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Attenuated Response to Stress and Novelty and Hypersensitivity to Seizures in 5-HT_4 Receptor Knock-Out Mice

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To study the functions of 5-HT_4 receptors, a null mutation was engineered in the corresponding gene. 5-HT_4 receptor knock-out mice displayed normal feeding and motor behaviors in baseline conditions but abnormal feeding and locomotor behavior in response to stress and novelty. Specifically, stress-induced hypophagia and novelty-induced exploratory activity were attenuated in the knock-out mice. In addition, pentylenetetrazol-induced convulsive responses were enhanced in the knock-out mice, suggesting an increase in neuronal network excitability. These results provide the first example of a genetic deficit that disrupts the ability of stress to reduce feeding and body weight and suggest that 5-HT_4 receptors may be involved in stress-induced anorexia and seizure susceptibility.

Key words: **anorexia; stress; locomotion; epilepsy; 5-HT_4 receptor; knock-out**

Introduction

Serotonin (5-HT) modulates feeding behavior as shown by the potent anorectic properties of 5-HT releasers (Bonasera and Tecott, 2000). The most widely prescribed drug for obesity was fenfluramine, a 5-HT uptake inhibitor and releaser, until negative cardiovascular side effects were reported. Fenfluramine exerts potent anorectic effects because of an indirect activation of 5-HT_{1B} and 5-HT_{2C} receptors in rodents (Lucas et al., 1998; Vickers et al., 1999, 2001). This conclusion was reached using both pharmacological and knock-out strategies. The 5-HT_{1B} receptor-null mice are insensitive to fenfluramine-induced anorexia (Lucas et al., 1998), and the 5-HT_{2C} receptor-null mice show reduced sensitivity to the same treatment (Vickers et al., 1999, 2001). It has been suggested recently that the lack of response to fenfluramine of 5-HT_{1B} knock-out mice may be attributable to a downregulation of 5-HT_{2C} receptors (Hewitt et al., 2002). The hypophagic action of the hallucinogenic drug 1-(2, 5-Dimethoxy-4-iodophenyl)-2-aminopropane appears to be mediated by the activation of 5-HT_{2C} as well as 5-HT_{2A} receptors (Schechter and Simansky, 1988). The important contribution of 5-HT_{2C} receptors in feeding behavior is also supported by the obesity of 5-HT_{2C} receptor-null mice (Tecott et al., 1995).

Less investigated is the phenomenon of stress-induced hypophagia. Hypothalamic-pituitary-adrenal axis hormones have been suggested to be involved in this stress response, at least in part, via the release of 5-HT in the medial prefrontal cortex, nucleus accumbens, amygdala, and dorsal hippocampus (Inoue et al., 1994; Ge et al., 1997; Konstandi et al., 2000). Only one study proposed that 5-$HT_{2A/2C}$ receptors could be involved in this cascade of events (Grignaschi et al., 1993).

5-HT_4 receptors are well known for their peripheral effects on the gastrointestinal tract, where they may serve as targets for therapeutic drugs used to treat dyspepsia, gastroesophageal reflux disease, gastroparesis (Eglen et al., 1995), or irritable bowel syndrome (Callahan, 2002). They are also expressed in limbic brain structures (hypothalamus, nucleus accumbens, amygdala), olfactory tubercles, hippocampus, basal ganglia (striatum, globus pallidus), and substantia nigra. Their involvement in learning and memory is well documented (Eglen et al., 1995; Bockaert et al., 1998). However, nothing is known about their contribution to feeding behavior and, furthermore, in stress-induced anorexia. Given the fact that knock-out phenotypes are often best revealed after an environmental or pharmacological challenge, we decided to investigate a series of behaviors after such challenges. Several studies have revealed disorders in novelty-induced locomotion in 5-HT receptor knock-out mice: 5-HT_{1A} (Ramboz et al., 1998), 5-HT_{1B} (Brunner et al., 1999), 5-HT_{2C} (Rocha et al., 2002), and 5-HT_{5A} (Grailhe et al., 1999); whereas these mutant mice did not exhibit any locomotion impairments in their home cage, reinforcing the view that (1)

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adaptive mechanisms are limited in such mice and (2) serotoninergic systems modulate stress responsiveness in situations such as reactivity to a novel environment.

Deficits in adaptation to stress and novel environments may be related to change in neuronal network excitability. Interestingly, 5-HT$_{2C}$ receptor-null mice exhibit an increased sensitivity to convulsant pentylenetetrazol (PTZ), which induces seizures (Tecott et al., 1995). This suggests that 5-HT inhibits neuronal network excitability, as reported previously (Jobe et al., 1973; Sparks and Buckholtz, 1985; Dailey et al., 1992). We generated mice lacking the gene encoding 5-HT$_4$ receptors by homologous recombination to study the various functions of this receptor.

Materials and Methods

5-HT$_4$ gene targeting. The gene-encoding 5-HT$_4$ receptors was cloned from a 129 Sv-J genomic library (Genome Systems, St. Louis, MO) using mouse 5-HT$_4$ cDNA probe. DNA *Pst*I fragments from 5-HT$_4$ receptor gene were then cloned in pGEM-5Zf(−). Genes encoding neomycin phosphotransferase (Neo), under the control of the phosphoglycerate kinase I promoter, were inserted into a created *Xba*I site in the exon encoding the third transmembrane domain. The DNA construct was transfected in W9.5 embryonic stem cells (800 V and 3 μF; Gene Pulser; Bio-Rad, Hercules, CA) with 30 μg of the targeting construct. The transfected embryonic stem cells were then plated onto mitomycin C-treated mouse embryonic fibroblasts for 1 week in the presence of G418 (150 μg/ml of active substance). The G418-resistant clones were screened by Southern blot with an AseI digest and a ^{32}P-labeled outside probe (300 bp *Bgl*II/*Pst*I fragment). Positive cells for the targeting event were injected into C57BL6/J blastocysts and implanted in B6CBAF1/J foster mothers, which gave birth to chimeric mice. Chimeras were mated with 129/Sv females to generate heterozygous mutant (+/−) mice on a pure 129/Sv genetic background. The resulting heterozygous mice were bred and generated 18% homozygous mutant mice (Table 2). The experiments were performed in accordance with the National Institutes of Health's *Guide for Care and Use of Laboratory Animals*.

