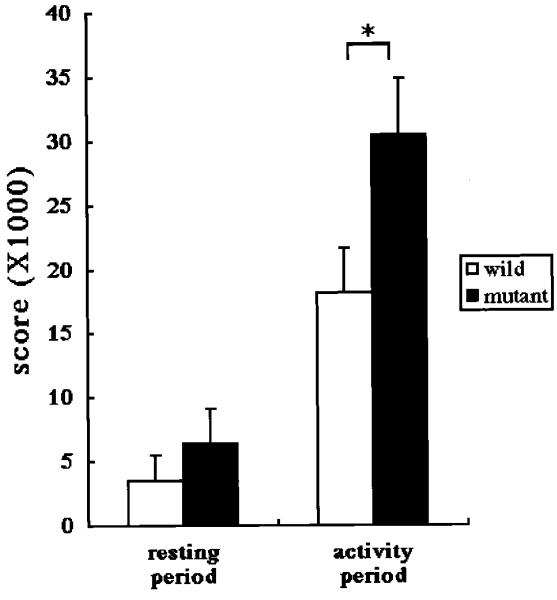

FIG. 4. Increased spontaneous locomotor activity during the active period in mutant mice. Spontaneous locomotor activity under dark/dark cycle of wild-type (open boxes) and mutant mice (closed boxes) is represented as mean ± SEM scored by the sensor of the magnetic field. In the active period, spontaneous locomotor activity of mutant mice was significantly increased ($P<0.01$, Student's *t*-test). In contrast, the mutant mice exhibited a trend toward increased activity in the resting period compared with wild-type mice, but this result did not show a significant difference ($p>0.05$). Asterisks indicate significant difference (*, $p<0.01$, Student's *t*-test).

tern of the track was not perturbed. Rearing observed by elevated plus-maze was not perturbed in the mutant mice. Mutant mice did not show any changes of scratching and grooming behaviors, rota rod test, and prepulse inhibition test. These results suggest that simple horizontal movement of spontaneous locomotor activity was increased in the mutant mice.

Contents of monoamine in mutant mice brain. Monoamines including dopamine and serotonin have been reported to be regulators of spontaneous locomotor activity (24, 25). Immunohistochemical staining for TH, 5-HT, and ChAT did not show any marked difference of staining between the brains of mutant and wild-type mice. Tissue contents of norepinephrine, dopamine, 5-HT, and their metabolites including 3-methoxy-4-hydroxyphenylglycerol, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid, and 3-methoxy-4-hydroxyphenylacetic acid were measured in the brain. However, they were not perturbed in the mutant mice.

Blood and physiological analyses on GRPR mutant mouse. Originally, GRP was purified from porcine gastric tissue using an *in vivo* bioassay for gastrin release (2). Many pharmacological studies have reported that bombesin-like peptides including GRP regulate digestion and metabolism (4). We examined blood levels of sugar, gastrin, amylase, insulin, GH and TSH. However, they were not perturbed in GRPR deficient mice.

In the cardiovascular and respiratory systems, the contraction of smooth muscle by bombesin has been reported (4). However, blood pressure, heart rate and breathing were not perturbed in GRPR deficient mice. Pharmacological effects of bombesin and GRP on behavior other than locomotor activity have been reported (4). After the administration of exogenous bombesin, decreased food intake and perception of pain have been observed. However, food intake and the tail flick test (an assay for pain perception) were not perturbed in GRPR mutant mice.

DISCUSSION

In this study, we have generated mice lacking GRPR which is one of the mammalian bombesin-like peptide receptors. Using RT-PCR, we did not detect GRPR gene expression in the mutant mouse brain. In addition, following intracerebroventricular administration of GRP and receptor autoradiography using ^{125}I-GRP, lack of functional GRPR was confirmed in the mutant mouse.

The present results indicate that GRPR mutant mice showed abnormal social behavior and hyperactivity. We observed the same phenotype in two different murine lines derived from two independent ES cells as well as in all tested generations. Although genetic effects of 129 ES strain linked to the targeted GRPR locus can not be completely ruled out, it is likely that GRPR is involved in the regulation of social responses and locomotor activities. In contrast to the hyperactivity observed in GRPR deficient mice, mice from strain 129 are reported to show significant hypoactivity compared to mice from B6 strain (26).

It is of particular interest that the GRPR mutant mice showed differences in their social and aggressive responses. Whereas some other mutant mice reported elsewhere do demonstrate aggressive behavior (27), few mutant mice are known to show abnormalities in social behavior. In a patient with autism and multiple exostoses, *de novo* balanced X chromosomal translocation which disrupted the GRPR coding region was reported (10). The GRPR gene is also shown to be located a region where Rett syndrome mapped. The syndrome is characterized by autism, apraxia, seizures and stereotypic hand movements. Autism is characterized by loss of communication skills. Together with our results of GRPR null mutant mice, these observations suggest that GRPR plays a role in regulating behavior and mentality.

Many pharmacological studies have shown that bombesin-like peptides including mammalian GRP regulate exocrine and endocrine secretions, digestion and metabolism as well as behavior (4). However, secretion of gastrin, amylase, insulin, prolactin, GH, and TSH were not perturbed in GRPR deficient mice. These

results suggest that factors other than GRP could be involved in these physiological processes. Administration of bombesin has been reported to decrease body temperature (4). However, hypothermia induced by the exogenous GRP into the mouse brain has not been reported. In the present study, we demonstrate that intracerebroventricular administration of GRP caused hypothermia in wild-type mice. This result suggests that GRP could be a major endogenous regulator of body temperature in mammalians.

At present, three mammalian bombesin-like peptide receptors, GRPR, NMBR and BRS3, have been identified. The phenotype of GRPR deficient mice differs from that of other bombesin receptor deficient mice (ref. 28 and our unpublished observation). Increased locomotor activity and abnormal social behavior were only observed in the GRPR null mutant but not in the NMBR or BRS3 null mutant mice. Although bombesin-like peptides elcit similar physiological effects *in vivo*, each bombesin receptor subtype is likely to mediate the specific function of the peptides. Further analysis of null mutant mice for each receptor subtype should provide insight into the role of the mammalian bombesin system *in vivo*.

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