Emergence of anxiety-like behaviours in depressive-like Cpe\textsuperscript{fat\_fat} mice

Ramona M. Rodriguiz\textsuperscript{1}, John J. Wilkins\textsuperscript{1}, Thomas K. Creson\textsuperscript{1}, Reeta Biswas\textsuperscript{2}, Iryna Berezniiuk\textsuperscript{2}, Arun D. Fricker\textsuperscript{3}, Lloyd D. Fricker\textsuperscript{4} and William C. Wetsel\textsuperscript{1,5,6,7}

\textsuperscript{1} Department of Psychiatry and Behavioral Sciences, Mouse Behavioral and Neuroendocrine Analysis Core Facility, Duke University Medical Center, Durham, NC, USA
\textsuperscript{2} Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA
\textsuperscript{3} Departments of Cell Biology, Neurobiology and Medicine (Endocrinology), Duke University Medical Center, Durham, NC, USA
\textsuperscript{4} Departments of Neurobiology, Mouse Behavioral and Neuroendocrine Analysis Core Facility, Duke University Medical Center, Durham, NC, USA
\textsuperscript{5} Departments of Medicine (Endocrinology), Mouse Behavioral and Neuroendocrine Analysis Core Facility, Duke University Medical Center, Durham, NC, USA

Abstract

Cpe\textsuperscript{fat\_fat} mice have a point mutation in carboxypeptidase E (Cpe), an exopeptidase that removes C-terminal basic amino acids from intermediates to produce bioactive peptides. The mutation renders the enzyme inactive and unstable. The absence of Cpe activity in these mutants leads to abnormal processing of many peptides, with elevated levels of intermediates and greatly reduced levels of the mature peptides. Cpe\textsuperscript{fat\_fat} mice develop obesity, diabetes and infertility in adulthood. We examined whether anxiety- and/or depressive-like behaviours are also present in these mutants. Anxiety-like responses are not evident in young Cpe\textsuperscript{fat\_fat} mice (<60 d), but appear in older animals (>90 d). These behaviours are reversed by acute treatment with diazepam or fluoxetine. In contrast, increased immobilities in forced swim and tail suspension are evident in all age groups examined. These behaviours are reversed by acute administration of reboxetine. In comparison acute treatments with fluoxetine or bupropion are ineffective; however, immobility times are normalized with 2 wk treatment. These data demonstrate that Cpe\textsuperscript{fat\_fat} mice display depressive-like responses aged ~60 d, whereas anxiety-like behaviours emerge ~1 month later. In tail suspension, the reboxetine findings show that noradrenergic actions of antidepressants are intact in Cpe\textsuperscript{fat\_fat} mice. The ability of acute fluoxetine treatment to rescue anxiety-like while leaving depressive-like responses unaffected suggests that serotonin mechanisms underlying these behaviours are different. Since depressive-like responses in the Cpe\textsuperscript{fat\_fat} mice are rescued by 2 wk, but not acute, treatment with fluoxetine or bupropion, these mice may serve as a useful model that resembles human depression.

Key words: Anxiety, carboxypeptidase E, depression, encephalin convertase, neuropeptides, obesity.

Introduction

Neuropeptides function in cell–cell communications in many physiological systems (Strand, 2003). In mammals, peptides are involved in regulating feeding, anxiety, depression, arousal, sexual behaviour, reproduction and many other functions. Neuropeptides are produced from precursor proteins through selective actions of peptidases (Seidah and Chrétien, 1999; Zhou et al., 1999). Endopeptidases initially cleave precursors at sites containing basic amino acids. Two endopeptidases, prohormone convertase (PC1) (also known as PC3) and PC2 cleave neuropeptide precursors on the C-terminal side of basic amino acids (Seidah and Chrétien, 1999; Zhou et al., 1999). A carboxypeptidase then removes the basic residues, generating the bioactive peptide. The major peptidase-processing carboxypeptidase is carboxypeptidase E (Cpe; Fricker, 2002, 2004a). Cpe is enriched in peptide-containing secretory vesicles in neuroendocrine tissues and it removes C-terminal lysine/arginine residues from a broad range of peptides (Fricker, 2002, 2004a). Mice lacking Cpe activity are viable, suggesting that another enzyme participates in peptide processing (Naggert et al., 1995). Although carboxypeptidase D (Cpd), a trans Golgi network enzyme (Fricker, 2002, 2004b) partially contributes to peptide processing, the mature forms of many peptides are greatly reduced in Cpe-deficient mice (Naggert et al., 1995; Fricker et al., 1996; Rovere et al., 1996; Cain et al., 1997; Shen and Loh, 1997; Udupi et al., 1997; Nillni et al., 2002; Cawley et al., 2004; Srinivasan et al., 2004; Che et al., 2005;
show a phenotype similar to Cpefat/fat

Two distinct lines of mice lack Cpe activity. One line has a spontaneous mutation (Ser to Pro) causing the complete loss of enzymatic activity and a decrease in protein stability (Naggert et al., 1995; Varlamov et al., 1996). This mutation, named fat, was discovered in 1973 at The Jackson Laboratory and was renamed Cpefat/fat after the genetic basis of the mutation was determined (Naggert et al., 1995). Homozygotes for fat are subfertile and overweight in all strains which carry the mutation. When placed onto the C57BKS background, Cpefat/fat males from age 14–38 wk are hyperglycaemic (Leiter et al., 1999). Another line of mice was recently created by disruption of the Cpe gene (Cavley et al., 2004) and they show a phenotype similar to Cpefat/fat animals. The reproductive dysfunction of male Cpefat/fat mice is due primarily to abnormal sexual behaviour (Srinivasan et al., 2004). Young Cpefat/fat animals are not hyperphagic (Leiter et al., 1999), whereas older Cpefat/fat mice (11–15 wk) show elevated food consumption (Yuan et al., 2004). In addition, dysfunction of the hypothalamic–pituitary–thyroid axis is evident in Cpefat/fat mice (Nillni et al., 2002).