Animals. All experiments were performed on male wild-type and 5-HT$_4$ receptor knock-out mice (4–6 months of age) on a 129/Sv genetic background. All were obtained from heterozygous breeding at the transgenic animal facility of Columbia University and Unité Propre de Recherche Centre National de la Recherche Scientifique 2580. Each mouse was identified using PCR technique. Mice were housed with food and water available *ad libitum* (n = 5 per cage) and maintained in a temperature-controlled environment on a 12 hr light/dark cycle with light onset at 6 A.M. For each experimental paradigm, we used different groups of wild-type and mutant mice.

Feeding paradigms test. In a first set of experiments, the body weight of mice of both genotypes was measured when housed in their home cage (n = 5), from days 21 to 52 after birth. A second group of mice (4 months of age) was housed individually to measure body weight, food intake, and metabolism parameters.

In a third set of experiments, a new set of mice was used to analyze their feeding responses after the restraint stress. On the basis of a previous study of rats (Rybkin et al., 1997), each experiment was divided into three periods: baseline (7 d), day of restraint stress, and recovery period (10 d after stress). Food intake (normal chow) and body weight of isolated mice were measured daily at 9:00 A.M., 2 hr after the start of light cycle for both periods. In each experiment, mice were divided into control and restraint groups matched for average weight. On the day of stress, day 8, control wild-type mice (n = 17) and 5-HT$_4$ receptor-null animals (n = 13) were individually placed in regular cages without any food or water for 110 min. Thirteen additional wild-type mice and 15 mutant animals were restrained in ventilated tubes of 50 ml of polypropylene. An interval of 3 min was maintained between each mouse to measure its body weight, and the stress was applied when necessary. After 110 min of a stress period, restrained animals returned to their regular cages with free access to classic food (16.5% crude proteins, 3.6% crude fat, 4.6% crude fiber, 5.2% Ash) and water. Food and water were provided to unrestrained animals 110 min after their manipulation.

Novelty tests. Open-field test: mice were tested for 30 min on three consecutive test days. The open-field test environment is a square chamber with an inside area that measures 43.2 × 43.2 × 30.5 cm. Mice were placed in the center and monitored with 32 infrared light sources spaced 0.5 inches apart (1.25 cm) (Med Associates, Georgia, VT) specifically adapted to record the location and the traveled path length.

Pentamethylenetetrazol-induced seizure. After a systemic injection of PTZ (60 mg/kg, 10 ml/kg) in mice of both genotypes, a trained observer evaluated their seizure profile and recorded the latency-to-seizure: latency to the first twitch, clonic–tonic seizure >5 sec with a loss of righting reflex, full tonic seizure. Finally, the duration to death was recorded. Seizure profiles were assessed through 30 min after PTZ dosing (no response, ear and facial twitching, myoclonic body jerks, clonic forelimb, generalized convulsions with tonic extension episode, status epilepticus). A score to each seizure was assigned (0, no motor seizure; 1, prone stretch posture, ataxia, tremors, immobile with hindlimb splay; 2, head search, twitches, tonic tremors, immobility; 3, slight clonic–tonic seizure <5 sec, chomping; 4, pawing, praying, trumpet, pop slight clonic–tonic seizures, increased frequency and duration; 5, clonic–tonic seizure >5 sec with loss of righting reflex; 6, crazy and tonic seizures with extension of hindlimbs, death). The occurrence of death was recorded during 30 and 60 min after injection.

Autoradiography. Frontal brain sections (15 μm) from adult (60 d) wild-type, heterozygous, and 5-HT$_4$ receptor-null mice (n = 6 mice per genotype) were thaw-mounted on gelatin-coated slides and stored (−80°C). 5-HT$_4$ receptor sites were labeled using the specific [^{3}H]-GR113808 5-HT$_4$ receptor antagonist, as reported previously (Compan et al., 1996). Briefly, sections were incubated for 30 min at 37°C in the following medium: [^{3}H]-GR113808 (Amersham Biosciences, Little Chalfont, UK; specific activity, 83 Ci/mmol; final concentration, 0.1 nM), 50 mM HEPES, pH 7.4, 10 μM pargyline (Sigma, St. Louis, MO), and 0.01% ascorbic acid. Nonspecific binding was determined on consecutive sections incubated in the presence of 10 μM 5-HT (Sigma). The labeled sections were exposed to [^{3}H]-Hyperfilms alongside tritiated polymer standards (Amersham Biosciences). The films were developed in Kodak (Rochester, NY) D-19 after a 2 month exposure at 4°C. Sections from all genotypes were processed together to obtain corresponding radiograms on the same films.

