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PACAP and VIP expression in the periaqueductal grey of the rat following sciatic nerve constriction injury

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ABSTRACT

Nerve injuries often result in neuropathic pain with co-morbid changes in social behaviours, motivation, sleep-wake cycles and neuroendocrine function. In an animal model of neuropathic injury (CCI) similar co-morbid changes are evoked in a subpopulation (~30%) of injured rats. In addition to anatomical evidence of altered neuronal and glial function, the periaqueductal grey (PAG) of these rats shows evidence of cell death. These changes in the PAG may play a role in the disruption of the normal emotional coping responses triggered by nerve injury. Cell death can occur via a number of mechanisms, including the disruption of neuroprotective mechanisms. Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two endogenous neuropeptides whose activities are tightly regulated by two receptors subtypes, namely the PAC1 and VPAC receptors. These peptides and their receptors exert robust neuroprotective roles. In these studies, we hypothesized that rats expressing disabilities following CCI showed altered expression of PACAP and VIP in the PAG. Rats were categorized as having either Pain alone, Transient or Persistent disability, based on changes in social behaviours pre- and post-CCI. Social interaction behavioural tested (BT), sham-injured and naïve untested rats were also included. For measurements of mRNA and protein expression we utilised micro-dissected PAGs blocks taken from each group. At the mRNA level, VIP was downregulated and PAC1 was up-regulated in BT animals, whilst VPAC1 mRNA was specifically increased in the Pain alone group. Interestingly, protein levels of both PACAP and VIP were remarkably increased in the Persistent Disability group. Taken together, sciatic nerve CCI that triggers neuropathic pain and persistent disability results in abnormally increased VIP and PACAP expression in the PAG. Our data also suggest that these effects are likely to be governed by post-transcriptional mechanisms.

1. Introduction

The neuropeptides pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are each localised within the periaqueductal grey region of the rat (Paspalas et al., 2000; Smith et al., 1994; Cauvin et al., 1991; Fukuchi et al., 2005; Jaworski, 2000). Both PACAP and VIP have been shown to be involved in neuroprotection (Chen et al., 2006; Shioda and Gozes, 2011; Shioda et al., 2006; Tominaga et al., 2008; Castorina et al., 2012; Castorina et al., 2008; Castorina et al., 2015), stress-related neuroendocrine regulation (Hashimoto et al., 2011; Iemolo et al., 2015; Tsukiyama et al., 2011) and immunomodulatory functions (Delgado et al., 2002; Michalski et al., 2008).

VIP in particular, has been strongly implicated in immune tolerance maintenance (Gonzalez-Rey et al., 2006) and reduced inflammatory response both in the central (Ganea et al., 2015; Waschek, 2013) and in the peripheral nervous system (Delgado and Ganea, 2013).

The activities of PACAP and VIP are mediated by three G protein-coupled receptors, namely PAC1, VPAC1 and VPAC2 (Giunta et al., 2010). PAC1 has lower affinity for VIP than for PACAP, whereas VPAC subtypes recognize with high and similar affinity both neuropeptides (Vaudry et al., 2009).

Recent data from our laboratory has shown that rats with neuropathic pain (NP) and co-morbid disruptions in social behaviours after sciatic nerve constriction injury have both neurochemical and neuroanatomical evidence of astroglial activation and cell death in the PAG (Mor et al., 2010; Mor et al., 2011). These evidences are corroborated by findings demonstrating an association between the heightened astrocyte activity in the PAG and the appearance of behavioural disruptions (referred to as depressive-like behaviours) in the spared nerve

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injury model, which were aggravated by chronic exposure to prior stressful stimuli (Norman et al., 2010). These rats also show evidence of altered neuroendocrine function; specifically they show altered hypothalamic-pituitary thyroid and adrenal axes activity, and exhibit distinct neuro-immune signatures (Kilburn-Watt et al., 2010, 2014). This constellation of changes does not however appear in nerve-injured rats with neuropathic pain but no co-morbid disabilities. These co-morbidities appear in the rat model with a frequency 25–30%, which is remarkably similar to that seen in the human clinical population.

To date, despite the involvement of the PAG in NP has been partly established, the exact reason for the appearance of disrupted behaviours remains elusive. Current evidence supports the idea that NP generates a moderate but chronic astrocyte activation in the PAG that may be associated with neuronal cell death, and this seems to correlate with the appearance of disrupted behaviours in NP animal models (Austin et al., 2010; Mor et al., 2010, 2015). These data raise issues on whether or not a disturbed local endogenous activity of neuroprotective/immune modulatory effectors in the PAG may be responsible for the development of such behavioural consequences in experimental NP.

Since PACAP and VIP neuroprotective activities are tightly linked to the fine-tuning of their endogenous expression levels, in the present studies we hypothesized that the reduction of PACAP or VIP expression in rats with NP correlate with the occurrence of behavioural disruptions. For this purpose, we aimed at investigating the expression profile of PACAP, VIP and related receptors in rats subjected to sciatic nerve chronic constriction injury (CCI), an experimental model of NP, and in behaviourally characterized CCI animals displaying different patterns of social interaction behaviours, as determined using the resident-intruder social interaction test.

2. Materials and methods

All experiments were conducted in line with the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes”. All procedures were approved by the University of Sydney, Animal Care and Ethics Committee (approval No: 2016/972).

2.1. Experimental design

Twenty-eight outbred male Sprague Dawley rats weighing about 250–350 g at the time of surgery were purchased from ARC (Perth, WA, Australia). Rats were maintained on a reversed 12/12 light/dark cycle with food and water available ad libitum. Room temperature was monitored and maintained at 22 ± 1 °C. Resident-intruder testing, as described by (Monassi et al., 2003) was carried out during the dark phase, a period where rats are naturally active. After 7 days of single housing, testing periods commenced, occurring at the same time each day for six consecutive days. Male “*residents*” were exposed for 6 min daily to an “*intruder*” rat: an age matched, weight matched male, an interaction which evokes social interactions and displays of dominance behaviours from the resident towards the intruder. On day 7, chronic constriction injury of the sciatic nerve (CCI) was performed (see the related subsection below), but no resident-intruder testing was carried out in order that the rats recovered from the surgery. On day 8, a further 6 days of behavioural testing commenced. Resident rats were never exposed to the same intruder more than twice, and never saw the same intruder on consecutive days. The day after behavioural testing was completed, resident animals were deeply anaesthetized with sodium pentobarbitone (Nembutal: 120 mg/kg i.p.) and perfused intra-cardially with 0.9% saline (500 mL at room temperature). Brains were removed and snap frozen at −80 °C, with micro-dissection of a midbrain block containing the PAG performed soon after. The isolated PAG from individual animals were then used for mRNA and protein extraction for subsequent downstream molecular analyses (the experimental timeline is shown in Fig. 1).