Since Cpe is involved in processing numerous peptides with diverse functions, it was anticipated that Cpefat/fat mice would show additional phenotypes. During routine handling of Cpefat/fat mice, they appeared to be more docile than wild-type (WT) littermates and rarely struggled when handled. Since this response could be due to altered emotional responses, we examined Cpefat/fat mice in several behavioural paradigms.

Materials and method

Animals

Two colonies of BKS.HRS-Cpefat/J mice were used in this study; one was housed at Albert Einstein College of Medicine (AECOM) and the other at Duke University Medical Centre. Both colonies used male and female Cpe+/fat mice that were obtained from Dr Edward Leiter at the Jackson Laboratory (Bar Harbor, USA). Parenthetically, this mouse line has been maintained in both laboratories through heterozygous matings and the Cpefat/fat mice from both laboratories display the same obesity, diabetes and reproductive phenotypes. In the AECOM facility, animals were group-housed in a barrier facility with lights on 08:00–20:00 hours with food (5058 diet; PMI Nutrition International, USA) and water freely available. At Duke, animals were housed in groups of three to five in a temperature- (22 °C) and humidity- (45%) controlled room with a 14:10 h light–dark cycle (lights on 06:00 hours) and provided food (5001 diet; PMI Nutrition International) and water ad libitum. The sites where the various behavioural assessments were made can be found in Supplementary Table S1. Cpe+/fat mice were mated and the WT and homozygous mutant offspring were identified by polymerase chain reaction analysis of tail DNA (see Srinivasan et al., 2004). All experiments were conducted with approved protocols from the AECOM and Duke University Institutional Animal Care and Use Committees in accordance with NIH guidelines for the care and use of laboratory animals.

Zero maze

The dimensions of the maze and test conditions have been described (Pogorelov et al., 2005). Briefly, the maze consisted of a 5.5 cm-wide circular platform (34 cm, inner diameter) located 43 cm from the floor with the closed areas facing each other and enclosed by 11 cm walls on the inner and outer edges of the platform. The areas of the closed and open quadrants were equal. The maze was illuminated at 50–60 lux; a video-camera was positioned 100 cm directly over the centre of the maze. One group of naive mice were aged 2, 3 or 5 months at the time of testing. A second group of naive mice aged 3 months were injected (i.p.) with vehicle, diazepam (Sigma-Aldrich, USA) or fluoxetine (Sigma-Aldrich) and tested 30 min later. Animals were placed into the closed area of the maze between 13:00 and 16:00 hours and allowed 5 min free investigation. Behaviour was videotaped and scored with Noldus Observer (Noldus, USA) for the latency to enter the open area and the percent time in the open areas. Animals were tested for only one session.

Light–dark exploration test

This test was conducted between 13:00 and 16:00 hours with naive mice aged 3 months (see Crawley and Goodwin, 1980; Fukui et al., 2007). At Duke University, the apparatus consisted of two chambers (20 × 16 × 21 cm/chamber) that were separated by an automated sliding door (MedAssociates, USA). The mouse was placed into the illuminated chamber (~ 600 lux) to begin the test. After 5 s the door was opened to the darkened chamber (~ 5 lux illumination) and the mouse was given free access to both chambers for 5 min. Latency to exit the illuminated chamber, time spent in the illuminated chamber, latency to re-enter the illuminated chamber, number of transitions between chambers and activities (beam-breaks) in the illuminated and darkened chambers were scored with infrared diodes interfaced to a computer running MedPC software. At AECOM, a rectangular polypropylene apparatus (42 × 35 × 12 cm) was used. The apparatus was divided into two chambers by a wall with a small opening that was 12 cm wide and 7 cm high. The illuminated area, which represented two-thirds of the total area, was illuminated by a 75 W fluorescent bulb positioned 40 cm above the apparatus. The darkened area was covered with cardboard and aluminium foil. Initially the naive mouse was placed into the centre of the illuminated chamber and the time to enter the darkened chamber, the percent total time in the illuminated
chamber, latency to return to the illuminated chamber and number of transitions between the two chambers were recorded for 5 min. Mice were tested between 12:00 and 16:00 hours.

**Forced swim**

The forced swim test was conducted at 13:00–16:00 hours similarly to that previously described (Fukui et al., 2007). Briefly, the apparatus consisted of a 4-litre Pyrex beaker (15 cm diameter) that contained 15 cm water (25 °C). Naïve mice were tested (no injection) aged 2, 3 or 5 months and were given vehicle or imipramine (i.p.) and tested 30 min later. Behaviour was videotaped for 6 min from the side of the beaker and scored subsequently using Noldus Observer by observers blinded to the genotypes of the mice. Immobility refers to the time the animal spent floating or engaged in minimal activity to stay afloat for ≥3 s. The parameters scored included total time in immobility, latency to first immobility bout, duration of first immobility bout and frequency of immobility bouts.