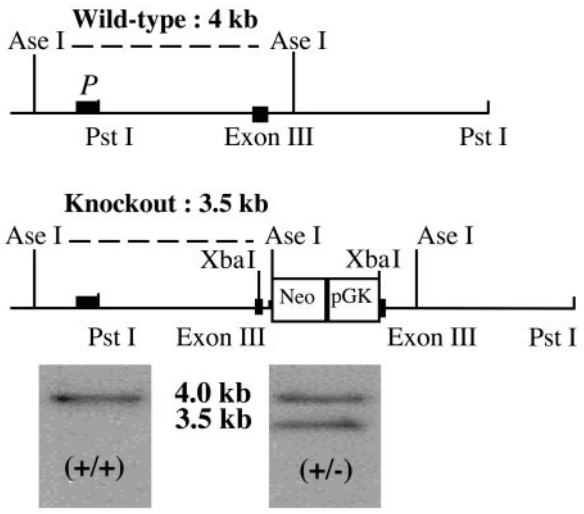

Figure 1. Targeted mutation of the 5-HT$_4$ receptor gene. *a*, Schematic diagram of a 6.5 kb 5-HT$_4$ genomic fragment encoding for the transmembrane domains II and III of 5-HT$_4$ receptors (exon III). The solid line (P) represents the external probe to detect Ase I-hybridizing restriction fragments (4 kb) from wild-type genomic DNA. *b*, Schematic representation of the 5-HT$_4$-targeting vector. The neomycin phosphotransferase gene (Neo) under the control of the phosphoglycerate kinase I promoter (pGK) is inserted in an engineered–created *Xba*I site localized in the DNA sequence encoding for the transmembrane domain III. The size of Ase I-hybridizing restriction fragments is 3.5 kb to identify the knock-out genomic DNA. *c*, Southern blot of genomic DNA from embryonic stem cells digested with Ase I and hybridized by the external probe.

Radioimmunoassay: corticosterone. Animals were singly housed for 4 d. A first blood sample was collected after a small tail incision on the fourth day. After 24 hr, the same animals were subjected to the elevated plus maze (EPM; square center platform and four arms, 30 cm above the floor), and, 30 min later, a second blood sample was taken from the incised tail over 10 min period. Blood samples were immediately centrifuged (4°C), and plasma samples were stored in −80°C until use. The concentration of corticosterone was measured using appropriate [^{125}I]-radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). In parallel, two other groups of mice were used to evaluate the amount of food consumed after a simple handling (Procedure 1) or handling and EPM (Procedure 2). Food consumption was also measured 3 hr after the beginning of the EPM (2.30 hr after the second tail incision; Procedure 3).

Data analysis. Data were analyzed using Statview 5 (Abacus Concepts, Calabasas, CA). A repeated measures ANOVA was performed on data that were obtained in multiple sessions over time. Genotype and stress were used as independent variables, if necessary. Food intake, body weight, or parameters from the elevated plus maze or open-field test session were used as dependant variables. If significant effects of genotype or stress or a genotype *x* treatment interaction, overtime or not, were found, the independent variables were split for a two-way (genotype and stress) or one-way ANOVA (genotype or treatment) analysis. For multiple comparisons, we used the Scheffé *F* test with a probability of 0.01 and 0.05 as a significant difference.

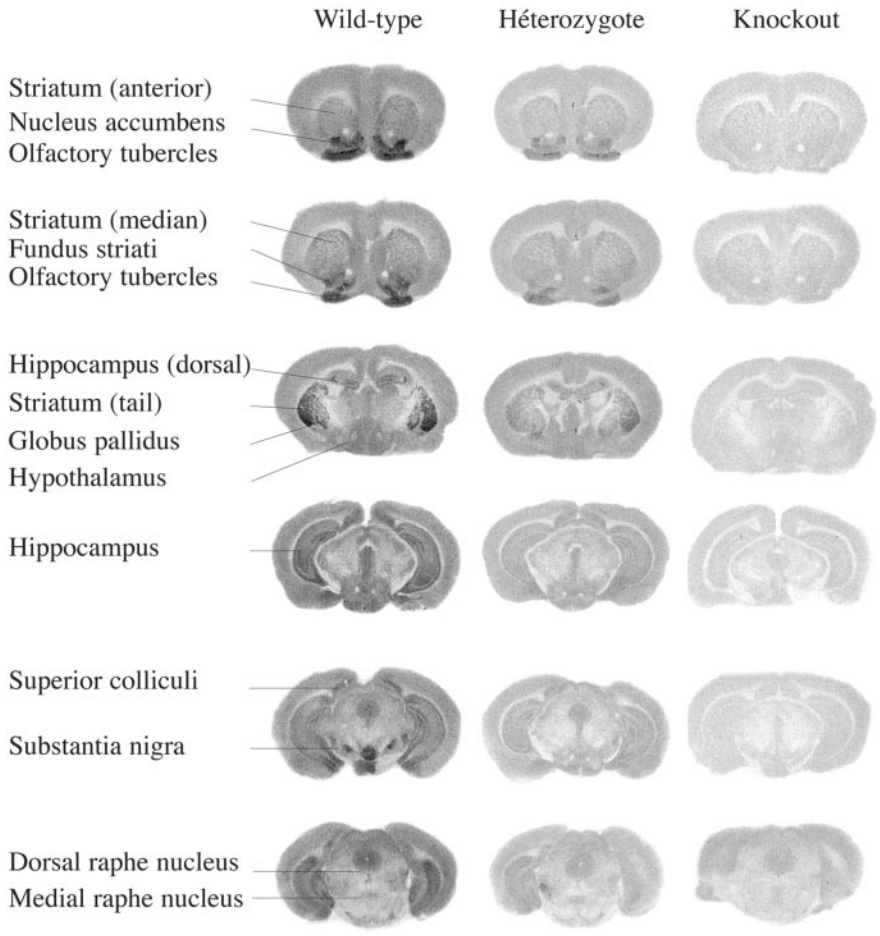

Figure 2. Radiograms of 5-HT$_4$ receptor-binding sites labeled with [^{3}H]GR113808 in the frontal brain sections from wild-type, heterozygoous, and 5-HT$_4$ receptor-null mice. In wild-type animals, the [^{3}H]GR113808 binding is more intense in the nucleus accumbens shell than the core, as analyzed previously in rat [24]. At the same levels, the olfactory tubercles and fundus striati, like the striatum tail in a next posterior level, exhibit strong labeling. The various hypothalamic nuclei show moderate density (medial preoptic area). The concentration of binding sites covered an intermediate range, although a classic distinct laminar pattern is observed in the hippocampal formation: it is weak in the dorsal and medial raphe nuclei.

