Alterations in the central CRF system of two different rat models of comorbid depression and functional gastrointestinal disorders

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Abstract

Clinical evidence suggests comorbidity between depression and irritable bowel syndrome (IBS). Early-life stress and genetic predisposition are key factors in the pathophysiology of both IBS and depression. Thus, neonatal maternal separation (MS), and the Wistar–Kyoto (WKY) rat, a genetically stress-sensitive rat strain, are two animal models of depression that display increased visceral hypersensitivity and alterations in the hypothalamic–pituitary–adrenal axis. Corticotrophin-releasing factor (CRF) is the primary peptide regulating this axis, acting through two receptors: CRF₁ and CRF₂. The central CRF system is also a key regulator in the stress response. However, there is a paucity of studies investigating alterations in the central CRF system of adult MS or WKY animals. Using in-situ hybridization we demonstrate that CRF mRNA is increased in the paraventricular nucleus (PVN) of WKY rats and the dorsal raphe nucleus (DRN) of MS animals, compared to Sprague–Dawley and non-separated controls, respectively. Additionally, CRF₁ mRNA was higher in the PVN, amygdala and DRN of both animal models, along with high levels of CRF₁ mRNA in the hippocampus of WKY animals compared to control animals. Finally, CRF₂ mRNA was lower in the DRN of MS and WKY rats compared to control animals, and in the hippocampus and amygdala of MS rats. These results show that the central CRF system is altered in both animal models. Such alterations may affect HPA axis regulation, contribute to behavioural changes associated with stress-related disorders, and alter the affective component of visceral pain modulation, which is enhanced in IBS patients.

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Introduction

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders, typically presenting alterations in gut motility, visceral sensitivity and hypothalamus–pituitary–adrenal (HPA) axis function (Cervero & Janig, 1992; Mayer & Collins, 2002). Such alterations can be comorbid with chronic stress-related psychiatric disorders (Clarke et al. 2009; Gros et al. 2009). It is also becoming clear that a number of animal models for the study of depression and anxiety also display some of the key IBS phenotypes (Clarke et al. 2009).

Corticotrophin-releasing factor (CRF) is one of the most important neuropeptides modulating the HPA axis, stimulating the release of adrenocorticotropic hormone (ACTH) into the bloodstream, which induces the secretion of glucocorticoids (de Kloet, 2000; de Kloet et al. 2005). Furthermore, changes in CRF expression and release regulate several physiological responses during stress (for a review see Bale & Vale, 2004). The CRF system has also been shown to be dysfunctional in psychiatric disorders such as anxiety and depression (Arborelius et al. 1999; Bale & Vale, 2004) and functional gastrointestinal disorders (Tache et al. 2009; Zorrilla et al. 2003). Within the central nervous system (CNS) expression of CRF is not only limited to the hypothalamus with CRF mRNA detected in many regions including the hippocampus, amygdala and dorsal raphe nucleus (DRN) (Chalmers et al. 1995; Reul & Holsboer, 2002; Van Pett et al. 2000).
brain regions are of importance, as the hippocampus is involved in the control of emotions in stressful situations, learning and memory processes and HPA axis regulation (Jacobson & Sapolsky, 1991; Vizi & Kiss, 1998). The amygdala is also important in the control of emotions, and moreover, for modulating fear-related information (LeDoux, 2007). Finally, the DRN is the major source of serotonergic input to the forebrain, and alterations in the serotonergic system have been linked to mood disorders such as major depression (Michelsen et al. 2007). Growing evidence suggests that the extra-hypothalamic CRF system is also key to the manifestation of the stress response (Lowry & Moore, 2006) and therefore is poised to play a critical role in both psychiatric and brain–gut axis disorders.

CRF signals through two different receptor subtypes: CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2) (Bale & Vale, 2004). Both receptors have also been found in different structures of the CNS (Chalmers et al. 1995; Reul & Holsboer, 2002; Van Pett et al. 2000), and have been involved in behavioural modulation during stressful situations. CRF1 null mice have reduced anxiety-like behaviours (Smith et al. 1998), while CRF2 knock-out mice show increased anxiety-like behaviours (Bale et al. 2000; Bale & Vale, 2003, 2004) and display increased immobility in the forced swim test (Bale & Vale, 2003), which indicates a depression-like behaviour (Cryan et al. 2002). However, the behavioural effects mediated by CRF2 are not entirely understood as activation of this receptor in the lateral septum produces anxiogenic effects under stressful situations (Henry et al. 2006; Takahashi et al. 2001).

Neonatal maternal separation (MS) is a well-validated model of depression and increases anxiety resulting in behavioural alterations (Lippmann et al. 2007) and functional changes in the HPA responsiveness in adulthood (Ladd et al. 1996; O’Mahony et al. 2009; Schmidt et al. 2004). More recently MS animals have been proposed as a model of IBS as they display increased visceral hypersensitivity (Barreau et al. 2007; O’Mahony et al. 2009; Ren et al. 2007), alterations in colonic morphology (O’Malley et al. 2010c), function (O’Mahony et al. 2009), and immune parameters (Hyland et al. 2009; McKernan et al. 2009).

