

# Significant Increase in the Aggressive Behavior of Transgenic Mice Overexpressing Peripheral Progastrin Peptides: Associated Changes in CCK<sub>2</sub> and Serotonin Receptors in the CNS

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The gastrin precursor peptide, progastrin (PG), is secreted from enteroendocrine cells in the intestine and increased in patients with hypergastrinemia and colorectal cancers. In recent years, we and others have demonstrated an important role of PG peptides in colorectal carcinogenesis, and were surprised to note significant changes in the behaviors of transgenic mice overexpressing PGs. In the present studies, we examined emotional behaviors of transgenic mice overexpressing PG in the intestinal and peripheral circulation. Aggression, locomotor activity and anxiety-like behaviors of the homozygous transgenic (Tg/Tg) mice and the wild-type (WT) littermates were examined by intruder/resident test, open field and elevated plus maze, respectively. A significant increase in the aggression, locomotor activity, and anxiety-like behaviors was detected in the Tg/Tg vs WT mice. As CCK, CCK<sub>2</sub> receptors (CCK<sub>2</sub>R), and 5-HT<sub>1A</sub> receptors (5-HT<sub>1A</sub>R) in the CNS play an important role in these behaviors, possible changes in the expression of CCK and CCK<sub>2</sub>R and the density of CCK<sub>2</sub>R and 5-HT<sub>1A</sub>R were determined by either real-time RT-PCR or autoradiography of ligand binding assays. The results suggest that the expressions of CCK and CCK<sub>2</sub>R were increased in the hypothalamus, and the density of CCK<sub>2</sub>R were increased in the hypothalamus and amygdala of Tg/Tg vs WT mice. Similarly, the density of 5-HT<sub>1A</sub>R was increased in the hypothalamus. Our results suggest that an upregulation of the CCK response system and 5-HT<sub>1A</sub>R in the hypothalamus of Tg/Tg mice may mediate the alterations in the observed behaviors of these mice.

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**Keywords:** anxiety-like; locomotor activity; aggression; CCK; CCK<sub>2</sub> receptors; 5-HT<sub>1A</sub> receptors

## INTRODUCTION

The gastrin gene is expressed normally by enteroendocrine (G) cells in the antrum of the stomach (Dockray, 1999). Gastrin gene products are processed within the G cells, and the full-length precursor peptide (progastrin<sub>1–80</sub>, PG) is converted into glycine extended gastrins (G-gly). PG and G-gly represent the non-amidated forms of gastrins. They are subsequently amidated at the carboxy-terminal end, generating amidated forms of gastrins (G17 and G34). Under physiological conditions, the amidated gastrins represent the major circulating forms of gastrins in humans and rodents. Amidated gastrins play a critical role in meal-

stimulated acid response (Dockray, 1999) and in the renewal of gastrointestinal mucosal cells (Johnson, 1981).

Under physiological conditions, the full-length precursor peptide, PG, is generally not detected in the circulation (Dockray, 1999). In certain diseased states, including hypergastrinemia, elevated levels of circulating PG (0.1–> 1.0 nM) are measured, as recently reviewed (Rengifo-Cam and Singh, 2004; Konturek *et al*, 2002). We and others have demonstrated that non-amidated gastrins, including PG, exert growth factor and antiapoptotic effects on small and large intestinal mucosal cells, *in vitro* (Baldwin *et al*, 2001; Singh *et al*, 2003; Wu *et al*, 2003) and *in vivo* (Cobb *et al*, 2004; Ottewell *et al*, 2003; Singh *et al*, 2000a, b; Wang *et al*, 1996). Transgenic mice overexpressing either pharmacological levels of PG (> 10–100 nM; hGAS mice) or pathophysiological levels of PG (0.1–1 nM; fatty acid-binding protein (Fabp)-PG mice), demonstrate a significant increase in the proliferation of the colonic crypt cells (Cobb *et al*, 2004; Singh *et al*, 2000a; Wang *et al*, 1996) and a significant increase in colon carcinogenesis in response to the colon

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specific carcinogen azoxymethane (Cobb *et al*, 2004; Ottewell *et al*, 2003; Singh *et al*, 2000a,b).

During the course of our studies, we observed a behavior alteration in the transgenic mice overexpressing PG (hGAS and Fabp-PG mice). For example, male transgenic mice demonstrated extensive fighting when placed together from different cages, as discussed previously (Singh *et al*, 2000a,b). We hypothesized that the alteration in the behaviors is mediated by the adaptive changes-induced by increased expression of PG in the intestine and/or in the circulation. In the present studies, we examined aggression and anxiety-like behaviors in Fabp-PG mice and their wild-type (WT) littermates. Several receptor subtypes are known to mediate the biological effects of PGs and gastrins, including CCK<sub>2</sub>R (cholecystokinin-2 receptor) and PG preferring receptors (Rengifo-Cam and Singh, 2004; Singh and Cobb, 2004), such as Annexin II (Singh *et al*, 2006). CCK and CCK<sub>2</sub>R in the brain are known to regulate anxiety, locomotor activity, and defensive behaviors (Hokfelt *et al*, 2002; Rotzinger and Vaccarino, 2003; Wang *et al*, 2005; Zanoveli *et al*, 2004; Rehfeld, 2000). In order to examine the mechanisms that may be mediating the significant differences in the behaviors of Fabp-PG *vs* WT mice, CCK, CCK<sub>2</sub>R, and PG-R were measured in the brains of these mice. Furthermore, as hypergastrinemia increases plasma serotonin concentration in human (Norton *et al*, 2004) and 5-HT is related to emotional regulation (Graeff *et al*, 1996), we additionally determined 5-HT<sub>1A</sub> receptor in the brains of Fabp-PG *vs* WT mice.

