Altered behavioural adaptation in mice with neural corticotrophin-releasing factor overexpression

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Overproduction of corticotrophin-releasing factor (CRF), the major mediator of the stress response, has been linked to anxiety, depression and addiction. CRF excess results in increased arousal, anxiety and altered cognition in rodents. The ability to adapt to a potentially threatening stimulus is crucial for survival, and impaired adaptation may underlie stress-related psychiatric disorders. Therefore, we examined the effects of chronic transgenic neural CRF overproduction on behavioural adaptation to repeated exposure to a non-home cage environment. We report that CRF transgenic mice show impaired adaptation in locomotor response to the novel open field. In contrast to wild-type (WT) mice, anxiety-related behaviour of CRF transgenic mice does not change during repeated exposure to the same environment over the period of 7 days or at retest 1 week later. We found that locomotor response to novelty correlates significantly with total locomotor activity and activity in the centre at the last day of testing and at retest in WT but not in CRF transgenic mice. Mice were divided into low responders and high responders on the basis of their initial locomotor response to novelty. We found that differences in habituation and re-exposure response are related to individual differences in locomotor response to novelty. In summary, these results show that CRF transgenic mice are fundamentally different from WT in their ability to adapt to an environmental stressor. This may be related to individual differences in stress reactivity. These findings have implications for our understanding of the role of CRF overproduction in behavioural maladaptation and stress-related psychiatric disorders.

Keywords: Adaptation, corticotrophin-releasing factor, individual differences, open-field, stress, transgenic mice

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Corticotrophin-releasing factor (CRF) is involved in regulating adaptive responses to stress. Besides activating the hypothalamic–pituitary–adrenal axis and the autonomic nervous system, CRF also modulates behavioural responses to a wide range of stressful stimuli (Hillhouse & Grammatopoulos 2006, for recent review). In experimental animals, CRF administration results in behavioural alterations reminiscent to the effects of stress (Koob & Heinrichs 1999). Inhibition of CRF neurotransmission can normalize a number of stress-induced behavioural changes (Koob & Heinrichs 1999). Increased activity of the brain CRF system has been suggested to play a role in stress-related psychopathology, including affective disorders (Holsboer 1999; Nemeroff 1996) and substance abuse (Sarnyai *et al.* 2001; Steckler & Dauntzenberg 2006).

To gain more insight into the relationship between central CRF hyperactivity and behavioural changes associated with chronic stress and psychopathology, a transgenic mouse model of global CRF overproduction has been developed (Stenzel-Poore *et al.* 1992). Detailed phenotyping of this mouse line has shown a number of abnormalities, including anxiety-like behaviour (Stenzel-Poore *et al.* 1994; van Gaalen *et al.* 2002) and impaired sexual behaviour, learning and attention (Heinrichs *et al.* 1996, 1997; van Gaalen *et al.* 2003). To overcome some of the drawbacks of the global CRF overexpression starting during the prenatal period (e.g. the early appearance of Cushing's syndrome), our group has more recently developed another CRF transgenic line using a chimeric CRF transgene comprising the murine Thy-1.2 promoter and regulatory regions driving the rat CRF gene (Dirks *et al.* 2002a). In these mice, overexpression starts 4–8 days after birth (Luthi *et al.* 1997) and is restricted to the central nervous system due to the promoter and regulatory region used. CRF overexpression is found not only in the paraventricular nucleus of the hypothalamus but also in extrahypothalamic areas relevant to stress-related behaviours, such as central nucleus of amygdala, bed nucleus of stria terminalis, hippocampus and cerebral cortex (Dirks *et al.* 2002a). These mice show a number of behavioural, physiological and endocrine abnormalities that correspond well with the central elevation of CRF activity, partially supporting previous findings obtained in mice with global CRF overexpression (Groenink *et al.* 2003 for review). Some unexpected findings in these mice, such as reduced startle reactivity, impaired startle habituation and prepulse inhibition of the acoustic startle response (Dirks *et al.* 2002b), raised the intriguing possibility that central CRF overproduction may reduce behavioural reactivity to environmental stimuli and impair information processing mechanisms.

Habituation refers to the reduction in behavioural response after initial and repeated exposure to the same environment, which is a measurable net result of two independent and

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opposing mechanisms, habituation and sensitization (Groves & Thompson 1970). This habituation occurs during a single session (short-term or within-session habituation) and across several sessions (long-term or between-session habituation). Habituation represents a fundamental form of nonassociative learning in which the organism learns to differentiate behaviourally meaningful from irrelevant stimuli not to respond to redundant nonsignificant stimuli (Groves & Thompson 1970; Thorpe 1956). An impaired habituation and resulting maladaptation to environmental stimuli may represent a central feature of stress-related behavioural pathology. Therefore, in our present study, we set out to investigate in detail the acute response to novelty, habituation to repeated exposure to the same, initially threatening, environment and the effect of re-exposure to the same stimuli after a period of 'extinction' in mice with central CRF overexpression. We also investigated the relationship between different aspects of open-field (OF) activity to shed light on the contribution of CRF overproduction in changes of stress reactivity and behaviour directed towards the anxiety-inducing elements of the environment over time.

The existence of marked individual differences in behavioural responsiveness to novel, stressful stimuli has been recently recognized. In a series of studies Piazza and coworkers have shown that outbred Sprague-Dawley rats characterized as 'high responders' (HR) on the basis of their locomotor response to a novel environment fundamentally differ from 'low responders' (LR) in a number of behavioural and physiological indices related to stress pathology, such as stress-induced corticosterone response, propensity to self-administer drugs of abuse, cognitive function in later life and brain neurochemistry (Dellu *et al.* 1993, 1994; Piazza *et al.* 1989). It has been proposed that genetically or environmentally encoded individual differences in behavioural and hormonal stress response may play a major role in the development of susceptibility to psychiatric disorders (Meaney & Szyf 2005). Therefore, we also aimed to study individual differences in locomotor response to novelty in CRF overexpressing transgenic (CRF-OE) mice and in their wild-type (WT), inbred C57BL/6 littermates and to study their relationship to behavioural adaptation.