Results

Generation of 5-HT$_4$ receptor-null mice

We generated 5-HT$_4$ receptor-null mice following standard procedures (Fig. 1). In this study, we used only male mice (4–6 months of age) born from couples of heterozygous mice. To verify that 5-HT$_4$ receptors were absent in homozygous mutant animals, we performed coronal brain sections from wild-type, heterozygous, and homozygous mutant (null) mice using the selective 5-HT$_4$ antagonist [^{3}H]GR113808. In wild-type mice, a heterogeneous distribution of [^{3}H]GR113808-binding sites was observed in the limbic system, hippocampal formation, or basal ganglia, in agreement with previous observations (Waeber et al., 1994; Compan et al., 1996). No specific binding site was found in 5-HT$_4$ receptor-null mice, confirming the absence of receptor proteins (Fig. 2). In heterozygous mice, the density of [^{3}H]GR113808-binding sites was decreased in all examined brain structures when compared with control animals (Table 1). In the absence of compensations in the heterozygous mice, we would expect 50% of mRNA, as described in the 5-HT$_{1A}$ receptor knock-out mice (Ramboz et al., 1998). This 50% of mRNA may translate in 50% of protein (ventral pallidum, rostral striatum). However, in some structures, such as the hippocampus, the values are considerably lower. This result may be explained by nonlinear changes in conformation of the receptor (such as dimer formation) or by a nonlinear trafficking of the receptor to various intracellular compartments (axons vs dendrites), as described previously for the 5-HT$_{1B}$ receptors (Ghavami et al., 1999).

A small Mendelian bias against null mutant mice was observed over several generations, with only 18% of the offspring of heterozygous crossings resulting in homozygous mutant mice, over a 3 year period (Table 2). As yet, we have no explanation for this unexpected non-Mendelian ratio. A cardiovascular or respiratory problem may have occurred (Edwards and Paton, 1999; Eftekhari et al., 2001; Manzke et al., 2003). Despite this bias, the mice lacking 5-HT$_4$ receptors appeared to develop normally. When grouped in their home cages, no difference in body weight was detected between wild-type and null mice during development (Fig. 3*a*) or at 4 months of age (Fig. 3*b*). In their home cage, no differences in eating (Fig. 3*c*), drinking (Fig. 3*d*), or metabolism (Fig. 3*e*,*f*) have been found between mice of both genotypes at 4 months of age. In addition, no differences in social behavior, aggression, or sleep have been observed between mice of both genotypes (data not shown).

Abnormal consumption of food after stress in 5-HT$_4$ receptor-deficient mice

We tested the motivation of wild-type and 5-HT$_4$ receptor-null mice for foods after restraint stress, a proposed animal model of anorexia nervosa (Rybkin et al., 1997). Daily total food intake and

Table 1. Densities of specific binding sites for [^{3}H]GR113808 (0.1 nM) in adult wild-type and 5-HT$_4$ receptor heterozygote mice

Regions	Bound receptor (mean ± SEM) in fmol/mg protein*	
	Wild types	Heterozygotes
Olfactory tubercles	201 ± 34	66 ± 16 (67%)
Pallidum ventral	171 ± 37	53 ± 21 (70%)
Nucleus accumbens	116 ± 28	36 ± 16 (68%)
Rostral striatum	115 ± 15	48 ± 11 (48%)
Caudal striatum	142 ± 25	18 ± 10 (85%)
Globus pallidus	90 ± 20	14 ± 8 (85%)
Hippocampus	104 ± 11	9 ± 8 (91%)

*Assuming an even concentration of 1 mg of protein per 10 mg of tissue throughout the brain.

Table 2. Ratio of wild-type, heterozygote, and homozygote mutants derived from heterozygote crossings

Wild types	Heterozygotes	Homozygotes
36% (414)	46%(532)	18%(209)

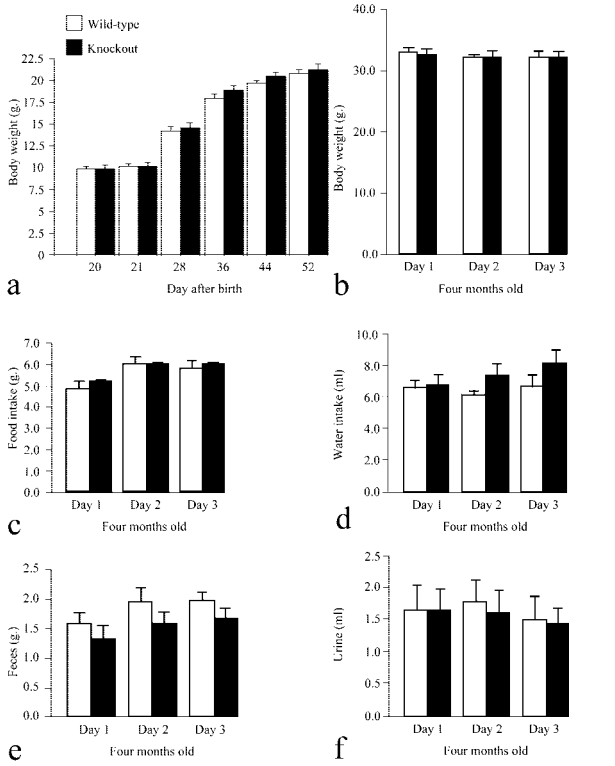

Figure 3. The body weight of 5-HT$_4$ receptor-null and wild-type mice is similar during development. *a*, Data are mean ± SEM in body weight gain for a group of 20 wild-type and knock-out animals. Repeated measures ANOVA revealed no significant difference in body weight between mice of both genotypes from day 21 to 52 after birth. *b–f*, No significant difference between wild-type ($n = 14$) and 5-HT$_4$ receptor-null ($n = 13$) mice has been found at 4 months of age in body weight (*b*), feeding (*c*), water intake (*d*), or metabolism (*e*,*f*).

body weight were measured during 8 d before the stress control period (habituation) and 10 d recovery period. The restraint stress was applied for duration of 110 min at the end of the habituation period. For all the following experiments, the animals were separated from their congeners and housed in individual cages.