## MATERIALS AND METHODS

### Fabp-PG Mice

Fabp-PG mice were generated as described recently (Cobb *et al*, 2004). The fatty Fabp promoter was used to drive the expression of human gastrin cDNA in the small and large intestines. The expression of Fabp requires the presence of fatty acids to active their promoter. Thus, Fabp is physiologically expressed postnatally and selectively in the intestinal cells. By using Fabp promoter to drive the expression of hPG, we expected that the overexpression of hPG would occur postnatally and selectively in the intestinal cells. We have previously confirmed that Fabp-PG mice express the human gastrin gene (and hence PG), mainly in the small and large intestine, with no expression of human gastrin gene in the brain and other tissues (Cobb *et al*, 2004). The Fabp-PG mice were positive for 0.5–2.0 nM PG in the plasma, whereas the WT mice were negative for detectable levels of hPG, as reported previously (Cobb *et al*, 2004). Circulating levels of total amidated gastrins (including human and mouse) (G17 and G34) were similar in Fabp-PG and WT mice (~30 pM) (Cobb *et al*, 2004).

The Fabp-PG transgenic mice and their WT littermates were bred and housed in separate cages in the animal housing facility at UTMB, using protocols approved by the Institutional Animal Care and Use Committee (IACUC) at UTMB, as described previously (Cobb *et al*, 2004). Age matched (2–4-month-old) male homozygous Fabp-PG mice (Tg/Tg) and WT mice were used (Cobb *et al*, 2004). Different groups of mice were used for the behavioral tests, real-time PCR and autoradiographic assays, described below.

### Behavioral Tests

**Resident-intruder test.** Intruder/resident test was used to evaluate aggression behavior. The test was conducted as described by Holmes *et al* (2002). Briefly, male mice (resident) were individually caged for 1 week before the test. On the test day, an intruder (WT FVB/N mice purchased from Taconic farm) was placed in the resident home cage for 15 min. The latency of the first attack (bites and sideway threats), the number of attacks, the duration of attack (total time of attacks), and the duration of social interaction (sniffing and grooming) were recorded by a video camera and then scored by an investigator who was blinded to the group being examined, using behavioral scoring software ('Hindsight', Scientific Programming Services, Wokingham, UK).

**Open field test.** Open field test was conducted as described previously (Holmes *et al*, 2003). The open field was a square arena (40 × 40 × 35 cm) with clear Plexiglas walls and floor, evenly illuminated by white overhead fluorescent lighting. Mice were individually placed in the center of the open field and left to freely explore for 45 min. Activity was measured by a computer-assisted Photobeam Activity System (PAS) with Flexfield (San Diego Instruments, Inc., San Diego, CA). The distance of travel and the duration of activity were recorded in every 5 min interval. The program was not set up for dividing the central and peripheral movements. Thus, the data indicated the total locomotor activity.

**Elevated plus maze.** The elevated plus-maze test was conducted as previously described (Holmes *et al*, 2003). The elevated plus-maze apparatus (San Diego Instruments, San Diego, CA) was comprised of two opened arms (30 × 5 × 0.5 cm) and two enclosed arms (30 × 5 × 15 cm) that extended from a common central platform (5 × 5 cm). A small raised lip (0.5 cm) around the perimeter of the open arms prevented the mouse from falling. The apparatus was constructed from polypropylene and Plexiglas, with a white floor and clear walls, and elevated to a height of 38 cm above floor level. Mice were individually placed on the center square, facing an open arm, and allowed to freely explore the apparatus under an even overhead fluorescent lighting (200 lux) for 5 min. Open and closed arm entries (all four paws in an arm), time spent in the open arms and rears within the closed arms were scored by an investigator who was blinded to the group being examined, using behavioral scoring software ('Hindsight', Scientific Programming Services, Wokingham, UK). Time spent (as seconds and % of total testing time) and entries (number and % of total entries) into the open arms were used as parameters for exploratory anxiety-like behavior. The longer the time spent in open arms, the less anxious the animal is. The entries to the enclosed arms and rearing were the references for locomotor activity.

### Real Time RT-PCR Analysis for Measuring mRNA of Mouse Gastrin, CCK, and CCK<sub>2</sub> R in Brain Tissues

The hypothalamus, hippocampus, midbrain, cortex, and cerebellum were carefully dissected out within a few minutes after decapitating the mice, samples placed in

pre-labeled vials, and immediately frozen in liquid nitrogen. The frozen tissues were pulverized and processed for making cDNA from cellular RNA by our published methods (Cobb *et al*, 2002). Real time RT-PCR was conducted with the cDNA samples for measuring relative levels of CCK<sub>2</sub>R transcripts in the brain regions of Fabp-PG and WT mice. The Applied Biosystem assay-on-demand 20 × assay mix of primers and TaqMan MGB probes (FAM<sup>TM</sup> dye-labeled) for mouse gastrin cDNA (Mm00439059\_g1 based on mCG20525), mouse CCK cDNA (Mm00446170\_ml based on NM\_031161 accession number), and mouse CCK<sub>2</sub>R cDNA (Mm06432329\_m1, based on NM007627 accession number) were used. The pre-developed 18S rRNA (VIC<sup>TM</sup>-dye labeled probe) in TaqMan<sup>®</sup> assay reagent (P/N 4319413E) was used as an endogenous control. In the above assay kit, the primers are designed to span exon-exon junctions in order to avoid the detection of genomic DNA. The TaqMan one-step PCR master mix reagent kit (P/N 4304437) was used as the major reagent in these assays. The cycling parameters used for PCR amplification of the indicated transcripts from cellular cDNA (equal to 20–50 ng RNA) included: UNG activation for 2 min at 50°C, AmpliTaq activation for 10 min at 95° and annealing/extension at 60°C for 1 min × 40 cycles, using the Applied Biosystems AB17000 cycler. Duplicate C<sub>T</sub> values were analyzed in Microsoft Excel using the comparative C<sub>T</sub>( $\Delta\Delta C_T$ ) method as per the instructions of the manufacturer (Applied Biosystems). The relative amounts of target transcript ( $2^{-\Delta\Delta C_T}$ ) were obtained by normalizing the values to an endogenous reference (18S).

