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Sciatic nerve injury rebalances the hypothalamic–pituitary–adrenal axis in rats with persistent changes to their social behaviours

M. Karmina Sosa | Damien C. Boorman | Kevin A. Keay

School of Medical Sciences and the Brain and Mind Centre, The University of Sydney, Camperdown, New South Wales, Australia

Correspondence
Kevin A. Keay, Brain and Mind Centre, 100 Mallet Street, Camperdown, NSW 2050, Australia.
Email: kevin.keay@sydney.edu.au

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Abstract

Increased glucocorticoids characterise acute pain responses, but not the chronic pain state, suggesting specific modifications to the hypothalamic–pituitary–adrenal (HPA)-axis preventing the persistent nature of chronic pain from elevating basal glucocorticoid levels. Individuals with chronic pain mount normal HPA-axis responses to acute stressors, indicating a rebalancing of the circuits underpinning these responses. Preclinical models of chronic neuropathic pain generally recapitulate these clinical observations, but few studies have considered that the underlying neuroendocrine circuitry may be altered. Additionally, individual differences in the behavioural outcomes of these pain models, which are strikingly similar to the range of behavioural subpopulations that manifest in response to stress, threat and motivational cues, may also be reflected in divergent patterns of HPA-axis activity, which characterises these other behavioural subpopulations. We investigated the effects of sciatic nerve chronic constriction injury (CCI) on adrenocortical and hypothalamic markers of HPA-axis activity in the subpopulation of rats showing persistent changes in social interactions after CCI (Persistent Effect) and compared them with rats that do not show these changes (No Effect). Basal plasma corticosterone did not change after CCI and did not differ between groups. However, adrenocortical sensitivity to adrenocorticotropic hormone (ACTH) diverged between these groups. No Effect rats showed large increases in basal plasma ACTH with no change in adrenocortical melanocortin 2 receptor (MC_2R) expression, whereas Persistent Effect rats showed modest decreases in plasma ACTH and large increases in MC_2R expression. In the paraventricular nucleus of the hypothalamus of Persistent Effect rats, single labelling revealed significantly increased numbers of corticotropin releasing factor (CRF) +ve and glucocorticoid receptor (GR) +ve neurons. Double-labelling revealed fewer GR +ve CRF +ve neurons, suggesting a decreased hypothalamic sensitivity of CRF neurons to circulating corticosterone in Persistent Effect rats. We suggest that in addition to rebalancing the HPA-axis, the increased CRF expression in Persistent Effect rats contributes to changes in complex behaviours, and in particular social interactions.

KEYWORDS
ACTH, CRF, glucocorticoid receptor, neuropathic pain, social interactions

1 | INTRODUCTION

Acute pain, or the anticipation of pain, quickly and reliably activates a range of physiological responses, mediated by both autonomic and neuroendocrine systems. These responses prepare an individual for dealing with the impending challenge to homeostasis,[1,2] and return quickly to baseline once the challenge is met. For instance, activation of the hypothalamic–pituitary–adrenal (HPA) axis, and the resultant release of glucocorticoids, contributes to reducing the unpleasantness of pain, while increasing pain tolerance.[3–6] Curiously, however, the chronic pain state is not reliably associated with a corresponding chronic activation of the HPA-axis. Studies in clinical populations most often find that chronic pain patients have similar levels of basal cortisol as healthy controls.[4,5,7–14] Although a number of studies have reported alterations to HPA-axis function in chronic pain, which include increases to cortisol levels,[15–17] its magnitude is incomparable to those seen in response to acute pain. These findings suggest neuroendocrine adaptations, preventing increased cortisol release, are a feature of HPA-axis function in people with a persistent pain state. Even more remarkably, recent research has demonstrated that chronic pain patients are still able to mount normal cortisol responses to acute stressors, including acute pain,[4,18,19] indicating that these adaptations are not simply a desensitization of the HPA-axis.

These findings have also been replicated in preclinical animal models. In response to acute stressors, such as acute pain, mice and rats release glucocorticoids in a similar time-course and to a similar degree to that seen in humans.[20–23] Additionally, in the preclinical chronic constriction injury (CCI) model of neuropathic pain, rats do not show changes to basal corticosterone levels, while retaining normal adrenocorticotropic hormone (ACTH) and corticosterone responses to acute stressors.[24–28] However, individual differences in an animal's responses to stressors, threats and motivational cues have led to the classification of a range of behavioural subpopulations. The most well characterized examples of these include reactive versus proactive coping styles,[29] sign-tracking versus goal tracking (autoshaping),[30] short versus long attack latency,[31] high versus low avoidance,[32] and short versus long latency to social defeat.[33] Importantly, many of these subpopulations are associated with divergent activity of the HPA-axis. For instance, higher glucocorticoid responses have been associated with reactive coping, short-latency defeat, long-latency to attack and low-avoidance,[31–34] suggesting the possibility that these subpopulations may reflect components of a single underlying behavioural phenotype. Studies of HPA-axis function in the context of chronic pain have not investigated the possibility that subpopulations of rats may have divergent adaptations to their HPA-axis in response to the injury. Supporting this possibility, these studies often report increased within-group variability in corticosterone and ACTH levels after injury.

Our laboratory has previously demonstrated that sciatic nerve CCI also manifests behaviourally distinct subpopulations of rats, which do not correlate with the severity of sensory changes from the injury (allodynia and hyperalgesia).[35–37] These subpopulations are remarkably similar to those seen in clinical cohorts, which likewise show dissociations between the sensory effects and behavioural consequences of neuropathic injury.[38–46] One of these subpopulations (~30% of rats) is characterized by persistent changes to their social interactions during daily Resident-Intruder tests; specifically, a reduction in the time spent performing dominance behaviours and an increase in the time spent performing nonsocial behaviours. In comparison to rats that do not show these changes in social behaviours, these "Persistent Effect" rats also show changes to a range of other complex behaviours, including disruptions to their sleep/wake cycles and decreased motivation to reward.[36,47] In regard to endocrine function, we have previously found that Persistent Effect rats have decreased plasma thyroid hormone concentrations, but no changes to their plasma corticosterone levels.[48] However, we have yet to investigate other markers of HPA-axis function such as ACTH levels, adrenocortical sensitivity to ACTH, hypothalamic corticotropin releasing factor (CRF) regulation or hypothalamic glucocorticoid sensitivity, each of which can independently alter HPA-axis function.

In this study we investigated individual differences in HPA-axis function following nerve injury to determine whether behavioural subpopulations may have distinct patterns of HPA-axis activity or HPA-axis adaptations in response to the injury. First, we conducted Resident-Intruder testing in a population of rats and selected specifically the Persistent Effect and No Effect behavioural phenotypes for comparison. We then sought to determine if there were any differences in the pre-CCI or post-CCI levels of both plasma corticosterone and ACTH between Persistent Effect and No Effect rats. Next, using both reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry, we determined any changes in hypothalamic activity and sensitivity to glucocorticoids by comparing the expression of CRF and glucocorticoid receptors (GR) between these groups and with uninjured (naïve) control rats. Finally, as we identified elevations in ACTH in the No Effect animals, we explored the consequences of these changes further by immunohistochemically quantifying its adrenocortical receptor, the melanocortin 2 receptor (MC_2R) in the adrenal gland.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Experiments and procedures were designed, developed and performed in accordance with the guidelines set out by the National Health and Medical Research Council (Australia) in the Australian code for the care and use of animals for scientific purposes eighth edition (2013). All procedures were approved by the University of Sydney Animal Care and Ethics Committee (AEC protocol #3920) and

followed the IASP's Ethical guidelines for investigations of experimental pain in conscious animals.[49] The data that support the findings of this study are available from the corresponding author upon reasonable request.

Our previous studies have demonstrated that ~30% of rats that receive a peripheral nerve injury develop persistent changes in social behaviours, sleep and motivation for natural reward.[36,37,47] Since these behaviours are known to be modulated by HPA-axis function, the experiments presented here were designed to investigate whether the animals that demonstrate these persistent changes also have alterations to their HPA-axis activity. Specifically, we first investigated the consequences of nerve injury on plasma concentrations of circulating hormones ACTH and corticosterone using enzyme-linked immunosorbent assay (ELISA). We then sought to identify any changes in adrenocortical sensitivity via immunohistochemical quantification of the ACTH receptor MC_2R in the adrenal gland. Finally, we assessed central changes in the HPA-axis by determining the gene expression (RT-PCR) and protein expression (immunohistochemistry) of GR and CRF in the paraventricular nucleus of the hypothalamus (PVH). For all experiments, comparisons were made between rats that demonstrated persistent changes to their social behaviours (Persistent Effect) with those that showed no changes (No Effect), to highlight the potential relationships between HPA-axis activity and these behavioural phenotypes.

2.2 | Animals and housing

Experiments were performed using outbred male Sprague–Dawley rats (ARC), weighing between 250 and 350 g at the time of CCI surgery. Upon arrival rats were assigned arbitrarily to be either a "Resident" or an "Intruder." Resident rats were housed individually in clear Perspex cages, while Intruder rats were group housed 4–6 per cage in a separate room. All rats had ad libitum access to standard laboratory chow and water. Both animal housing rooms were on a reversed 12-h light–dark cycle (lights off at 08:00 h), with the ambient room temperature maintained at 22°C. All rats were given 2 weeks to acclimatize to these conditions, after which time rats showed normal sleep–wake cycles.[36] All experimental procedures and behavioural testing were performed in the dark phase of the rats' circadian cycle, when rats are most active.

2.3 | Resident–Intruder testing and analysis

A total of 63 rats were used in this study. Forty-seven underwent Resident–Intruder testing, while 16 rats were used for control tissues. Of the rats used for Resident–Intruder testing, 17 had been implanted with a venous jugular cannula 4 days prior to the beginning of behavioural testing, the remaining 32 were noncannulated. Resident–Intruder tests were conducted every day at the beginning of the dark cycle, at least 2 h prior to sensory testing, and at least 5 h prior to blood samples being drawn from the cannulated rats. After 5 days of behavioural testing, each rat was given a unilateral sciatic nerve CCI. Resident–Intruder testing recommenced the following day and continued for a further 6 days. On each of these days testing began by recording the behaviour of the Resident (in its home cage) immediately prior to the introduction of an age, weight and sex matched conspecific Intruder. The interactions of the Resident and Intruder rats were digitally recorded for 6 min for later scoring, then the Intruder was removed. Each Resident only met the same Intruder rat twice throughout experiment and never on consecutive days. The recordings also contained both sonic and ultrasonic vocalisations (22–28 kHz, made audible by means of a bat detector). Behavioural records were always scored by at least two experimenters, who were trained to identify four mutually exclusive behavioural categories. These were:

- Dominance behaviours: Standing on top of the Intruder, biting to the back, neck or tail, lateral attack or chasing of the Intruder.
- Social behaviours: General sniffing and investigation of the Intruder, often focused around the anogenital region.
- Nonsocial behaviours: Exploration of the cage and self-grooming, or behaviours not directed towards the Intruder.
- Submissive behaviours: Supine posture, defensive retreat movement, "freezing."