Another animal model for the study of mood disorders is the Wistar–Kyoto (WKY) rat, a selectively bred strain that is hyper-responsive to stress compared to Sprague–Dawley (SD) rats commonly used as control (Armario et al. 1995; De La Garza & Mahoney, 2004; Lahmanne et al. 1997; Lopez-Rubalcava & Lucki, 2000; Pare, 1992; Rittenhouse et al. 2002). As with MS animals, WKY rats show alterations in the HPA axis (Rittenhouse et al. 2002; Solberg et al. 2001), visceral hypersensitivity (Gibney et al. 2010; Greenwood-Van Meerveld et al. 2005; Gunter et al. 2000; O’Mahony et al. 2010), immune alterations in the colon (McKernan et al. 2009) and altered colonic morphology (O’Malley et al. 2010c) which has led to them being proposed as an animal model of brain–gut axis dysfunction.

Given the phenotype described for both models we hypothesized that MS and WKY rats would have similar alterations in the CRF system at the hypothalamus and in extra-hypothalamic structures. Therefore, the aim of this work was to evaluate the expression levels of CRF, CRF1 and CRF2 mRNA using in-situ hybridization in the paraventricular nucleus (PVN) of the hypothalamus, hippocampus, amygdala and DRN of adult WKY rats compared to SD rats, and in adult MS rats compared to non-separated (NS) controls.

Materials and methods

Animals

For the maternal separation (MS) protocol, adult male Sprague–Dawley (SD) rats were obtained from Biological Service Unit, University College Cork, Cork, Ireland [n = 6 MS rats from three different litters and n = 6 non-separated (NS) controls from three different litters]. Additionally adult male WKY (n = 6) and SD (n = 6) rats were obtained from Harlan, UK. These were allowed to acclimate for > 7 d in the housing facility before the experiment. Animals were group-housed (4–6 animals per cage) in standard conditions (room temperature 21 °C, with a 12-h light/dark cycle, lights on 07:00 hours) with access to regular chow and water ad libitum. Cages were cleaned once weekly to avoid excessive handling. Rats were of comparable weight (250–350 g) and age (11–13 wk) when sacrificed. All experimental procedures were performed in accordance with the protocols approved by the Ethics Committee, University College Cork under a license issued from the Department of Health and Children (Cruelty to Animals Act 1876, Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [89/609/EEC]).

Maternal separation

Early-life stress was performed as described previously (Hyland et al. 2009; O’Mahony et al. 2009). Briefly, the litters that were randomly assigned to undergo MS, were removed from the home cage, placed into a smaller cage on heating pads set at 30–33 °C for 3 h (09:00–12:00 hours). After that time,
pups were returned to the original home cage in the main colony room. This procedure was repeated from post-natal day 2 (PD 2) to PD 12. Control, NS litters remained undisturbed except for routine cage cleaning performed once a week. At PD 21, pups were weaned and group-housed (3–5 per cage), and left undisturbed until adulthood (11–13 wk). We have previously shown that this MS protocol induces an array of behavioural and physiological changes that are indicative of increased anxiety and altered HPA and brain–gut axis function (Hyland et al. 2009; O’Mahony et al. 2009).

Sacrifice and in-situ hybridization
Animals were lightly anaesthetized with isofluorane, and killed by decapitation. The brain was immediately extracted, snap-frozen in cold isopentane and stored at −80 °C before being processed for in-situ hybridization.

The in-situ hybridization was conducted as previously described (Bravo et al. 2006, 2009), using oligodeoxynucleotide (cDNA) probes complementary to CRF mRNA (738–782 bp access no. X03036.1), CRF1 mRNA (1174–1218 bp access no. NM_030999.1) and CRF2 mRNA (781–824 bp access no. U6253.1), labelled with a digoxigenin (DIG) oligonucleotide 3'-OH tailing kit (Roche, Molecular Biochemicals, Germany). Briefly, 10-μm coronal brain sections were mounted on glass slides and post-fixed for 30 min in 4% paraformaldehyde. After treatment with Proteinase-K, acetic anhydride and delipidation in chloroform, the tissues were incubated overnight at 37 °C with the hybridization solution (formamide 50%, saline and sodium citrate buffer (SSC) 4×, sheared salmon DNA 6.25 mg/ml, TRNA 125 μg/ml, and cDNA probe at a fixed concentration of 100 pmol/ml). Hybridization detection was performed with anti-DIG antibody, conjugated with an alkaline phosphatase (Roche, Molecular Biochemicals). Finally, substrate solution was added (NBT/BCIP; Sigma, USA) and when a violet/blue precipitate was present on the tissues, the reaction was stopped. The slides were then left to air-dry, coverslipped, and photographed. Specificity of the hybridization was verified in brain regions where a differential expression of CRF1 and CRF2 has been previously shown (Chalmers et al. 1995; Van Pett et al. 2000). In addition, negative controls were generated by using 100-fold excess of the respective unlabelled oligodeoxynucleotide. For semi-quantitative analysis, densitometric measurements of each PVN, hippocampus, amygdala and DRN microphotograph were performed using FujiFilm’s Science Laboratory Multi Gauge v. 2.2 software (Fuji Photo Film Co. Ltd). Images were analysed in greyscale and values correspond to the intensity of pixels (the darkest staining corresponding to the highest intensity) in a given area (density of pixels). Values for each animal represent the average from 4–5 brain sections (analysed on both brain hemispheres for hippocampus and amygdala).