### Autoradiography of Receptor Binding

**Tissue preparation.** Mice were killed by rapid cervical dislocation. Brains were removed and frozen immediately in dry ice-cold isopentane for 10 s, transferred to dry ice for 10 min until completely frozen, and then stored at ~80°C. The brains were cut into 15- $\mu$ m-thick coronal sections in a cryostat (Vibratome, St Louis, MO). The sections containing hypothalamus (bregma -0.7 to -1.94 mm) were thaw-mounted onto chromalum/gelatin-coated glass slides and stored at ~80°C.

**CCK<sub>1/2</sub> receptor binding.** Autoradiography of <sup>125</sup>I-CCK8 binding was used for examining relative levels of CCK<sub>1/2</sub> receptors, as described by Reubi *et al* (1997). Briefly, the brain sections were pre-incubated for 30 min at room temperature in a pre-assay buffer (50 mM Tris-HCl pH 7.4, 130 mM NaCl, 5 mM MgCl<sub>2</sub>, 4.7 mM KCl, and 0.2% BSA). The slides were washed with the assay buffer (50 mM Tris-HCl pH 7.4, 130 mM NaCl, 5 mM MgCl<sub>2</sub> and 4.7 mM KCl) to remove BSA, and were then incubated with 0.0125 nM <sup>125</sup>I-CCK-8 (sulfated, labeled with Bolton and Hunter reagent, 2000 Ci/mmol = 0.5 pmol/ $\mu$ Ci, Amersham Biosciences, Piscataway, NJ) at room temperature for 90 min in a buffer containing 50 mM Tris-HCl (pH 7.4), 130 mM NaCl, 5 mM MgCl<sub>2</sub>, 4.7 mM KCl, 1 mM dithiothreitol, 0.25 TIU/ml aprotinin, and 0.0025% bacitracin. The nonspecific binding was defined by additionally incubating the slides in the presence of 1  $\mu$ M CCK-8. The brain sections were washed four times with 50 mM Tris-HCl (pH 7.4) for 10 min at 4°C and rinsed with ice-cold ddH<sub>2</sub>O, followed by rapid air

drying with a hair drier. The slides were then exposed to Kodak Biomax MR film at -80°C for 7 days. A set of <sup>125</sup>I microscales (Amersham Biosciences, Piscataway, NJ) was exposed with the slides to calibrate the optic density into fmol/mg of tissue equivalent. Following exposure, brain images were digitized and analyzed using AIS image software (Imaging research Inc., Ontario, Canada). The gray scale density readings were calibrated to fmol/mg of tissue equivalent using the <sup>125</sup>I microscale. Specific <sup>125</sup>I-CCK8 binding in each brain region was determined by subtracting the nonspecific binding from the total binding in each region. Data for each individual subject and brain region are the mean of four-six adjacent sections.

**PG binding in the brain.** In order to analyze possible presence of specific PG binding sites in the brain, brain sections were incubated with <sup>125</sup>I-rhPG in the absence or presence of thousand-fold excess non-labeled rhPG. The full-length recombinant human PG peptide (rhPG) was generated in our laboratory as described previously (Singh *et al*, 2003), and the purified rhPG peptide was radio-labeled with <sup>125</sup>I as described previously (Singh *et al*, 2003). The assay procedure for measuring relative binding of <sup>125</sup>I-rhPG to the tissue sections was similar to that described above for <sup>125</sup>I-CCK8 binding assay. Briefly, the tissue sections were incubated with ~3  $\mu$ Ci/ml of <sup>125</sup>I-rhPG in the presence or absence of 1  $\mu$ M rhPG, in order to measure total vs nonspecific binding. The brain sections were exposed to the Kodak film for 2 weeks, and the autoradiographic data analyzed as described above for CCK<sub>1/2</sub>R.

**5-HT<sub>1A</sub> receptor binding.** Autoradiography of <sup>125</sup>I-MPPI binding for the density of 5-HT<sub>1A</sub> receptors was conducted as described previously (Li *et al*, 2004). The brain sections were pre-incubated for 30 min in an assay buffer (50 mM Tris-HCl, pH 7.4, containing 200 nM MgCl<sub>2</sub>) and then incubated with <sup>125</sup>I-MPPI (0.14 nM in the assay buffer) for 2 h at room temperature. Nonspecific binding was defined in the presence of 10<sup>-5</sup> M 5-HT. Slides were then washed twice with the assay buffer at 4°C for 15 min and rinsed with cold ddH<sub>2</sub>O. After being air blow-dried, the slides were exposed to Kodak Biomax MR film. The films were exposed at -80°C for 3 days. A set of <sup>125</sup>I microscales (Amersham Biosciences, Piscataway, NJ) was exposed with the slides to calibrate the optic density into fmol/mg of tissue equivalent. Following exposure, brain images were digitized and analyzed using AIS image software (Imaging research Inc., Ontario, Canada). The gray scale density readings were calibrated to fmol/mg of tissue equivalent using the <sup>125</sup>I microscale. Specific <sup>125</sup>I-MPPI binding in each brain region was determined by subtracting the nonspecific binding sites from the total binding sites in each region. Data for each individual subject and brain region are the mean of four adjacent sections.

### Data Analysis and Statistics

The data are presented as mean  $\pm$  SEM of values from 10 separate mice, unless otherwise noted. All of the data were analyzed by one-way analysis of variance, followed by Student Newman-Keuls *post hoc* test. A computer program,

StatView (Abacus Concepts Inc., Berkeley, CA) was used for all the statistical analysis.

## RESULTS

### Behavioral Alterations

**Resident-intruder test.** The male Fabp-PG transgenic mice demonstrated a significant decrease in the latency of the first attack ( $F_{(1,15)} = 38.17$ ,  $P < 0.0001$ ), an increase in the number of bite attacks ( $F_{(1,15)} = 12.93$ ,  $P = 0.0027$ ), and an increase in the duration of attacks ( $F_{(1,15)} = 8.25$ ,  $P = 0.0117$ ) (Figure 1), compared to that measured for the male WT mice. On the other hand, the social interaction behaviors (sniffing and grooming) were not significantly different between the male Fabp-PG and the corresponding WT mice (Figure 1).