The cumulative time spent on these four behavioural categories was calculated for each Resident rat. The order of scoring of these 6-min recordings were randomized for each experimenter, and the inter-scorer reliability was determined to be at least 95%. For each behavioural category, a pre-CCI average was calculated for each rat by taking the mean of the final three tests prior to surgery (i.e., days −2, −1 and 0 from surgery). On all post-CCI days, the duration of each behaviour was compared to its pre-CCI average. Rats that met the following criteria were identified:

- Persistent Effect: A decrease of at least 30% in the duration of dominance behaviour displayed for at least five of the six post-CCI surgery days.
- No Effect: Minimal or no change between pre-and post-CCI dominance levels.

2.4 | Sensory testing

Sensory testing was conducted approximately 1 h after Resident–Intruder social interactions testing. Mechanical (von Frey) and thermal (cold plate) tests were performed on alternating days beginning with mechanical sensitivity testing 5 days before CCI. Sensory testing was not performed on the CCI surgery day.

2.4.1 | Mechanical von Frey testing

Mechanical von Frey testing began by placing the rat inside a custom-built Plexiglass chamber (12 × 15 × 15 cm), which had a mesh floor

that allowed access to the plantar surface of the paws, for a 20-min habituation period. After habituation, von Frey filaments with a bending force ranging from 0.217 to 15.00 *g* were applied perpendicularly to the mid-plantar surface of each hind paw in ascending order until a withdrawal response was observed. The test was repeated five times for each hind paw. The threshold force for an evoked withdrawal for each hind paw was calculated by taking the mode of these five repetitions. Similar procedures were used by Chen et al.[50] and Monassi et al.[36]

2.4.2 | Thermal cold plate testing

To measure thermal sensitivity, rats were placed inside a transparent, open-topped Plexiglass cylinder (15 cm diameter × 30 cm height), which sat atop a glass plate cooled to 11°C. The total number and cumulative duration of hind paw withdrawals in 6 min was recorded. This testing method has been described previously by Jasmin et al.[51] and Monassi et al.[36]

2.5 | Venous cannulation

Rats were anaesthetized with an intramuscular injection (1 ml/kg) of ketamine and xylazine (75 mg/ml ketamine:4 mg/ml xylazine). A small subcutaneous injection of atropine (0.25 ml of 0.65 mg/ml) was also administered to reduce mucous secretion during surgery. The right jugular vein was isolated, just inferior to the junction of the cephalic and external jugular branches, and a custom-built cannula, filled with heparinised saline (100 IU/ml) and gentamicin (20 mg/ml) was inserted. The cannulae were constructed from polyethylene tubing (20 cm length, I.D. 0.58 mm, O.D. 0.96 mm), attached to a length of Silastic tubing (3.5–4.0, 30–32 mm length, I.D. 0.64 mm, O.D. 1.19 mm). The cannula was then exteriorised in the midline of the neck between the scapulae, and the open end occluded. Postoperative analgesia was provided (0.05–0.075 ml/kg; temgesic, s.c.). Animals were returned to their home cage and monitored continuously until they had regained mobility and started to eat autonomously, after which time they were observed twice daily for overall health and wellbeing (i.e., general appearance, posture, coat condition, response to handling, demeanour, temperament and bodyweight). Each rat was then habituated to handling—specifically to manipulation of the indwelling cannula—for a period of 3 days. Following this habituation period, daily blood sampling commenced. At approximately 18:00 h each day (2 h prior to light on), each rat was restrained gently, and a 1 ml blood sample was removed, and the volume replaced with artificial plasma (Plasma-Lyte; Baxter International) plus red cell fraction following centrifugation at 2,000 *g*. Plasma was stored at −80°C until assay. Cannula patency was maintained by ensuring the cannula was refilled with heparinised saline/gentamicin solution following each blood sample removal. A total of 17 rats underwent this procedure, but samples from two of these animals were unfortunately not suitable for analysis.

2.6 | Chronic constriction injury

Rats were anaesthetized with halothane in 100% oxygen (5% for induction, 2%–3% for maintenance). Once a surgical plane of anaesthesia was achieved, a small incision was made in the right hindlimb, and gentle, blunt dissection through the biceps femoris revealed ~10 mm of the sciatic nerve. Four chromic catgut ligatures (5-0; Johnson & Johnson) were tied around the exposed nerve, each approximately 1 mm apart. These ligatures gently compressed the nerve, yet still permitted epineural circulation. The incision was cleaned and closed, and the skin treated with a topical antibiotic powder (Tricin; Jurox). Each rat was returned to their home cage and monitored continuously until they had regained mobility and eating, after which time they were observed twice daily for overall health and wellbeing. This nerve injury procedure replicates exactly the methodology first described by Bennett and Xie,[52] which is used extensively in preclinical neuropathic pain research.

2.7 | Plasma ACTH and corticosterone measurement

Adrenocorticotropic hormone concentrations were determined in triplicate using a commercially available ELISA assay (Bioamerica). The concentration of the biologically active, 39 amino acid chain of ACTH was determined in each sample using a two-site binding process (C-terminal of ACTH [34–39] and midregion and N-terminal of ACTH [1–24]). In addition to calibration standards and controls provided by the manufacturer, we also verified our interassay reliability by including plasma samples from the total exsanguination of a single rat in each ELISA plate tested. Absorbances for each sample were calculated using a microplate reader (Polar Star Galaxy; BMG Labtech) and the concentration of ACTH (pg/ml) in each sample was calculated from standard curves verified across plates using our single exsanguinated rat control samples.

Plasma corticosterone concentrations were determined in triplicate using competitive enzyme immunoassay (Octeia; IDS). Commercially provided calibration samples were used to construct a standard curve, the reliability of which was tested using our exsanguinated control samples. Plasma samples from injured and uninjured (naïve) control rats were incubated with HRP-labelled corticosterone in micro-titre wells coated with a polyclonal corticosterone antibody for 24 h at 2°C. The wells were washed, and the bound labelled antibody was visualised using tetra-methyl benzidine. Absorbances were determined using a microplate reader (Polar Star Galaxy). Corticosterone concentrations (ng/ml) were determined for each of the plasma samples from the calibration curve (semi-log).

2.8 | Euthanasia, perfusion and tissue collection

On the day following the final Resident–Intruder test (i.e., post-CCI day 7), 17 of the noncannulated Resident rats were deeply

anaesthetized with sodium pentobarbital (Nembutal i.p 120 mg/kg; Boehringer Ingelheim) and perfused transcardially with 500 ml of heparinised sterile saline (0.9%, 4°C), followed by fixation with 500 ml of 4% paraformaldehyde (PFA) in acetate-borate buffer (pH 9.6, 4°C), and their brains and sciatic nerve injury sites extracted and post-fixed for 48 h in the same fixative. After post-fixation, brains and nerves were transferred to 30% sucrose in 0.1 M phosphate buffered saline (PBS) and stored at 4°C until processing. The remaining 30 Resident rats were rapidly decapitated, and their brains and adrenal glands immediately extracted. These unfixed brains were placed in TRI-reagent (Sigma-Aldrich) and immediately frozen in liquid nitrogen and stored at −80°C for RT-PCR analysis. The adrenal glands were post-fixed in 4% PFA prior to being paraffin embedded for histological processing.

2.9 | Quantitative real time RT-PCR

The frozen brains were thawed, and the hypothalamus was isolated by microdissection. Total RNA was extracted using TRI-reagent and purified using the GenElute Mammalian Total RNA Kit (Sigma). RNA concentrations for each sample were determined by spectrophotometry (ND-1000 Spectrophotometer; Nanodrop), and quality verified using the 2100 Bioanalyser (Agilent Technologies). Extracted RNA showed high levels of purity with clear and defined peaks for 18S and 28S ribosomal RNA (Figure S1). The absence of background detection showed there was no genomic or protein contamination. Gels identified only two bands representing 18S and 28S rRNA. Negative reverse transcriptase controls did not yield amplification products, further confirming the absence of genomic contamination. Each assay comprised 3 μg of RNA, 1 μl of random hexamers, and 4 μl of 2.5 mM dNTP mix. The volume was then made up to 20 μl using RNAse-free water. The assays were incubated for 15 min at 65°C, then chilled on ice for 1 min, followed by centrifugation. Four microlitres of buffer, 1 μl of RNAse OUT, 1 μl of dithiolthreitol, and 1 μl of reverse transcriptase enzyme (SuperScriptIII), was then added to each assay tube. These were then incubated for 5 min at 25°C, 50 min at 50°C, and then 10 min at 70°C. The resulting cDNA was then cooled for 5 min at 4°C and then stored at −20°C until RT-PCR was performed. Negative controls were run using the same RNA samples without the addition of the reverse transcriptase enzyme, which was replaced by RNA-free water. These controls were used to determine genomic DNA contamination of our RNA samples.

Glucocorticoid receptor and CRF mRNA expression were measured using the TaqMan quantitative RT-PCR system, using the Rotor Gene 2000 Real-Time PCR machine (Corbett Research). Housekeeping gene standards, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 s were used to calculate relative mRNA expression. The following TaqMan probes were used:

- GR: Rn00561369_m1.
- CRF: Rn01462137_m1.
- 18s: Hs99999901_s1.
- GAPDH: Rn99999916_s1.

RT-PCR reactions were carried out in 20 μl reaction volumes (0.2 ml tubes). Each assay contained 10 μl TaqMan Universal PCR Master Mix (Roche Molecular Systems), 0.6 μl template cDNA, 8.4 μl RNAse-free H_2O, and 1 μl of probe/primer master mix containing unlabelled amplification primers and FAM dye-labelled TaqMan minor groove binder probe (Applied Biosystems). Each sample was assayed in triplicate, including the housekeeping genes. Primer-dimer formation was not considered to be a problem based on the use of the TaqMan platform. The RT reaction cycles were 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min of 60°C. Detection of probe FAM fluorescence was acquired at 60°C.

Each run contained standardised control samples and experimental samples. For each gene, average amplification values were calculated as the mean of the triplicate. Relative quantification of gene expression was calculated using the relative expression software tool (REST[53]). Gene expression for each sample was expressed in fold change relative to the average of the control samples.

