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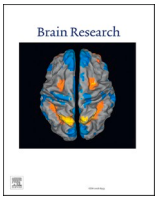

The impact of sciatic nerve injury and social interactions testing on glucocorticoid receptor expression in catecholaminergic medullary cell populations

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ABSTRACT

Paradoxically, while acute pain leads to transiently elevated corticosterone, chronic pain does not result in persistently elevated corticosterone. In the sciatic nerve chronic constriction injury (CCI) model of chronic pain, we have shown that the same nerve injury produces a range of behavioural outcomes, each associated with distinctive adaptations to the HPA-axis to achieve stable plasma corticosterone levels. We also demonstrated that CRF and GR expression in the paraventricular hypothalamus (PVH) was increased in rats that showed persistent changes to their social behaviours during Resident-Intruder testing ('Persistent Effect' rats) when compared to rats that showed no behavioural changes ('No Effect' rats). In this study, we investigated whether these changes were driven in part by altered sensitivity of the brainstem catecholaminergic pathways (known to regulate the PVH) to glucocorticoids. GR expression in adrenergic (C1,C2) and noradrenergic (A1,A2) cells was determined using immunohistochemistry in behaviourally tested CCI rats and in uninjured controls. We found no differences between Persistent Effect and No Effect rats in (1) the glucocorticoid sensitivity of these cells, or (2) the numbers of adrenergic and noradrenergic cells in each region. However, we discovered an overall reduction in GR expression in the non-catecholaminergic cells of these regions in both experimental groups when compared to uninjured controls, most likely attributable to the repeated Resident-Intruder testing. Taken together, these data suggest strongly that brainstem mechanisms are unlikely to play a key role in the rebalancing of the HPA-axis triggered by CCI, increasing the probability that these changes are driven by supra-hypothalamic regions.

1. Introduction

We have previously demonstrated that a sciatic nerve chronic constriction injury (CCI) results in distinct behavioural subpopulations – 'Persistent Effect' rats, who show long-term reductions in dominance behaviours during Resident-Intruder interactions, and 'No Effect' rats, who do not show any changes to their dominance behaviours (Monassi et al., 2003; Keay et al., 2021). We have recently reported that these behavioural subpopulations also show divergent neuroendocrine phenotypes, characterized by distinctive adaptations in the HPA axis (Sosa et al., 2022). Specifically, Persistent Effect rats selectively show increased adrenocortical MC_2R expression as well as increased paraventricular hypothalamic GR and CRF expression. Whereas No Effect rats did not show changes to GR, CRF or MC_2R expression, but showed elevated basal plasma ACTH levels in response to CCI. Despite these changes, basal plasma corticosterone levels remained stable in all injured animals.

In the discussion of Sosa et al. (2022), we proposed that this injury-induced rebalancing of the HPA-axis might be initiated by changes to afferent signals to the PVH caused by increased ascending nociceptive transmission. Since the PVH only receives sparse or no inputs directly from the spinal cord (Lumb & Wolstencroft, 1985; Werner & Bienek, 1985; Kai et al., 1988; Cliffer et al., 1991), it is more likely that one or more nociceptive recipient brainstem regions with strong projections to the PVH would drive these changes. One particularly strong candidate was the collection of catecholaminergic cell groups in the medulla,

Abbreviations: ACTH, adrenocorticotropic hormone; *CCI*, chronic constriction injury; *CRF*, corticotropin releasing factor; *DAB*, 3-3′-diaminobenzidine tetrahydrochloride; *GR*, glucocorticoid receptor; *PBS*, 0.1 M phosphate buffered saline (pH 7.4); *PVH*, paraventricular nucleus of the hypothalamus; *RRID*, research resource identifier; *RT-PCR*, reverse-transcriptase polymerase chain reaction.