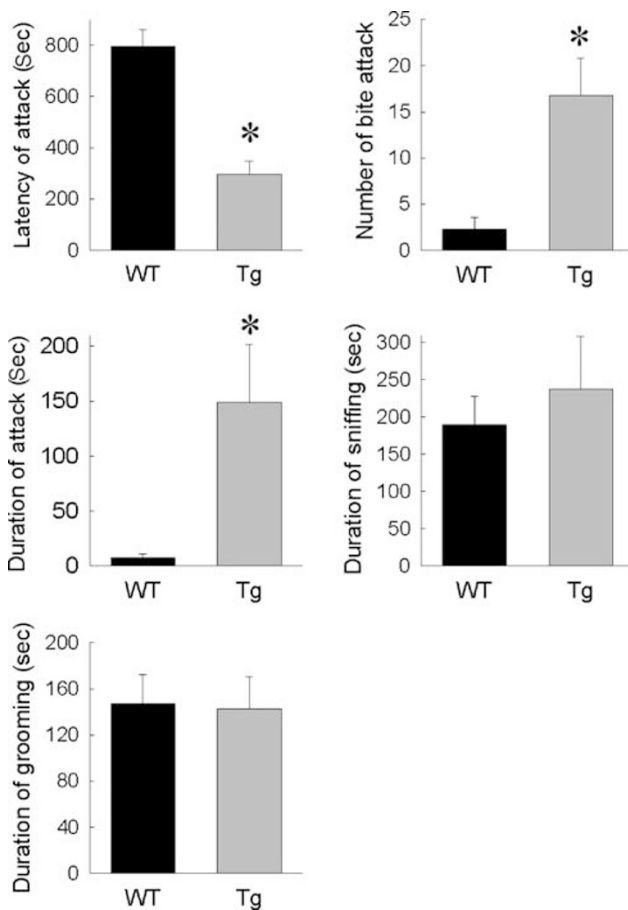
**Open field test.** The locomotor activity of the Fabp-PG mice was significantly increased compared to that of the WT mice in an open field test. The total distance traveled (WT  $8641 \pm 284$  cm vs Fabp-PG  $11629 \pm 289$  cm), and the duration of locomotor activity (WT  $1947.1 \pm 49.6$  s vs Fabp-PG  $2143.2 \pm 37.4$  s) were significantly higher for the Fabp-PG vs WT mice. As shown in Figure 2, the locomotor activity of WT mice was gradually reduced during the 45 min testing

period. On the other hand, the locomotor activity of the transgenic mice was not reduced until 40 min after the initiation of the test. As a result, the difference in the locomotor activity of Fabp-PG vs WT mice was especially evident at later time periods of testing. Overall, the locomotor activity of the transgenic mice was significantly higher both in the initial time points and throughout the testing period, compared to that of the WT mice (Figure 2).

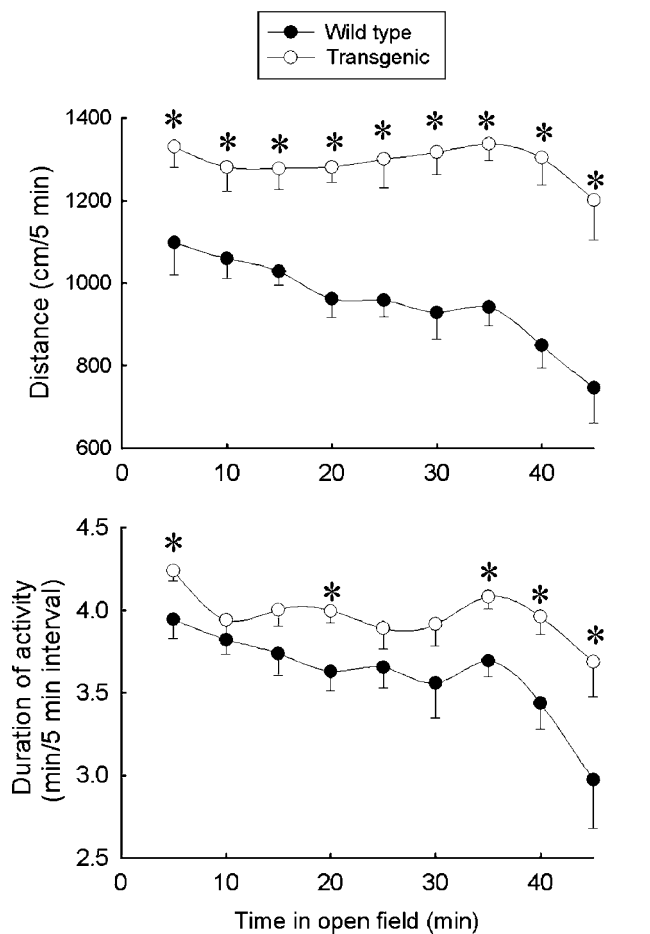
**Elevated plus maze.** The number of entries and the time spent in open arms of the elevated plus maze, by the Fabp-PG mice, were significantly reduced compared to that measured for the WT mice (Figure 3), suggesting an increase in the anxiety-like behavior of the Fabp-PG vs WT mice. At the same time, entries to closed arms of the plus maze by the Fabp-PG mice were significantly increased compared to that of the WT mice, confirming an increase in the locomotor activity of the Fabp-PG mice as measured in the open field test. No significant alteration in the vertical activity (rearing) of the Fabp-PG vs WT mice was detected in the elevated plus-maze test (data not shown).