2.2. Chronic constriction injury (CCI) of the sciatic nerve

Chronic constriction injury (CCI) was performed on male Sprague Dawley rats following the procedures described by Bennett and Xie (1988). Animals were placed into a clear perspex box and anaesthesia induced by exposure to 5% halothane in O_2 with a flow rate of 2 L /min. Once anaesthetised the rat was removed and anaesthesia was maintained using 2.5–3.5% halothane in 1.5 L of 100% O_2/min via a custom-made face mask. Body temperature of 37 °C was maintained using a homoeothermic blanket.

The right sciatic nerve was exposed at the level of the mid-thigh by blunt dissection laterally through the *biceps femoris* muscle. Approximately 10 mm of sciatic nerve just proximal to the trifurcation into the common peroneal, tibial and sural nerves was loosened from surrounding connective tissue. Four single chromic gut ligatures were then tied around the nerve at intervals of approximately 1 mm. The sciatic nerve was then replaced below the overlying muscle, and the skin sutured. Triple antibiotic powder (Tricin®) was then applied topically to the surgical wounds.

2.3. Mechanical threshold withdrawal test

To perform the mechanical threshold withdrawal test, rats were placed in the dark under the illumination of a red light and allowed habituate to the test apparatus for 30 mins before testing commenced. Following the application of ascending series of Von Frey filaments (0.16 g to 15 g) to the mid-plantar surface of each hind paw, evidence of retraction of the hind paw was considered a response. Three baseline mechanical sensory measurements were performed on separate days before surgery, and three measurements were taken on separate days post-surgery. We have reported that both allodynia, and hyperalgesia occurs in all CCI animals (Monassi et al., 2003). To confirm that hyperalgesia occurred in all CCI animals, sensory testing was performed on nine CCI rats, four of which were later characterized as Pain alone rats and five were characterized as Persistent Disability rats (Fig. 2).

2.4. Resident-Intruder testing – Measuring the behavioural ‘disability’

The resident-intruder test was adapted to quantify changes in social interactions in the resident rat, rather than the usual focus on the behaviours of the intruder. The following mutually exclusive behaviours were scored: Dominance, Social, Non-Social and Submissive behaviours, as previously detailed in Monassi et al. (2003). Behaviours were evaluated in resident rats in the 6 days following sciatic nerve constriction injury and were compared to the average scores obtained in the 3 days prior to injury (i.e. pre-CCI days 3–5). On the basis of these comparisons, rats were categorized into one of three distinct behavioural groups: “Pain alone” rats, which showed no changes or differences in dominance behaviour, “Transient Disability” rats, which showed an initial decrease in dominance behaviour, but then recovered to > 70% pre-CCI behaviour by day 6 and “Pain and Disability” rats, which showed a persistent reduction (of at least 30%) of their dominance behaviour in the 6 days post CCI, along with increases of both the mutually exclusive submissive and non-social behaviours.

2.5. Microdissection of the PAG

Dorsal midbrain blocks encompassing the PAG were isolated from each of the frozen brains for subsequent biochemical analyses as previously described (Mor et al., 2015). Briefly, the PAG region surrounding the mesencephalic aqueduct was dissected free under a binocular microscope at 10× magnification. The rostral boundary of the block was at the approximate coronal level of −5.3 mm caudal to bregma and the caudal boundary at approximately −8.8 mm caudal to bregma, according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986).

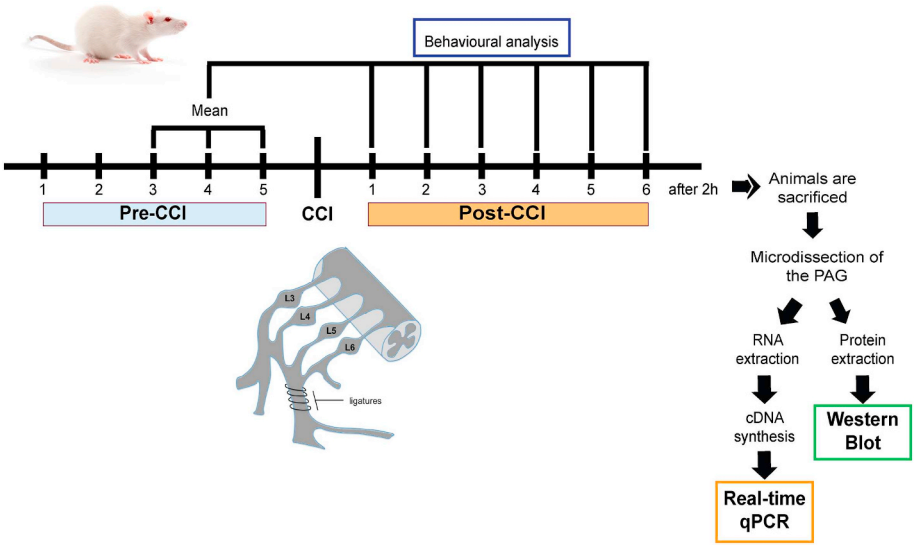

Fig. 1. Experimental timeline for behavioural testing and downstream analyses. Following one-week acclimation, animals were tested for social interactions using the resident-intruder test. The test was adapted to score the following behavioural landmarks: Dominance, Social, Non-Social and Submissive behaviours, as previously detailed by Monassi et al. (2003). Behaviours were evaluated in resident rats in the 6 days following sciatic nerve constriction injury and were compared to the average scores obtained in the 3 days prior to injury (i.e. pre-CCI days 3–5). Based on these comparisons, rats were categorized as detailed in the related Materials & Methods – Section 2.3. Two hours after the last session of behavioural testing, rats were euthanized, brains were removed; PAG tissue microdissected and processed for downstream molecular analyses as illustrated.