**Tail suspension**

Animals were naïve (aged 2, 3 or 5 months), or aged 3 months and had been examined previously in the zero maze (no drugs) 3 months and had been examined previously in the zero maze. As onset of obesity in Cpefat/fat animals becomes apparent after adulthood, mice were tested aged 2 (body weights: WT, 17–27 g; Cpefat/fat, 17–28 g), 3 (WT, 22–28 g; Cpefat/fat, 22–30 g), or 5 months (WT, 23–29 g; Cpefat/fat, 30–36 g). Aged 2 months, both genotypes responded similar to that previously described (Fukui et al., 2007). In a separate set of experiments, mice were given daily injections of vehicle, 7.5 mg/kg fluoxetine or 5 mg/kg bupropion (i.p.) for 2 wk and tested 30 min after the final injection. Immobility times were determined using MedAssociates software.

**Locomotor activity**

The assessment of activity was conducted at 13:00–16:00 hours as outlined (Fukui et al., 2007). The apparatus consisted of an open field (21 × 21 × 30 cm) illuminated at 340 lux. Locomotor activity was monitored by eight photo-beams spaced 2.5 cm apart, positioned 2.25 cm from the floor and equally distributed along the perimeter of the open field (AccuScan Instruments, USA). Naïve mice aged 2, 3 or 5 months was tested for 30 min in the open field. Alternatively, naïve mice or animals aged 3 months that had been tested 2 wk previously in tail suspension (no drugs) were acclimated to the open field for 30 min, injected (i.p.) with vehicle, 1 mg/kg diazepam, 20 mg/kg fluoxetine, 10 mg/kg reboxetine, 20 mg/kg bupropion or 20 mg/kg imipramine and immediately returned to the open field for 30 min. Horizontal distance travelled (cm) was monitored over 5 min segments.

Statistics

The data are presented as means and standard errors of the mean. All analyses were performed with the Statistical Package for the Social Sciences, version 11.0 (SPSS; Chicago, USA). Initially, all statistical analyses were conducted using sex as a variable. Since neither main effects of sex nor interactions with sex as a variable were observed in any statistical analysis, the data were collapsed across sex and analysed as a single group. To evaluate genotypic responses in the light–dark emergence test, ANOVA was used to examine responses in the zero maze, open field, forced swim and tail suspension tests. Repeated-measures ANOVA was used to analyse effects on locomotion in the open field over time. Bonferroni’s corrected pair-wise comparisons were used as the post hoc tests. A p < 0.05 was considered significant.

**Results**

**An anxiety-like phenotype develops in Cpefat/fat mice**

To determine whether mutants displayed anxiety-like behaviours, naïve WT and Cpefat/fat mice were evaluated in the zero maze. As onset of obesity in Cpefat/fat animals becomes apparent after adulthood, mice were tested aged 2 (body weights: WT, 17–27 g; Cpefat/fat, 17–28 g), 3 (WT, 22–28 g; Cpefat/fat, 22–30 g), or 5 months (WT, 23–29 g; Cpefat/fat, 30–36 g). Aged 2 months, both genotypes responded similarly in the maze according to latency to first enter the open areas (Fig. 1a) and the percent time spent in the open areas (Fig. 1b). By age 3 and 5 months, the latencies to exit the closed area were prolonged and the percent times in the open areas were decreased in Cpefat/fat mice.

To further confirm that the Cpefat/fat mice aged 3 months show anxiety-like behaviours, responses were analysed in the light–dark exploration test (WT, 21–29 g; Cpefat/fat, 22–29 g). In this test, Cpefat/fat animals exited the illuminated chamber more quickly (Fig. 2a) and spent less time in this chamber than the WT controls (Fig. 2b). Upon entering the darkened chamber, the latency to leave the chamber was prolonged (Fig. 2c) and Cpefat/fat animals engaged in fewer transitions between chambers than their WT littermates (Fig. 2d). Overall activity was also reduced in both chambers for Cpefat/fat mice (Fig. 2e, f). Despite a decrease in activity, the initial rapid exit from the illuminated chamber demonstrates that the mutants can move very quickly in the apparatus. Similar results were obtained in the light–dark exploration test with WT and Cpefat/fat mice at AECOM (data not shown). Collectively, results from the zero maze and light–dark box suggest anxiety-like behaviour emerges in Cpefat/fat mice between ages 2 and 3 months.

**Cpefat/fat mice are responsive to anxiolytic drugs**

To ascertain whether the anxiety-like behaviours in Cpefat/fat mice could be alleviated by anxiolytic drugs,
WT and Cpefat/fat mice displayed significant main effects of genotype (F<sub>1,84</sub> = 22.445, p < 0.001) and age (F<sub>2,84</sub> = 9.436, p < 0.001), and a significant genotype × age interaction (F<sub>2,84</sub> = 4.881, p < 0.010). Bonferroni’s corrected pair-wise comparisons indicated no genotype differences were evident at 2 months and no behavioural changes were observed at 2, 3 or 5 months for wild-type (WT) mice. By contrast, 3 and 5 month Cpefat/fat animals took longer to enter the open areas than WT animals at all ages (p < 0.002 for all) and mutants aged 2 months (p < 0.001 for all). ANOVA revealed significant main effects of genotype (F<sub>1,84</sub> = 18.725, p < 0.001) and age (F<sub>2,84</sub> = 7.774, p < 0.001) and a significant genotype × age interaction (F<sub>2,84</sub> = 3.236, p < 0.044). Bonferroni’s tests failed to discern any genotype differences at age 2 months and responses of WT mice aged 2, 3 or 5 months were undifferentiated. Percent open area times were decreased for Cpefat/fat animals aged 3 and 5 months relative to WT controls at the same ages (p < 0.002 for all) and to WT and mutant mice aged 2 months (p < 0.001 for all). N = 8–18 mice/age/genotype. * p < 0.05, compared to WT mice (within age).