### Alteration in Mouse Gastrin mRNA in the Brain

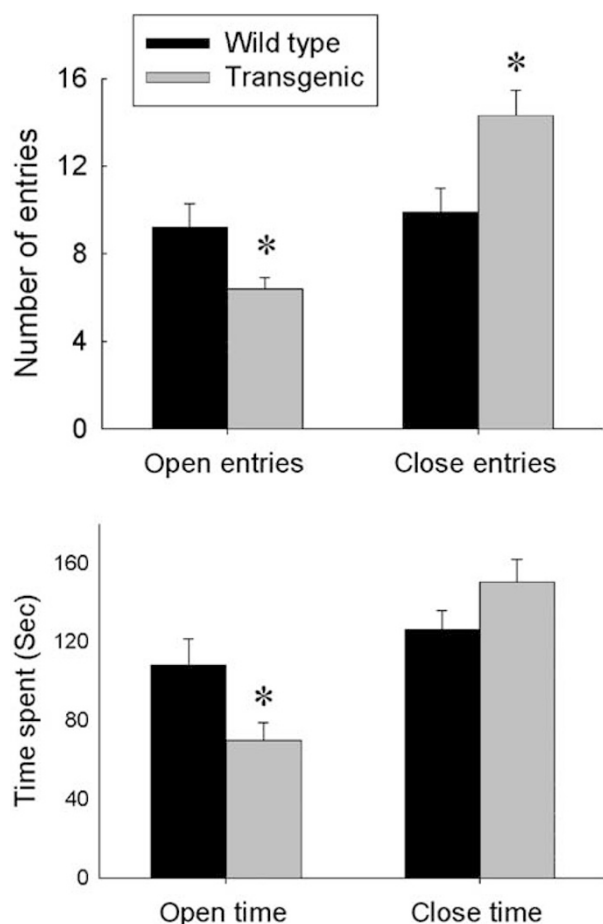
The expression of mouse gastrin in the brain regions of Fabp-PG and WT mice was determined by real-time PCR.



**Figure 1** Resident-intruder test for aggressive behavior in Fabp-PG mice (Tg). The data are presented as mean  $\pm$  SEM ( $n = 9-10$ ). \*Significantly different from wild-type mice (WT).



**Figure 2** Time course of locomotor activity during the open field test in Fabp-PG mice. The data are presented as mean  $\pm$  SEM ( $n = 10$ ). \*Significantly different from WT mice at same time point.



**Figure 3** Elevated plus-maze test in Fabp-PG mice. The data are presented as mean  $\pm$  SEM ( $n = 10$ ). \*Significantly different from WT mice.

The highest gastrin mRNA was in the midbrain and the lowest in the cerebellum. The hypothalamus, hippocampus, and cortex contained moderate level of gastrin mRNA. The relative levels of gastrin mRNA were significantly increased in the hypothalamus ( $F_{(1,5)} = 8.712$ ,  $P = 0.026$ ), but significantly reduced in the midbrains ( $F_{(1,7)} = 5.823$ ,  $P = 0.047$ ) of Fabp-PG *vs* WT mice (Table 1). No significant change in gastrin mRNA was detected in other brain regions (Table 1).

#### Alteration in CCK mRNA in the Brain

The expression of CCK was determined in several brain regions of Fabp-PG and WT mice using real-time RT-PCR. In WT mice, CCK mRNA was highly expressed in the hippocampus and cortex, moderately expressed in the midbrain and was low in the hypothalamus and cerebellum. Relative levels of mouse CCK mRNA were significantly increased in the hypothalamus of Fabp-PG mice compared to that in the hypothalamus of WT mice ( $F_{(1,13)} = 4.8$ ,  $P = 0.04$ , Table 3). This elevation was not observed in other brain regions (Table 2).

#### Alterations in Receptors

**CCK<sub>2</sub> receptors and PG binding in the brain.** Using real-time RT-PCR, we examined the expression of CCK<sub>2</sub>

**Table 1** Mouse Gastrin mRNA in the Brain Regions

	Wildtype	Transgenic
Cortex	0.9 $\pm$ 0.33 (4)	1.35 $\pm$ 0.31 (6)
Hypothalamus	0.97 $\pm$ 0.08 (3)	5.0 $\pm$ 1.02 (5) <sup>a</sup>
Hippocampus	0.81 $\pm$ 0.04 (3)	1.47 $\pm$ 0.43 (7)
Midbrain	12.36 $\pm$ 0.9 (3)	2.47 $\pm$ 0.57 (6) <sup>a</sup>
Cerebellum	0.6 $\pm$ 0.38 (3)	0.32 $\pm$ 0.07 (7)

The data are presented as mean  $\pm$  SEM ( $n$ ) of gastrin mRNA levels normalized by S18 mRNA and a WT hypothalamus sample.

<sup>a</sup>Significantly different from corresponding WT values ( $P < 0.05$ ).

**Table 2** CCK mRNA in the Brain Regions

	Wildtype	Transgenic
Cortex	4.1 $\pm$ 1.4 (5)	5.7 $\pm$ 1.0 (9)
Hypothalamus	0.73 $\pm$ 0.05 (5)	2.63 $\pm$ 0.6 (10) <sup>a</sup>
Hippocampus	6.5 $\pm$ 0.2 (4)	8.81 $\pm$ 1.66 (7)
Midbrain	2.74 $\pm$ 1.29 (5)	3.04 $\pm$ 0.75 (9)
Cerebellum	1.09 $\pm$ 0.1 (4)	1.34 $\pm$ 0.36 (9)

The data are presented as mean  $\pm$  SEM ( $n$ ) of CCK mRNA levels, normalized by S18 mRNA and a WT hypothalamus sample.

<sup>a</sup>Significantly different from WT values ( $P < 0.05$ ).