Although food intake in 5-HT$_4$ receptor-null mice did not significantly differ from wild-type mice during the habituation period, they gained less body weight than wild-type mice over this same period ($F_{(6,360)} = 2.46$; $p < 0.03$). Separate ANOVAs confirmed that body weight gain was significant for the wild-type mice ($F_{(6,174)} = 4.45$; $p < 0.001$) but did not reach significance for 5-HT$_4$ receptor-null mice ($F_{(6,186)} = 0.94$) (Fig. 4*c*,*d*). Discrepancy between food intake and body weight, as reported previously for mice (Yamada et al., 2000), may be associated with changes in metabolism, resulting in an alteration in fat content. In a series of new experiments, perigonadic adipose tissue was carefully dissected. We found that, after stress, the ratio of white adipose tissue weight/body weight was lower in null than wild-type mice (−38%; 0.021 ± 0.001 vs 0.013 ± 0.013; $F_{(1,23)} = 15.92$; $p < 0.001$), again with no difference in their total body weight (wild-type, 27.32 ± 0.649, $n = 16$; knock-out, 26.38 ± 0.853, $n = 9$).

Restraint stress induced a marked decrease of food intake in wild-type mice, as compared with their unrestrained genotype counterparts (−36.4%) (Fig. 4*a*); however, this effect was lost, in part, in mutant animals (−19.6%) (Fig. 4*b*). A *post hoc* comparison between mice of both genotypes revealed that 5-HT$_4$ receptor-null mice consumed significantly more food than wild-type animals during the first 24 hr after the restraint stress (+24%; $p < 0.05$). No significant effect on food intake was detected in 5-HT$_4$ receptor-null mice 48 hr after restraint stress as compared with their baseline level (Fig. 4*b*).

Restraint stress also provoked a significant body weight loss in wild-type animals for the first 4 d of the recovery period (Fig. 4*c*). In contrast, restraint stress did not induce body weight loss in 5-HT$_4$ receptor-null mice (Fig. 4*d*). They were not totally insensitive to immobilization, because a significant difference between the body weight changes of restrained and unrestrained mutant mice was detected 24 hr after stress (Fig. 4*d*). This was because of a slight body weight gain of unrestrained mutant animals, which was also found in unrestrained wild-type mice (Fig. 4*c*,*d*). This effect may be related to the 110 min of food deprivation in both restrained and unrestrained animals (see Materials and Methods).

The reduced sensitivity of 5-HT$_4$ receptor-null mice to stress-induced anorexia was also found in a gradual stressful task (handling, elevated plus maze combined, or not, with a small tail incision) (Table 3). We then tested whether the lack of 5-HT$_4$ receptors would alter the activity of the hypothalamo-pituitary axis in response to stress. However, no difference in corticosterone levels was found between wild-type and mutant mice before or after the elevated plus maze (Fig. 5).

5-HT$_4$ receptor-null mice are less reactive in novel environments

5-HT$_4$ receptor-null mice were placed into an open field, and their locomotion was monitored for 30 min. The test was repeated for three consecutive days to evaluate habituation to a novel environment. The mutant mice displayed an overall decrease in the traveled path length on the first day of exposure, as compared with wild-type animals (−45%; $F_{(1,14)} = 6.76$; $p < 0.05$) (Fig. 6*a*,*b*) in both the periphery and center of the open field (Fig. 6*b*,*c*,*d*). During the second and third day, the horizontal activity of 5-HT$_4$ receptor-null and wild-type mice was similar. Less reactivity in 5-HT$_4$ receptor-null mice has also been found in the elevated plus maze and alley tests (data not shown). In contrast, there was no difference in locomotion between the mutant and wild-type animals in their home cages (data not shown). In addition, the mutant mice did not exhibit impairment in the rotarod test, which demonstrates that there is no deficit in motor