Statistical analysis
Results are expressed as mean ± S.E.M. Data were analysed with a two-tailed Student’s t test (GraphPad Prism 4, GraphPad Software Inc. USA). Statistical significance was accepted at the p < 0.05 level.

Results
Specific in-situ hybridization signal for CRF1 mRNA was detectable in the cortex (Fig. 1a) and cerebellum (Fig. 1g), with little or no signal in the lateral septum (Fig. 1e) and ventromedial hypothalamus (Fig. 1c). Specific CRF2 mRNA signal was observed in the septum (Fig. 1f) and ventromedial hypothalamus (Fig. 1d), with very low levels in the cortex (Fig. 1b) and cerebellum (Fig. 1h). Additionally, signal for CRF, CRF1 and CRF2 mRNAs was detectable in the suprapyramidal (SupDG) and infrapyramidal (InfDG) layers of the dentate gyrus (DG); the cornus ammon fields CA1–CA3 of the hippocampus; basolateral amygdala (BLA) and central amygdala (CeA), the PVN and DRN. The level of staining in hippocampus, hypothalamus, amygdala and DRN allowed densitometric analysis of CRF, CRF1 and CRF2 mRNA expression. In addition to the areas shown in Fig. 1, negative controls were performed using an excess of unlabelled cDNA probe during the hybridization stage for each detection (Figs 4n, 5n, 6n, 7n, 8n, 9n).

CRF mRNA expression
Densitometric analysis of the in-situ hybridization revealed no differences between MS and NS animals at the PVN (Fig. 2a–c). However, higher levels of CRF mRNA were detected in the PVN of WKY rats compared to SD rats (SD vs. WKY: 9.33 ± 0.24 vs. 11.96 ± 0.38; p < 0.001) (Fig. 3a–c). In addition, there were no differences in CRF mRNA between MS and NS rats in hippocampus and amygdala (Fig. 4a–j). However, higher levels of this transcript were found in the DRN of MS animals compared to NS rats (NS vs. MS: 7.41 ± 0.38 vs. 9.81 ± 0.51; p < 0.005) (Fig. 4k–m). No differences were found between SD and WKY animals in hippocampus, amygdala and DRN (Fig. 5a–m).
Expression of CRF1 mRNA of the rat brain. Representative images of specific CRF hybridization signal performed in areas of the rat brain where CRF1 and CRF2 are differentially expressed: cortical expression of CRF1 mRNA (a) and CRF2 mRNA (b) (scale bar, 50 μm). Expression of CRF1 (c) and CRF2 mRNA (d) in the ventromedial hypothalamus (VMH) (scale bar, 1 mm). Expression of CRF1 (e) and CRF2 (f) in the lateral septum (LS) (scale bar, 1 mm). Expression of CRF1 (g) and CRF2 (h) in the cerebellum. (Representative images of three Sprague–Dawley rats. Scale bar, 50 μm.)

**CRF1 mRNA expression**

As shown in Fig. 2, CRF1 mRNA levels were higher in the PVN of MS rats compared to NS animals (NS vs. MS: 11.64 ± 0.40 vs. 15.59 ± 0.29; p < 0.001) (Fig. 2d–f). However, no differences were found in hippocampal CRF1 mRNA of MS animals when compared to NS rats (Fig. 6a–f). On the other hand, the levels of this transcript were significantly higher in the BLA and CeA of MS animals compared to NS rats (BLA, NS vs. MS: 7.17 ± 0.34 vs. 9.53 ± 0.16; p < 0.0001; CeA, NS vs. MS: 4.36 ± 0.34 vs. 5.73 ± 0.40; p < 0.05) (Fig. 6g–j). Additionally, higher levels of CRF1 mRNA were found in the DRN of MS rats compared to NS rats (NS vs. MS: 14.23 ± 0.73 vs. 19.73 ± 0.61; p < 0.001) (Fig. 6k–m).