2.6. RNA extraction and cDNA synthesis

For RNA extraction we used micro dissected rat PAG tissue samples ($n = 4$–6 *per* group), taken from animals falling into the following categories: (1) Naïve (home cage control, $n = 6$); (2) Behaviourally tested (BT, n = 6); (3) Sham injured (surgery without ligatures, $n = 6$); (4) CCI rats without 'behavioural disability' (Pain alone, $n = 6$); (5) CCI rats showing recovery (Transient Disability, $n = 4$) and (6) CCI rats with 'disability' (Pain & Disability, $n = 6$). PAG tissue samples were weighed and total RNA was extracted using 1 ml TRI reagent (Sigma-

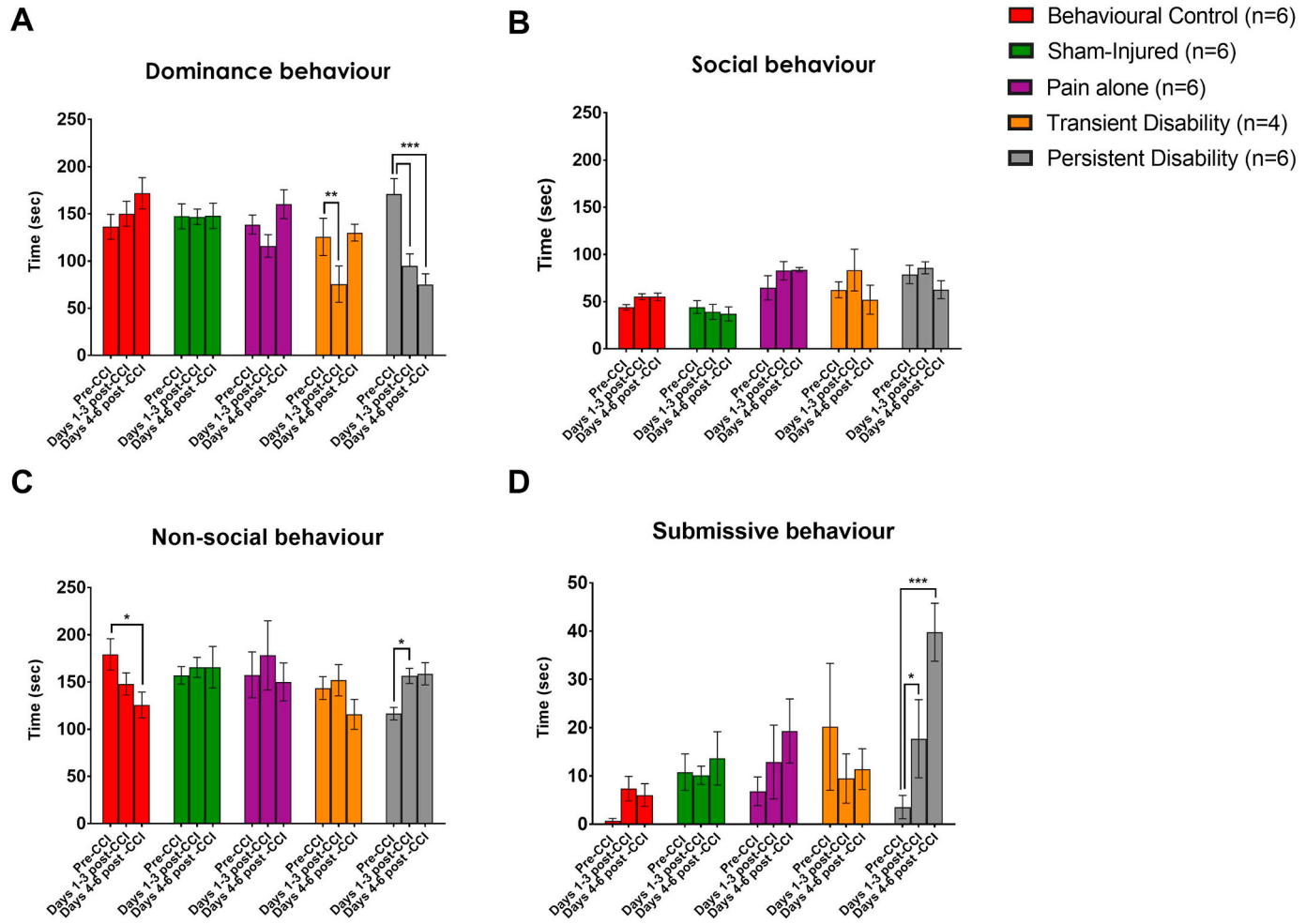

Fig. 2. CCI injury-evoked alterations in social behaviour. Bar graphs displaying the mean ± SEM durations (sec) of Dominance, Social, Non-social and Submissive behaviours on the 3 days preceding nerve injury (Pre-CCI) and Post-CCI days 1–3 and 4–6, in groups as indicated in the legend. Significant differences (Mixed ANOVA, with pairwise comparisons) between groups are indicated by $*p < 00.05$, $**p < .001$ and $***p < .0001$.

Table 1
Primers sets used in end-point and real-time qPCR analyses.

Accession #	Gene	Primer Sequence	Length
NM_016989	PACAP Peptide	Fwd 5′ GAGGCTTACGATCAGGACGG 3′ Rev. 5′ TCCTGTCGGCTGGGTAGTAA 3′	121 bp
NM_053991	VIP Peptide	Fwd 5′ CATTGGCAAACGAATCAGCAGT3′ Rev. 5′ CTCACTGCTCCTCTTCCCATTTAG 3′	157 bp
NM_001270579	PAC1 receptor	Fwd 5′ TTCCACTACTGCGTGGTGTC 3′ Rev. 5′ CACACACAGTAGGTGTCCCC 3′	145 bp
NM_012685	VPAC1 Receptor (VIPR1)	Fwd 5′ AAGCTGCACTGTACCCGAAA 3′ Rev. 5′ CGCTGTTGAAGAGGGCCATA 3′	103 bp
NM_017238	VPAC2 Receptor (VIPR2)	Fwd 5′ ACCCAGAATGCCGGTTTCAT 3′ Rev. 5′ GCCAGCATGTGATGTTGTCC 3′	123 bp
NM_213557	18S ribosomal subunit	Fwd 5′ CCTGCGAGTACTCAACACCA 3′ Rev. 5′ CTGCTTTCCTCAACACCACA 3′	110 bp

Forward and reverse primers were selected from the 5′ and 3′ region of each gene mRNA. The expected length of each amplicon is indicated in the right column.