Materials and methods

Animals

CRF-OE mice were generated as previously described (Dirks *et al.* 2002a). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF complementary DNA (cDNA) [0.6 kilo base (kb) fragment], which was inserted at the *Xho*I restriction site into an 8.2 kb EcoR1 genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons (Luthi *et al.* 1997; Morris & Grosveld 1989). The Thy-1 CRF transgene was prepared for microinjection by isolating a 9.0 kb *Not*I fragment containing the fusion gene, which was purified from conventional agarose gel by electroelution. The fragment was microinjected into fertilized eggs (C57BL/6J), and the injected cells were transplanted into pseudopregnant foster mothers. To identify transgenic founder animals, tail DNA from offsprings was screened by standard Southern dot blot analysis using the 6.0 kb CRF cDNA fragment as a probe. These procedures yielded founder animals, which gave rise to a line (CRF-OE 2122 line), that was further bred at the local breeding facilities (Central Laboratory Animal Institute, Utrecht University, the Netherlands) and used for this study. Breeding consisted of mating between transgenic male and C57BL/6J female mice. Tail DNA from offsprings, extracted with High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany), was screened by polymerase chain reaction using transgene-specific primers. The forward primers were specific for rat CRF and the reverse primers originated from the Thy-1 promoter, thus excluding the possibility that the endogenous CRF and Thy-1 genes were amplified.

Fifteen male CRF-OE (aged 26–31 weeks and with body weight 25.6–38.5 g at the beginning of experiment) and fifteen male littermate C57BL/6J WT mice (aged 23–31 weeks and with body weight 29.8–36.9 g at the beginning of experiment) were used in this experiment. Mice were housed ($n = 2$–3 per cage) in plastic cages (12 $\times$ 22 $\times$ 15 cm; Tecniplast, Buguggiate, Italy) enriched with bedding (EnviroDri®; BMI, Helmond, The Netherlands), a piece of PVC tubing (diameter 5 cm) and a nesting material at constant room temperature (21 $\pm$ 2°C) and relative humidity (40–50%). Standard rodent food pellets (Special Diet Services Ltd., Witham, Essex, UK) and tap water were freely available. Animals were maintained on a 12-h light–12-h dark cycle (lights on from 0600 h until 1800 h). All experimental procedures were conducted during the light phase of the cycle, between 0900 h and 1630 h. All studies and procedures were approved by the ethical committee on animal experiments of the Faculties of Pharmacy, Biology, and Chemistry of Utrecht University, The Netherlands, according to the Dutch law for animal experimentation and the Declaration of Helsinki.

Apparatus and experimental protocol

Spontaneous locomotor behaviour was quantified in an OF (Fig. 1). Four OFs, round opaque plastic boxes, diameter 42 cm and height 29.5 cm, with centre circles of diameter 21 cm, were placed in a sound-attenuated room to allow recording of undisturbed behaviour (illumination approximately 400–600 lux at floor level). At the beginning of each session, mice were put individually into one of the four circular compartments of the OF and were allowed to freely explore for 30 min. During 30 min, the behaviour of the mouse was recorded using Ethovision® automated tracking programme (Noldus Information Technology, Wageningen, The Netherlands). Total distance moved (TD), distance moved in the centre circle (CD), distance moved in the periphery, frequency of entry to the centre circle (CE), time spent in the centre circle, time spent in the periphery, average velocity in the centre circle and average velocity in the periphery were measured. At the end of the 30-min period on each experimental days, all mice received a saline injection and placed back into the OF for another 60 min (not reported). This procedure was repeated daily for 7 days, followed by a 7-day period when animals were not subjected to any experimental manipulations and were left undisturbed in their home cages ('extinction'). Then, mice were subjected to re-exposure to the OF on Day 15 for 30 min to test their activity as described above. To identify individual differences in novelty-induced locomotor reactivity, the group (WT and CRF-OE) medians were calculated for the TD during 30 min on the first day of the OF study. Animals that showed TD above or below the group median (8685.68 and 8128.70 cm/30 min for WT and CRF-OE, respectively) and were classified as HR or LR, respectively (Piazza *et al.* 1989). Mice with values at the median were excluded from the analyses involving individual differences.

Data analysis

Data were analysed by two-way analysis of variance (ANOVA) on genotype and time unless stated otherwise. Appropriate post hoc tests were used for paired comparison. The statistical analyses were performed by StatView software (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$.

Results

Locomotor activity: TD

Upon first exposure to the OF (Day 1; Fig. 2a) repeated measure ANOVA did not find a significant genotype effect

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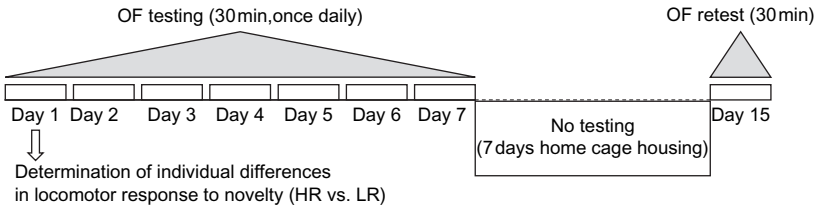

Figure 1: Experimental design.

($F_{1,28} = 2.417$, $P = 0.131$). However, there was a highly significant time effect ($F_{5,140} = 13.931$, $P < 0.0001$) and interaction between genotype and time ($F_{5,140} = 5.569$, $P = 0.0001$). Post hoc paired comparisons showed that TD during the first 5 min differed significantly from all that during later time points in WT, but not in CRF-OE, mice. Furthermore, CRF-OE mice were significantly less active during the first 5 min than WT animals (TD: $P < 0.0001$). On Day 7 of the repeated OF exposure (Fig. 2b), there was no significant main genotype effect ($F_{1,28} = 3.183$, $P = 0.085$), but there was a significant time effect ($F_{5,140} = 2.26$, $P = 0.05$) and genotype × time interaction ($F_{5,140} = 6.87$, $P < 0.0001$). While WT mice did not show significant differences, CRF-OE mice were significantly more active overall at all later time points than during the first 5 min. On the Day 15 retest (Fig. 2c), after 7 days of home cage housing with no testing following the 7 days repeated OF testing, there was no overall genotype difference ($F_{1,28} = 0.353$, $P = 0.557$), but there was a significant main time effect ($F_{5,140} = 3.781$, $P = 0.003$) and genotype × time interaction ($F_{5,140} = 2.243$, $P = 0.05$). These differences were probably contributed by a somewhat lower activity at min 20 of the WT mice compared with that of CRF-OE mice ($P < 0.05$).

Comparing overall TD (30 min OF) on Days 1, 7 and 15 (Fig. 2d) showed no main genotype effect ($F_{1,28} = 0.03$, $P = 0.863$), but a significant main effect of day ($F_{2,56} = 42.108$, $P < 0.0001$) was found. Genotype × day interaction was significant ($F_{2,56} = 5.528$, $P = 0.006$). Post hoc analysis showed that both WT and CRF-OE mice exhibited significantly less activity on day 7 ($P < 0.0001$ and $P = 0.008$, for WT and CRF-OE mice, respectively). A similar pattern emerged when Day 1 activity was compared with Day 15 ($P < 0.0001$ and $P = 0.01$, for WT and CRF-OE mice, respectively). No significant difference was found between WT and CRF-OE mice at any time-point.