2.10 | Immunohistochemistry

2.10.1 | MC_2R expression in the adrenal gland

Immersion-fixed adrenal glands were dehydrated in ascending alcohols (50%, 70%, 95%, 100% 45 min each), cleared in xylene (2 × 45 min), then infiltrated with paraffin wax (60°C) overnight. The paraffin embedded adrenals were then sectioned (10 μm) into two series on a rotary microtome (American Optical), mounted from warm water (40°C) onto cleaned glass slides and dried overnight at 40°C. The slides were deparaffinised by washing in xylene (3 × 5 min) and the sections were rehydrated in descending alcohols (3 min at 100%, 95%, 80%, 70%). One series of sections was Nissl stained. The second series were rinsed in PBS (2 x 3 min). These sections were incubated in goat anti-MC_2R (1:1000; RRID:AB_2266374) overnight at 4°C in 10% normal horse serum in PBS (PBSH). The following day slides were washed in PBS (3 × 5 min) at room temperature and then incubated for 2 h at room temperature in biotinylated anti-goat IgG (1:500 in PBSH, RRID:AB_2336126; VectorLabs). Slides were washed in PBS, then incubated in ExtrAvidin peroxidase (1:1000 in PBS; Sigma) for 2.5 h at room temperature. Slides were then washed once more in PBS and the bound anti-MC_2R was visualised using 0.05% 3-3′ diaminobenzidine tetra-hydrochloride (DAB), following our previously published procedures.[54] For each animal, the five sections that encompassed the largest cross-sectional area of each gland were selected for analysis. An experimenter blinded to the status of each rat captured photomicrographs of each section using a digital camera (Olympus DP-70) connected to a light microscope (Olympus BX-51). Four images of the adrenal cortex were captured from each section, one from each quadrant. These images were converted to greyscale (8-bit) and the average pixel value of the image was calculated (Adobe Photoshop CS3). These values were averaged for all images isolated for each gland and were used to determine the MC_2R staining density for each rat.

2.10.2 | GR and CRF expression in the hypothalamus

A block containing the hypothalamus was isolated from the fixed brains. Serial coronal sections were cut on a freezing microtome (50 μm at 18°C) as a one in five series and collected into PBS. Single label GR was investigated in one cohort of CCI rats ($n = 6$ Persistent Effect and $n = 6$ No Effects). Single label CRF and double label GR + CRF was investigated in a second cohort of CCI rats ($n = 6$ Persistent Effect and $n = 6$ No Effect). Uninjured (naïve) control rats ($n = 6$) were used for both single and double labelling procedures. Immunohistochemistry was performed on series of free-floating sections were washed in 50% ethanol (30 min), and endogenous peroxidase activity was quenched by washing in 3% H_2O_2 in 50% ethanol (30 min). Sections were then rinsed in PBS, before being blocked in 1% PBSH (5 min). Sections were incubated overnight in either rabbit-anti-GR (1:5000; RRID:AB_2155786, Santa Cruz Biotechnology) at 4°C, or goat anti-CRF (1:1000 in PBSH, RRID:AB_631299; Santa Cruz) at 4°C. The following day each series of sections was rinsed in PBS, then incubated for 2 h in a biotinylated secondary antibody targeting the species of their primary antibody (1:500 in either anti-rabbit IgG, RRID:AB_2336201, or anti-goat IgG; Vector Laboratories). Sections were rinsed in PBS (3 x 10 min) and incubated in ExtrAvidin Peroxidase at 1:1000 in PBS for 2.5 h at room temperature. The bound antibodies were visualised using the DAB visualisation process as described above. The CRF and GR single labelled sections were then mounted onto glass slides, dried overnight, dehydrated in ascending alcohols and coverslipped with DPX. The series of sections that underwent double labelling procedures to identify neurons that contained both GR and CRF received the same GR and CRF staining procedures as described, except the visualisation of CRF was performed using 0.04% 3-amino-9-ethylcarbazole (AEC) in 0.015% H_2O_2 in 50 mM acetate buffer (pH 5.0) as the chromogen instead of DAB. These sections were mounted onto glass slides and coverslipped with glycerol gelatin (Sigma).

For the single label GR series, five equidistant coronal sections through the hypothalamus between −2.00 and −1.00 mm caudal to bregma were analysed. For both the single and double labelled CRF series, the section at approximately −1.80 mm from bregma, anatomically verified to contain the four subdivisions of the PVH, was chosen for analysis. Single or double labelled cells were verified at a range of magnifications (40–200×) and manually counted using a light microscope (Olympus BX-51). Cells counts within the medial parvocellular (PaMP); lateral magnocellular (PaLM); parvocellular dorsal cap (PaDC) and ventral (PaV) divisions of each side of the paraventricular hypothalamus were performed.

2.11 | Statistical analysis

Statistical tests were conducted in R version 3.6.1 (R Core Team, 2021) using RStudio version 1.4.1106 (RStudio Team), the afex package (v1.0–1; Singmann et al., 2021),[55] the tidyverse package (v1.2.1; Wickham, 2017),[56] and the emmeans package (v1.5.5–1; Lenth, 2021).[57] For all statistical tests $\alpha = .05$. To evaluate whether the average durations of nonsocial, social and submissive behaviours differed between Persistent Effect and No Effect rats, a two-way, repeated measures (time × behavioural group) analysis of variance (ANOVA) was conducted for each behavioural category. Post hoc multiple pairwise comparisons (Sidak correction) were used to compare differences between groups at each time point. No statistical test was performed to assess differences between groups for dominance duration as this variable was used to categorize the rats into the groups. Separate, unpaired *t*-tests were used to compare pre-CCI ACTH and corticosterone levels between behavioural groups, while two-way, repeated measures (time x behavioural group) ANOVAs with multiple pairwise comparisons (Sidak correction) were used to compare post-CCI ACTH and corticosterone plasma concentrations. Separate, one-way ANOVAs were used to assess differences between Persistent Effect, No Effect and uninjured (naïve) control groups for: (i) MC_2R staining intensity in the adrenal cortex, (ii) GR and CRF mRNA expression in the hypothalamus, and (iii) the numbers of GR, CRF or double labelled GR + CRF neurons in each of the subdivisions of the PVH. If an ANOVA detected significant differences between groups, then post hoc multiple pairwise comparisons (Tukey's correction) were performed. See Supplementary Tables 1-6 for full details of the results for all statistical tests. Additionally, simple linear regressions were used to determine whether the change in a rat's post-CCI dominance on the final Resident–Intruder test was correlated with its MC_2R staining in the adrenal cortex, or the numbers of glucocorticoid receptor immunoreactivity (GR-ir) neurons per section in the PVH. In all Figures $^*p < .05$, $^{**}p < .001$, $^{***}p < .0001$.

3 | RESULTS

3.1 | Resident–Intruder behaviour in CCI rats

The behavioural testing reported here was conducted in two cohorts of rats by two investigators (M.K.S. and P.M.W.). Identical to our previous reports,[36,37] sciatic nerve CCI caused persistent reductions in dominance behaviour during Resident–Intruder social interactions in a subpopulation of animals. These Persistent Effect rats demonstrated reduced dominance behaviours (i.e., below 70% of the pre-CCI baseline) on all days tested after CCI. The total time spent performing dominance, nonsocial, social and submissive behaviours are illustrated in Figure 1. Prior to nerve injury, all rats showed similar levels of dominance behaviours (143.4 ± 6.3 s SEM), and importantly, there were no differences between rats subsequently categorised into either the No Effect or Persistent Effect groups (141.5 ± 7.02 s vs. 146.2 ± 11.93 s). The post-CCI reduction in dominance in Persistent Effect rats was accompanied by a corresponding increase in nonsocial behaviours. This reduction was statistically significantly different when compared to the No Effect rats as revealed by a two-way ANOVA: behavioural group ($F_{1,44} = 12.19$, $p = .001$), *day* ($F_{4.6,200.3} = 22.77$, $p < .001$), behavioural group × day ($F_{4.6,200.3} = 6.172$, $p < .001$). In contrast, social and submissive behaviours remained similar throughout the testing period, and two-way ANOVAs did not find any main effect of behavioural group (social: $F_{1,44} = 0.394$, $p = .53$; submissive: $F_{1,44} = 3.309$, $p = .08$)