In WKY rats the level of CRF1 mRNA expression in the PVN was significantly higher than in SD rats (SD vs. WKY: 11.11 ± 0.28 vs. 13.31 ± 0.47; p < 0.005) (Fig. 3d–f), and there were also higher levels of CRF1 mRNA expression in the SupDG of the hippocampal formation compared to SD rats (SD vs. WKY: 29.75 ± 1.71 vs. 36.68 ± 2.01; p < 0.05) (Fig. 7c), and a higher, but not significant, level of expression in the CA1 layer of WKY rats compared to SD rats (SD vs. WKY: 19.14 ± 1.43 vs. 22.95 ± 1.13; p = 0.063) (Fig. 7c). In the amygdala, WKY rats had higher levels of CRF1 expression in the BLA compared to BLA of SD rats (SD vs. WKY: 7.43 ± 0.18 vs. 8.54 ± 0.35; p < 0.05) (Fig. 7i), with no differences in the CeA. Regarding the expression of CRF1 mRNA in the DRN, WKY rats had significantly higher levels of this transcript compared to SD rats (SD vs. WKY: 12.77 ± 0.77 vs. 20.29 ± 1.55; p < 0.005) (Fig. 7m).

**CRF2 mRNA expression**

The expression of CRF2 mRNA in the PVN of MS rats was not different from NS animals (Fig. 2g–i). However, rats subjected to MS showed significantly lower levels of CRF2 mRNA expression in hippocampal CA1 (NS vs. MS: 23.98 ± 1.90 vs. 14.42 ± 2.37; p < 0.05) (Fig. 8c), SupDG (NS vs. MS: 44.49 ± 3.79 vs. 27.15 ± 4.08; p < 0.05) (Fig. 8e), and InfDG (NS vs. MS: 58.95 ± 4.18 vs. 37.67 ± 6.14; p < 0.05) (Fig. 8f). Moreover, in the same animals, a significant reduction in the CRF2 transcript was observed in the BLA (NS vs. MS: 5.17 ± 0.13 vs. 4.02 ± 0.22; p < 0.005) (Fig. 8i), and the DRN (NS vs. MS: 15.21 ± 1.80 vs. 9.99 ± 0.53; p < 0.05) (Fig. 8m).

In WKY animals there were no differences in PVN (Fig. 3g–i) and hippocampal CRF2 mRNA expression compared to SD rats (Fig. 9a–f). However, in WKY rats CRF2 mRNA expression was significantly lower in the CeA of the amygdala (SD vs. WKY: 2.73 ± 0.20 vs. 1.93 ± 0.17; p < 0.05) (Fig. 9j) and in the DRN (SD vs. WKY: 10.32 ± 0.53 vs. 6.60 ± 0.70; p < 0.05) compared to the same regions of SD rats (Fig. 9m).

**Discussion**

Understanding how various susceptibility factors can converge in inducing maladaptive stress responses will significantly aid our understanding of the...
pathophysiology of stress-related disorders such as major depression and disorders of brain–gut axis such as IBS. The present work shows that animals subjected to early-life stress, and animals that are genetically prone to higher indices of anxiety and stress have overlapping alterations in the expression of genes relevant to the CRF system in the PVN of the hypothalamus and in extrahypothalamic regions of the CNS, such as the hippocampus, amygdala and DRN.

The two animal models used in this work have been described as having altered HPA axis function (Ladd et al. 1996; O’Mahony et al. 2009; Rittenhouse et al. 2002; Schmidt et al. 2004; Solberg et al. 2001), probably due to alterations in CRF and CRF1 mRNA expression in the PVN. Our findings suggest that a higher genetic susceptibility to anxiety and/or the effects of early-life stress affect gene expression in the CNS with similar functional consequences throughout life: inability to cope with stress and visceral hypersensitivity. However, some of these changes, particularly those induced by MS are regionally dependent. For instance, in the present study CRF mRNA expression in the amygdala and hippocampus was not affected by MS. However, there is a non-significant increase in CRF mRNA in the PVN of MS rats compared to NS rats, which could underlie our previous observations of MS-induced HPA axis alterations (O’Malley et al. 2009). On investigating CRF receptors in the PVN there was a significant increase in the expression of CRF1 mRNA in MS rats, with no differences in CRF2 mRNA expression, findings that could account for the alteration in HPA function and that are in line with protein levels shown in a recent study from our group (O’Malley et al. 2010b). Similarly, Plotsky et al. (2005) demonstrated that MS (3 h daily, PD 2–14) not only induces an increase in CRF mRNA in the PVN, but