Aldrich) and 0.2 ml chloroform and precipitated with 0.5 ml 2-propanol following established protocols (Castorina et al., 2014). Pellets were washed with 75% ethanol and air-dried. RNA concentrations were calculated using spectrophotometry (Nanodrop ND-1000® spectrophotometer, Wilmington, DE, USA). Single-stranded cDNAs were then synthesized using the Tetro cDNA synthesis kit (Bioline, Sydney, NSW, Australia). Briefly, total RNA (2 µg) was incubated with the Tetro reverse transcriptase (200 U/µl); Oligo-$(dT)_{18}$ primer (100 nM); 0.5 mM dNTP mix, RNase-inhibitor (10 U/µL) at 45 °C for 40 min in a final volume of 20 µL. The reaction was terminated by incubation of samples at 85 °C for 5 min.

2.7. End-point reverse transcription polymerase chain reaction (RT-PCR)

Aliquots of cDNA were amplified using specific primers designed to recognize PACAP, VIP and the three high affinity receptors (PAC1, VPAC1 and VPAC2). Primer pairs for the 18S ribosomal subunit were used as an internal control. Oligonucleotide sequences are listed in Table 1. Each PCR reaction contained 0.4 µM specific primers, 200 µM dNTPs, 1.25 U AmpliTaq Gold DNA polymerase and GeneAmp buffer containing 2.5 mM $MgCl^{2+}$ (Applied Biosystem). PCR was performed using the following three cycle programs: (1) denaturation (1 cycle: 95 °C for 12 min); (2) amplification (40 cycles: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s); (3) final extension (1 cycle: 72 °C for 7 min). End-point PCR products were finally separated by electrophoresis in a 1.8% agarose gel in 0.045 M Tris–borate/1 mM EDTA buffer and visualized under UV light in the presence of the DNA-intercalating dye, ethidium bromide.

2.8. Quantitative real time polymerase chain reaction (qPCR)

Real-time qPCR was performed to analyse the steady-state mRNA levels of 6 genes: PACAP, VIP, PAC1, VPAC1, VPAC2, and the ribosomal protein 18S was used as the housekeeping gene. Each qPCR experiment was carried out using the LightCycler® 480 instrument (Roche Diagnostics), following the protocol detailed in Castorina et al. (2013), with minor adjustments. Each reaction consisted of 4 µL cDNA (final concentration 100 ng), 4.4 µL of SensiFAST SYBR® No-ROX Master mix (Bioline, Australia), 0.8 µL forward and reverse primers (final concentration = 400 nM). To examine changes in expression, we analysed the mean fold change values of each sample, calculated using the ΔΔCt method as previously described by Schmittgen and Livak (2008). PCR product specificity was evaluated by melting curve analysis, with each gene showing a single peak (data not shown).

2.9. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and Western blotting

PAG protein extraction was performed using TRI Reagent® protocol provided by the manufacturer (Sigma-Aldrich) and protease (Complete Mini, Roche Diagnostics) and phosphatase inhibitors (PhosStop, Roche Diagnostics) were added to preserve protein integrity. Protein concentrations were then measured using the Direct Detect® infrared spectrophotometer (Merck- Millipore). Equal amounts of proteins (30 µg) were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gradient Criterion™ mini gels (10 well, Bio-Rad). A molecular weight ladder (Precision Plus Protein™ Prestained Standard in All Blue, Kaleidoscope™) was included for comparison. Transfer to PVDF membranes was performed using the semi-dry method (BioRad Trans-Blot® Turbo™ Transfer System). The membrane was then placed in TBS + 0.1% Tween 20 (TBST) and blocked for 1 h in 5% skim milk in TBST at room temperature on an oscillator (~80 rpm). Incubation with primary antibodies was performed O/N in a cold room (4 °C). The following antibodies were used: PACAP (H-76, Rb polyclonal, Santa Cruz Biotechnologies, 1:400), VIP (Immunostar, Rb polyclonal, 1:800), PAC1 (H-55, Rb polyclonal, Santa Cruz, 1:500), VPAC1 (H-130, Rb polyclonal, Santa Cruz Biotechnologies, 1:800), VPAC2 (H-50, Rb Polyclonal, Santa Cruz Biotechnologies, 1:400), GAPDH (0411, Ms. monoclonal, Santa Cruz Biotechnologies, 1:500). Secondary antibodies raised against Ms. or Rb were used at a dilution of 1:5000. Western blots were visualized using the chemiluminescence BioRad Clarity™ Western ECL Blotting Substrate solution. Images were acquired using the Bio-Rad ChemiDoc™ MP System. Images were analysed using Fiji ImageJ, and ratios were normalized to GAPDH, which was used as a loading control.

2.10. Statistical analyses

All data are reported as mean ± S.E.M. For the resident-intruder tests, the duration of time spent in each category of behaviour for each behavioural group were expressed as means ± (SEM). Comparisons between groups were made with a mixed ANOVA and significant differences between groups were determined by Bonferroni pairwise comparisons. In qPCR studies, One-Way ANOVA followed by Tukey *post-hoc* test was used to determine differences among groups. *P*-values $\leq .05$ were considered statistically significant.

3. Results

3.1. CCI injury generates three different alterations in social interactions

Fig. 2A-D shows the traits of dominance and non-social behaviours, respectively, in each behavioural group following CCI. Consistent with our previous findings (Mor et al., 2015), the behavioural phenotypes of the selected CCI rats fell into three subcategories: Pain alone, Transient disability and Persistent disability groups, as detailed in *Section 2.3*.

In the Pain alone group, dominance behaviour was not affected at days 1–3 post-CCI nor, at days 4–6 post-CCI when compared to pre-CCI

levels ($p > .05$). In contrast, in the Transient disability group, a significant reduction was seen at days 1–3 post-CCI (**$p < .01$), which returned to pre-CCI levels in the following three days ($p > .05$). In the Persistent disability group, a reduction of dominance was observed both after days 1–3 and days 4–6 post-CCI (***$p < .001$, Fig. 2A). Changes in dominance inversely correlated with non-social behaviours (Fig. 2). Controls for the behavioural testing procedures, showed increases in dominance over the testing period, these increases were coupled with a progressive decrease in non-social behaviours, which became statistically significant in the last three days of resident-intruder testing (*$p < .05$). Similarly, the decline of dominance in the Persistent disability group was paralleled by increased levels of non-social interactions at days 1–3 post-CCI (*$p < .05$, Fig. 2C). Small, yet statistically significant increases in submissive behaviours was recorded in the Persistent disability group both at days 1–3 (*$p < .05$) and days 4–6 post-CCI (***$p < .001$, Fig. 2D).