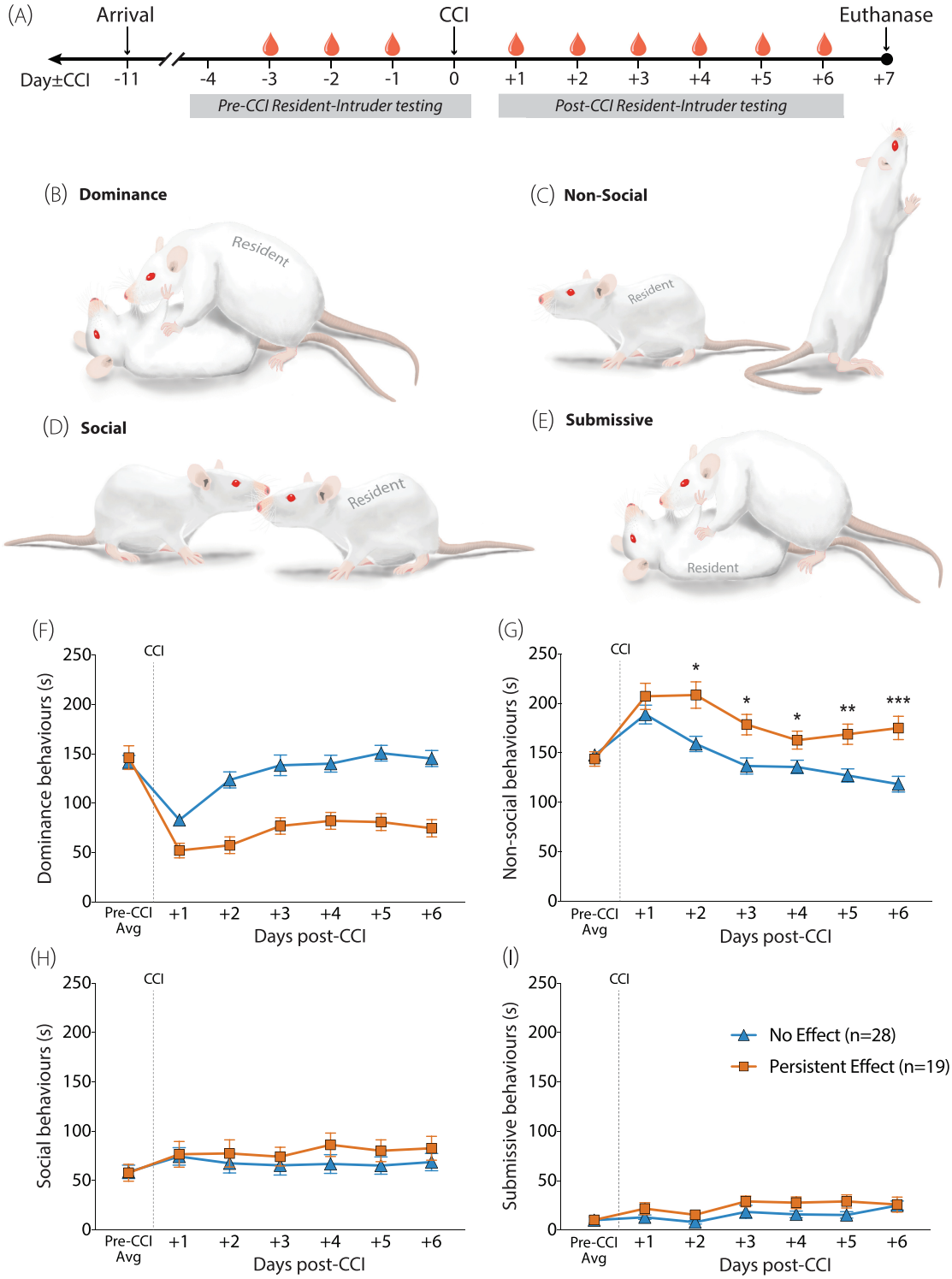

FIGURE 1 Experimental timeline and Resident–Intruder behaviours. (A) Experimental timeline. Male rats ($n = 47$) underwent Resident–Intruder social interactions testing for 11 consecutive days. Droplets indicate the days on which blood was collected. After the fifth Resident–Intruder test, rats received a unilateral CCI of the sciatic nerve. The time spent performing four stereotyped, mutually exclusive behaviours was recorded: (B) dominance behaviours, (C) social behaviours, (D) nonsocial behaviours or (E) submissive behaviours. (F) Time spent performing dominance behaviours during Resident–Intruder testing. While all rats developed similar levels of thermal and mechanical allodynia (see Figure S2), rats either demonstrated a persistent reduction in dominance behaviours post-CCI (Persistent, $n = 19$) or little to no change (No Effect, $n = 28$) compared to their pre-CCI baseline levels. This was most often accompanied by a corresponding increase in nonsocial behaviours (G), and modest or no changes to the time spent performing social behaviours (H) and submissive behaviours (I). Error bars represent ± SEM. $^{*}p < .05$, $^{**}p < .001$, $^{***}p < .0001$, Tukey's pairwise comparisons, two-way repeated measures ANOVA. ANOVA, analysis of variance; CCI, chronic constriction injury; SEM, standard error of mean

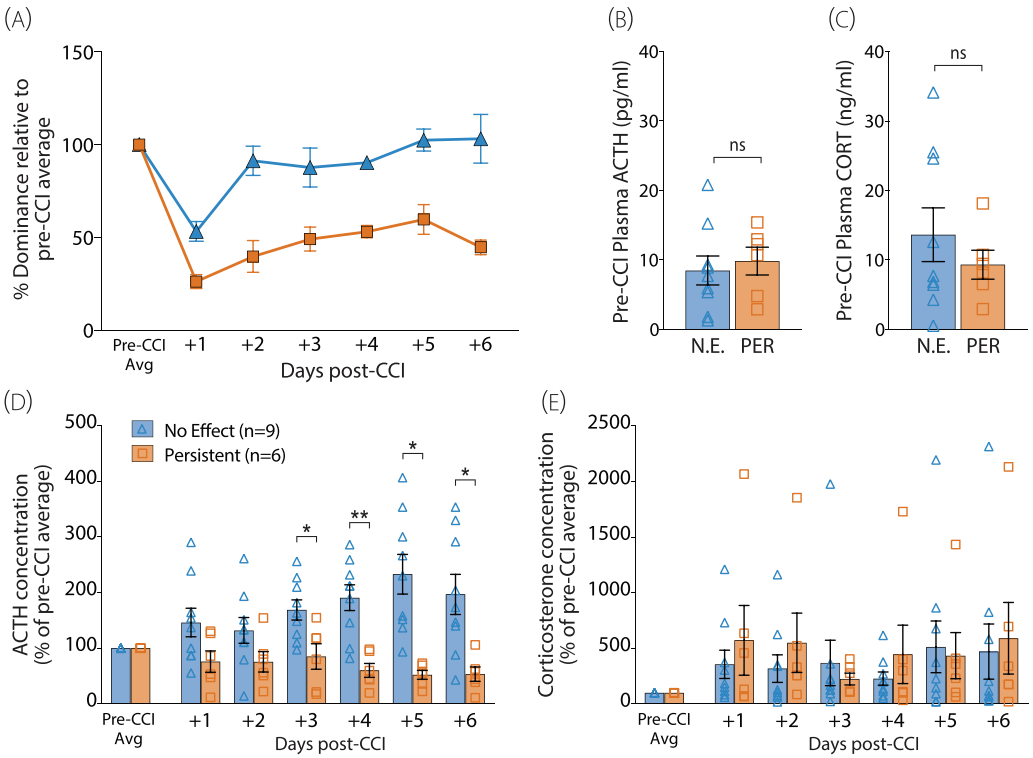

FIGURE 2 Plasma corticosterone and ACTH levels in No Effect ($n = 9$) and Persistent Effect ($n = 6$) rats. (A) Dominance behaviour during Resident–Intruder testing of rats whose plasma corticosterone and ACTH concentrations were measured. Prior to CCI, there were no differences in (B) plasma ACTH or (C) plasma corticosterone concentrations between rats in the No Effect (N.E.) group and the Persistent Effect (PER) group. Unpaired *t*-tests. (D) Post-CCI, No Effect rats had increased plasma ACTH concentrations compared to their Persistent Effect counterparts. (E) In contrast, plasma corticosterone concentrations were similar between the two groups at all post-CCI time points measured. Error bars represent ± SEM. $^{*}p < .05$, $^{**}p < .001$, Tukey's pairwise comparisons, two-way repeated measures ANOVA. ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; SEM, standard error of mean

or any interaction effects between behavioural group and time (social: $F_{5.1,223.8} = 1.428$, $p = .21$; submissive: $F_{4.1,179.2} = 1.079$, $p = .37$). The reduction in dominance behaviours was due largely to decreases in the frequency, intensity and duration of lateral and back attacks, while the increase in nonsocial behaviours consisted mainly of self-grooming focused on the CCI-injured limb. These behavioural changes occurred despite sensory testing revealing identical increases in both mechanical and thermal (cold) sensitivity in all injured rats following CCI (Figure S2). Additionally, post-mortem macroscopic inspections of the injured sciatic nerves confirmed that all four ligatures remained intact and the presence of local inflammatory responses in all rats tested.

3.2 | Plasma ACTH and corticosterone

Figure 2A shows the post-CCI changes in dominance behaviour of the rats whose plasma ACTH and corticosterone concentrations were measured. Prior to nerve injury, there were no significant differences in plasma ACTH concentrations between No Effect and Persistent Effect rats (8.49 ± 2.08 vs. 9.85 ± 2.01 pg/ml, $t = 0.448$, $p = .66$, unpaired *t*-test) (Figure 2B). These concentrations are within the normal circadian range for rats approximately 2 h prior to sleep.[58–60] After CCI, rats in the No Effect group showed large increases in plasma ACTH concentrations on each of the 6 days sampled, while rats in the Persistent Effect group showed a clear trend toward decreased plasma ACTH concentrations (Figure 2D). As such, a two-way ANOVA detected a significant main effect of behavioural group ($F_{1,13} = 16.777$, $p = .001$), and an interaction effect between behavioural group and time ($F_{4.2,54.5} = 5.079$, $p = .001$). These differences became progressively larger over time and were statistically significantly different on post-CCI days +3 ($p = .013$), +4 ($p = .0009$), +5 ($p = .0014$) and +6 ($p = .0079$). Time spent performing dominance behaviours did not correlate significantly with these changes in plasma ACTH for either behavioural group (data not shown).

Similar to plasma ACTH, there were no significant differences in plasma corticosterone concentrations between the No Effect and Persistent Effect rats prior to CCI ($t = 0.847$, $p = .41$) (Figure 2C). Despite an observation of increased variability post-CCI, a two-way ANOVA did not detect statistically significant differences in plasma corticosterone concentrations over time, or between pre- and post-CCI timepoints for either group. Additionally, there were no differences between groups on any of the post-CCI days (behavioural group:

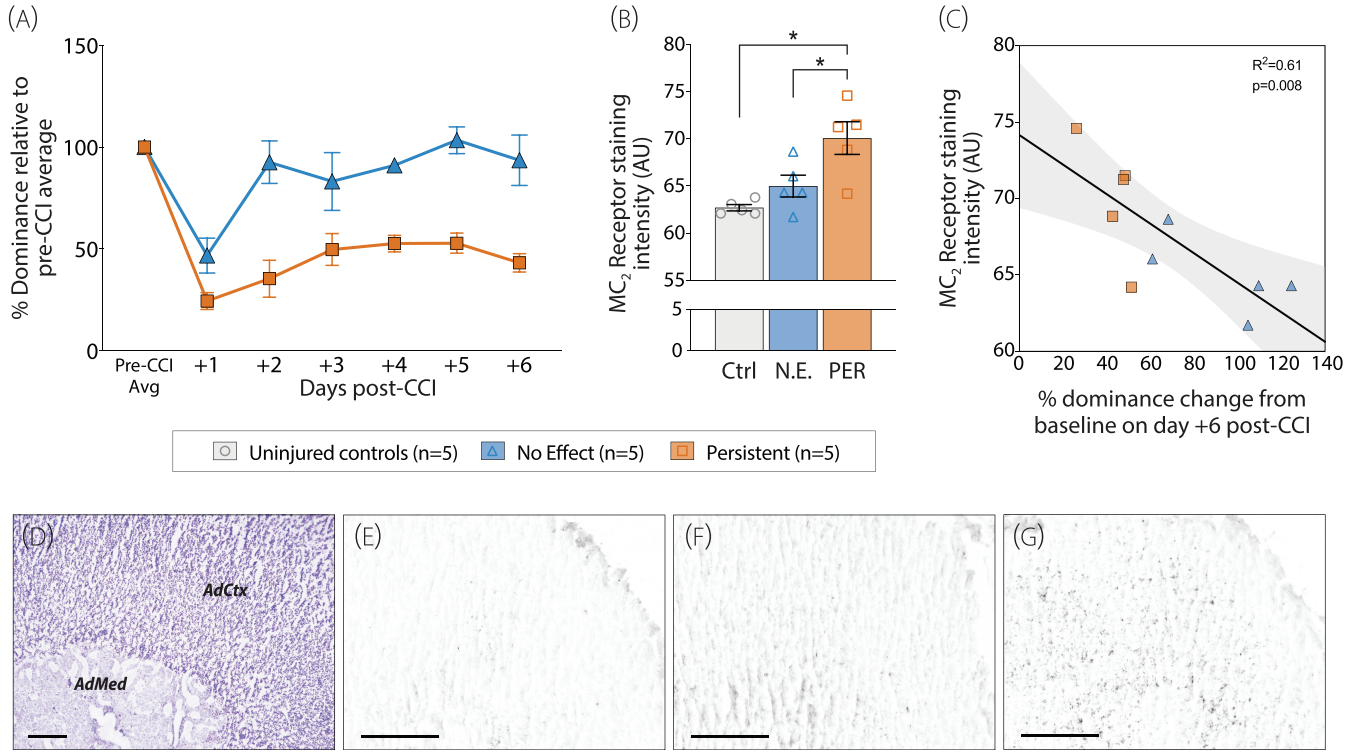

FIGURE 3 MC_2R expression in the adrenal cortex of control rats ($n = 5$), No Effect rats ($n = 5$), and Persistent Effect rats ($n = 5$). (A) Dominance behaviour during Resident–Intruder testing of rats whose tissue was used to assess MC_2R staining in the adrenal cortex. (B) Persistent Effect rats had increased MC_2R expression compared to both No Effect rats and uninjured (naïve) controls. *$p < .05$, one-way ANOVA with post hoc pairwise comparisons (Tukey's correction). Error bars represent ± SEM. (C) A linear regression revealed a significant correlation between MC_2R expression and changes in the levels of dominance behaviours performed on the final day of Resident–Intruder testing (+6 days post-CCI). (D) Nissl stain of the adrenal gland showing both the adrenal medulla (AdMed) and adrenal cortex (AdCtx). (E–G) Example photomicrographs of MC_2R expression in the adrenal cortex from rats in the uninjured (naïve) control group (E), No Effect group (F), and the Persistent Effect group (G). Scale bars represent 50 μm in all images. ANOVA, analysis of variance; CCI, chronic constriction injury; MC_2R, melanocortin receptor 2; SEM, standard error of mean

$F_{1,13} = 0.17$, $p = .69$; time: $F_{2.6,34.2} = 1.846$, $p = .163$; behavioural group × time: $F_{2.6,34.2} = 0.516$, $p = .65$). The outlier points seen in Figure 2E are accounted for by four animals, two from each group.