Fig. 2. CRF, CRF1 and CRF2 mRNA expression in the paraventricular nucleus (PVN) of maternally separated (MS) and non-separated (NS) rats. Maternal separation did not affect the level of CRF mRNA in the PVN (a) [representative microphotographs (b) and (c)] compared to NS animals. Expression of CRF1 mRNA in the PVN of MS animals was significantly higher than NS rats (**p < 0.001) (d) [representative microphotographs (e) and (f)]. There are no differences in the PVN expression of CRF2 mRNA between MS and NS rats (g) [representative microphotographs (h) and (i)]. A small region surrounding the analysed areas was considered as background and was subtracted from the value obtained in the region of interest. In the microphotographs the scale bar is 1 mm (NS, n = 6; MS, n = 6).
also increases CRF₁ mRNA and CRF₁ total binding in the PVN of adult Long-Evans rats compared to animals that were subjected to 15 min of manipulation and/or non-handling (Plotsky et al. 2005). Interestingly, previous studies found no differences in CRF₁ mRNA expression in the PVN of male MS rats. On the other hand, Griesen and colleagues using a similar experimental strategy as Plotsky et al. (2005) observed a reduction in CRF₁ mRNA expression in complete hypothalamus homogenates (Greisen et al. 2005). Other authors also report similar reductions in CRF mRNA at the PVN of MS rats, although separation was performed for 24 h on PD 3 (Worke et al. 2001) or PD 13 (Vázquez et al. 2003). Therefore the present results suggest that alterations in the hypothalamic CRF system arise as a result of early-life stress, and could induce alterations in HPA axis function. The PVN of the WKY rat shows higher levels of CRF and CRF₁ mRNA than SD rats, with no difference in CRF₂ mRNA expression. This is the only analysed brain structure that showed a difference in CRF mRNA expression between rat strains. The change in CRF₁ mRNA expression found in WKY rats complements our findings in the early-life stress model, and therefore could be viewed as a common neurobiological substrate for the manifestation of stress-related psychiatric and/or functional gastrointestinal disorders. Moreover, as with the MS model, there are contradictory findings when comparing CRF expression in the PVN between WKY and other rat strains. Spontaneously hypertensive rats (SHR) show higher levels of CRF mRNA in the PVN than WKY rats (Imaki et al. 1998; Krukoff et al. 1999). Additionally, levels of total CRF protein in the hypothalamus of WKY rats were similar to those found in Brown–Norway, Fisher,

Fig. 3. CRF, CRF₁, and CRF₂ mRNA expression in the paraventricular nucleus (PVN) of Sprague–Dawley (SD) and Wistar–Kyoto (WKY) rats. WKY rats have higher CRF mRNA levels than SD rats (** p < 0.005) (a) [representative microphotographs (b) and (c)]. The levels of CRF₁ mRNA in the PVN of WKY are higher compared to SD rats (** p < 0.005) (d) [representative microphotographs (e) and (f)]. However, there are no differences in CRF₂ mRNA expression between WKY and SD rats (g) [representative microphotographs (h) and (i)]. A small region surrounding the analysed areas was considered as background and was subtracted from the value obtained in the region of interest. In the microphotographs the scale bar is 1 mm (SD, n = 6; WKY, n = 6).
Lewis and SHR rats (Lahman et al. 1997). Other studies have shown that hypothalamic levels of CRF mRNA of WKY rats are similar to those of Wistar (Gutierrez-Mariscal et al. 2008) and SD rats (Gomez et al. 1996; Krukoff et al. 1999). Although discrepancies exist between this and other studies, our observations strongly suggest that increased CRF and CRF1 mRNA expression in the PVN of WKY rats could account for some of the HPA axis alterations already described (Rittenhouse et al. 2002; Solberg et al. 2001), similar to what was observed in the MS model.

The hippocampus is a structure involved in the control of emotions, learning and memory processes and HPA axis regulation (Anacker et al. 2010; Herman et al. 1992; Jacobson & Sapolsky, 1991; van Haarst et al. 1997; Vizi & Kiss, 1998), although the hippocampal control of the HPA axis is mostly due to glucocorticoid receptor activation (Boyle et al. 2005; Furay et al. 2008; van Haarst et al. 1997). Nonetheless, the differential expression of CRF receptors observed in the two animal models could affect other neurotransmitter systems within the hippocampus, as shown by a microdialysis study in which intracerebroventricular (i.c.v.) administration of CRF enhanced the release of hippocampal serotonin and noradrenaline (de Groote et al. 2005). In the present study we found that MS reduced hippocampal expression of CRF2 mRNA, while WKY rats had higher levels of CRF1 mRNA in
the SupDG of the hippocampus (and in the CA1 area, although this increase was not statistically significant) compared to SD rats, with no differences between the two strains in hippocampal CRF2 mRNA levels. These alterations suggest that hippocampal CRF would preferentially signal through CRF1, therefore contributing to alterations in hippocampal neurotransmitter release, and affecting the behavioural response to stress. In a recent study, we found that CRF1 and CRF2 protein levels from the hippocampus of MS rats were not significantly different from NS rats (O’Malley et al. 2010b), but these observations were performed in complete hippocampal homogenates, which could mask specific changes in discrete hippocampal areas.