3.2. Mechano-sensory testing responses in pain alone vs persistent disability rats following CCI injury

Consistent with previous studies (Keay et al., 2004; Monassi et al., 2003), mechano-sensory thresholds of rats were similarly reduced in animals classified as Persistent Disability and those classified as Pain Alone. Specifically, there was a significant main effect for post-CCI sensory thresholds ($F_{3, 28} = 5.937$, **$p < .01$) but no difference between sensory thresholds of the no disability and the disability rats ($F_{1, 28} = 2.979$, $p = .095$). *Post-hoc* analyses showed a significant reduction in sensory thresholds developed three days after CCI surgery (pre-CCI vs. 3 days post-CCI, $p < .05$; Fig. 3).

3.3. Social interaction testing and CCI alter the mRNA expression of PACAP family members

An end-point PCR analysis was carried out using Naïve control PAG cDNA templates to investigate whether transcripts for PACAP, VIP and receptors (PAC1, VPAC1 and VPAC2) could be identified in the isolated PAG tissue. As displayed in Fig. 4A, all transcripts were expressed in the PAG, as amplicons of the expected molecular weight were visible in the gel (please refer to Table 1). A fragment corresponding to the 18S ribosomal subunit mRNA was also amplified and included as internal control.

Relative quantitative real time RT-PCR analyses were used to measure changes in gene expression among the groups. As depicted in Fig. 4B, PACAP mRNA steady-state levels were stable among the experimental groups, showing no statistical significance ($p > .05$). On the other hand, VIP mRNAs were significantly reduced both in behavioural controls, sham operated (*$p < .05$) and Transient disability groups (**$p < .01$).

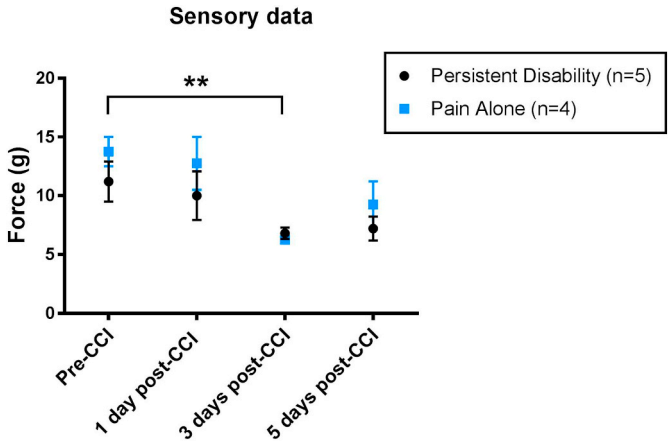

Fig. 3. Mechanical sensitivity following CCI injury. Dot plot graph showing the mean (± SEM) of sensory thresholds for Persistent Disability ($n = 5$) and Pain alone rats ($n = 4$). Significant differences were computed using 2-way ANOVA, followed by Tukey post-hoc comparisons. Significant differences are indicated by **$p < .01$.

Expression levels of the receptors mRNAs are shown in Fig. 4C. The expression of the stress-sensitive PAC1 receptor gene was augmented by > 2-fold both in behavioural tested and in Transient disability animals compared to Naïve (*$p < .05$), without showing significant changes among the different behavioural CCI subgroups ($p > .05$). Conversely, VPAC1 receptor mRNA expression was not affected by social interaction testing, but gene expression was significantly increased in the Pain alone group (#$p < .05$ Vs Naïve and Behavioural tested), showing about 1.8-fold increase when compared to Naïve. In the Transient disability group, VPAC1 mRNAs were reduced at levels comparable to Controls, whereas in the Persistent disability group VPAC1 mRNA expression showed a slight but not significant increase (about 1.5fold). VPAC2 receptor mRNAs were heterogeneously expressed in-between the groups. Expression levels were slightly increased in behavioural controls (about 1.8-fold) and further increased in Sham injured rats (about 2-fold). In the CCI groups, VPAC2 receptor transcripts were further augmented, with fold change values of about $2.3\times - 2.7\times$ that of controls but were not statistically significant ($p > .05$).

3.4. Protein expression profile of PACAP, VIP and related receptors following social interaction testing and CCI

In order to investigate levels of protein expression, we carried out Western blot analyses. Semi-quantitative densitometry analyses of protein levels were performed using the ImageJ software, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping control (Fig. 5A). Protein expression studies revealed some inconsistencies with qPCR data, as illustrated in the bar graphs in Fig. 5B. Indeed, PACAP protein expression was downregulated both in behavioural tested, sham controls and Pain alone animals. However, PACAP expression increased in Transient and more remarkably, in Persistent disability groups. VIP protein levels slightly reduced following social interaction testing and surgery, but expression levels increased by > 2.5-fold in the Persistent disability group.

PAC1 receptor levels showed minimal changes among the groups, with a minor increase of protein expression in behavioural controls and a slight decrease in the Pain alone group when compared to Naïve. VPAC1 expression was > 2-fold higher in social interaction tested animals compared to Naïve controls. However, in sham, Pain alone and Transient disability groups VPAC1 receptor levels were comparable to Naïve. Interestingly, VPAC1 protein expression was upregulated (about 2.2-fold) in the Persistent Disability group, reaching levels similar to social interaction tested rats. By contrast, VPAC2 protein expression was downregulated about 2-fold in response to behavioural testing, and further decreased in sham, Pain alone, Transient and Persistent disability groups (about 3-fold). Finally, to define whether the pattern of changes in the expression of PACAP, VIP and receptors correlated with the changes in dominance behaviour, hence with the degree of behavioural disability, we sought to conduct linear regression analyses. We identified two significant and inverse correlations: one with PACAP protein expression ($r^2 = 0.307$, $p = .032$, Fig. 6A) and a very robust correlation with VIP expression ($r^2 = 0.686$, $p = .0001$, Fig. 6B). No further significant correlations were found with any PACAP/VIP receptors (Fig. 6C–E).