**Table 3** CCK<sub>2</sub>R mRNA in the Brain Regions

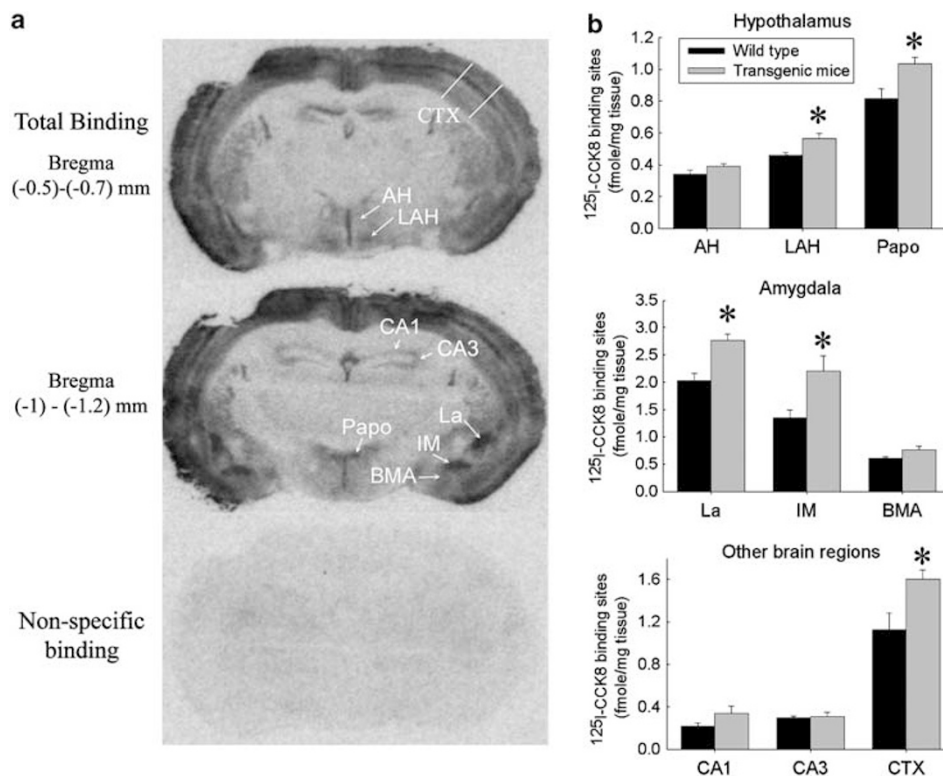
	Wildtype	Transgenic
Cortex	1.37 $\pm$ 0.17 (4)	1.16 $\pm$ 0.14 (9)
Hypothalamus	0.68 $\pm$ 0.22 (4)	2.15 $\pm$ 0.35 (6) <sup>a</sup>
Hippocampus	0.97 $\pm$ 0.10 (4)	0.69 $\pm$ 0.10 (6)
Midbrain	1.10 $\pm$ 0.12 (4)	1.57 $\pm$ 0.26 (7)
Cerebellum	0.3 $\pm$ 0.06 (4)	0.11 $\pm$ 0.02 (9) <sup>a</sup>

The data are presented as mean  $\pm$  SEM ( $n$ ) of CCK<sub>2</sub>R mRNA levels, normalized by S18 mRNA and a WT hypothalamus sample.

<sup>a</sup>Significantly different from corresponding WT values ( $P < 0.05$ ).

receptors in several brain regions. Levels of CCK<sub>2</sub>R mRNA were relatively high in the cortex and midbrains of WT mice, followed by lower levels in the hypothalamus and hippocampus and relatively low levels in the cerebellum (Table 3). A significant increase in the relative levels of CCK<sub>2</sub>R mRNA was detected in the hypothalamus of Fabp-PG mice relative to that in the WT mice ( $F_{(1,8)} = 10.02$ ,  $P = 0.013$ , Table 2). On the other hand, relative levels of CCK<sub>2</sub>R mRNA in the cerebellum were significantly reduced in Fabp-PG mice compared to that in the WT mice ( $F_{(1,11)} = 20.52$ ,  $P < 0.001$ , Table 3).

Autoradiographic analysis of <sup>125</sup>I-CCK-8 binding determined the density of CCK<sub>1</sub> and CCK<sub>2</sub> receptors. As the majority of CCK receptors in the brain are CCK<sub>2</sub> receptors, the data obtained from the autoradiographic binding of <sup>125</sup>I-CCK-8 mainly presented the density of CCK<sub>2</sub> receptors. The results demonstrated a high density of CCK<sub>2</sub> receptors in



**Figure 4** Autoradiography of  $^{125}\text{I}$ -CCK8 binding to CCK2 receptors in Fabp-PG and WT mice. (a) Representative autoradiographic data from a WT mouse, demonstrating binding of  $^{125}\text{I}$ -CCK8 to coronal sections of the brain at the hypothalamic level. Top and middle panels: total binding in two coronal sections; bottom panel: nonspecific binding defined by presence of excess CCK8 ( $10^{-6}\text{M}$ ). (b) The density of CCK2 receptors in the hypothalamus, amygdala, hippocampus, and cortex. The data are presented as mean  $\pm$  SEM ( $n = 4-7$ ). \*Significantly different from WT mice. Abbreviations: AH, anterior hypothalamic nucleus; BMN, basal medial amygdaloid nucleus; CA1, CA1 area of hippocampus; CA3, CA3 area of hippocampus; CTX, cortex; IM, intercalate main; La, lateral amygdaloid nucleus; LAH, lateral anterior hypothalamic nucleus, and Papo, paraventricular nucleus, posterior.

the layer four of cortex, lateral amygdaloid nucleus (La), main intercalated amygdaloid nucleus (IM), and posterior paraventricular hypothalamic nucleus (PaPo) (representative data from WT mice are presented in Figure 4a). Moderate density of CCK<sub>2</sub> receptors was found in lateral anterior hypothalamic nucleus (AHL) and basal medial amygdala (BMA). The density of CCK<sub>2</sub> receptors was relatively low in the hippocampus. The densities of CCK<sub>2</sub> receptors in the lateral anterior hypothalamus, PaPo, lateral amygdala, IM and cortex were increased in Fabp-PG mice compared to that in the WT mice (Figure 4b). On the other hand, the density of CCK<sub>2</sub> receptors in the hippocampus was similar in the Fabp-PG and WT mice.