The current data is also relevant in the context of higher pain processing. Hippocampal activation also occurs in response to nociceptive stimulus such as mild and moderate heat-induced pain (Derbyshire et al. 1997) and pain-learning paradigms involving an unexpected stimulus (Ploghaus et al. 2000). Moreover, induction of visceral pain by colorectal distension increases the release of hippocampal noradrenaline in animal models (Saito et al. 2002) and clinical studies report hippocampal activation after painful rectal balloon distension in IBS patients (Kwan et al. 2005). These findings suggest that the hippocampus is important to the affective processing of pain, evocating high levels of anxiety in the case of visceral pain.
The WKY rat and the early-life stress paradigm have been used as models of IBS-induced brain–gut axis dysfunction, as they display visceral hypersensitivity (Gibney et al. 2010; Gosselin et al. 2009; Gunter et al. 2000; Hyland et al. 2009; O’Mahony et al. 2009; O’Malley et al. 2010). In addition, changes in CRF receptor expression found in these animal models are consistent with increased anxiety and depression-like behaviours (Bale & Vale, 2003, 2004; Coste et al. 2000). Thus alterations in the hippocampal CRF system may have an important role in some of the behavioural aspects of the stress response and may also influence the affective processing of visceral pain and contribute to visceral hypersensitivity.

The amygdala is involved in the central processing of stressful or emotionally charged stimuli (LeDoux, 2007). This structure integrates excitatory input from several regions including the hippocampus (LeDoux, 2007; Sandi et al. 2008; Ugolini et al. 2008). CRF in the amygdala has an important effect modulating behaviour: when rats are administered CRF directly into the BLA social interaction is reduced (Sajdyk et al. 1999) and the i.c.v. administration of the non-selective CRF receptor antagonist alpha-helical CRF9-14 reverses this condition (Heinrichs et al. 1992). Moreover, it has
been reported that MS rats have an increased acoustic startle response compared to non-separated animals (Caldji et al. 2000) suggesting an alteration in amygdala-modulated behaviour induced by early-life stress. There is evidence that i.c.v. injection of CRF potentiates the acoustic startle response (Liang et al. 1992a, b; Servatius et al. 2005) and lesions of the CeA block the CRF potentiation of the acoustic startle response (Valentino & Foote, 1987). Although we did not find differences in CRF mRNA between MS rats and their controls in both regions of the amygdala, the altered acoustic startle response observed by Caldji et al. (2000) could be due to changes in CRF receptor expression within this structure. Similarly, there is evidence showing that WKY rats have an increased acoustic startle response compared to SD rats (Martin et al. 2004; McAuley et al. 2009), suggesting alterations in amygdala-modulated behaviours in WKY rats, which could be attributable to differences in CRF receptor expression within this structure.
In addition, the amygdala is recognized as an important site of CRF-mediated pain modulation (Fu & Neugebauer, 2008). Pain carries a negative affective valence and is closely related to anxiety and depression (Fu & Neugebauer, 2008). There are direct pain-related inputs to the CeA, which contains nociceptive neurons (Bourgeais et al., 2001), and is an important mediator in the emotional-affective dimension of pain (Fu & Neugebauer, 2008). Activation of CRF1 in the CeA contributes to pain-related synaptic facilitation, and CRF2 activation within the BLA exerts a latent inhibitory influence over the same response (Fu & Neugebauer, 2008). We have reported that early-life stress reduces the pain perception threshold in a model of visceral pain (O’Mahony et al., 2009), and in the present work MS increased the expression of CRF1 mRNA in BLA and CeA, while reducing the expression of CRF2 mRNA only in the BLA. These changes in the expression of both receptors as a consequence of early-life stress, may contribute to the altered visceral pain responses in this animal model. In addition, we have recently shown that the protein levels of CRF1 and CRF2 from the amygdala of MS rats were not significantly different from NS rats (O’Malley et al., 2010b). However, as with the hippocampal levels of protein discussed earlier, these observations were

Fig. 8. CRF2 mRNA expression in hippocampus, amygdala and dorsal raphe nucleus (DRN) of maternally separated (MS) and non-separated (NS) rats. Maternal separation induced a reduction in CRF2 mRNA in the hippocampus [representative microphotographs (a, b), graphs (c) CA1, * p < 0.05, (d), (e) SupDG, * p < 0.05, and (f) InfDG, * p < 0.05]; amygdala [representative microphotographs (g, h); graphs (i) BLA, ** p < 0.005, and (j)] and DRN [representative microphotographs (k, l); graph (m) p < 0.05] compared to NS animals. Representative microphotograph of a negative control (n) showing the hippocampus of a NS animal. This control was performed using an excess of unlabelled CRF2 cDNA probe during the hybridization stage. In the hippocampus, the signal in the stratum radiatum was considered as background and was subtracted from the value obtained in the hippocampal cell layers. Regarding the amygdala, a small region between the analysed areas was considered as background, and for the DRN a small region surrounding these structures was taken as background. In hippocampus and amygdala microphotographs the scale bar is 1 mm, and in DRN microphotographs it is 500 μm. CA1, cornus ammon field 1; CA3, cornus ammon field 3; SupDG, suprapyramidal layer of the dentate gyrus; InfDG, infrapyramidal layer of the dentate gyrus; BLA, basolateral amygdala; CeA, central amygdala (NS, n = 6; MS, n = 6).
performed in homogenates of complete amygdala, and therefore reflect the contribution of more cellular areas than the ones discussed in the present study.