3.3 | MC_2-R immunoreactivity in the adrenal cortex

Figure 3A shows the post-CCI changes in dominance behaviour of the rats whose adrenal glands were stained for MC_2R expression. There were no significant differences between Persistent and No Effect rats in adrenal gland weights (two-way ANOVA: behavioural group $F_{1,15} = 0.49$, $p = .49$, anatomical side $F_{1,15} = 1.71$, $p = .21$, behavioural group × anatomical *side* $F_{1,15} = 0.001$, $p = .97$), or when adrenal gland weights were normalised to bodyweight (two-way ANOVA: behavioural group $F_{1,15} = 1.94$, $p = .18$, anatomical side $F_{1,15} = 1.48$, $p = .24$, behavioural group × anatomical side $F_{1,15} = 0.001$, $p = .96$) (Figure S3). MC_2R immunoreactivity was only present within the adrenal cortex, not in the adrenal medulla, and appeared as punctate deposits with clear cellular distribution (Figure 3E–G). Rats that received CCI had increased MC_2R-ir when compared to uninjured controls (one-way ANOVA $F_{2,12} = 9.67$, $p = .003$). Persistent Effect rats had significantly greater MC_2R-ir compared to both the No Effect group ($p = .03$) and the uninjured control group ($p = .003$), as revealed by post hoc pairwise comparisons with Tukey's correction (Figure 3B). Additionally, a linear regression between MC_2R staining intensity and the changes in post-CCI dominance duration on the final Resident–Intruder test (post-CCI day +6) found a significant correlation equation (Figure 3C): Δdominance = 74.15 + −0.1 * intensity ($R^2 = .61$, $F_{1,8} = 12.43$, $p = .008$), indicating that the rats who had greater MC_2R-ir in the adrenal cortex tended to show greater reductions in dominance behaviours at this time point. This correlation appears to be driven largely by differences between behavioural groups, rather than at an individual level within each group.

3.4 | GR mRNA and protein expression in the PVH

Figure 4A shows the post-CCI changes in dominance behaviour of the rats whose tissue was used to quantify glucocorticoid receptor expression in the PVH. Compared to uninjured controls, GR-mRNA expression was increased selectively in No Effect rats following CCI (1.49-fold increase, one-way ANOVA, $F_{2,12} = 4.25$, $p = .04$, with Tukey's pairwise comparison) (Figure 4B). GR-ir was identical to that

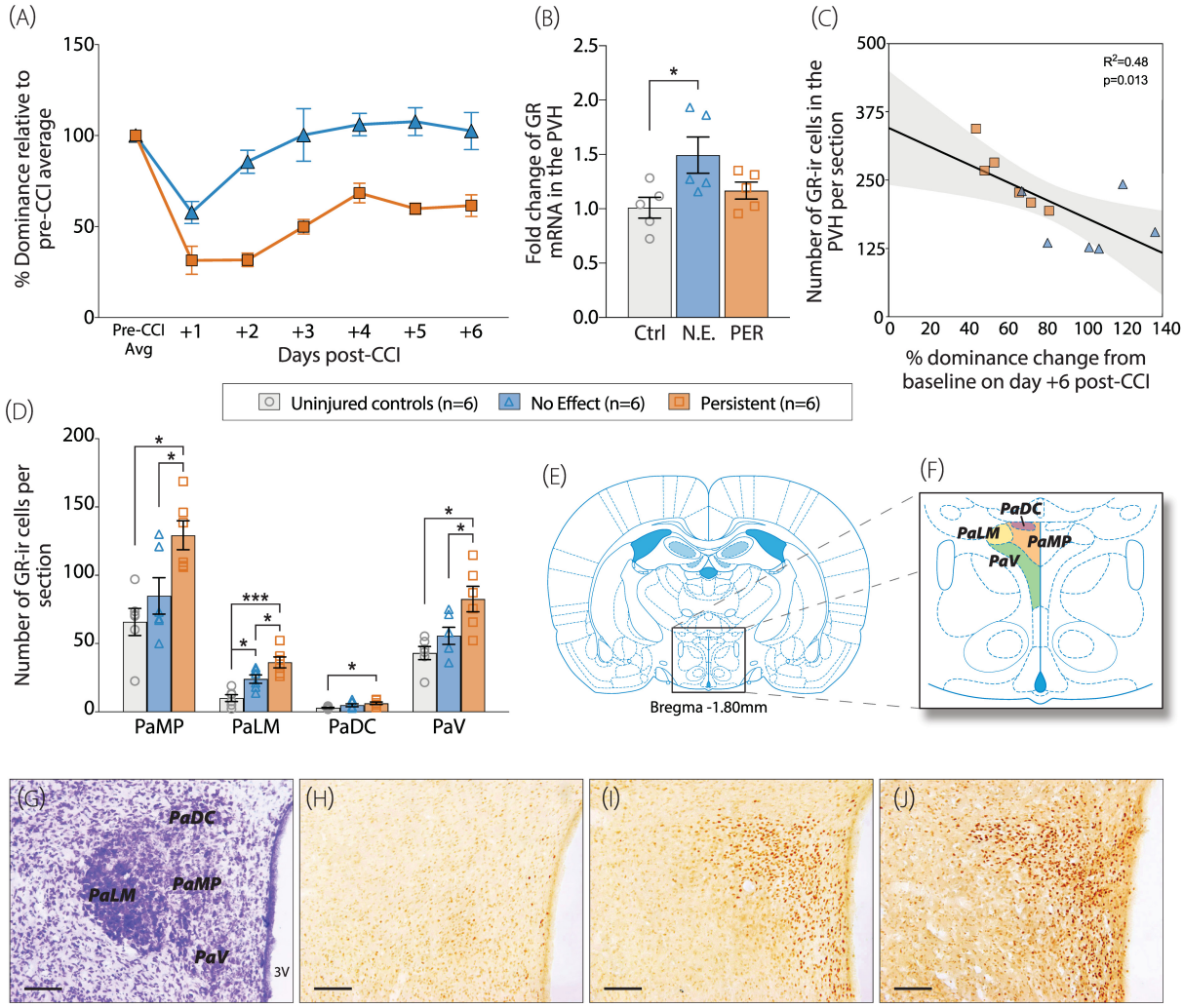

FIGURE 4 Glucocorticoid receptor expression in the PVH. (A) Dominance behaviour during Resident–Intruder testing of rats whose tissue was used to quantify GR-ir cells in the PVH. (B) Quantitative RT-PCR comparing GR mRNA expression levels between rats whose post-CCI dominance levels were persistently reduced (PER, $n = 5$), rats who displayed no change in dominance post-CCI (N.E., $n = 5$) and uninjured (naïve) controls who did not undergo Resident-Intruder testing (Ctrl, $n = 5$). (C) A linear regression revealed a significant correlation between the total numbers of GR-ir cells in the PVH and changes in the levels of dominance behaviours performed on the final day of Resident–Intruder testing (+6 days post-CCI). (D) Numbers of GR-ir cells in each subdivision of the PVH in control ($n = 6$), Persistent Effect ($n = 6$) and No Effect ($n = 6$) rats. Error bars represent ± SEM. $^{*}p < .05$, $^{***}p < .0001$, one-way ANOVAs with post hoc pairwise comparisons (Tukey's correction). PaDC, paraventricular hypothalamus dorsal cap, PaLM, paraventricular hypothalamus lateral magnocellular part, PaMP, paraventricular hypothalamus medial parvocellular part, PaV, paraventricular hypothalamus, ventral part. (E, F) Schematic of a coronal section and (G) Nissl stain illustrating the paraventricular hypothalamic subdivisions, 3V, third ventricle. (H–J) Example photomicrographs of GR expression in the PVH from rats in the uninjured (naïve) control group (H), No Effect group (I), and the Persistent Effect group (J). Scale bars represent 200 μm. ANOVA, analysis of variance; GR-ir, glucocorticoid receptor immunoreactive; PVH, paraventricular nucleus of the hypothalamus; RT-PCR, reverse-transcriptase polymerase chain reaction; SEM, standard error of mean

seen by Morimoto et al.,[61] appearing as a dark brown nuclear stain (Figure 4H–J). In contrast to the levels of GR-mRNA regulation on post-CCI day 7, the number of GR immunoreactive cells in the PVH was increased in CCI animals and was greatest in Persistent Effect rats. In fact, a linear regression between the number of GR-ir cells per section and the changes in post-CCI dominance duration on the final Resident–Intruder test found a significant correlation equation: $\Delta\text{dominance} = 345.8 + -1.63 * \text{cells/section}$ ($R^2 = .48$, $F_{1,10} = 9.18$, $p = .013$) (Figure 4C). Similar to the MC_2R expression, this correlation appears to be driven largely by differences between behavioural groups, rather than at an individual level within each group. Detailed analysis of the distribution of the GR-ir cells in the PVH did not detect any lateralisation effects in any of the four major subdivisions of the PVH for any group (Figure S4), and cell numbers from left and right were summed for statistical analysis. One-way ANOVAs found significant differences between Persistent Effect, No Effect and uninjured controls in the paraventricular nucleus medial parvocellular part (PaMP; $F_{2,15} = 8.204$, $p = .004$), the paraventricular nucleus lateral