Fig. 1. Anxiety-like behaviours emerge with age in Cpefat/fat mice. (a) Latency to enter the open areas of the elevated zero maze. ANOVA demonstrated significant main effects of genotype (F<sub>1,84</sub> = 22.445, p < 0.001) and age (F<sub>2,84</sub> = 9.436, p < 0.001), and a significant genotype × age interaction (F<sub>2,84</sub> = 4.881, p < 0.010). Bonferroni’s corrected pair-wise comparisons indicated no genotype differences were evident at 2 months and no behavioural changes were observed at 2, 3 or 5 months for wild-type (WT) mice. By contrast, 3 and 5 month Cpefat/fat animals took longer to enter the open areas than WT animals at all ages (p < 0.002 for all) and mutants aged 2 months (p < 0.001 for all). ANOVA revealed significant main effects of genotype (F<sub>1,84</sub> = 18.725, p < 0.001) and age (F<sub>2,84</sub> = 7.774, p < 0.001) and a significant genotype × age interaction (F<sub>2,84</sub> = 3.236, p < 0.044). Bonferroni’s tests failed to discern any genotype differences at age 2 months and responses of WT mice aged 2, 3 or 5 months were undifferentiated. Percent open area times were decreased for Cpefat/fat animals aged 3 and 5 months relative to WT controls at the same ages (p < 0.002 for all) and to WT and mutant mice aged 2 months (p < 0.001 for all). N = 8–18 mice/age/genotype. * p < 0.05, compared to WT mice (within age).

animals aged 3 months were treated with diazepam or fluoxetine and evaluated in the zero maze. The body weights of Duke University Cpefat/fat mice tested under different treatment conditions were similar between genotypes (WT, 22–26 g; Cpefat/fat, 21–26 g). To ascertain whether diazepam could normalize these responses, naive WT and Cpefat/fat animals were administered vehicle, or 0.5, 1 or 2 mg/kg diazepam 30 min before testing. Vehicle-treated Cpefat/fat mice took longer to exit the closed areas and they spent less time in the open areas than the WT controls (Fig. 3a, b). Diazepam influenced WT and Cpefat/fat responses on both zero-maze parameters. Importantly, this benzodiazepine was successful in altering the anxiety-like behaviours of the mutants in a dose-dependent fashion, such that diazepam normalized Cpefat/fat responses to levels similar to those of the vehicle-treated WT controls. These data indicate that the anxiety-like behaviours of Cpefat/fat mice can be alleviated with diazepam.

Besides benzodiazepines, selective serotonin reuptake inhibitors can be used to treat anxiety disorders in human patients (Tollefson and Rosenbaum, 1998) and they can alleviate anxiety-like behaviours of mice in the open field (Chen et al., 2006) and the plus maze (Holmes and Rodgers, 2003). To determine whether the naive WT and Cpefat/fat animals aged 3 months would respond to these drugs, they were given 5 or 10 mg/kg fluoxetine and tested 30 min later in the zero maze. Again, vehicle-treated Cpefat/fat mice took longer to enter the open areas and they spent less time in the open areas than WT controls (Fig. 4a, b). WT animals showed some responses to fluoxetine. For Cpefat/fat animals fluoxetine dose-dependently decreased the latency to enter the open areas and it increased percent time in the open areas of the maze. Moreover, it normalized responses to those of the WT vehicle-controls. The responses to diazepam and fluoxetine appear to be specific in the zero maze because both drugs had anxiolytic activity while in the open field diazepam increased locomotor activity while fluoxetine reduced it in the Cpefat/fat mice (data not shown). Hence, the results from the zero maze support an anxiety-like phenotype in the Cpefat/fat animals.

Cpefat/fat mice display abnormal responses to antidepressants

Anxiety and major depression are frequently co-morbid in human patients (Zimmerman et al., 2002). To determine whether the Cpefat/fat animals displayed depressive-like behaviours, mice were examined in the forced swim test aged 2 (WT, 18–27 g; Cpefat/fat, 17–28 g), 3 (WT, 21–27 g; Cpefat/fat, 22–29 g) and 5 months (WT, 22–29 g; Cpefat/fat, 29–38 g) at Duke University. Time spent in immobility during the 5 min test was quite different for WT and Cpefat/fat animals where it was prolonged in Cpefat/fat mice at all ages examined (Fig. 5a). Similar results were obtained with AECOM WT and Cpefat/fat animals. Naive mice or mice previously tested in the zero maze with vehicle were examined in tail suspension aged 2 (WT, 17–26 g; Cpefat/fat, 17–27 g), 3 (WT, 20–28 g; Cpefat/fat, 22–28 g) and 5 months (WT, 22–29 g; Cpefat/fat, 28–38 g). Cpefat/fat mice spent more time in immobility in tail suspension than WT controls at each of the three ages (Fig. 5b). Hence, increased immobilities in the forced swim and tail suspension tests appear to be pre-existing conditions in Cpefat/fat animals.
To analyse whether immobilities of Cpe\textsuperscript{fat/fat} animals were responsive to antidepressant drugs, animals aged 3 months were given an acute injection of vehicle or imipramine and tested in forced swim. Parenthetically, no differences in body weight were evident between genotypes (WT, 21–26 g; Cpe\textsuperscript{fat/fat}: 22–27 g). Vehicle-treated mutants spent more time in immobility, and their latencies to first immobility were shorter and their durations of first immobility longer than those of the WT controls (Fig. 6\textsubscript{a}–\textsubscript{c}). Additionally, Cpe\textsuperscript{fat/fat} mice had fewer overall numbers of immobility bouts during the 6 min test than WT animals (Fig. 6\textsubscript{d}), because the durations of their bouts were longer (Fig. 6\textsubscript{e}). Imipramine served to decrease total immobility times and to reduce duration of the first immobility bouts for both genotypes (Fig. 6\textsubscript{a},\textsubscript{c}). Furthermore this antidepressant increased the overall frequencies of immobility bouts for the mutant mice (Fig. 6\textsubscript{d},\textsubscript{e}). These findings show that imipramine can successfully ameliorate many behaviours of Cpe\textsuperscript{fat/fat} mice in the forced swim test.