Similar to MS animals, WKY rats also display visceral hypersensitivity to colorectal distension (Gibney et al. 2010; Greenwood-Van Meerveld et al. 2005; Gunter et al. 2000; O’Mahony et al. 2010), which has been suggested to be mediated through central and peripheral activation of CRF$_1$ (Greenwood-Van Meerveld et al. 2005). However, the present data show that the pattern of the altered expression of CRF$_1$ and CRF$_2$ in the amygdala of WKY rats is not the same as in the MS rat model with CRF$_1$ being higher only in BLA, and CRF$_2$ lower in CeA but not BLA in WKY rats. It is plausible that lower levels of CRF$_2$ mRNA in the CeA of WKY compared to SD rats could favour the signalling of CRF$_1$ in that same area and as a result this activation could play an important role in pain-related synaptic facilitation (Fu & Neugebauer, 2008) with no inhibition mediated by CRF$_2$ in the BLA.

Serotonergic neurotransmission can be markedly affected by CRF (Cryan et al. 2005; Lowry & Moore, 2006; Valentino & Commons, 2005) and injection of low doses of CRF (up to 1 µg) in the DRN reduces the

Fig. 9. CRF$_2$ mRNA expression in hippocampus, amygdala and dorsal raphé nucleus (DRN) of Wistar–Kyoto (WKY) and Sprague–Dawley (SD) rats. There are no differences between WKY and SD rats in hippocampal CRF$_2$ mRNA expression [representative microphotographs (a, b), graphs (c–f)]. However WKY rats had lower levels of CRF$_2$ mRNA expression in the amygdala [representative microphotographs (g, h); graphs (i) and (j)] and in the DRN [representative microphotographs (k, l); graph (m)] compared to SD rats. Representative microphotograph of a negative control (n) showing the hippocampus of a SD rat. This control was performed using an excess of unlabelled CRF$_1$ cDNA probe during the hybridization stage. In the hippocampus, the signal in the stratum radiatum was considered as background and was subtracted from the value obtained in the hippocampal cell layers. Regarding the amygdala, a small region between the analysed areas was considered as background, and for the DRN a small region surrounding these structures was taken as background. In hippocampus and amygdala microphotographs the scale bar is 1 mm, and in DRN microphotographs it is 500 µm. CA1, cornus ammon field 1; CA3, cornus ammon field 3; SupDG, suprapyramidal layer of the dentate gyrus; InfDG, infrapyramidal layer of the dentate gyrus; BLA, basolateral amygdala; CeA, central amygdala (SD, n = 6; WKY, n = 6).
discharge rate of serotonergic neurons in the striatum (Kirby et al. 2000; Price et al. 1998). However, a higher dose of CRF (3 μg) increased striatal serotonin (5-HT) release (Price et al. 1998). Additionally, 5-HT levels in the hippocampus are increased by i.c.v. administration of low and high doses of CRF (Peñalva et al. 2002). These effects of CRF on the serotonergic system are mediated through the activation of CRF₁ and CRF₂, as it has been reported that CRF₁-deficient mice have an enhanced hippocampal serotonergic neurotransmission, after being subjected to forced swim stress (Peñalva et al. 2002). In other studies, injection of low doses of CRF (100 ng) into the DRN induced a reduction in 5-HT release in the nucleus accumbens, while a higher dose (500 ng) caused an increase in the release of this neurotransmitter (Lukkes et al. 2008). These data suggest that 5-HT neurotransmission is tightly regulated by the CRF system, and that any alteration on CRF and/or its receptors affects the neurotransmission, after being subjected to forced swim stress (Lowry & Moore, 2006; Valentino & Commons, 2005).

In the present work, results suggest a differential effect of CRF on CRF₁ and CRF₂ (Brunnhuber et al. 2006). Of particular interest, as evidence shows that i.c.v. administration of low doses of CRF in the DRN, as both models display an increase in CRF₁ mRNA and a reduction in CRF₂ mRNA. This preferential CRF₁ signalling could be particularly favoured in MS rats as early-life stress increased the levels of CRF mRNA expression. However, the increased levels of CRF mRNA in MS animals could also be a mechanism to counterbalance CRF₁ activation, as higher levels of CRF are required to induce CRF₂ activation (Bale et al. 2000, 2002; Bale & Vale, 2004; Lukkes et al. 2008; Smith et al. 1998; Van Pett et al. 2000).