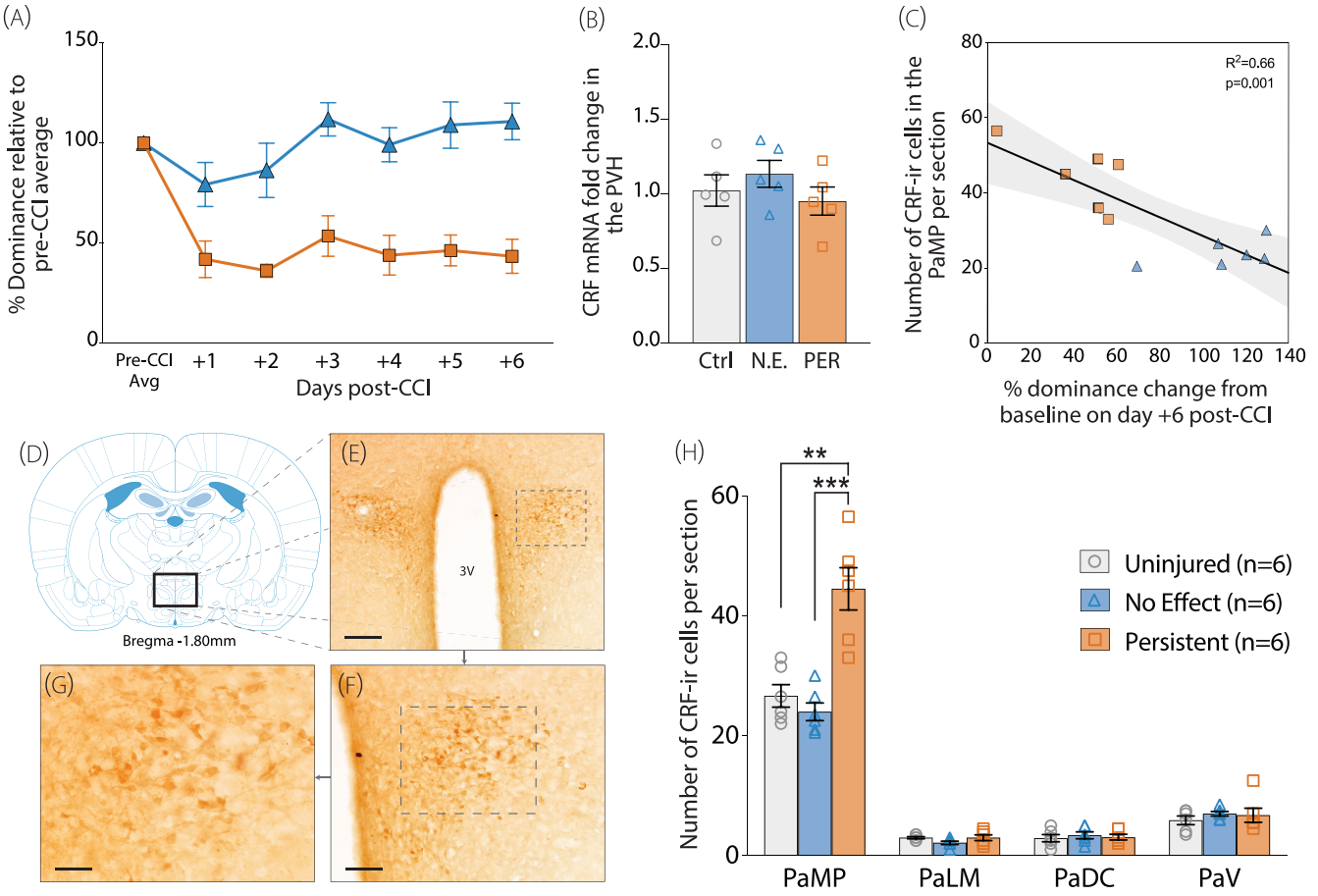

FIGURE 5 Corticotropin releasing factor in the PVH. (A) Dominance behaviour during Resident–Intruder testing of rats whose tissue was used to quantify CRF-ir in the PVH. (B) Quantitative RT-PCR comparing CRF mRNA expression levels between Persistent Effect rats (PER, $n = 5$), No Effect rats (N.E., $n = 5$) and uninjured (naïve) controls who did not undergo Resident–Intruder testing (Ctrl, $n = 5$). (C) A linear regression revealed a significant correlation between the numbers of CRF-ir cells in the PaMP subdivision of the PVH and changes in the levels of dominance behaviours performed on the final day of Resident–Intruder testing (+6 days post-CCI). (D) Schematic of a coronal section containing the PVH, and (E–G) example photomicrographs of CRF-ir in the PVH. Dashed boxes indicate the image area shown in (G). 3V, third ventricle. Scale bars represent 400, 200, 100 μm respectively. (H) Numbers of CRF-ir cells in each subdivision of the PVH in control ($n = 6$), Persistent Effect ($n = 6$) and No Effect ($n = 6$) rats. Error bars represent ± SEM. $^{**}p < .001$, $^{***}p < .0001$, one-way ANOVAs with post hoc pairwise comparisons (Tukey's correction). PaDC, paraventricular hypothalamus dorsal cap, PaLM, paraventricular hypothalamus lateral magnocellular part, PaMP, paraventricular hypothalamus medial parvocellular part, PaV, paraventricular hypothalamus, ventral part. ANOVA, analysis of variance; CRF-ir, corticotropin releasing factor immunoreactivity; PVH, paraventricular nucleus of the hypothalamus; RT-PCR, reverse-transcriptase polymerase chain reaction; SEM, standard error of mean

magnocellular part (PaLM; $F_{2,15} = 16.47$, $p = .0002$), the paraventricular nucleus dorsal cap (PaDC; $F_{2,15} = 4.07$, $p = .039$) and the paraventricular nucleus ventral part (PaV; $F_{2,15} = 8.129$, $p = .004$) (Figure 4D). The majority of GR-ir cells were located within PaMP and PaV, with fewer cells in PaLM and the least number of cells in PaDC (Figure 4G–J).

3.5 | CRF mRNA and protein expression in the PVH

Figure 5A shows the post-CCI changes in dominance behaviour of the rats whose tissue was used to quantify CRF expression in the PVH. CRF-mRNA expression was unaffected by nerve injury at day 7, and a one-way ANOVA found no significant differences between groups ($F_{2,12} = 0.908$, $p = .43$) (Figure 5B). CRF immunoreactivity (CRF-ir) was visible in the cytoplasm of neurons as brown reaction product. As with the GR-ir, there was no lateralisation in the numbers of CRF-ir cells in any of the four major subdivisions of the PVH for any group (Figure S4), and cell numbers from left and right were summed for statistical analysis. Labelled cells were found almost exclusively in the PaMP (Figure 5E–G), in which there were twice the number of CRF-ir neurons in the Persistent Effect rats compared to either the No Effect rats or uninjured controls ($F_{2,15} = 20.34$, $p < .0001$). Additionally, a linear regression between the number of CRF-ir

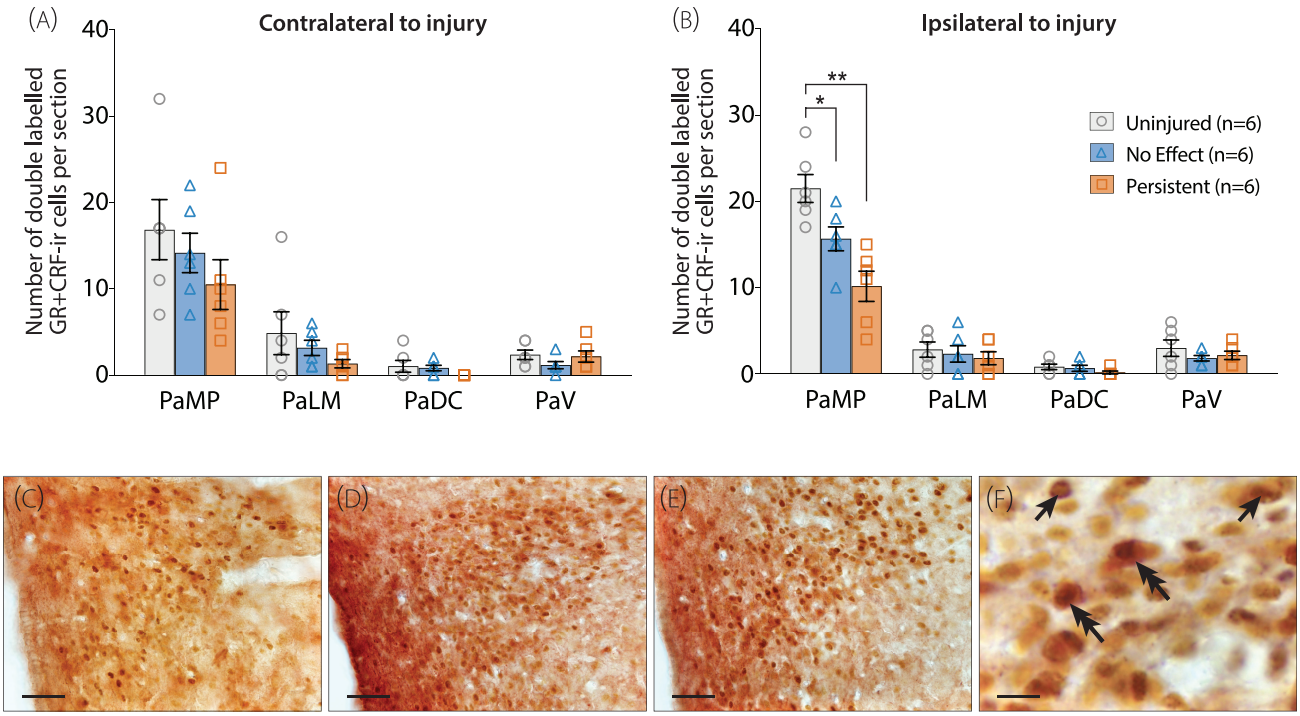

FIGURE 6 Double-label GR + CRF in the PVH in control ($n = 6$), Persistent Effect ($n = 6$) and No Effect ($n = 6$) rats. (A, B) Numbers of double labelled GR and CRF immunoreactive (GR + CRF-ir) cells per section in each subdivision of the PVH (A) contralateral to CCI, and (B) ipsilateral to CCI. Error bars represent ± SEM. *$p < .05$, **$p < .001$, one-way ANOVAs with post hoc pairwise comparisons (Tukey's correction). PaDC, paraventricular hypothalamus dorsal cap, PaLM, paraventricular hypothalamus lateral magnocellular part, PaMP, paraventricular hypothalamus medial parvocellular part, PaV, paraventricular hypothalamus, ventral part. (C–E) Example photomicrographs of GR expression in the PVH from rats in the uninjured (naïve) control group (C), No Effect group (D), and the Persistent Effect group (E). Scale bars represent 150 μm. (F) Photomicrograph showing examples of double labelled GR + CRF-ir cells (double arrows), and single labelled GR-ir cells (single arrows). Scale bar represents 50 μm. ANOVA, analysis of variance; CCI, chronic constriction injury; CRF-ir, corticotropin releasing factor immunoreactivity; GR-ir, glucocorticoid receptor immunoreactive; PVH, paraventricular nucleus of the hypothalamus; SEM, standard error of mean

neurons in the PaMP and the changes in post-CCI dominance duration on the final Resident–Intruder test (post-CCI day +6) found a significant correlation equation (Figure 5C): Δdominance = 53.31 + −0.25 * cells/section ($R^2 = .66$, $F_{1,10} = 19.01$, $p = .001$), indicating that the rats who had greater CRF-ir neurons in this subdivision tended to show greater reductions in dominance behaviours at this time point. Once again, this correlation seems to be driven largely by differences between behavioural groups, rather than at an individual level within each group. There were few labelled cells seen in the rest of the PVH and one-way ANOVAs did not find significant differences between groups in the PaLM ($F_{2,15} = 2.016$, $p = .17$), PaDC ($F_{2,15} = 0.219$, $p = .81$) or the PaV ($F_{2,15} = 0.459$, $p = .64$) (Figure 5H).