To examine responses with additional antidepressant drugs, animals aged 3 months (WT, 22–28 g; Cpe\textsuperscript{fat/fat}, 23–29 g) were tested in tail suspension with fluoxetine, reboxetine, or bupropion. Immobility times for vehicle-treated Cpe\textsuperscript{fat/fat} animals were prolonged compared to those for WT vehicle-controls (Fig. 7\textsubscript{a}–\textsubscript{c}). Compared to these controls, immobility times were decreased for WT animals with 10 mg/kg fluoxetine and 10 or 20 mg/kg bupropion (Fig. 7\textsubscript{a},\textsubscript{b}). In contrast, immobility times for mutants were unaffected by fluoxetine (Fig. 7\textsubscript{a}) or bupropion (Fig. 7\textsubscript{b}). Nevertheless, acute administration of 10 mg/kg reboxetine reduced immobility times in

**Fig. 2.** Responses of wild-type (WT) and Cpe\textsuperscript{fat/fat} mice in the light–dark exploration test. (a) Cpe\textsuperscript{fat/fat} mice exited the illuminated and entered the darkened chamber more quickly than WT controls ($t_{1,16}=3.851, p<0.001$). (b) Cpe\textsuperscript{fat/fat} animals spent less time in the light compartment than WT mice ($t_{1,16}=2.280, p<0.037$). (c) The latency to re-enter the illuminated after crossing into the darkened chamber was prolonged for Cpe\textsuperscript{fat/fat} mice ($t_{1,16}=3.280, p<0.002$). (d) The number of transitions between the illuminated and darkened chambers was decreased for Cpe\textsuperscript{fat/fat} animals ($t_{1,16}=4.858, p<0.001$). (e) Activity in the illuminated chamber was reduced for Cpe\textsuperscript{fat/fat} mice ($t_{1,16}=2.184, p<0.044$). (f) Cpe\textsuperscript{fat/fat} animals were less active in the darkened chamber than WT controls ($t_{1,16}=2.254, p<0.039$). $N=9$ mice/genotype. * $p<0.05$, compared to WT controls.
Fig. 3. Effects of diazepam on anxiety-like behaviour of Cpefat/fat mice in the zero maze. (a) Latency to enter the open areas. Analysis of variance (ANOVA) found a main effect of genotype ($F_{1,88} = 7.668, p < 0.007$) and the treatment × genotype interaction to be significant ($F_{3,88} = 11.027, p < 0.001$). Bonferroni’s comparisons observed that vehicle- and 0.5 mg/kg diazepam-treated Cpefat/fat mice took longer to enter the open areas than similarly treated WT mice ($p < 0.003$ for all). Mutants given 0.5 or 1 mg/kg diazepam took longer to enter the open areas ($p < 0.039$ for all), but those given 2 mg/kg diazepam did not differ from the WT vehicle-controls. In Cpefat/fat animals 1 or 2 mg/kg diazepam reduced open entry times relative to their vehicle-controls ($p < 0.014$ for all). For WT animals, 1 and 2 mg/kg diazepam prolonged their latencies to enter the open areas relative to their vehicle-controls ($p < 0.003$ for all). (b) Percent time in the open areas. ANOVA revealed only a significant genotype × treatment interaction ($F_{3,87} = 7.336, p < 0.001$). Bonferroni’s tests noted genotype effects for vehicle and 0.5 and 2 mg/kg doses of diazepam ($p < 0.003$ for all). WT mice showed a reduction in open area times with 1 and 2 mg/kg diazepam compared to their vehicle-controls ($p < 0.014$ for all), while mutants given these doses spent significantly more time in the open areas than their vehicle-controls ($p < 0.050$ for all). Cpefat/fat mice administered 0.5 mg/kg diazepam spent less time in the open areas than WT vehicle-controls ($p < 0.036$), whereas mutants given 1 or 2 mg/kg were not different from these WT animals. N = 9–13 mice/treatment group. * $p < 0.05$, compared to WT controls (within treatment); † $p < 0.05$, compared to WT vehicle-controls; ‡ $p < 0.05$, compared to vehicle-treated Cpefat/fat mice (within genotype).