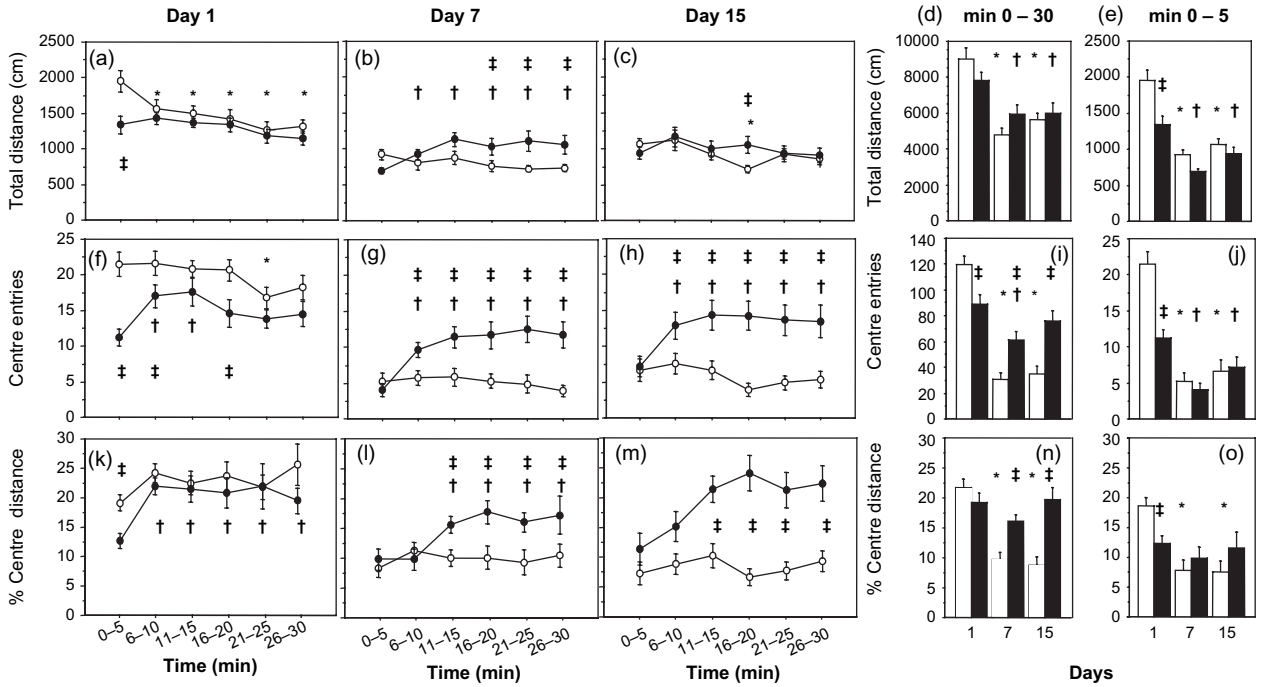

Figure 2: OF activity during the daily 30-min sessions and total activity of WT and CRF-OE mice on Days 1, 7 and 15. (a–e) Total Distance (cm), (f–j) number of centre entries, and (k–o) relative distance moved in centre (per cent centre distance). First three panels from the left: open circles represent WT mice and filled circles, CRF-OE mice. *$P < 0.05$ compared with min 0–5 in WT, †$P < 0.05$ compared with min 0–5 in CRF-OE mice and ‡$P < 0.05$ CRF-OE vs. WT mice. In two right panels: open bars represent WT mice and filled bars, CRF-OE mice. *$P < 0.05$ compared with Day 1 in WT, †$P < 0.05$ compared with Day 1 in CRF-OE and ‡$P < 0.05$ CRF-OE vs. WT on Day 1, 7 or 15.

Because the novelty stimulus of the OF is the strongest during the initial first 5 min, we separately analysed the TD during this period on Days 1, 7 and 15 (Fig. 2e). ANOVA revealed significant main genotype ($F_{1,28} = 8.208$, $P = 0.008$) and day ($F_{2,56} = 70.378$, $P < 0.0001$) effects. Genotype × day interaction was significant ($F_{2,56} = 6.038$, $P = 0.004$). Post hoc analysis showed that both WT and CRF-OE mice exhibited significantly less TD on Day 7 ($P < 0.0001$ for both WT and CRF-OE) and Day 15 ($P < 0.0001$ and $P < 0.01$, for WT and CRF-OE mice, respectively) than on Day 1. Furthermore, CRF-OE mice were significantly less active than WT animals on Day 1 ($P < 0.001$), whereas on Days 7 and 15, CRF-OE no longer differed from WT mice.

Anxiety-related measures: centre entries (CE) and per cent distance in centre

The number of entries into the centre area (CE) of the OF and the per cent distance travelled in the centre area (%CD) were analysed across the 30-min period on Days 1, 7 and 15 (Fig. 2f, g and h and Fig. 2k,l and m). Two-way ANOVA with repeated measures identified significant main effects for genotype ($F_{1,28} = 8.711$, $P = 0.006$) and time ($F_{5,140} = 4.516$, $P = 0.0008$) and significant genotype × time interaction ($F_{5,140} = 3.132$, $P = 0.01$) on Day 1 (Fig. 2f) for CE, and no significant genotype effect ($F_{1,28} = 1.69$, $P = 0.204$), a significant main effect of time ($F_{5,140} = 4.29$, $P = 0.0012$) and no significant interaction between genotype and time ($F_{5,140} = 1.105$, $P = 0.361$) for %CD (Fig. 2k) was identified. Post hoc paired comparisons showed that CE was significantly lower at 25 min than during the first 5 min in WT mice ($P < 0.05$). For CRF-OE mice, CE increased after the first 5 min, and they were significantly higher at 10 and 15 min than during the initial 5-min period ($P = 0.01$ and $P < 0.005$, respectively). Whereas %CD remained constant during the 30 min of observation for WT, CRF-OE mice showed significantly higher %CD values at all time points compared with those during the initial 5-min period.