3.6 | Double labelled GR + CRF expression in the PVH

Figure 5A shows the post-CCI changes in dominance behaviour of the rats whose tissue was used to quantify double-labelled GR + CRF immunoreacted neurons in the PVH. Cells were considered to be double labelled if a brown/black stained nucleus (GR-ir, DAB reaction product) was surrounded by a red/pink stained cytoplasm (CRF-ir, AEC reaction product). Examples of double-labelled cells are shown in Figure 6F. Double-labelled cells were found in the PVH of all animals, with the PaMP subdivision containing more than 90% of these cells. The PaLM, PaDC and PaV subdivisions had few double-labelled cells, which were similar in numbers across these regions. When compared to uninjured control rats, CCI caused a bilateral reduction in the mean numbers of double-labelled cells, most notably in the PaMP (Figure 6A,B). This effect was strongest in the PaMP ipsilateral to nerve injury ($F_{2,15} = 12.81$, $p = .0006$, one-way ANOVA), with larger variance seen within groups in the contralateral PaMP ($F_{2,15} = 1.186$, $p = .33$, one-way ANOVA). No significant differences between groups were observed in the ipsilateral or contralateral PaLM (ipsilateral, $F_{2,15} = 0.336$, $p = .72$; contralateral, $F_{2,15} = 1.283$, $p = .31$), PaDC (ipsilateral, $F_{2,15} = 1.548$, $p = .25$; contralateral, $F_{2,15} = 1.535$, $p = .25$) or PaV (ipsilateral, $F_{2,15} = 0.863$, $p = .44$; contralateral, $F_{2,15} = 1.327$, $p = .30$).

4 | DISCUSSION

In this study, animals that showed persistent changes to social interactions following a peripheral nerve injury (Persistent Effect) were deliberately selected for comparison with those whose social interactions were unchanged (No Effect). Consistent with other studies, and our previous work, we found that CCI did not have significant effects on basal plasma corticosterone levels in either of these groups.[24–28,48] These findings have led to a more global suggestion that neuropathic pain in rats is not associated with changes to the HPA axis. However, data presented in this study warrants a revision of this view. We found that CCI resulted in distinct changes within the HPA-axis that were related to a rat's behavioural phenotype.

Prior to injury, plasma ACTH and corticosterone concentrations were similar between Persistent Effect and No Effect rats and were within normal ranges as reported previously.[58–60] Following CCI, although there were no changes in plasma corticosterone levels, plasma ACTH concentrations of these groups diverged for the remainder of the experiment, whereby rats in the No Effect group showed elevated levels, while Persistent Effect rats showed modest decreases. This observation suggests a divergence of adrenocortical sensitivity to ACTH. Our finding that MC_2R expression increased in Persistent Effect, but not in the No Effect rats, supports this view. Additionally, central changes to the HPA-axis of Persistent Effect rats included increased numbers of GR-positive cells in the PVH, and increased numbers of CRF-expressing neurons in the PaMP subdivision of the PVH. Remarkably, these increases in GR and CRF were not within the same population of cells. Our results therefore reveal significant CCI-induced HPA-axis changes, which were most prominent in Persistent Effect rats, characterized by increased adrenocortical sensitivity to ACTH and decreased hypothalamic sensitivity of CRF neurons to circulating corticosterone.

4.1 | The paradoxical relationship of chronic pain and glucocorticoids

It is well established that acute painful events lead to HPA-axis activation, with the resultant spike in plasma glucocorticoids suggested to reduce the unpleasantness of pain and increasing pain tolerance,[3–6] both key features of the phenomenon of stress-induced analgesia. This observation has led to a widely accepted suggestion that chronic pain leads to chronic HPA-activation and sustained elevations in basal cortisol levels. As Vachon-Presseau[62] surmises: "the unpredictable and uncontrollable nature of persistent pain represents a recurrent stressor challenging the homeostasis of the organism." Surprisingly, however, studies that have investigated whether chronic pain patients have sustained increases in basal cortisol levels have produced mixed results. In comparison to healthy controls, basal salivatory cortisol levels in chronic pain patients have been found to be increased,[15–17] decreased,[63–65] or not different,[4,5,7–14] across a range of chronic pain conditions.

However, while evidence for altered basal glucocorticoid levels in chronic pain patients is weak or equivocal, numerous studies have demonstrated that cortisol responses to acute stressors remain largely intact in these people. Vachon-Presseau et al.[4] have demonstrated that a noxious thermal stimulus evokes cortisol release in chronic back pain patients of a similar magnitude and time course as healthy controls. Geiss et al.[18] found patients with fibromyalgia mounted an acute cortisol response to a pressure pain threshold test, and Yoshihara et al.[19] have shown intact or exaggerated cortisol, adrenaline and noradrenaline responses to psychological stressors in patients with chronic myofascial pain. Taken together, these findings suggest specific modifications to the HPA-axis that prevents the persistent nature of chronic pain from elevating basal glucocorticoid levels. Importantly, these modifications do not prevent individuals from mounting the adaptive cortisol increases required for dealing with acute stressors. The data presented in this study support this view and suggest that these modifications include increased adrenocortical sensitivity as well as changes in hypothalamic CRF- and GR-signalling.

4.2 | Rebalancing the HPA-axis after nerve injury

The homeostatic functions of the HPA-axis are incredibly well understood: stressor-induced CRF release drives increased ACTH production, which in turn, elevates glucocorticoids.[66,67] In this context, our observation that Persistent Effect rats had increased numbers of CRF neurons in the PVH, yet reduced plasma ACTH levels seems paradoxical. We suggest three likely explanations for this finding. Firstly, the increased numbers of CRF neurons are accounted for by de novo synthesis in cell populations that do not project to the median eminence, but instead use CRF as a neuropeptide transmitter, and thus do not directly stimulate ACTH release. Outputs of these neurons could include the periaqueductal gray (PAG),[68,69] the parabrachial nucleus,[70] and the ventral tegmental area.[71] Another possibility is that despite increased CRF release into the hypophyseal portal system, CRFR1/2 receptor expression on corticotrophs in the pituitary has been selectively downregulated by the nerve injury. Supporting this possibility, a number of physiological challenges have been shown to persistently downregulate the genes for CRFR1 and CRFR2 expression in the pituitary.[72–76] Finally, pituitary corticotrophs may have upregulated GR expression, desensitizing them to the effects of CRF.[77] It should also be noted that these explanations are not mutually exclusive, and the relative contributions of each of these suggestions remains to be experimentally determined.

Taken together, our observations of increased hypothalamic CRF expression, increased adrenocortical MC_2R expression, yet normal basal corticosterone levels indicates that the HPA-axis has been rebalanced by the nerve injury in some animals. This finding invites two important questions: (i) how does CCI trigger the upregulation of CRF and GR in the hypothalamus as well as MC_2R expression in the adrenal cortex, and (ii) why are these changes strongest in the rats that show alterations in social interactions and other complex behaviours?

4.3 | CCI evoked CRF, GR and MC_2R expression

Importantly, it should be noted that whilst our behavioural and endocrine data were collected daily over a period 11 days, the PCR and immunohistochemical data reflect mRNA and protein expression levels at a single timepoint, which was 7 days after the nerve injury and 24 h after the last Resident–Intruder test. This temporal differential must be taken into consideration when interpreting the findings. While we found no changes to CRF mRNA expression in the PVH at 7 days following surgery, CRF protein expression was increased at this time. When evaluated 3–4 weeks after CCI, others have also reported CRF mRNA expression levels to be the same as those in uninjured controls.[27,78] Additionally, although CCI has a strong inflammatory component, the long-term downregulation of CRF mRNA seen in other inflammatory stress models was not observed.[79] Therefore, the gene upregulation that resulted in greater CRF protein expression is likely to have occurred at an earlier timepoint post-injury. Acute upregulation of CRF mRNA in the PVH has been reported following restraint, footshock, intraperitoneal injection of hypertonic saline and forced swimming.[80,81] Interestingly, the acute increase in CRF gene expression to these stressors still occurs even in the face of the enhanced negative feedback signal from elevated stress-induced corticosterone release (which would normally dampen down CRF release), suggesting a neural rather than endocrine driver of these changes. Further supporting this idea, Kiss et al.[82] showed that a unilateral disconnection of the brainstem prevented restraint-evoked increases in CRF mRNA expression in the PVH ipsilateral to the lesion, highlighting the importance of ascending brainstem projections in the regulation of hypothalamic CRF levels.

Glucocorticoid receptor regulation in the CNS is exceptionally complex, with a range of stimuli and physiological challenges altering the expression of GR in a site-specific manner.[83,84] For example, the persistent reduction of circulating glucocorticoids after adrenalectomy results in upregulated GR expression in multiple brain sites including the hippocampus and amygdala,[84,85] but not in the PVH, thalamus or cortex.[86,87] Conversely, reduced GR expression in the hippocampus, amygdala and pituitary is seen after the persistent elevation of circulating corticosteroids due to chronic stress,[83,88] repeated dexamethasone treatment,[89,90] or an early life stress.[91] Based on these studies, and the absence of change in plasma corticosterone in rats in this study, the GR upregulation we observed is most likely due to altered afferent drive on PVH neurons[84] in a similar manner to that described for CRF above. Likely sources of these afferents include the medullary noradrenergic pathways, nociceptive recipient midbrain structures (such as the PAG) and direct spinal projections, each of which are significantly impacted by neuropathic injury.[54,92–96]

The third change that we saw in the HPA-axis was increased MC_2R expression in the adrenal cortex. Unlike typical gene regulation, MC_2R expression is upregulated by its own ligand (ACTH),[97] which is essential for adrenocortical maintenance, as hypophysectomised animals develop adrenocortical atrophy.[98] Given that our data reflect a single snapshot in time, we are unable to evaluate the temporal relationships between ACTH release and MC_2R protein expression. Our observations indicate that basal ACTH levels are different between Persistent Effect and No Effect groups, and that these levels do not correlate with the functional regulation of adrenocortical MC_2R expression described previously. It is possible that the increase in MC_2R expression seen in Persistent Effect rats is due to an acute ACTH pulse that occurred between daily blood sampling. Such a pulse may have been triggered by the Resident-Intruder encounter, which took place ~5 h before blood was drawn and ~24 h before euthanasia. There is also some evidence that adrenocortical sensitivity can be regulated by neural inputs carried within splanchnic nerves,[99] as thoracic splanchnic nerve transection attenuates stress-induced corticosterone release.[100]

Finally, whether the changes we have reported across the HPA-axis are each directly triggered by the CCI, or whether they are the consequences of a set of cascading homeostatic adaptations due to a perturbation of the HPA axis at a single point remains to be answered.