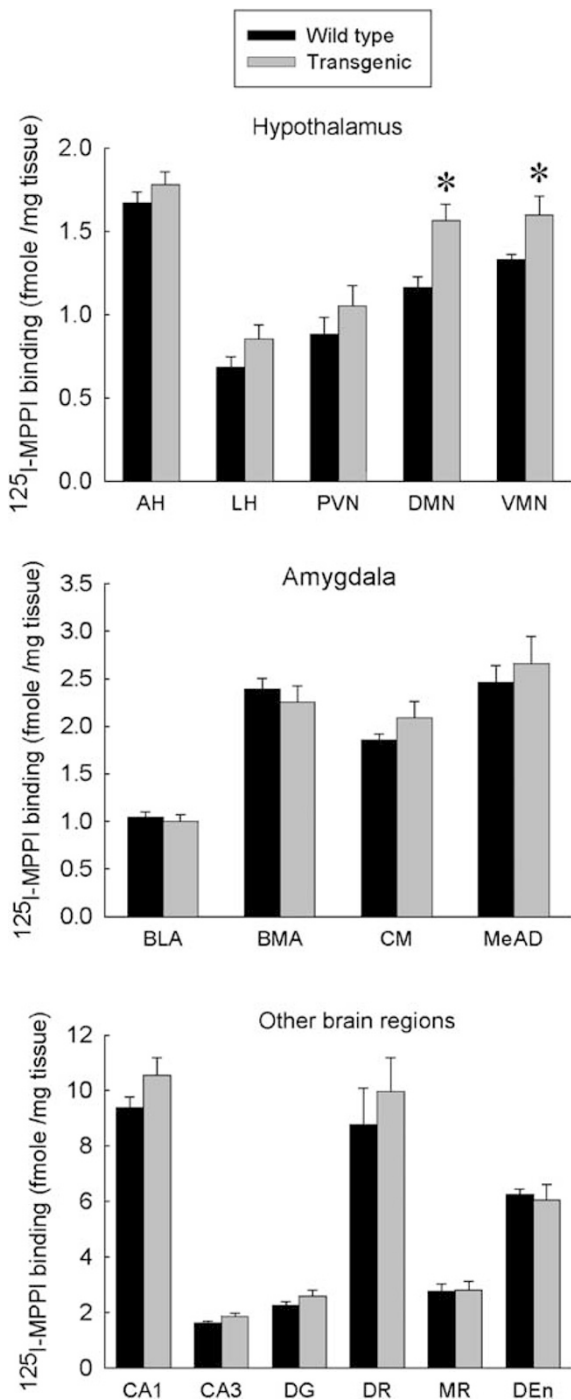
Specific binding of  $^{125}\text{I}$ -rhPG to the brain slices was not detected either in the Fabp-PG or the WT mice. These results suggest that the PG preferring receptors (such as Annexin II), as measured in the colonic epithelial cells (Singh *et al*, 2003, 2006), are absent in the brain. The latter results were not surprising, as extracellular Annexin II is primarily expressed in proliferating cells; the results further confirm that PG peptides do not bind CCK<sub>2</sub> receptors in the brain, as previously reported for peripheral tissues (discussed by Singh *et al*, 2006).

**5-HT<sub>1A</sub> receptors.** Autoradiographic analysis of  $^{125}\text{I}$ -MPPI binding revealed that 5-HT<sub>1A</sub> receptors in the hypothalamus were significantly increased in the Fabp-PG vs WT mice

(Figure 5). The density of 5-HT<sub>1A</sub> receptors in the dorsomedial and ventromedial nuclei of the hypothalamus was significantly elevated in Fabp-PG compared to that in the WT mice. However, there was no change in the binding of  $^{125}\text{I}$ -MPPI to all other regions of the brain in Fabp-PG vs WT mice (Figure 5).

## DISCUSSION

The results of the present studies demonstrate for the first time, that elevated levels of PG in the circulation of transgenic mice overexpressing PG in the intestinal mucosal cells, results in significant alterations in the emotional behaviors of mice. A significant increase in the aggressive and locomotor activity, in association with an increase in anxiety-like behavior of Fabp-PG mice, represents a new and interesting aspect of our findings. Mechanistically, the alterations in the behavior of the mice could be related to an observed increase in the expression of gastrin and CCK and/or an increase in the relative levels of CCK<sub>2</sub> receptors and 5-HT<sub>1A</sub> receptors in the hypothalamus, amygdala, and cortex of Fabp-PG mice. An important role of gut-brain peptides in behavioral sciences is receiving an increasing attention in recent years. The results of the present study provide yet another example of important behavioral affects of a gastrointestinal peptide on central nervous system.



**Figure 5** Autoradiographic density of 5-HT<sub>1A</sub> receptors, examined using <sup>125</sup>I-MPPPI binding to sections of the brain from Fabp-PG and WT mice. The data are presented as mean ± SEM (*n* = 4–7). \*Significantly different from WT mice. Abbreviations: BLA, basal lateral amygdala; CM, central medial amygdala; DEn, dorsal endoperiform; DG, dentyl gyrus of hippocampus; DMN, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; LH, lateral hypothalamus; MeAD, medial amygdaloid dorsal nucleus; MR, medial raphe nucleus; PVN, paraventricular nucleus; VMN, ventromedial hypothalamic nucleus and others are as listed in Figure 4.

However, the mechanism by which peripheral PG alters the CCK and 5-HT systems in the brain, as measured in the present studies, remains speculative.

Evidence strongly suggests that gastrin and CCK are related to anxiogenic effects, which are mediated by CCK<sub>2</sub>R (Bradwejn, 1993; Rehfeld, 2000; Singh *et al*, 1991). The increased brain CCK level was reported in a rat model of anxiety (Pavlovic *et al*, 1993). Stimulation of CCK<sub>2</sub> receptors in the brain increases anxiety and panic-like behaviors (Zanoveli *et al*, 2004; Abelson *et al*, 2005). The hypothalamus and amygdala are known to be involved in the regulation of defensive behaviors (Choi *et al*, 2005). The amygdala is a key region that regulates fear and anxiety behaviors (LeDoux, 2000). Furthermore, the paraventricular nucleus in the hypothalamus contains corticotrophin releasing factor neurons that control hypothalamic-pituitary-adrenal axis (HPA axis) (Van de Kar and Blair, 1999). Reports in literature suggest that CCK<sub>2</sub> receptor-related alterations in the anxiety and panic-like behaviors may be mediated by stimulation of hypothalamic-pituitary-adrenal axis (Abelson and Young, 2003; Abelson *et al*, 1994; Zwanzger *et al*, 2003). It is thus possible that the significant increase in the levels of gastrin and CCK in the hypothalamus and the density of CCK<sub>2</sub>R in the hypothalamus and amygdala may have contributed to the behavioral changes observed in the PG-expressing mice in the present studies.