On Day 7 (Fig. 2g and l), the analysis of CE and %CD showed significant main effects for genotype (CE: $F_{1,28} = 13.6$, $P = 0.001$; %CD: $F_{1,28} = 5.54$, $P = 0.026$) and time (CE: $F_{5,140} = 4.442$, $P = 0.0009$; %CD: $F_{5,140} = 3.436$, $P = 0.006$) and significant genotype × time interaction (CE: $F_{5,140} = 5.307$, $P = 0.0002$; %CD: $F_{5,140} = 3.047$, $P = 0.012$). The relatively low number of centre entries and activity in centre did not change during the 30-min period in WT mice, but CE increased significantly after the first 5 min and remained higher throughout in the CRF-OE mice ($P < 0.005$, compared with first minute values). Similarly, relative activity in the centre (%CD) from min 15 was also higher than during the initial 5 min in CRF-OE mice. Furthermore, CRF-OE mice entered more frequently into the centre area than WT animals from min 10 to 30 ($P < 0.005$ throughout) and travelled more there from min 15 to 30 ($P < 0.05$ at min 15 and 25; $P < 0.01$ at min 20 and 30).

On Day 15 (Fig. 2h and m), the analysis of CE and %CD showed significant main effects for genotype (CE: $F_{1,28} = 16.00$, $P = 0.0004$; %CD: $F_{1,28} = 21.202$, $P < 0.0001$) and time for %CD ($F_{5,140} = 4.715$, $P = 0.0005$) but not for CE ($F_{5,140} = 2.067$, $P = 0.073$) and significant genotype × time interaction (CE: $F_{5,140} = 3.87$, $P = 0.002$; %CD: $F_{5,140} = 4.156$, $P = 0.002$). At this time-point, similarly to Day 7, the relatively low number of centre entries and activity in centre did not change during the 30-min period in WT mice, but CE increased significantly after the first 5 min and remained higher throughout in the CRF-OE mice. Similarly, relative activity in the centre (%CD) from min 15 was also higher than during the initial 5 min in CRF-OE mice. Furthermore, CRF-OE mice entered more frequently into the centre area than WT animals from min 10 to 30 ($P < 0.05$ at min 10, $P < 0.01$ from min 15 throughout) and travelled more there from min 15 to 30 ($P < 0.0007$ at min 15, $P < 0.0001$ from min 20 to 30).

Analysis of the sum of the CE and the mean of %CD during the 30-min period in OF in Days 1, 7 and 15 (Fig. 2i and n) showed no main genotype effect for CE ($F_{1,28} = 2.887$, $P = 0.1$) but a significant genotype effect for %CD ($F_{1,28} = 13.639$, $P = 0.001$). A significant main effect of day was found in both measures (CE: $F_{2,56} = 83.159$, $P < 0.0001$; %CD: $F_{2,56} = 16.194$, $P < 0.0001$). Genotype × day interaction was significant in both measures (CE: $F_{2,56} = 31.778$, $P < 0.0001$; %CD: $F_{2,56} = 11.675$, $P < 0.0001$). Post hoc analysis showed that both WT and CRF-OE mice exhibited significantly less number of entries into the centre and less relative activity in the centre on Days 7 and 15 than on Day 1 ($P < 0.0001$ for CE from Day 1 in both measures and time points). However, CE on Day 1 differed from that only on Day 7 but not on Day 15 and %CD on Days 7 and 15 did not differ from that on Day 1 for CRF-OE mice. CRF-OE mice entered significantly less than WT mice into the centre on Day 1 ($P = 0.002$) and significantly more on Day 7 ($P = 0.002$) and Day 15 ($P < 0.0001$). Mean relative activity in the centre did not differ between WT and CRF-OE on Day 1 (Fig. 2g) but was higher in CRF-OE than in WT mice on Day 7 ($P = 0.003$) and Day 15 ($P < 0.0001$).

Focusing on the period when the novelty stimulus is the strongest in the OF during the initial 5 min, we separately analysed the CE and %CD during this period on Days 1, 7 and 15 (Fig.2j and o). ANOVA showed significant main genotype effect for CE ($F_{1,28} = 5.928$, $P = 0.022$) but not for %CD ($F_{1,28} = 0.002$, $P = 0.969$) and significant main effect for day (CE: $F_{2,56} = 75.664$, $P < 0.0001$; %CD: $F_{2,56} = 8.564$, $P = 0.0006$). A significant genotype × day interaction was also shown for both measures (CE: $F_{2,56} = 16.473$, $P < 0.0001$; %CD: $F_{2,56} = 4.835$, $P < 0.01$). Post hoc analysis showed that WT mice exhibited significantly less CE and %CD on Day 7 ($P < 0.0001$) and Day 15 ($P < 0.0001$) than on Day 1. In contrast, CRF-OE mice showed less CE but unchanged %CD on Days 7 and 15 than on Day 1 (Day 1 vs. Day 7: $P = 0.0003$; Day 1 vs. Day 15: $P < 0.05$). Furthermore, CRF-OE mice were significantly less active in both CE and %CD than WT animals on Day 1 only ($P < 0.0001$ for CE, $P < 0.05$ for %CD).

Relationship between initial reactivity to novelty and later activity

Spontaneous locomotor activity in the OF when rodents are first exposed to it as novel environment has been considered

as a measure of the behavioural stress response. First, we were interested in studying the relationship between the initial behavioural stress response (TD on Day 1) and the number of entries into the centre area (CE), considered to be a measure of anxiety/emotionality (Fig. 3a). We found significant positive correlations between TD on Day 1 and CE on Day 1 ($r^2 = 0.380$, $P = 0.014$), Day 7 ($r^2 = 0.75$, $P < 0.0001$) and Day 15 ($r^2 = 0.527$, $P = 0.002$) in WT mice, suggesting that the less stress responsive the WT mice were the more anxiety-related behaviour they exhibited. Surprisingly, we found a different pattern in CRF-OE mice. While the initial behavioural stress response correlated positively to CE on Day 1 ($r^2 = 0.395$, $P = 0.012$), similar to that of WT mice, no correlations was found between TD on Day 1 and CE on Day 7 ($r^2 = 0.035$, $P = 0.502$) or Day 15 ($r^2 = 0.193$, $P = 0.101$). This finding suggests that upon repeated exposure to the same environment, the behavioural response of CRF-OE mice towards the centre of the OF changed considerably and the initial relationship between behavioural stress response and anxiety-related behaviour is lost. Furthermore, we found (Fig. 3b) that only in WT mice, but not in CRF-OE animals, the initial behavioural stress response correlates with relative activity in the centre (%CD; $r^2 = 0.641$, $P = 0.0003$ and $r^2 = 0.484$, $P = 0.004$, for Day 1 vs. Day 7 and Day 1 vs. Day 15, respectively) on both Day 7 and Day 15. The more active behavioural stress response the WT mice showed initially the more active they remained overall and in the centre of OF throughout the entire experiment.