4. Discussion

Evidence from both our laboratory and others support a critical role for the PAG in co-ordinating the distinctive emotional coping responses evoked by acute painful stimulation of different tissues. Recent work

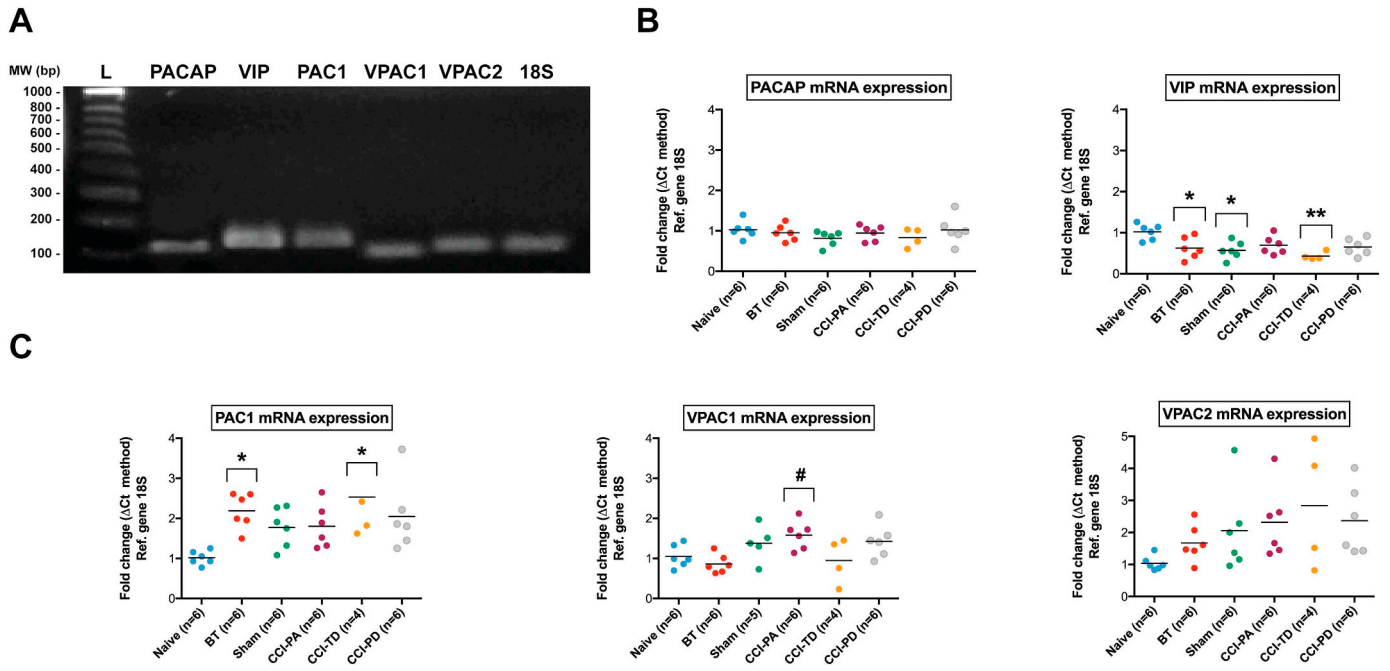

Fig. 4. Gene expression profile of PACAP, VIP and related receptors following social interaction testing and CCI. (A) Representative photomicrograph of a 1.8% agarose gel run to identify the presence of RT-PCR end-products relative to PACAP, VIP, PAC1, VPAC1, VPAC2 amplicons. 18S ribosomal protein cDNAs were used as an internal control. Each band confirmed the predicted amplicon size. A molecular weight (MW) ladder is shown on the left side of the gel, with MW indicated in base pairs (bp). (B, C) Real-time qPCR data showing the differential expression of transcripts for the indicated genes in the PAG of rats pertaining to the indicated subcategories. The number of animals per group is shown. Amplifications were performed using selected primers optimized for qPCR analyses (< 155 bp length) which recognize fragments within the coding sequence of the gene of interest (for details refer to Table 1). Results are presented as mean fold changes with respect to Naïve ± SEM. Fold changes of each gene were obtained after normalization to the endogenous reference gene and calculated using the comparative ΔΔCt method. Baseline expression levels of the Naïve groups were set to 1. *$p < .05$ or **$p < .01$ Vs Naïve, #$p < .05$ Vs Behavioural tested, as determined using the unpaired One-way ANOVA followed by Tukey *post-hoc* test. BT = Behavioural tested, CCI-PA = CCI animals exhibiting pain alone, CCI-TD = CCI animals exhibiting transient disability, CCI-PD = CCI animals exhibiting persistent disability.

has focussed on the role of the PAG in the altered emotional coping responses seen in some individuals with chronic pain. Using a rat model of neuropathic pain, we have identified a correlation between the expression of behavioural and endocrine disturbances in a subgroup of nerve-injured rats and neuroanatomical markers of cell death in the PAG (Mor et al., 2010; Mor et al., 2015; Mor and Keay, 2013). The emerging understanding of the role of PACAP/VIP peptides in modulating neuronal death due to their key neuroprotective actions in the central nervous system led us to ask whether neuropathic pain with comorbid behavioural disruptions might be related to a reduction of the neuroprotective activity of VIP/PACAP peptides in the PAG (Tamas et al., 2012; Tsuchikawa et al., 2012).

The data we report provides evidence for distinct patterns of the neuropeptides VIP/PACAP in the PAG of rats that develop pain comorbid with changes in behaviour following CCI and support the idea that the behavioural abnormalities triggered by experimental neuropathic pain may either depend on or cause changes to VIP protein expression and although to a lesser extent, PACAP.

These data also reveal that exposure to resident intruder, social interactions testing procedure, is also able to modulate the regulation of the endogenous VIP/PACAP peptidergic system.

Moreover, we sought to profile the expression of PACAP, VIP and receptors (i.e. PAC1, VPAC1 and VPAC2) for each subcomponent of the experimental procedure (i.e. effects of the social interaction testing and/or of the surgical procedure). The idea was to identify if the pattern of change (if any) correlated with the repertoire of events, as often experienced by patients whose NP develops following surgery (i.e. pre- and post-operative stress, anaesthesia, pain).

5. Nerve injury effects on VIP/PACAP in PAG

VPAC1 mRNA was selectively increased in the Pain alone group, supporting an involvement of the receptor in the PAG during NP. On the contrary, VPAC2 mRNA revealed a high noise-to-gene expression ratio, possibly due to the relatively low abundance of transcripts or the individual variability of gene expression within the selected cohort.