Fig. 4. Effects of fluoxetine on anxiety-like behaviour of Cpefat/fat mice in the zero maze. (a) Latency to enter the open areas of the maze. Analysis of variance (ANOVA) revealed main effects of genotype ($F_{1,88} = 25.129, p < 0.001$) and treatment ($F_{2,88} = 13.116, p < 0.001$), and a significant treatment × genotype interaction ($F_{2,88} = 7.624, p < 0.001$). Bonferroni’s corrected pair-wise comparisons observed that latencies to enter the open areas were prolonged for Cpefat/fat mice relative to WT animals given vehicle or 5 mg/kg fluoxetine ($p < 0.001$ for all). WT responses were not significantly affected by fluoxetine. Mutants given 5 or 10 mg/kg doses had shorter latencies to enter the open areas than their vehicle-controls ($p < 0.050$ for all). Latencies were still longer for Cpefat/fat animals than WT vehicle-controls ($p < 0.036$), whereas mutants given 1 or 2 mg/kg were not different from these WT animals. N = 9–13 mice/treatment group. * $p < 0.05$, compared to WT controls (within treatment); † $p < 0.05$, compared to WT vehicle-controls; ‡ $p < 0.05$, compared to vehicle-treated Cpefat/fat mice (within genotype).

WT and Cpefat/fat animals (Fig. 7c). Importantly, this dose of reboxetine was sufficient to decrease immobility times of mutants to those of the WT vehicle-controls. Despite the lack of response by Cpefat/fat mice to fluoxetine and bupropion in tail suspension, both genotypes responded to fluoxetine, reboxetine and bupropion in the open field.
Anxiety-like responses in the mutants were observed (Srinivasan et al., 2004). In the present experiments, fertility is decreased in mutants at 60 d, dramatically to the in the mutants given either antidepressant relative consecutive days. This regimen reduced immobility times to those of the WT controls with acute administration of fluoxetine or bupropion. To determine whether these mutants present with depressive-like behaviours, animals were evaluated in the forced swim and tail suspension tests. In both assays, immobility times were prolonged. Interestingly, the immobility in mutants aged 5 months compared to WT mice aged 2 months and to mutants aged 2 or 3 months (p < 0.003 for all), which were not different from each other. N = 9–12 mice/genotype/age. In tail suspension, ANOVA revealed significant main effects of genotype (F_{2,83} = 33.127, p < 0.001) and age (F_{2,83} = 9.846, p < 0.001), with a trend for the genotype × age interaction (F_{2,83} = 2.667, p < 0.076). Bonferroni’s corrected pair-wise comparisons showed that Cpefat/fat mice spent more time in immobility at all ages than WT littermates (p < 0.038 for all). N = 9–17 mice/age/genotype. * p < 0.05, compared to WT controls (within age); † p < 0.05, compared to vehicle-treated WT mice aged 2 months; ‡ p < 0.05 compared to vehicle-treated Cpefat/fat mice aged 2 and 3 months.

Discussion

The fat mutation was first identified at the Jackson Laboratories in 1973 (Leiter et al., 1999). Hyperglycaemia, obesity and subfertility are present in adulthood and they become more severe with age (Leiter et al., 1999; Srinivasan et al., 2004). With regard to obesity, food consumption of WT and Cpefat/fat mice is similar at age 2 months (Leiter et al., 1999), whereas it is increased in mutants aged > 3 months (Yuan et al., 2004). Similarly, fertility is decreased in mutants at 60 d, dramatically reduced at 90 d, and completely lost by age 5 months (Srinivasan et al., 2004). In the present experiments, anxiety-like behaviours were not observed in Cpefat/fat animals at age 60 d; however, they emerged with time. Anxiety-like responses in the mutants were observed both in the zero maze and the light–dark box. Importantly, the responses of the mutants in the zero maze to pharmacological therapy support the contention that the older Cpefat/fat mice display an anxiety-like phenotype. Diazepam decreased in a dose-dependent manner the latency to enter the open areas of the maze and it increased time in the open areas. Although diazepam increased locomotor activity of the Cpefat/fat mice in the open field, it is unlikely that the reduced anxiety-like behaviours in the zero maze to this drug were due to a general enhancement in motor activity. Previous studies from our laboratory (Pogorelov et al., 2005) and others (Crawley and Goodwin, 1980) have found that increased exploration in the open field following diazepam treatment may be due to a suppression of anxiety-like behaviours. Collectively, these findings strongly suggest that Cpefat/fat mice display anxiety-like behaviours that emerge over time and that diazepam can alleviate these responses.

Anxiety and major depression are frequently co-morbid in human patients (Zimmerman et al., 2002) and they may be ameliorated with selective serotonin re-uptake inhibitors. In our experiments, the anxiety-like behaviours of Cpefat/fat mice responded in dose-dependent manners to diazepam and fluoxetine. To determine whether these mutants present with depressive-like behaviours, animals were evaluated in the forced swim and tail suspension tests. In both assays, immobility times were prolonged. Interestingly, the immobility in mutants was alleviated with acute doses of imipramine and reboxetine, but not with fluoxetine or bupropion. Although acute antidepressant treatment is often sufficient to normalize behaviour in tail suspension in rodents (see Fukui et al., 2007), human depressed patients frequently...
Bonferroni’s corrected pair-wise comparisons found that compared to vehicle (Veh) treatments, immobility was reduced for wild-type (WT) and Cpefat/fat treatments compared to WT animals. Importantly, immobility time for Cpefat/fat mutants was similar to that of their Veh-controls. The 10 mg/kg dose clearly increased the frequencies and reduced the overall time in immobility in the mutants. The 5 mg/kg imipramine reduced duration but increased frequency of immobility bouts in mutants, while the total time spent immobile was similar to that of Veh-treated mutants that showed less frequent and more prolonged bouts of immobility. The 5 mg/kg imipramine reduced duration but increased frequency of immobility bouts in mutants, while the total time spent immobile was similar to that of their Veh-controls. The 10 mg/kg dose clearly increased the frequencies and reduced the overall time in immobility in the mutants.