Individual differences in reactivity to novelty and behavioural adaptation

WT and CRF-OE mice were divided into HR and LR on the basis of their initial locomotor response (sum of total activity in 30 min) to the novel environment in the OF (*Materials and methods*), then TD, CE and %CD on Days 1, 7 and 15 were analysed and compared (Fig. 4). For TD (Fig.4a), ANOVA showed no significant main effect for genotype ($F_{1,24} = 0.019$, $P = 0.891$) but a significant main effect for stress reactivity (LR vs. HR) ($F_{1,24} = 30.75$, $P < 0.001$) and day ($F_{1,48} = 44.994$, $P < 0.0001$). There was no significant genotype × stress reactivity ($F_{1,24} = 0.006$, $P = 0.999$) interaction, but a significant genotype × day ($F_{1,48} = 6.135$, $P = 0.004$) and stress reactivity × day interactions ($F_{1,48} = 7.130$, $P = 0.002$). The genotype × day × stress reactivity interaction was not significant ($F_{1,48} = 1.601$, $P = 0.212$). LR and HR animals differed significantly from each other in both genotypes on Day 1 by definition. At the end of the repeated OF exposure on Day 7, animals of different stress responsivity did not differ in either genotype. Upon re-exposure to the OF 7 days later (Day 15), a significant difference was found between LR and HR CRF-OE ($P < 0.005$), but not WT, animals.

ANOVA for CE (Fig. 4b) identified near-significant main effects for genotype ($F_{1,24} = 3.767$, $P = 0.064$) and highly significant effects for stress reactivity ($F_{1,24} = 17.454$, $P = 0.0003$) and day ($F_{1,48} = 81.396$, $P < 0.0001$). No significant interactions was found for genotype × stress reactivity ($F_{1,48} = 0.000$, $P = 0.993$), day × stress reactivity ($F_{1,48} = 1.218$, $P = 0.305$) or genotype × stress reactivity × day ($F_{1,48} = 1.183$, $P = 0.315$), but a significant genotype × day interaction ($F_{1,48} = 31.193$, $P < 0.0001$) was found. On Day 1, HR mice exhibited more centre entries than LR mice in both genotypes. At the end of the repeated OF exposure on Day 7, HR had higher CE than LR among WT, but not CRF-OE, animals. Upon re-exposure to the OF 7 days later (Day 15), significant difference was found between LR and HR mice in both genotypes ($P < 0.005$), HR being significantly more active moving into the centre.

When relative activity in the centre area (%CD) was analysed (Fig. 4c), ANOVA identified a significant main effect for genotype ($F_{1,24} = 12.521$, $P = 0.0017$) and day ($F_{1,48} = 18.783$, $P < 0.0001$) but nonsignificant main effects for stress reactivity ($F_{1,24} = 1.588$, $P = 0.219$). Significant interactions were found between genotype and day ($F_{1,48} = 14.367$, $P < 0.0001$) and genotype × stress reactivity × day ($F_{1,48} = 4.951$, $P = 0.01$) but not between genotype and stress reactivity ($F_{1,24} = 1.659$, $P = 0.210$) and stress reactivity and day ($F_{1,48} = 1.975$, $P = 0.150$). On Day 1, post hoc analysis did not find any difference on the basis of initial stress response in either genotype. At the end of the repeated OF exposure on Day 7, HR showed higher relative activity in the centre than LR among WT, but not CRF-OE, animals. Upon re-exposure to the OF 7 days later (Day 15), significant difference was found between LR and HR only in WT mice ($P < 0.05$).

Discussion

In this study, we aimed to investigate the behavioural response to acute novelty stress and the adaptation to the repeated exposure to the same environment in CRF-OE mice and in their WT littermates. We also studied the relationship between initial reactivity to novelty and behavioural plasticity upon repeated exposure. Overall (Table 1), we have found that CRF-OE mice are initially less active and differ from WT in their within-session and between-session habituation, in particular in behaviours directed towards the centre of the OF. Furthermore, we have shown that the relationship between behavioural reactivity to novelty stress and anxiety-related activity is a stable trait in WT mice, whereas such relationship disappears upon repeated exposure in CRF-OE animals. Individual differences in locomotor response to novelty predict differential adaptation in WT and CRF-OE mice. These results suggest altered behavioural adaptation in mice overexpressing CRF in the brain.

In response to the first exposure to the novel OF, CRF-OE mice showed no overall difference in total locomotor activity from WT. However, they were clearly less active than WT during the first 5 min, when the novelty value of the experience is the strongest. This finding is in agreement with previous studies on the same line showing lower total and peripheral activity as measured by time spent moving in a novel OF during the 10 min of investigation (Dirks *et al.* 2001). Comparable results (Stenzel-Poore *et al.* 1994), or a similar trend (van Gaalen *et al.* 2002), were obtained previously using novel OF in a transgenic mouse strain with global CRF overexpression. Locomotor hypoactivity has also been shown in response to intracerebroventricular (i.c.v.) administration of CRF in a novel environment in rats (Sutton