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which include the noradrenergic A1 and A2 and adrenergic C1 and C2 cell populations in the ventrolateral and dorsomedial medulla. Each of these cell groups have large projections to the PVH (Cunningham & Sawchenko, 1988; Cunningham et al., 1990; Woulfe et al., 1990; Shioda & Nakai, 1993; Verberne et al., 1999; Morales et al., 2000; Cobos et al., 2003). Additionally, these ascending catecholaminergic inputs have been shown to influence the regulation of neuronal CRF in the PVH and exert control over the HPA-axis. For instance, Sawchenko (1988) found that severing these ascending catecholaminergic projections using medullary knife cuts reduced the numbers of CRF-immunoreactive cells (but not vasopressin-immunoreactive cells) in the ipsilateral PVH. While Kiss et al. (1996) demonstrated that a unilateral disconnection of the brainstem prevented restraint-stress evoked increases in CRF mRNA in the PVH.

Importantly, these medullary cell populations are known to respond to noxious stimuli. Electrophysiological recordings of cells in the A1-C1 regions reveal that noxious tail pinch, heat, cold and colorectal distension produce either excitatory or inhibitory responses in the majority of these neurons, depending on the stimulus modality (Sun & Spyer, 1991; Hathaway et al., 1995; Ness et al., 1999; Lyubashina et al., 2019). Furthermore, in monoarthritic rats these neurons display spontaneous activity and increased discharge to noxious stimulation (Pinto-Ribeiro et al., 2011). Supporting these observations, a range of noxious stimuli have been shown to induce c-Fos expression in the regions containing these catecholaminergic cell populations (Bullitt, 1990; Jones & Blair, 1995; Monnikes et al., 2003; Sabbatini et al., 2004; Pinto et al., 2006). Additionally, there is substantial evidence that these cell populations are highly innervated by spinal cord projections, from both the superficial dorsal horn (Lima et al., 1991; Craig, 1995), as well as deeper layers (Menetrey et al., 1984), see Tavares and Lima (2002) for review. Indeed, it has been shown that neurons in the superficial dorsal horn projecting to the ventrolateral medulla express c-Fos after noxious stimulation of the skin (Tavares et al., 1993).

These catecholaminergic cell populations could thus represent a crucial relay through which ascending spinal activity can affect the functioning of the PVH. In fact, it has previously been shown that pharmacological inhibition of the A1 cells blocked somatic nerve stimulation- and paw pinch- induced firing of hypothalamic neurons (supraoptic nucleus) (Day & Sibbald, 1990). Therefore, in this study, we investigated the possibility that a differential sensitivity of the ascending catecholaminergic pathways to the effects of CCI might underlie the increased numbers of CRF expressing neurons seen selectively in the Persistent Effect animals. Since our previous study found that part of the rebalancing of the HPA-axis included changes to GR expression in the hypothalamus, we also examined whether similar GR expression differences might exist in the ascending catecholaminergic projections, which would also exert a regulatory effect on CRF expression in the PVH. However, contrary to our hypothesis, we found no evidence for differences between the Persistent Effect and No Effect animals in either (1) the size of the catecholaminergic cell populations, or (2) the glucocorticoid sensitivity of these cells.

2. Results

2.1. GR and TH expression in the ventrolateral medulla

Rats that received CCI and behavioural testing showed decreased numbers of GR-immunoreactive cells bilaterally across the ventrolateral medulla (see Fig. 1A,D). In the region containing the A1 cell group and in the A1/C1 transition region this decrease was ~ 25–30%, whereas a much larger effect was observed in the region of the C1 cell group, in which the number of GR-ir cells decreased by ~ 75% (Fig. 1B). Welch's unpaired t-tests found statistically significant differences in the numbers of GR-ir cells between behaviourally tested CCI rats and uninjured control rats in the A1 region ($T_{12.4=}3.42$, $p = 0.005$**, Hedges' $g = 1.58$) and the C1 region ($T_{5.8} = 2.58$, $p = 0.04$*, Hedges' $g = 1.68$), but not in the A1/C1 transition region ($T_{10.4} = 1.95$, $p = 0.07$). There was no effect of nerve injury and behavioural testing detected on the numbers of TH-ir cells in the ventrolateral medulla (see Fig. 1C, E and Statistical Results Table 1). Despite the reduction in the number of GR immunoreactive neurons in the behaviourally tested CCI rats, there were no differences in the number of double labelled cells when compared to uninjured rats in either the A1 ($T_{8.1} = 0.39$, $p = 0.71$), the A1/C1 ($T_{7.8} = 1.64$, $p = 0.14$) or the C1 cell groups ($T_{10.2} = 0.56$, $p = 0.59$) (Fig. 1C, F), indicating that the reduction in GR expression was primarily in non-TH containing cells. Finally, we found no differences between the behavioural phenotypes in either GR or TH expression in this region.