**Fig. 6.** Effects of acute imipramine on immobility in forced swim for Cpefat/fat mice. (a) For the overall duration of immobility, analysis of variance (ANOVA) found only significant main effects of genotype ($F_{1,51} = 58.260, p < 0.001$) and treatment ($F_{2,51} = 5.810, p < 0.001$). Bonferroni’s corrected pair-wise comparisons found that compared to vehicle (Veh) treatments, immobility was reduced for wild-type (WT) and Cpefat/fat mice given 10 mg/kg ($p < 0.036$ for all). Despite this reduction, immobility in Cpefat/fat mice was enhanced under all treatments compared to WT animals. Importantly, immobility time for Cpefat/fat mice given 10 mg/kg imipramine did not differ from that of Veh-treated WT animals. (b) For latency to the first immobility bout ANOVA observed only the genotype effect to be significant ($F_{1,51} = 104.709, p < 0.001$). Bonferroni’s comparisons demonstrated that Cpefat/fat mice had shorter latencies under all treatment conditions compared to WT animals ($p < 0.001$ for all). (c) For the duration of the first immobility bout ANOVA revealed significant main effects of genotype ($F_{1,51} = 16.772, p < 0.001$) and treatment ($F_{2,51} = 12.073, p < 0.001$), and a significant genotype $\times$ treatment interaction ($F_{2,51} = 3.394, p < 0.041$). Bonferroni’s tests reported that Cpefat/fat mice given Veh had longer initial bouts of immobility than WT Veh-controls ($p < 0.001$). WT mice administered the 10 mg/kg dose showed a significant reduction in this parameter relative to WT Veh-controls ($p < 0.048$). The 5 or 10 mg/kg imipramine dose reduced this time for Cpefat/fat mice compared to their respective Veh-controls ($p < 0.001$) and these doses normalized their responses to the WT Veh-controls. (d) For the overall frequencies of immobility bouts during the 6 min test ANOVA noted the treatment effect ($F_{2,51} = 3.878, p < 0.0291$) and the genotype $\times$ treatment interaction to be significant ($F_{2,51} = 8.674, p < 0.001$). Bonferroni’s comparisons showed for Veh-treatments, Cpefat/fat animals had fewer bouts of immobility than WT mice ($p < 0.001$). While imipramine did not alter this frequency in WT mice under any treatment, the 5 and 10 mg/kg doses increased this frequency in Cpefat/fat animals compared to their Veh-controls ($p < 0.025$ for all). Interestingly, 5 mg/kg imipramine normalized the responses in Cpefat/fat mice, whereas the 10 mg/kg dose increased the bouts of immobility relative to WT controls given the same dose ($p < 0.025$). N=9–10 mice/genotype/treatment group. $^*$ $p < 0.05$, compared to WT controls (within treatment); $^+$ $p < 0.05$, compared to WT Veh-controls; $^+$ $p < 0.05$, compared to Veh-treated Cpefat/fat mice (within genotype).

(e) Representative time–event plots showing the time spent in immobility (black bars) and swimming (white bars) during the 6 min test for Veh-treated WT and Cpefat/fat mice, and for mutants given 5 or 10 mg/kg imipramine. WT mice spent most of the time swimming, while immobility bouts were brief but more frequent than Veh-treated mutants that showed less frequent and more prolonged bouts of immobility. The 5 mg/kg imipramine reduced duration but increased frequency of immobility bouts in mutants, while the total time spent immobile was similar to that of their Veh-controls. The 10 mg/kg dose clearly increased the frequencies and reduced the overall time in immobility in the mutants.
require more protracted therapy (Nemeroff and Owens, 2002). When Cpefat/fat mice were treated for 2 wk with fluoxetine or bupropion, both antidepressants reduced the immobility times of mutants. The response in tail suspension to acute administration of reboxetine indicates that noradrenergic neurotransmission is intact in Cpefat/fat mice, whereas the lack of response to acute fluoxetine treatment suggests that serotonergic neurotransmission may be perturbed in Cpefat/fat animals. Administration of fluoxetine for 2 wk was sufficient to overcome enhanced immobility in tail suspension. In a similar manner human patients typically require antidepressant treatment over several weeks to decrease their symptoms (Nemeroff and Owens, 2002). In contrast to fluoxetine, the response to 2 wk bupropion treatment suggests that several mechanisms may be perturbed in Cpefat/fat mice since this drug has multiple modes of action (Ascher et al., 1995). Nevertheless, the present results
Flux reduced immobility times to those of the WT controls. Analysis of variance revealed only significant main effects of treatment ($F_{3,48} = 40.498, p < 0.001$) and Bonferroni’s corrected pair-wise comparisons demonstrated that mutants given vehicle (Veh) spent more time in immobility than wild-type (WT) Veh-controls ($p < 0.001$). Both bupropion (5 mg/kg; 5 Bup) and fluoxetine (7.5 mg/kg; 7.5 Flux) reduced immobility times in Cpefat/fat mice compared to Veh-treated mutants ($p < 0.014$ for all; 7.5 Flux reduced immobility times to those of the WT controls. $N = 9-14$ mice/treatment group. $^* p < 0.05$ compared to WT Veh-controls; $^+ p < 0.05$ compared to Veh-treated Cpefat/fat mice. 