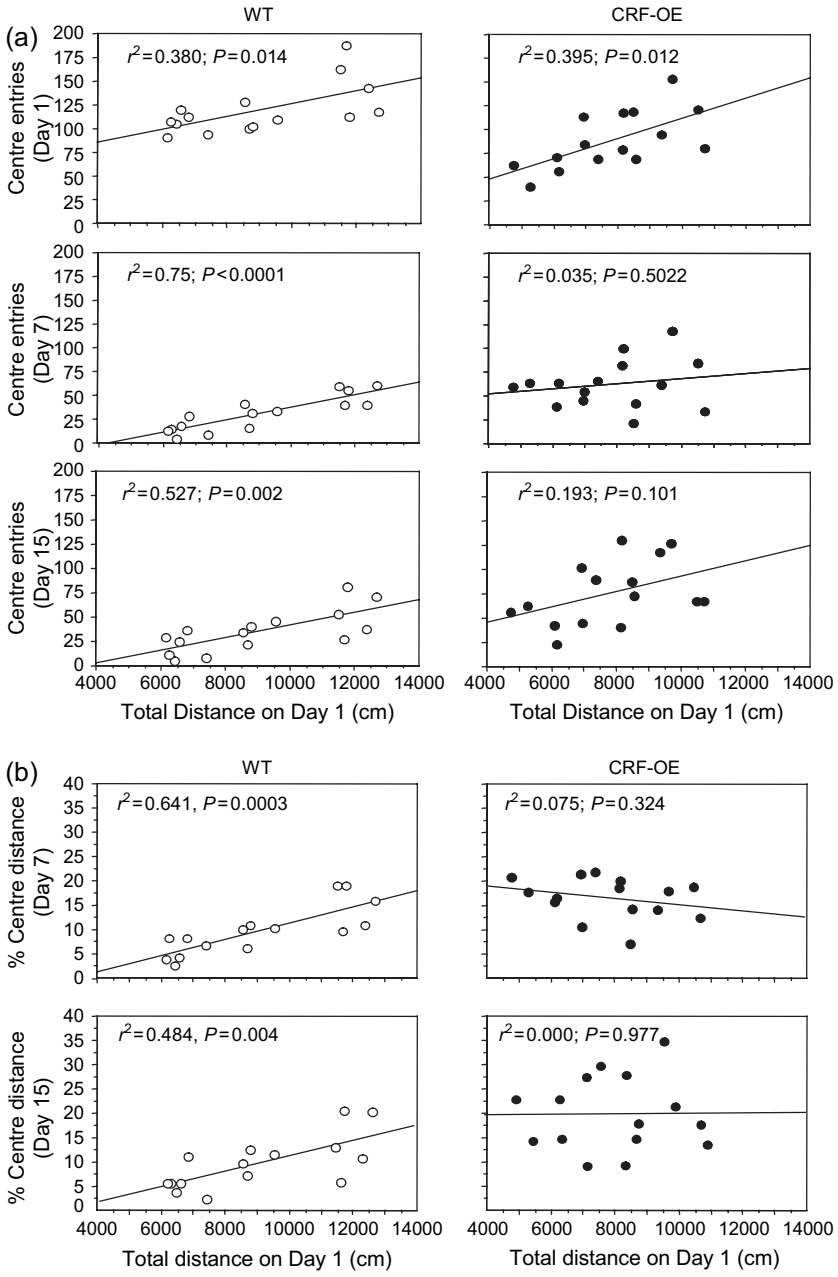

Figure 3: Correlations between initial locomotor response to novelty and later activities in the centre of OF. Correlations between the total distance moved in OF during the 30 min on Day1 and the number of centre entries on Days 1, 7 and 15 (a) and between the total distance moved in OF during the 30 min on Day1 and the per cent centre distance (relative activity in centre) on Days 7 and 15 (b) in WT (open circles) and CRF-OE (filled circles) mice.

et al. 1982). Therefore, our present finding is consistent with the view that an increased CRF drive results in locomotor hypoactivity in a novel environment.

In this study, CRF-OE mice showed higher 'anxiety'-related behaviour, such as lower number of entries into the centre area and relative distance travelled in the centre during the first 5 min. A similar trend, although nonsignificant, was observed by van Gaalen *et al.* (2002) in mice with global CRF overexpression. The use of a larger OF in the present experiment may explain its potentially more anxiety-inducing effects.

Within-session habituation was determined by comparing activity in the first 5 min to later time points during the initial 30-min novel OF exposure. Total distance travelled, a measure that reflects locomotor activity more than 'emotionality', decreases significantly after the initial novelty stress-induced hyperactivity in WT mice, and it gradually decreases throughout the 30-min period. This reflects efficient within-session habituation to the locomotor-stimulating effect of the novelty. However, CRF-OE mice do not show any signs of within-session habituation in this measure, with their total activity remaining unchanged throughout. This cannot be explained by a 'floor effect' (e.g. locomotor activity being already very low) as these mice are capable of exhibiting lower locomotor activity as evidenced by measures in the following days of repeated OF exposures. A different habituation pattern was found in anxiety-related measures. Number of entries into the

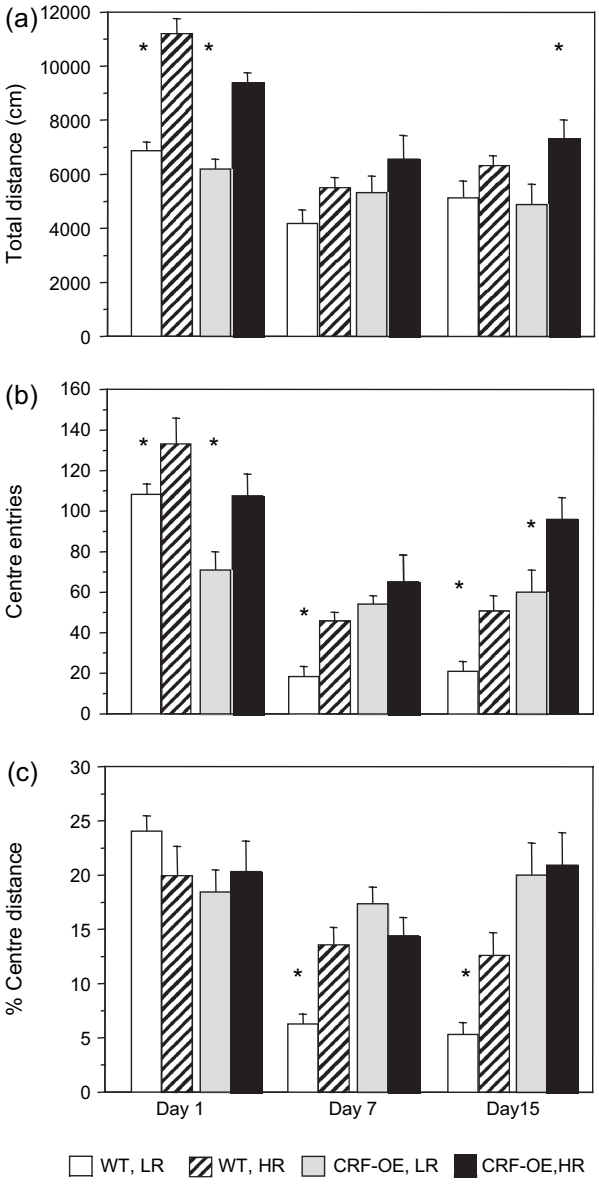

Figure 4: Individual differences in OF activity in WT and CRF-OE mice. Total distance moved (cm) (a), number of centre entries (b) and per cent centre distance (c) during the 30 min of OF exposure on Days 1, 7 and 15 in WT and CRF-OE mice. HR vs. LR mice were identified by their locomotor reactivity to novel OF on Day 1 (total distance moved during 30 min). Mice with activity above or below the appropriate group median were classified as HR and LR, respectively. WT, LR: open bars, WT, HR:dark grey bars, CRF-OE, LR: light grey bars, CRF-OE, HR:black bars. $*P <$ 0.05 in LR vs. HR within a particular genotype and day.