In contrast to mRNA studies, at the protein level we found a higher degree of association between the expression of the peptides PACAP and VIP and the 'disability' profile of CCI rats. Indeed, whilst social interaction and CCI downregulated the abundance of both peptides in the PAG, PACAP and VIP expression levels were remarkably increased in the CCI subgroups displaying persistent disability. A similar type of association was also observed with VPAC1 protein expression. In this case, however, VPAC1 levels also increased following the social interaction testing procedure, returned to baseline levels (Naïve) in rats that underwent sham-surgery, pain alone and transient disability, to increase by > 2-folds in the 'Persistent Disability' subgroup. In our opinion, VPAC1 protein expression in the PAG seems to be 'more' sensitive to the various subcomponents of the experimental model, including the stress caused by social interaction testing, but also to the long-lasting effects of anaesthesia, as previously reported for other trophic molecules (Dalla Massara et al., 2016; Fan et al., 2016). The exact reason for the specific upregulation of VPAC1 in the CCI subgroups manifesting persistent disability remains unknown. However, the general idea is that an abnormal and sustained increase (6 days post-CCI) in the levels of PACAP and VIP, along with VPAC1 proteins in this key area controlling affective responses may be associated with disruptions of the intrinsic protective activity mediated by the receptor, contributing to the appearance of behavioural abnormalities in NP rats.

In these studies, we have also highlighted how the expression profile

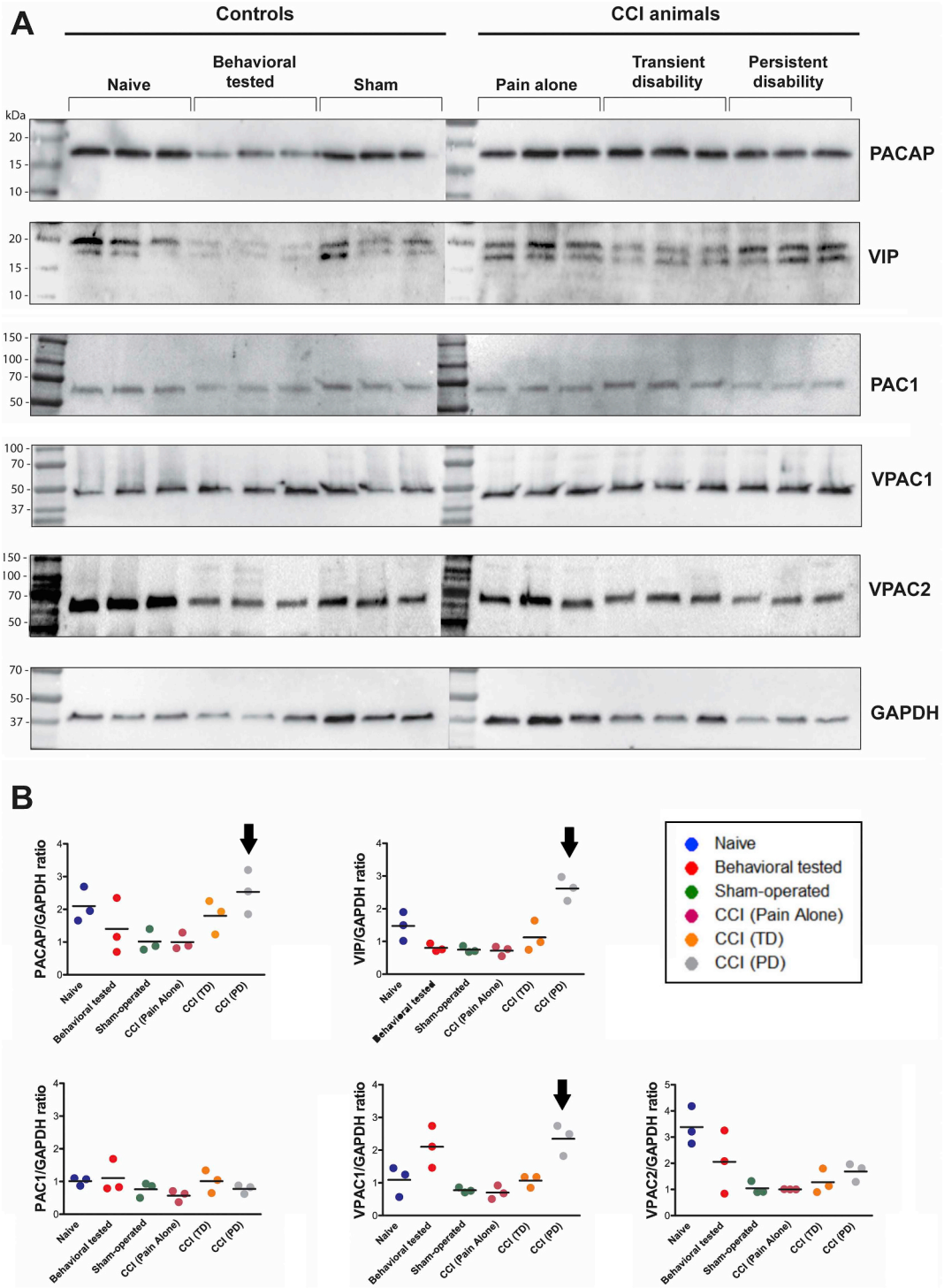

Fig. 5. Protein expression profile of PACAP, VIP and related receptors following social interaction testing and CCI. (A) Immunoblots showing the the differential expression of transcripts for the indicated genes in the PAG of rats pertaining to the indicated subcategories. (B) Scattered plots showing the results of densitometric analyses of bands intensities. Densitometry was performed using the ImageJ software and normalized values were calculated by dividing the mean optical density (OD) of bands over the corresponding GAPDH, which served as loading control. Each blot and corresponding value represents the OD intensity from an individual rat (n = 3 per group). Black arrows highlight the main changes observed.

of PACAP, VIP and receptors in the PAG is likely to be both pre- and post-transcriptionally regulated in our experimental model, often culminating in disparities between mRNA and protein profiles. Since the dynamics of gene and protein synthesis are not interdependent processes (Valencia-Sanchez et al., 2006; Costello et al., 2015), it is possible that epigenetic factors (i.e. changes in mRNA stability, protein turnover etc.) might contribute to the discrepancies between transcripts and proteins we observed at the time of analyses. Further understanding of the underlying mechanisms driving these changes will certainly provide important clues on the mechanisms that regulate PACAP system expression in the brain not only following NP, but also in other medical conditions.