4.4 | Are the behavioural and endocrine effects of CCI causally related to each other?

Our data show clear and significant correlations between the degree of disruption to Resident–Intruder social interactions and MC_2R, GR and CRF expression. However, the causal relationships between these variables remains unclear. We do not believe that the divergence in social behaviour (i.e., Persistent vs. No Effect) can be attributed to differences in the degree of damage at the site of injury, as sensory testing revealed similar changes to mechanical and thermal sensitivity in both subpopulations (Figure S2). Such a dissociation between behavioural phenotype and sensory sensitivity has been reported in our earlier preclinical work[36] as well as in a number of clinical studies.[42–46] Instead, we suggest that following nerve injury animals have an intrinsic predisposition towards one of several distinct behavioural phenotypes (see Monassi et al.[36]), some of which appear to have strong neuroendocrine correlates.

Individual differences in the response to stress, threat, pain and reward have been described previously, and well-characterized behavioural subpopulations have been identified. These include high versus low avoidance,[32] short versus long attack latency,[31] short versus long social defeat latency,[33] reactive versus proactive coping styles,[29] sign-tracking versus goal-tracking,[30] and high versus low responding to drug self-administration.[101] Most of these subpopulations also show differences in HPA-axis activity.[102] Higher basal and/or stress-induced corticosterone and ACTH levels have been associated with reactive coping, short-latency defeat, long-attack latency and low-avoidance phenotypes.[31–34]

A number of studies have implicated changes to CRF signalling as a critical factor that mediates both the changes to HPA-axis activity and differences in behavioural phenotype. For instance, high-responsive rats prone to drug self-administration[103] and mice more susceptible to social defeat[104] have increased CRF mRNA expression in the PVH, while long-attack latency mice show similar increases after an acute stress.[31] Furthermore,[104] demonstrated that knockdown of CRF specifically in the PVH attenuated social avoidance following repeated

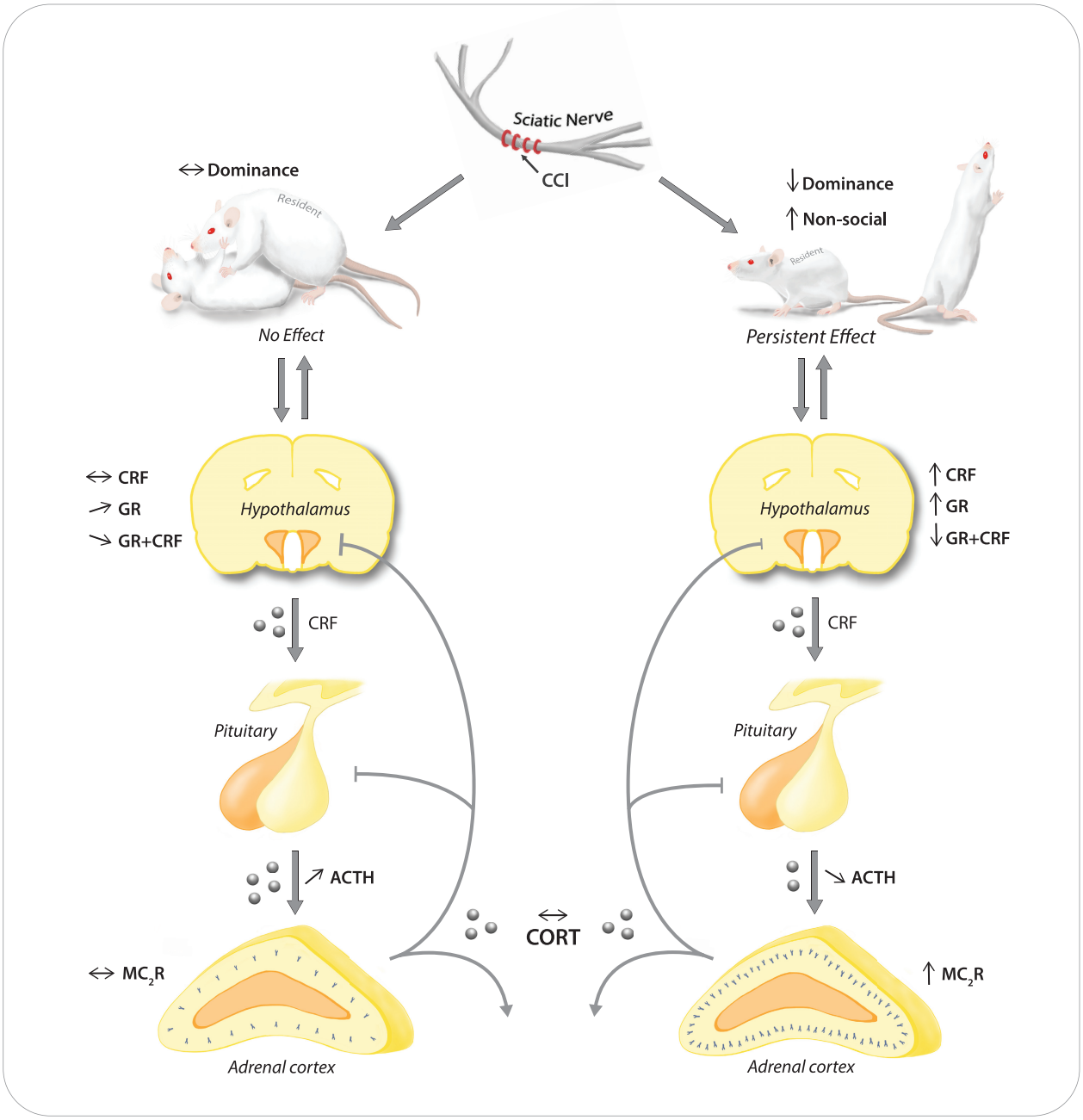

FIGURE 7 Summary of HPA-axis modifications after peripheral nerve injury in No Effect and Persistent Effect rats. Sciatic nerve CCI results in behaviourally distinct subpopulations of rats. Persistent Effect rats demonstrate consistent and long-lasting changes to their social interactions during a Resident-Intruder test as well as changes to other complex behaviours, while No Effect rats do not display these changes. These behavioural phenotypes are associated with distinctive modifications to the HPA-axis. Persistent Effect rats show increased numbers of CRF containing neurons and GR containing neurons in the PVH, but decreased numbers of double labelled CRF + GR containing neurons. These rats also have reduced plasma ACTH after CCI and increased MC_2R expression in the adrenal cortex. No Effect rats show little or no changes to the numbers of CRF, GR or CRF + GR containing neurons in the PVH, increased plasma ACTH after CCI, and no changes to MC_2R expression in the adrenal cortex. Both sets of modifications, however, appear to rebalance the HPA-axis so as to result in normal basal corticosterone levels, as both Persistent Effect and No Effect rats showed no changes to plasma corticosterone after CCI (Figure 2). ACTH, adrenocorticotropic hormone; CCI, chronic constriction injury; Cort, Corticosterone; CRF, corticotropin releasing factor; GR, glucocorticoid receptor; MC_2R, melanocortin receptor type-2; PVH, paraventricular nucleus of the hypothalamus

social defeat. Pharmacological interventions have also shown that the modulation of CRF signalling has direct impact on complex behaviours, including social interactions. Infusions of CRF into the amygdala have been shown to decrease immobility and increase exploration in Roman high-avoidance rats,[105] while a systemic CRF_1 receptor antagonist increases the latency to social defeat.[106] Additionally,[107] demonstrated

that intracerebroventricular CRF administration decreased the time rats spent in social interactions in a familiar environment while increasing the time spent self-grooming (i.e., non-social). For a comprehensive review of the associations between CRF activity and behavioural consequences see Packard et al.[67]

Given that we saw a doubling of the numbers of CRF containing cells in the PVH specific to the Persistent Effect rats we suggest that changes to CRF signalling might also underlie the differences we see in their changes in social behaviours. While we only investigated CRF expression in the PVH, based on the studies described above, it seems plausible to suggest that Persistent Effect rats would also have increased CRF expression in other extra-hypothalamic brain regions. Indeed, CCI-induced increases in CRF mRNA has been reported in the central amygdala and bed nucleus of the stria terminalis.[27,78] Persistent Effect rats may therefore have a genetic or epigenetic predisposition to injury-induced changes in CRF regulation; a suggestion we think warrants further investigation.

In addition to increased CRF expression, we also found that Persistent Effect rats showed greater CCI-induced increases in GR expression in the PVH. However, as we noted above, the precise neural mechanisms by which CCI is able to induce GR upregulation in the PVH remains to be determined. A greater population of GR containing cells in this region would usually suggest that these rats are more sensitive to the stress-induced negative feedback signal of corticosterone release; the net result being a hyper-suppression of HPA-axis activity after the stressful event.[108] However, given that we found fewer numbers of double labelled GR + CRF cells in the PVH of Persistent Effect rats, it would appear that this CRF-containing neuronal population is actually less sensitive to the negative feedback effects of stress-induced corticosterone release, which would instead result in a prolonged physiological stress response. To evaluate this suggestion, future studies could measure corticosterone levels immediately after the Resident-Intruder interaction or an acute stressor.

5 | CONCLUSIONS

Maintaining tight regulation over peripheral corticosterone or cortisol levels in the face of both acute and chronic stressors confers an adaptive advantage and is essential for both short-term and long-term homeostasis.[109,110] In response to a neuropathic injury, we have identified two sets of HPA-axis adaptations, involving both peripheral and central changes, that ultimately result in similar basal plasma corticosterone levels. As illustrated in Figure 7, these adaptations are strongly correlated with a rat's behavioural phenotype as determined by Resident–Intruder social interactions testing. Rats that show persistent changes in social interactions (Persistent Effect), which are also accompanied by reduced sleep, decreased motivation for reward, and changes to thyroid function, selectively show increased adrenocortical MC_2R expression as well as increased hypothalamic GR and CRF expression. In contrast, rats that do not display changes in social interactions (No Effect) did not show changes to GR, CRF or MC_2R expression, but show increased basal ACTH levels in response to CCI. We suggest that in addition to rebalancing the HPA-axis, the increased CRF expression in Persistent Effect rats may also contribute to their changes in complex behaviours, and in particular their social interactions. Studies in clinical populations have often failed to find relationships between cortisol levels and chronic pain or disability. However, based on the data presented in this study, it is possible that some of these people may undergo other HPA-axis adaptations that go undetected (such as altered CRF and GR expression), but which may underlie the disruptions to complex behaviours often experienced by those suffering with chronic pain.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jne.13131.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Damien C. Boorman https://orcid.org/0000-0003-2819-1948
Kevin A. Keay https://orcid.org/0000-0003-3934-4380

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