Fig. 8. Effects of daily injections of antidepressants for 14 d consecutively on tail suspension responses in Cpefat/fat mice.

Since Cpe is involved in processing many inter-mediates to bioactive peptides, the peptides which may be responsible for the depressive- and anxiety-like behaviours may be inferred from studies where a given peptide gene or its receptor have been genetically targeted for deletion or where a peptide receptor antagonist has been used. It is noteworthy that both anxiolytic and anxiogenic effects have been identified using genetic and pharmacologic paradigms (Millan, 2003). Although depressive-like responses of peptides have been investigated, possible antidepressant effects remain largely unexplored (Urani et al., 2005). Neuropeptides implicated in depressive-like responses include brain-derived neurotrophic factor, corticotrophin-releasing hormone, neuropeptide Y, substance P and thyrotropin-releasing hormone. Although deletion of the gene for each of these individual peptide precursors does not produce depressive-like responses in mice (Urani et al., 2005), the Cpefat/fat mouse may be expected to have greatly reduced levels of all of these peptides and this combined effect is likely to be more dramatic than individual gene deletions. Peptides that are known or predicted to be processed by Cpe and whose loss is anxiogenic include oxytocin (Mantella et al., 2003; Amico et al., 2004), urocortin I (Vetter et al., 2002), urocortin II (Valdez et al., 2002), neuropeptide Y (Bannon et al., 2000), encephalin (König et al., 1996), nocioceptin/orphanin FQ (Küster et al., 1999), neuropeptide S (Xu et al., 2004), cocaine- and amphetamine-related transcript (Stanek, 2006), gastrin (Singh et al., 2004), brain-derived neurotrophic factor (Chen et al., 2006) and ghrelin (Seoane et al., 2004). Gene-disruption and receptor antagonist studies have identified also some neuropeptides that have anxiolytic actions (see Millan, 2003). Regardless, in Cpefat/fat mice the net effect is clearly an increase in anxiety-like responses with age.

Since depressive-like behaviours are evident at all ages examined, they are likely to be due primarily to the Cpefat/fat mutation. The dependence of the anxiety-like behaviours on the Cpefat/fat mutation is less clear. One possibility is that the abnormal behaviours may be a direct result of the reduction of peptides produced by Cpe, with compensation from another carboxypeptidase at younger ages. While Cpe is inactive, unstable and rapidly degraded in Cpefat/fat mice (Naggert et al., 1995; Valamov et al., 1996), it has been proposed that Cpd may partially compensate for loss of Cpe in these mutants (Fricker, 2004b). If Cpd levels decrease over the first 2–3 months, this could explain the emergence of anxiety-like behaviour found in the present study, as well as the age-dependent decrease in processing of the reproductive neuropeptide gonadotropin-releasing hormone in Cpefat/fat mice (see Srinivasan et al., 2004). Although this is certainly a possibility, levels of Cpd protein are not up-regulated in whole brain of Cpefat/fat mice (see Fricker et al., 1996). Another possibility for the delayed emergence of anxiety-like behaviours is that the effect is secondary to the effect of Cpe on neuropeptide levels. While body weights are generally similar in WT and Cpefat/fat animals aged 3 months when anxiety-like responses are first observed, the mutant mice are beginning to gain weight and there may be metabolic changes that are occurring at this time. There is some support of this idea since a pilot study at AECOM found the anxiety-like behaviour in overweight Cpefat/fat mice was attenuated when they were feed-restricted. Additional secondary effects may include alterations in sex steroids or other hormonal changes associated with the early development of diabetes, obesity and/or subfertility.

In the present studies, we have shown that Cpefat/fat mice display both anxiety- and depressive-like behaviours before they develop obesity. Depression and anxiety are co-morbid in many patients (Zimmerman et al., 2002); however, it is unclear whether both abnormalities emerge together or have separate aetiologies. Since the depressive-like phenotype in Cpefat/fat animals precedes the appearance of their anxiety-like responses and because both behaviours show differential sensitivities to fluoxetine, it should be possible in the future to separate the mechanisms that control anxious from depressive states in this mutant. The delay in the response to treatment with fluoxetine in the tail suspension test...
clearly suggests that serotonin transmission is abnormal in Cpefat/fat mice and that continual treatment for ≥2 wk is required to alleviate the depressive-like behaviour. It should be emphasized that human patients also require prolonged treatment with antidepressants and many patients still fail to respond to treatment (Nemeroff and Owens, 2002). For this reason, it is recognized that new antidepressant drugs need to target pathways that are independent of norepinephrine and/or serotonin neurotransmission. From this perspective, the Cpefat/fat mouse may serve as a useful model to identify novel drug targets and to devise new strategies for treatment of anxiety and depression in humans.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713000059.

Acknowledgements

This work was supported in part by National Institutes of Health (NIH) grants DK-51271 and DA-04494 (L.D.F.) and by NIH grants HD36015 and MH60451 and unrestricted funds (W.C.W.). Some of the equipment used in the behavioural testing was purchased with a grant from the North Carolina Biotechnology Center. We thank Dr Ed Leiter for providing Cpe+/fat mice, Ms Jiechun Zhou for breeding and genotyping the mice, and Ms Dongqin Zhu, Ms Liping Du, and Ms Santowana Ayral for maintaining the mice at Duke University. We also thank Mr Michael Cools, Mr Matthew Pease, Ms Caroline Kim and Ms Adwanna Howard for their assistance in behavioural testing.

Statement of Interest

None.

References

Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity.


Nat Neurosci 5:1068–1070.