5-HT<sub>1A</sub> receptors are important in the regulation of anxiety. Several studies have shown that systemic administration of 5-HT<sub>1A</sub> agonists and 5-HT<sub>1A</sub> antagonists modulates defensive behaviors (Blanchard *et al*, 2003; Griebel *et al*, 1999). We recently reported that 5-HT<sub>1A</sub> receptors in the medial hypothalamus, regulate locomotor activity that may be related to defensive behaviors (Li *et al*, 2004). Furthermore, 5-HT<sub>1A</sub> receptors interact with CCK receptors to regulate anxiety behaviors (To and Bagdy, 1999). Therefore, we examined the density of 5-HT<sub>1A</sub> receptors in the hypothalamus and amygdala of PG-expressing vs WT mice. The results demonstrated that the density of 5-HT<sub>1A</sub> receptors in several nuclei of the hypothalamus is significantly increased in PG-expressing mice. These data suggest that the increase in locomotor activity in PG-expressing mice may be partly due to an increase in the relative levels of 5-HT<sub>1A</sub> receptors in the hypothalamus. As no changes were observed in 5-HT<sub>1A</sub> receptors in the amygdala of PG-expressing mice, it is unlikely that the increase in anxiety-like behavior of Fabp-PG mice is mediated by 5-HT<sub>1A</sub> receptors. As other serotonergic receptors, such as 5-HT<sub>2C</sub> and 5-HT<sub>3</sub> receptors, may also regulate anxiety-like behaviors, it will be worthwhile to examine those receptors as well in the Fabp-PG mice in the future.

A puzzling question that remains is how overexpression of PG affects the neurotransmitter and neuropeptide systems in CNS and subsequently alters emotional behaviors, as the overexpression of PG was limited to the intestinal mucosa (Cobb *et al*, 2004). Although PG levels in the circulation were significantly increased in the Fabp-PG mice, gastrin levels were not significantly elevated in PG-expressing mice (Cobb *et al*, 2004). To date, no data suggests that PG can cross the blood brain barrier. In the present studies, we were not able to detect the presence of high affinity PG binding sites in the brains of both the Fabp-PG and WT mice. Therefore, it is unlikely that the alteration in emotional behaviors of the Fabp-PG mice is due to a

direct effect of PG. Based on literature, several possible mechanisms are speculated. First, the behavioral changes may be mediated by an increase in the HPA axis. Studies have shown that peripheral PG and its products can activate CCK receptors in the pituitary and adrenal gland to increase ACTH and corticosterone release (Abelson *et al*, 1994). The increase in HPA axis activity could cause changes in emotional behaviors (Claes, 2004; Mello *et al*, 2003). Second, gastrin in the stomach and intestine can increase the neuronal activity in several brain regions via vagal stimulation (Yakabi *et al*, 2002). Studies have found that an increase in gastrin in the stomach and circulation stimulates c-fos expression in the nucleus of the solitary tract that receives vagal input (Danzer *et al*, 2004a, b). The vagal stimulation can further activate the hypothalamic and other limbic regions of the brain (Yakabi *et al*, 2002). It is possible that the activation of the limbic neurons results in the increase of the expression of gastrin, CCK, and CCK<sub>2</sub>R in the hypothalamus, which may mediate the observed changes in emotional behaviors. Although we cannot rule it out, it is unlikely that the alterations in the CNS are due to adaptive changes at early developmental stages (prenatally). As the expression of hPG is driven by Fabp promoter, the over expression of hPG is not expected to occur prenatally, in the absence of fatty acids in the intestines. However, developmental overexpression of PG in the intestinal mucosa of Fabp-PG mice has not been examined as yet.

Additionally, it is known that neurons in the brain process pro-cholecystokinin and pro-gastrin peptides in a cell-specific manner (Rehfeld *et al*, 1988). Owing to the presence of elevated PG in the gut and circulation of Fabp-PG mice (Cobb *et al*, 2004), it is possible that gut neurons process the available PG to gastrins; the local increase in gastrins in peripheral neurons may be directly or indirectly transmitted to specific areas of the brain, such as brainstem. This speculative pathway may explain the observed loss in gastrin expression in the midbrain of Fabp-PG mice (perhaps due to a negative feedback). These intriguing possibilities will be examined in future studies.

In previous studies, we reported that the loss of gastrin gene expression (as in GAS-KO mice) results in a profound loss in both the locomotor activity and aggressive behavior, accompanied by an increase in anxiety-like behavior (Singh *et al*, 2004). Importantly, a significant loss in the physical activity resulted in an obese phenotype of the GAS-KO mice (Cowey *et al*, 2005). Reduced effects of amphetamine on prepulse inhibition of startle in GAS-KO mice has also been reported (Van den Buuse *et al*, 2005), suggesting an important role of brain gastrin in emotional behavior of mice. These data and our present results provide evidence of gut-brain interaction. Therefore, an investigation of behavioral patterns of mice, mutant for specific gut peptide hormones, will allow us to discover the role of brain-gut peptides in dictating the behavioral phenotype, which can be expected to contribute to the health outcome of animals/humans. In the present studies, we demonstrate for the first time that genetically elevated levels of PG in the peripheral tissue, as measured in patients with hypergastrinemia and colon cancer (reviewed by Rengifo-Cam and Singh, 2004), may alter behaviors. A significant change in the behaviors (such as increased aggressiveness, paranoia, etc) can be expected to potentially impact the well being of the

individual in the long run. This intriguing possibility needs to be examined in patients with hypergastrinemia and/or elevated levels of PG.

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