centre area (CE) and relative activity in the centre (%CD) that are relatively high do not change during the first OF exposure in WT mice. This may be explained by the relatively low-anxiety-related behaviour (Paylor *et al.* 2006; Rodgers *et al.* 2002) and higher exploratory activity and risk taking (Augustsson & Meyerson 2004) found in the C57BL/6J mice. These and our present results suggest that WT C57BL/6J mice do not perceive the centre area as a highly 'anxiogenic' stimulus. However, the behaviour directed towards the centre changes considerably in CRF-OE mice over time, showing a significant increase after the initial 5-min period. This may reflect decreasing anxiety-like behaviour in CRF-OE mice. In summary, there seems to be a dissociation between two aspects of novelty stress-related within-session habituation in the transgenic animals, e.g. lack of habituation in the stress-induced locomotor reactivity and efficient habituation in novelty-induced anxiety-like behaviour. Interestingly, similar habituation deficits were found in adult rats subjected to neonatal (Dubovicky *et al.* 1999) or psychosocial (Park *et al.* 2001) stress, manipulations known to elevate the activity of the brain CRF systems (Funk *et al.* 2006; Plotsky *et al.* 2005). Habituation represents a fundamental learning process, in which the organism learns not to respond to redundant nonsignificant stimuli (Groves & Thompson 1970). However, this progressive disappearance of responses can also be considered as an indicator of a cognitive integration of the properties inherent to the stimuli present and the speed at which the animals become habituated, as an indication of the speed at which they process information (Chapillon & Roullet 1997). It is intriguing that the same CRF-OE mouse line shows impaired habituation of the startle response to repeated auditory stimuli (Dirks *et al.* 2002b). It has been recently shown that mice with global overexpression of CRF exhibit deficits in sustained and divided attention, measured by the five-choice serial reaction time task (van Gaalen *et al.* 2003). In the light of these results, it is tempting to speculate that the habituation deficit seen in CRF-OE mice in the OF may be related to cognitive/attentional factors.

As expected, repeated exposure to the same OF leads to decreased activity in WT mice, consistent with an efficient between-session habituation. This is already seen on the second day of exposure (data not shown) and remains constant throughout the 7 days of daily, repeated OF study. The lack of within-session habituation in either the activity-related or the anxiety-related measures indicates the familiarity of the OF environment, e.g. it is not perceived as stressful any longer by the WT mice. Although total activity does not differ in the WT and transgenic mice, the significant interaction between genotype and time indicates a differential response during the 30-min period of the OF on Day 7. Indeed, CRF-OE mice, although they were less active during the initial 5 min than they were upon the novel OF exposure, which may indicate between-session habituation, showed an increased activity after the initial 5 min. In fact, the total locomotor activity was higher towards the second half of the OF period in CRF-OE mice than in WT animals. One possible explanation is that, by Day 7, the OF is perceived as familiar for the mice and the decrease in the novelty/anxiety stimulus unmasks the effects of CRF overproduction in the brain. Indeed, i.c.v. administration of CRF has been shown to increase locomotor activity in a low-anxiety, familiar environment (Sutton *et al.* 1982). CRF-OE mice differ considerably from WT in their activity directed towards the centre area on Day 7. Although both genotypes show low number of entries into and relative activity in the centre initially (first 5 min), the centre-directed activity increases significantly over time and becomes two or three

Table 1: Summary of main findings

	Day 1		Day 7		Day 15	
OF activity	Overall	1st 5 min	Overall	1st 5 min	Overall	1st 5 min
Total distance (cm)	CRF-OE = WT	CRF-OE < WT	CRF-OE≥WT	CRF-OE = WT	CRF-OE = WT	CRF-OE = WT
Centre entries	CRF-OE < WT	CRF-OE < WT	CRF-OE > WT	CRF-OE = WT	CRF-OE > WT	CRF-OE = WT
Per cent centre distance	CRF-OE = WT	CRF-OE < WT	CRF-OE > WT	CRF-OE = WT	CRF-OE > WT	CRF-OE = WT
Individual differences in locomotor response to novelty	Day 1 WT	CRF-OE	Day 7 WT	CRF-OE	Day 15 WT	CRF-OE
Total distance (cm)	HR > LR	HR > LR	HR = LR	HR = LR	HR = LR	HR > LR
Centre entries	HR > LR	HR > LR	HR > LR	HR = LR	HR > LR	HR > LR
Per cent centre distance	HR = LR	HR = LR	HR > LR	HR = LR	HR > LR	HR = LR

times higher in CRF-OE mice than in the WT animals, mostly due to the considerable decrease in the centre-directed activity in the WT. One possible interpretation of this unexpected finding is that the higher centre activity in the transgenic animals is due to increased overall locomotor activity in the already familiar environment (see above). However, it seems that the opposite is true because transgenic and WT mice do not differ in their activity in the periphery (data not shown), and the slightly higher overall activity in the CRF-OE mice seems to be driven by the major increase in centre-directed activity. Another possibility is that, similar to the initial, novel OF experience, CRF-OE mice are more anxious at the beginning of the Day 7 testing, and the increase in centre-directed activity later is a reflection of habituation (e.g. gradually decreasing anxiety). This, however, is not likely, as the initial 5-min activity of CRF-OE mice is not only significantly lower than on Day 1, which clearly shows between-session habituation for number of entries into the centre, but also undistinguishable from that of the WT on Day 7. This provides further support for the notion that by Day 7, the environment is already familiar, not anxiety inducing, for mice of both genotypes. Interestingly, no between-session habituation from Days 1 to –7 is observed in relative activity in the centre in CRF-OE mice, which suggests that number of entries into and relative activity in the centre may measure different aspects of behaviour. It is, however, possible that neurobiological processes underlying spontaneous activity in a familiar environment are fundamentally different from those in a novel environment. An intriguing possibility is that the gradually increasing, high activity towards the centre of a familiar OF may reflect increased impulsivity/behavioural disinhibition. In fact, behavioural indices related to centre activity in the OF, for example per cent time spent in the centre area, have been considered measures of impulsivity-like behaviour (Colorado *et al.* 2006), especially when the environment is already familiar and devoid of the masking effect of the novelty-induced anxiety. Maternal separation, a profound early life stress that is associated with increased brain CRF gene expression in adulthood (Plotsky *et al.* 2005), has been shown to result in an increased impulsivity-like behaviour, as measured by the per cent time spent in the centre of the OF (Colorado *et al.* 2006). In fact, when we analysed the per cent time spent in the centre, we found that, on Day 7 but not on Day 1, CRF-OE mice spent relatively more time in the centre area than WT animals (CRF-OE: $11.3 \pm 1.7\%$; WT: $3.3 \pm 1.7\%$; $P < 0.05$), supporting the notion of a possible impulsivity-like behaviour in transgenic mice. Interestingly, anxiolytic diazepam administration induces a stronger behavioural disinhibition in CRF-OE mice than in WT (van Gaalen *et al.* 2003). Therefore, the most consistent explanation for our present findings is that under low-anxiety condition (e.g. in a familiar environment), CRF-OE mice may show an increased behavioural disinhibition/impulsivity-like phenotype.