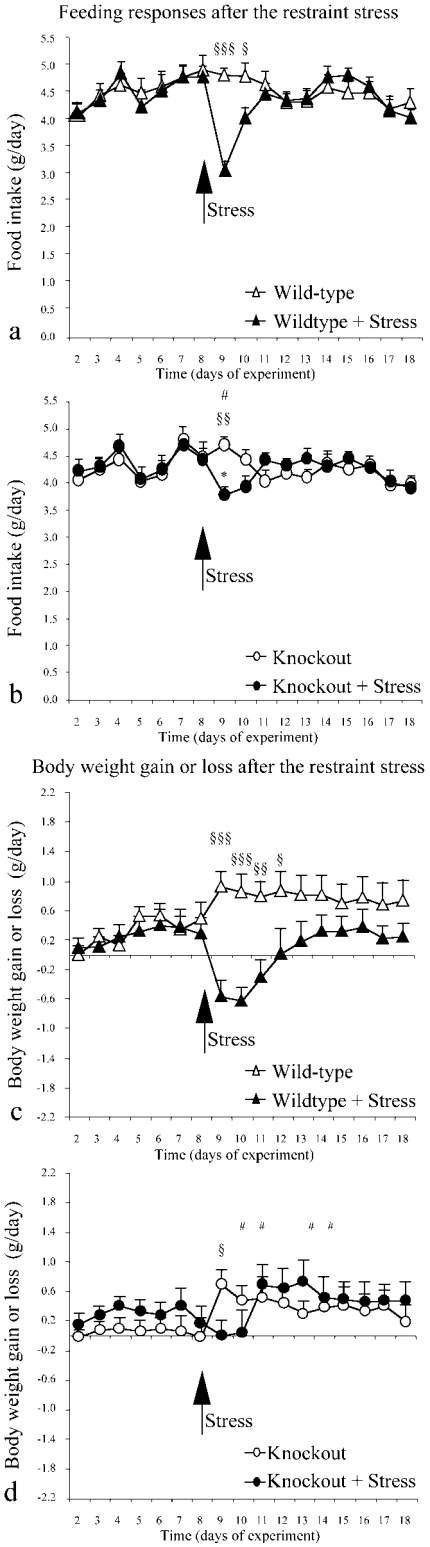

Figure 4. In 5-HT$_4$ receptor-null mice, the restraint stress lost its ability to decrease food intake and body weight. Data are means ± SEM daily total food intake and body weight gain or loss for groups of 14 wild-type (WT) and 18 WT mice plus stress (*a*,*c*), 17 knock-out (KO), and 16 KO animals plus stress (*b*,*d*). Data were measured from the first day (9:00 A.M. in the light cycle) for the habituation (8 d) and recovery periods (10 d). The 110 min acute restraint stress or immobilization was applied on day 8 (arrow). Repeated measures ANOVA indicated significant effects of stress on food intake over the recovery period ($F_{(9,567)} = 13.99$; $p < 0.0001$) and an interaction of genotype and time ($F_{(9,549)} = 2.2$; $p < 0.05$). Subsequently, ANOVA analysis for each genotype and day of experiment revealed a significant difference in food intake between genotypes 24 hr after stress ($F_{(1,61)} = 6.73$; $p < 0.05$). *a*, Stress-induced anorexia in WT animals for the 48 hr recovery period. *b*, This effect is less marked in KO mice. In parallel, repeated measures ANOVA revealed significant effects of stress on body weight gain over the recovery period ($F_{(9,522)} = 11.33$; $p < 0.0001$) and an interaction of genotype and time ($F_{(9,522)} = 2.38$; $p < 0.05$). *c*, *d*, Stress-induced, significant marked decreases in body weight gain in WT mice (*c*) but less or not effective in KO mice (*d*). Significant differences between restrained and unrestrained animals are marked (§§§$p < 0.0001$; §§$p < 0.001$; §$p < 0.05$). Significant genotype effect is noted (*$p < 0.05$), and significant genotype *x* stress interaction is noted (#$p < 0.05$).

Table 3. Feeding responses after a gradual stressful task

	Procedure 1	Procedure 2	Procedure 3
Wild type	0.82 ± 0.07 (n= 9)	0.64 ± 0.07 (n= 9)	0.20 ± 0.08**,*** (n= 8)
Knock-out mice	0.91 ± 0.08 (n=10)	0.89 ± 0.09* (n=10)	0.45 ± 0.09*,**,*** (n=10)

Data are mean ± SEM of total food intake, measured 3 hr after the beginning of adverse procedures using wild-type and 5-HT$_4$ receptor null mice. ANOVA revealed a significant effect of genotype ($F_{(1,50)} = 8.18$; $p < 0.01$) and procedure ($F_{(2,50)} = 21.81$; $p < 0.0001$). A gradual stressful paradigm (Procedure 1, isolation for 3 hr; Procedure 2, elevated plus maze, EPM 5 min and isolation for 3 hr; Procedure 3, isolation for 96 hr, small tail incision, saline injection, EPM) significantly reduced food intake in wild-type mice (**$p < 0.01$), whereas the anorectic effect was reduced in 5-HT$_4$ receptor null mice. The significance between Procedures 1 and 2 or 3 are respectively marked (**$p < 0.01$; ***$p < 0.01$). A significant genotype effect is noted (*$p < 0.05$).

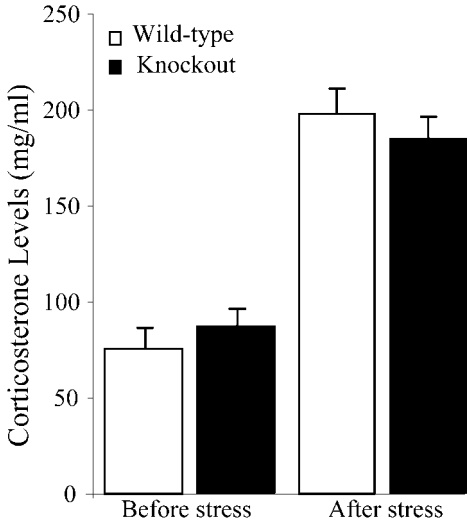

Figure 5. The absence of 5-HT$_4$ receptors did not alter a stress-induced increase in corticosterone levels. Data are mean ± SEM corticosterone levels in wild-type (n = 9) and mutant mice (n = 12) 24 hr before and 30 min after a 5 min trial in the elevated plus maze.

coordination in 5-HT$_4$ receptor-null mice (data not shown). Together, these results suggest that the 5-HT$_4$ receptor-null mice display a decreased reactivity to novelty rather than a locomotor impairment.

5-HT$_4$ receptor-null mice are hypersensitive to pentylenetetrazol-induced seizures

Beyond the possibility that inabilities to adapt may be related to impairments in neuronal network excitability, we noticed that 5-HT$_{2C}$ receptor (Tecott et al., 1995), norepinephrine (Thomas and Palmiter, 1997; Szot et al., 1999), and NPY (Erickson et al., 1996; Baraban et al., 1997) null mice exhibit both hypersensitivity to convulsants and changes in appetite. A better understanding of such coexisting mechanisms may provide clues to help prevent the increase in body weight often observed in humans treated with anticonvulsants (for review, see Jallon and Picard, 2001).