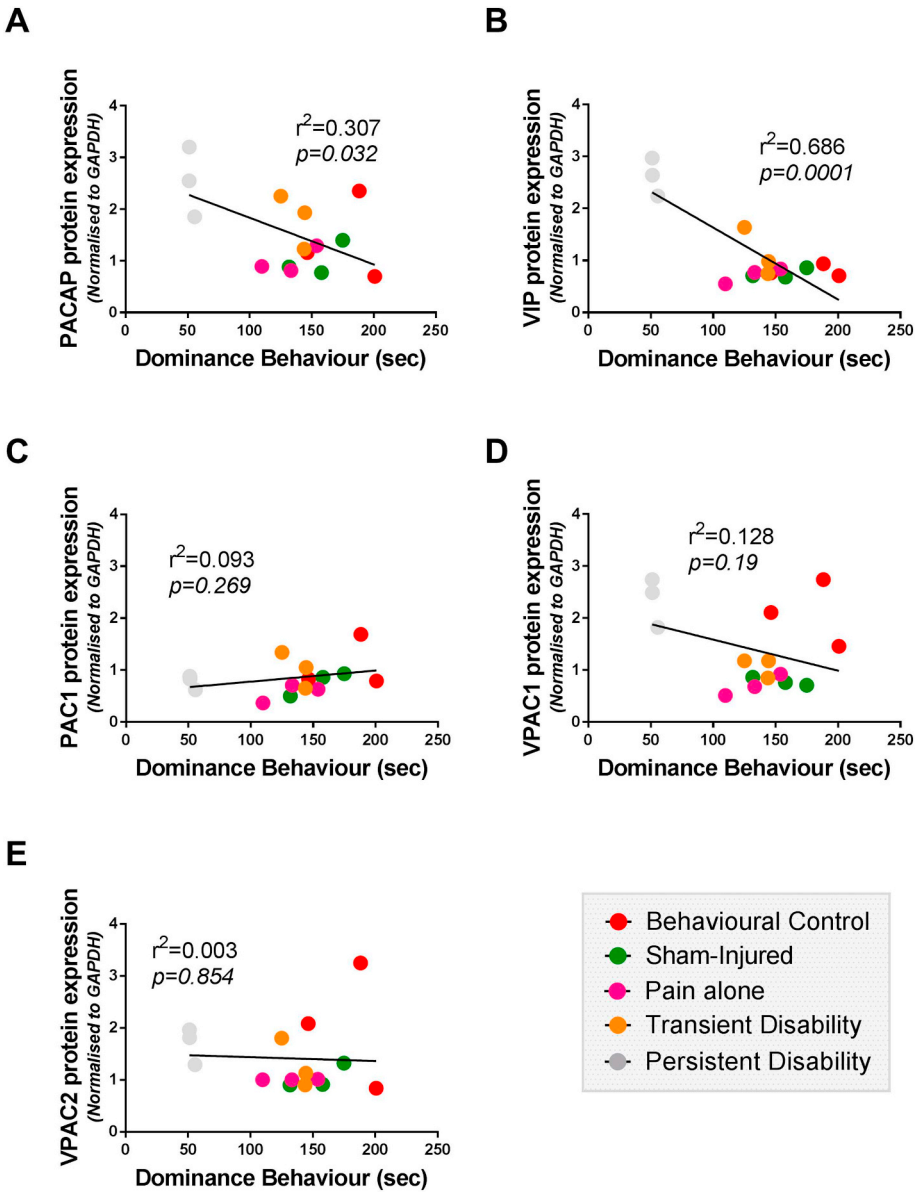

Fig. 6. Scatterplots and regression lines showing the relationship between dysregulated behaviours and the protein expression of PACAP/VIP system components following CCI. Significant inverse correlations between protein expression and the dominance behaviour were found only for (A) PACAP and (B) VIP. Correlations with (C) PAC1, (D) VPAC1 and (E) VPAC2 were not significant. Coefficients of determination (r^2) and related *p* values are shown in each panel.

6. Resident intruder interactions effects on VIP/PACAP in PAG

Our major findings at the mRNA level identified two main targets that were inversely regulated following the resident-intruder test (i.e. decreased VIP and increased PAC1 receptor expression, respectively) and one that was specifically upregulated by CCI-induced NP (VPAC1 receptor). These findings, particularly the increased PAC1 expression following social interaction testing, are consistent with the putative role of this receptor as a stress-sensor (Ressler et al., 2011; Mustafa et al., 2015). VIP and PAC1 mRNA expression were not further influenced by any other inherent components of our experimental model, like anaesthesia, nerve injury and/or the associated behavioural comorbidities.

7. Functional significance of the changes

It is ascertained that the PAG is a vital element within the pain/affective cascade. The PAG receives direct and topographically organised inputs from sciatic nerve recipient lumbar spinal segments. It also plays critical roles in regulating emotional coping responses to stressful, threatening and painful stimuli (Keay and Bandler, 2001; Keay et al., 2001). PAG projections partake in the regulation of the sleep–wake cycle (Lu et al., 2006; Sastre et al., 1996) and it projects into endocrine regulatory regions of the hypothalamus (Bereiter and Gann, 1990; Floyd et al., 1996).

Lately, we have identified sustained astroglial activation and signs of neuronal death in specific columns of the PAG in CCI rats that display behavioural disability (Mor et al., 2010, 2015). This suggests that there might be a set of neurochemical changes occurring in the PAG, potentially triggered by chronic local neuroinflammation, which trigger the sickness behaviour in some NP subgroups (~30%). In addition, a significant number of PACAP and VIP nerve terminals have been localised in the rat PAG (Paspalas et al., 2000; Smith et al., 1994), justifying the rationale for a potential link between the dysregulated expression of the peptides and the dysfunctional behavioural phenotype observed in a subset of CCI rats.

In summary, in the present manuscript we show that sciatic nerve injury but also social interaction testing both contribute to disrupt the expression of components of the PACAP system. The pattern of changes in VIP and PACAP expression seems to be distinct within the subgroup showing behavioural disability, suggesting that the fine-tuning of VIP and possibly PACAP may be implicated in the development of behavioural comorbidities following NP.

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