We tested whether the behaviour of mice changes upon re-exposure to the same OF environment 1 week after the end of the repeated, 7 days, OF sessions. If the behavioural pattern remains similar to what was seen at Day 7, it would indicate that mice do not perceive the OF differently from that at the end of the habituating schedule, e.g. they would likely have appropriate memory of the OF, the room and the procedure. In fact, total locomotor activity and indices of centre-directed behaviour (CE and %CD) were almost indistinguishable from the pattern seen at Day 7 in both WT and CRF-OE mice. Measures of locomotor activity stayed mostly unchanged throughout the 30-min OF session for both genotypes. These results suggest that mice of both genotypes remembered the OF experience they were habituated to a week before.

The basic premise of the exploration-based tests (Crusio 2001) is that the 'innate tendency to explore a novel place will be inhibited by increasing aversive nature of the environment, thereby producing a conflict between approach and avoidance' (Holmes 2001). In other words, high levels of exploration in a novel, aversive environment are interpreted as low levels of anxiety-like behaviour. Indeed, analysis of the relationship between total exploratory activity during the first exposure to the novel OF and number of centre entries (an anxiety-related measure) showed significant positive correlation in both WT and transgenic mice, supporting the notion that animals that show high exploratory activity in a novel environment are less 'anxious'. This seems to be a stable trait in WT mice because the relationship between the initial exploratory reactivity in the novel environment and the centre entries on Days 7 and 15 of the OF study remains significant.

This is in spite of the fact that total locomotor activity in WT mice is decreased about 50% and the number of entries is reduced by more than 75% of their Day 1 values on Days 7 and 15. However, in CRF-OE mice, there is no longer a significant association between these factors at the later time points. This may not be the result of decreased activity in the OF over time because these changes are much more modest (~25% for total distance and ~30% and ~15% for centre entries) in CRF-OE mice than in WT animals, where the relationship remains unchanged throughout. Rather, the neurobiological mechanisms underlying behavioural adaptation to novelty are regulated fundamentally differently in the transgenic animals. This is supported by a trend towards a stronger, although still nonsignificant, relationship between initial locomotor reactivity to novelty and centre entries upon re-exposure to the OF on Day 15 following a week of undisturbed home cage housing, when the degree of familiarity of the environment will presumably have decreased. Moreover, the finding that initial locomotor reactivity to a novel environment correlates to %CD on Days 7 and 15 in WT, but not in CRF-OE mice, suggests that behavioural stress response and habituation to the OF environment are regulated differently in CRF-OE animals. Overall, correlation analyses have shown that habituation and re-exposure to an initially aversive environment do not change the relationship between the locomotor reactivity to novelty (behavioural stress response) and the activity towards the centre area in WT mice, but they do in CRF-OE animals. Therefore, chronic, transgenic overproduction of CRF may alter basic processes related to habituation to stress.

The existence of marked individual differences in behavioural responsiveness has been recently recognized. In a series of studies, Piazza and coworkers have shown that outbred Sprague-Dawley rats characterized as HR on the basis of their locomotor response to a novel environment fundamentally differ from LR in a number of behavioural and physiological indices related to stress pathology, such as stress-induced corticosterone response, propensity to self-administer drugs of abuse, cognitive function in later life and brain neurochemistry (Dellu *et al.* 1993, 1994; Piazza *et al.* 1989). Therefore, we separated WT and CRF-OE mice on the basis of their locomotor reactivity to a novel environment on Day 1. Remarkably, we found 40% difference in activity between HR and LR in WT, inbred C57BL/6J mice (34% in CRF-OE), identical to what was found in outbred rats (Dellu *et al.* 1993). Furthermore, in line with earlier findings of Piazza in rats (Piazza *et al.* 1990), we found that this can be attributed to the novelty effect because the individual difference disappears in both genotypes after repeated exposure to the same environment, e.g. when the novelty decreases. However, the difference in reactivity returns upon re-exposure to the OF after 7 days of home cage housing only in CRF-OE mice, suggesting that they may again perceive the environment as novel. Individual differences in the number of centre entries and relative activity in the centre remain throughout in WT mice, whereas they disappear by habituation and partially return (CE only) by re-exposure in CRF-OE mice. This suggests, on one hand, that the fear-inducing and/or motivational effects of the centre area of the OF are different from the novelty-inducing effects for WT mice. On the other hand, individual differences in habituation profile and in recall to the past environmental context may differ in CRF-OE animals.

In our CRF-OE mice, the transgenic overexpression of CRF starts after birth. Therefore, the behavioural alterations observed may be the result of CRF-related changes during postnatal, rather than prenatal development. CRF transgenic mice have elevated basal plasma corticosterone levels but a normal corticosterone response to stress (Groenink *et al.* 2002). Therefore, it is conceivable that the sustained elevation in corticosterone levels may contribute to the observed behavioural phenotype. Although this possibility cannot be ruled out on the basis of our experiment, it seems somewhat unlikely. Previous studies have shown that either 10 days or 10 weeks of chronic corticosterone administration have failed to alter locomotor response in a novel OF in rats (Ehlers *et al.* 1992; van den Buuse *et al.* 2002). Furthermore, long-term, developmental exposure to corticosterone from late prenatal period to weaning resulted in an increased, rather than a decreased, OF activity in the mouse (Pechnick *et al.* 2006). It is, therefore, likely that the observed phenotype in CRF-OE mice can be best explained by the lifelong overexpression of the CRF gene and sustained overproduction of CRF peptide and by associated neurochemical alterations.

In summary, we found that mice with central, postnatal overexpression of CRF show abnormal behavioural habituation to environmental stimuli and altered plasticity in mechanisms that may underlie individual differences in behavioural responses to novelty. Some of these differences may be related to increased behavioural disinhibition/impulsivity or altered cognitive functions such as attention to environmental events. These findings may help better understand the role of CRF in stress-related behavioural pathology, such as drug addiction, depression and psychosis, where the role of genetically and/or environmentally encoded individual differences is becoming increasingly recognized.

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