To analyze the seizure susceptibility of 5-HT$_4$ receptor-null mice, we used the response to the convulsant PTZ (a GABA$_A$ receptor antagonist) as an overall index of neuronal network excitability. The 5-HT$_4$ receptor-null mice were more sensitive to

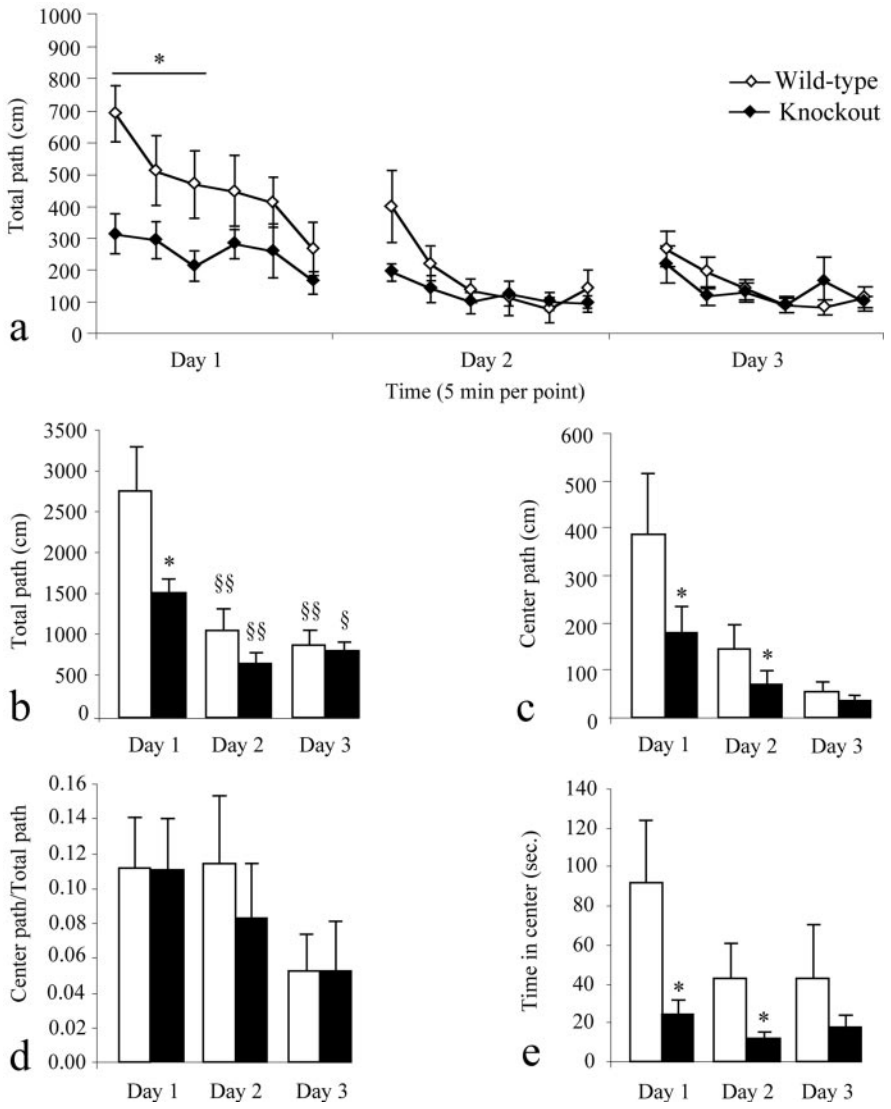

Figure 6. 5-HT$_4$ receptor-null mice are less reactive in the open field than wild-type animals. *a–e*, Naive wild-type (open bars or points) and mutant (filled bars or points) animals were placed in identical open fields, and their horizontal activity (*a–d*) and time in the center (*e*) were monitored for 30 min (5 min per point) for three consecutive days. *a*, The mutant males were less active in the open field for the first 15 min of day 1 in the open field as compared with wild-type mice, whereas this effect was not detected over the following days. *b*, In the same line, the total distance traveled was lower in mutant animals than wild-type mice on the first day of exposure but not day 2 or 3. *c*, *d*, The absence of significant difference in the center/total path ratio between mice of both genotypes (*d*) indicated that this lower activity was detected in any part of the open field, as illustrated for the center (*c*). The mutant mice spent less time in the center during the first two consecutive days (*e*), which suggests a slight hyperanxiety-like behavior in the absence of 5-HT$_4$ receptors. Significant genotype effect is noted (*$p < 0.05$). Significant differences in path between days for the wild-type or null mice are noted (§§$p < 0.01$; §$p < 0.05$).

PTZ-induced seizures compared with wild-type animals, as shown in an increased number of deaths at 60 min after treatment (Fisher exact test; $p < 0.04$) and decreased latency to both death and tonic seizure ($F_{(1,27)} = 6.44$; $p < 0.02$) (Fig. 7).

Discussion

A number of studies suggest that an increase in 5-HT neuromodulation may contribute to stress-induced anorexia. Stress-related behavioral paradigms, such as conditioned fear, increase 5-HT metabolism and release in the medial prefrontal cortex, nucleus accumbens, amygdala, and dorsal hippocampus (Inoue et al., 1994; Ge et al., 1997; Konstandi et al., 2000). In particular, restraint stress increases 5-HT turnover in the hypothalamus and amygdala in mice and rats (Konstandi et al., 2000). However, the identities of the 5-HT receptors involved in mediating stress-induced decreases in food intake are primarily unknown. This study provides the first evidence that stress-induced hypophagia may be mediated by 5-HT$_4$ receptors.

The ability of stress to decrease food intake is not only attributable to an increase in the activity of the serotonergic systems but also to the hyperactivity of hypothalamo-pituitary adrenal (HPA) axis (Beck, 2000). Peptides of the corticotropin-releasing hormone (CRH) family, such as stresscopin or urocortin, induce decreases in food intake (Momose et al., 1999). The reciprocal influences between serotonergic systems and HPA axis (Lopez et al., 1999) have made it difficult to identify a clear neurochemical cascade underlying the influence of stress on feeding behavior. A working hypothesis would be that an increase in the activity of the HPA axis could induce an elevation in 5-HT, which, in combination with stress hormones, induces a decrease in food intake. In keeping with this hypothesis, CRH has been shown to stimulate the activity of serotoninergic neurons (Kirby et al., 2000; Lowry et al., 2000). In addition, repeated injections of corticosterone enhance the excitatory effect of 5-HT$_4$ agonists on hippocampal CA1 neurons (Zahorodna et al., 2000). However, despite of the ability of CRH to decrease food intake (Momose et al., 1999), mutant mice lacking CRH are still sensitive to stress-induced hypophagia (Weninger et al., 1999). This finding suggests that other mechanisms are involved. We found that the absence of 5-HT$_4$ receptors did not affect the increase in corticosterone levels after stress. Our results could be explained by two related mechanisms. First, stress induces an increase in the levels of 5-HT that activates 5-HT$_4$ receptors and decreases food intake. Secondly, stress induces an increase in stress hormones, which indirectly increases the activity of 5-HT$_4$ receptors, which in turn results in a decrease in food intake. Numerous studies indicate that corticosterone levels can modify the density, as well as the mRNA levels of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. In particular, one recent study indicates that rats chronically treated with corticosterone develop some extra sensitivity of presynaptic 5-HT$_{1B}$ receptors in the hypothalamus (Gur et al., 2001).