The DRN is also strongly involved in pain modulation (Benarroch, 2006; Rodella et al. 1998; Wang & Nakai, 1994) and nociceptive neurons have been previously documented within this structure (Ren et al. 2007; Rodella et al. 1998; Sanders et al. 1980). Further, there is evidence supporting the involvement of the DRN in visceral pain modulation as the intraperitoneal injection of a 3.5% acetic acid solution increases cFos expression in several brainstem regions including the DRN (Rodella et al. 1998), and colorectal distension increases cFos immunostaining in the DRN (Ren et al. 2007). In relation to early-life stress, MS induces an increase in cFos immunostaining in the DRN (Ren et al. 2007) and there is high turnover of 5-HT in the brainstem of MS rats compared to NS animals (O’Mahony et al. 2008), suggesting an effect of early-life stress on the function of the DRN. The changes in CRF receptor expression for both models is similar: CRF₁ mRNA expression is increased in WKY and MS rats and CRF₂ mRNA is reduced in both models in relation to their respective controls. However, there is no information on the participation of these receptors in direct pain modulation within the DRN.

It is important to note that in the current studies we have focused our analysis of the impact of early-life stress and genetic background to unstressed, adult animals and to changes at the mRNA level. Future studies should investigate alterations in CRF and its receptors and also downstream signalling mechanisms. It is tempting to speculate that changes would have been observed in CRF mRNA expression in hippocampus and amygdala of both animal models if these had been exposed to a stressor, as there is evidence indicating that stress evokes enhanced cellular brain activation (Gibney et al. 2010; O’Malley et al. 2010b). In addition, other extrahypothalamic areas of the brain might also play an important role in the development of stress-related psychiatric and functional gastrointestinal disorders. For example, the bed nucleus of the stria terminalis (BNST) is a region of great interest for further analysis as it is recognized as part of the extended amygdala (De Olmos et al. 2004), with high expression of CRF and CRF₁ (Santibañez et al. 2005; Van Pett et al. 2000). Moreover, it has been shown to play a role in fear modulation and in the emotional aspect of visceral pain (Deyama et al. 2009).

CRF receptor activity can also be modulated by other peptides, such as urocortins (Ucn) (Bale & Vale, 2004; Tache & Brunnhuber, 2008). In the mammalian brain three Ucn peptides have been identified (Bale & Vale, 2004; Hsu & Hsueh, 2001; Lewis et al. 2001; Reyes et al. 2001): Ucn I binds to CRF₁ and CRF₂ with similar affinities, while Ucn II and Ucn III selectively bind to CRF₁ (Bale & Vale, 2004; Dautzenberg et al. 2004; Morin et al. 1999), which suggests that these two peptides are the endogenous ligands for CRF₂ (Bale & Vale, 2004; Dautzenberg et al. 2004; Kozicz et al. 1998; Morin et al. 1999; Reyes et al. 2001). Ucn peptides are highly expressed in cell bodies of the Edinger Westphal nucleus (Vaughan et al. 1995), hypothalamus, amygdala, brainstem and spinal cord (Hsu & Hsueh, 2001; Lewis et al. 2001; Reyes et al. 2001). Future studies directed at understanding the role of Ucn on the development of stress-related psychiatric disorders and alterations on the brain–gut axis are of interest, as evidence shows that i.c.v. administration of Ucn increases locomotor activity and anxiety like behaviours (Moreau et al. 1997; Slawecki et al. 1999; Vetter et al. 2002), suppression of food intake (Spina et al. 1996), modulation of gastric motility (Kihara et al. 2001) and centrally mediated alterations in colonic transit (Martinez et al. 2004). Moreover, Ucn-deficient mice display reduced levels of CRF₂ mRNA.
expression in different brain regions (Vetter et al. 2002), suggesting that some of the changes observed in the present work may be due to alterations in Ucn expression.

In conclusion, the data presented here show that early-life environment can permanently affect the developmental set-point of the central CRF system. In addition, differential expression of genes of the extrahypothalamic CRF system as part of the genetic background can predispose an individual to develop inadequate stress responses. These alterations on the central CRF system are very similar in both animal models, suggesting that the underlying mechanisms for inadequately stress responses and pain perception have a common neural substrate in unstressed situations. In addition, these overlapping changes were observed in brain structures involved in processes of memory, learning, control of emotions, responses to stress and pain perception in the two animal models, further supporting the idea of a common neural substrate between stress-induced psychiatric disorders and functional gastrointestinal disorders. Moreover, these findings add to other studies from our laboratory, showing a differential expression of CRF receptors in the colon of WKY and SD rats (O’Malley et al. 2010). Together, these observations could help to understand how early-life stress and genetic background contribute to the development of alterations that not only affect the stress response, but also the affective component of pain.

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Statement of Interest
None.

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