Other 5-HT receptors have also been shown to regulate feeding and body weight. For example, the body weight of adult 5-HT$_{2C}$ receptor-null mice is greater than that of wild-type animals (Tecott et al., 1995). These results suggest the presence of at least two modes of action of 5-HT to regulate body weight. In baseline conditions, the body weight is primarily regulated via the 5-HT$_{2C}$ receptors, whereas after an unusual stressful event, 5-HT$_4$ receptors may become involved.

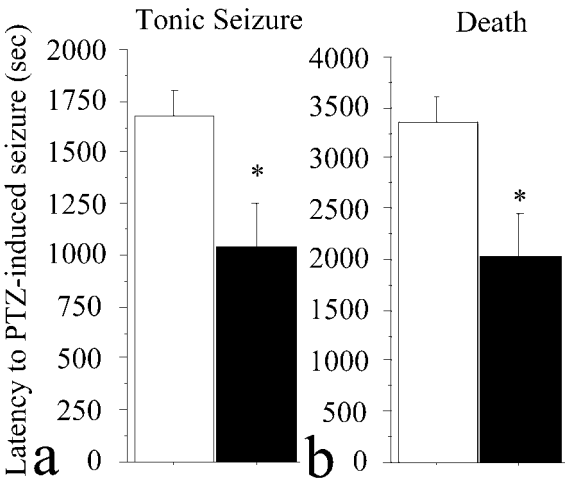

Figure 7. The 5-HT$_4$ receptor-null mice are hypersensitive to the convulsant GABA$_A$ receptor PTZ compared with wild-type animals (open bars). *a*, *b*, Data are means ± SEM latency (s.) after the PTZ administration-induced tonic seizures (*a*) and death (*b*) in mice of both genotypes. Significant genotype effect is noted (*$p < 0.05$).

We found that 5-HT$_4$ receptor-null mice are less reactive to different novel environments, whereas their locomotor activity was not altered in their home cages. These results suggest that the absence of 5-HT$_4$ receptors results in an attenuation of the motor responses induced by novel environments. 5-HT$_{1A}$ receptor-null mice are also less reactive to novelty-induced locomotion, whereas it is the inverse for 5-HT$_{1B}$, 5-HT$_{2C}$, or 5-HT$_{5A}$ receptor-null mice (Brunner et al., 1999; Grailhe et al., 1999; Rocha et al., 2002). These data suggest that a permanent absence of the genes encoding 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2C}$ 5-HT$_{5A}$, or 5-HT$_4$ receptors provokes either a hyporeactivity or hyperreactivity to novelty. Interestingly, a lesion of serotonergic neurons enhanced novelty-induced locomotion (Geyer et al., 1976). However, various modes of 5-HT depletion induce different changes in the density of 5-HT$_{1B}$ and 5-HT$_{2A/2C}$ receptors in the rat basal ganglia, nucleus accumbens, and frontoparietal cortex (Compan et al., 1998a,b), which may in turn induce different motor outcomes. Together, these findings suggest that 5-HT$_4$ receptors modulate the activity of neuronal circuits involved in the response to stress and novelty.

Similar to the 5-HT$_{2C}$ receptor-null mice (Tecott et al., 1995), we found that 5-HT$_4$ receptor-null mice were more sensitive to PTZ-induced seizure. The GABA$_A$ receptor antagonist PTZ blocks the neurotransmission of GABA, which in turn increases the excitability of neuronal networks. This result suggests a tonic inhibitory influence of 5-HT$_4$ receptors on the excitability of neurons either in adulthood or during development. Several studies have described a 5-HT$_4$ receptor-mediated increase in neuronal excitability via the blockade of K^+ channels in cortical and hippocampal neurons (Dumuis et al., 1988; Bockaert et al., 1990; Chaput et al., 1990; Fagni et al., 1992; Monferini et al., 1993; Ansanay et al., 1995). This could ultimately contribute to the facilitation of neuromediator release. Because 5-HT$_4$ receptors are localized on neurons expressing GABA (Siarey et al., 1995; Compan et al., 1996), decreased GABA release in 5-HT$_4$ receptor-null mice is likely. Indeed, activation of 5-HT$_4$ receptors increases the frequency of postsynaptic GABA$_A$ and GABA$_B$ IPSP recorded in the hippocampal dentate gyrus (Bijak and Misgeld, 1997). It is difficult to correlate a general decrease in GABA transmission and absence of 5-HT$_4$ receptors all over the brain and feeding behaviors, because stimulation of GABA receptors inhibits or activates food intake when respectively injected in the lateral hypothalamus (Maldonado-Irizarry et al., 1995) or in the shell of the nucleus accumbens and in the paraventricular nucleus of the hypothalamus (Stratford and Kelley, 1997; Pu et al., 1999). Decreased GABA transmission is not always correlated with increased body weight and eating, because hyperanxiety, related to a decrease in GABA transmission, coexists with both anorexia and bulimia (Chesters et al., 1998). This point opens the possibility that the inability of null mice to adapt to the restraint stress may be related to a higher level of anxiety after stress.

Our results suggest that 5-HT$_4$ receptors modulate the activity of neuronal circuits involved in stress-induced hypophagia, reactivity to novelty, and PTZ-induced seizures. It is therefore conceivable that 5-HT$_4$-specific ligands will be useful in treating eating disorders.

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