2.2. GR and TH expression in the dorsomedial medulla

Similar to GR expression in the ventrolateral medulla, rats that received CCI and behavioural testing showed decreased numbers of GR-ir cells bilaterally across the dorsomedial medulla between 25 and 32% (see Fig. 2A, D). Welch's unpaired t-tests found this decrease was statistically significant at the transition zone of the A2/C2 cell groups, as the central canal opens into the fourth ventricle (–13.7 mm to –12.7 mm from bregma; $T_{6.7} = 2.40$, $p = 0.049$*, Hedge's $g = 1.44$), but not in the A2 ($T_{7.1} = 1.71$, $p = 0.13$) or C2 regions ($T_{7.1} = 1.66$, $p = 0.14$) (Fig. 2B). We found a small reduction of ~ 20% in the numbers of TH containing neurons in behaviourally tested CCI rats compared to uninjured controls only in the A2 region ($T_{14.2} = 2.36$, $p = 0.03$* $g = 1.03$), but not in the A2/C2 transition zone ($T_{6.4} = 0.11$, $p = 0.92$) or the C2 ($T_{10.9} = 0.35$, $p = 0.73$) region. The numbers of double labelled GR + TH-ir neurons remained stable across the rostro-caudal extent of the dorsomedial medulla (Fig. 2C, F), with no differences in the number of double labelled cells between the behaviourally tested CCI rats and uninjured control rats in either the A2 ($T_{14.5} = 0.27$, $p = 0.79$), the A2/C2 ($T_{12.2} = 1.60$, $p = 0.14$) or the C2 cell groups ($T_{14.7} = 1.34$, $p = 0.20$) (Fig. 2C, F). Therefore, the reduction in GR expression appears to be accounted for primarily by non-TH containing cells. Once again, there were no differences between the behavioural phenotypes in either GR or TH expression in this region.

2.3. GR and PNMT expression in the ventrolateral and dorsomedial medulla

PNMT immunoreactive cells were localised predominantly within the C1 region and C1/A1 transition zone, and to a lesser extent the C2 and C2/A2 regions, consistent with its role in the synthesis of adrenaline. PNMT-ir neurons were seldom seen in the A1 region. Compared with TH expression in the same regions, PNMT expression was found in fewer cells, representing ~ 25% of the TH-ir cells in the A1/C1 and C1 regions, and ~ 10–50% of the TH-ir cells in the A2/C2 and C2 regions. An increase in the numbers of PNMT immunoreactive cells was found in the Persistent Effect rats compared to the No Effect rats in the C1 region ($T_{9.9} = 2.26$, $p = 0.047$* $d = 1.31$). This effect was not seen in the A1/C1, A2/C2 or C2 regions. The numbers of double labelled GR + PNMT immunoreactive cells remained stable between behaviourally tested CCI rats and uninjured controls across all regions (see Statistical Results Table 1).

2.4. GR and TH mRNA expression in the rostral vs. caudal medulla

Extracted RNA was of high purity, with clear peaks for 18S and 28S ribosomal RNA. Amplification product was observed in both 18S and GAPDH housekeeping genes. Mean amplification of 18S RNA occurred after 6.2 ± 0.05 cycles, while the mean amplification of GAPDH RNA occurred after 13.2 ± 0.7 PCR cycles. Amplification products for GR and TH in naïve animals occurred after 17.7 ± 0.9 and 18.6 ± 0.8 respectively in the caudal medulla and 18.2 ± 0.3 and 19.7 ± 0.1 respectively in the rostral medulla. Relative to uninjured control rats, CCI and behavioural testing did not alter GR or TH mRNA regulation in either the

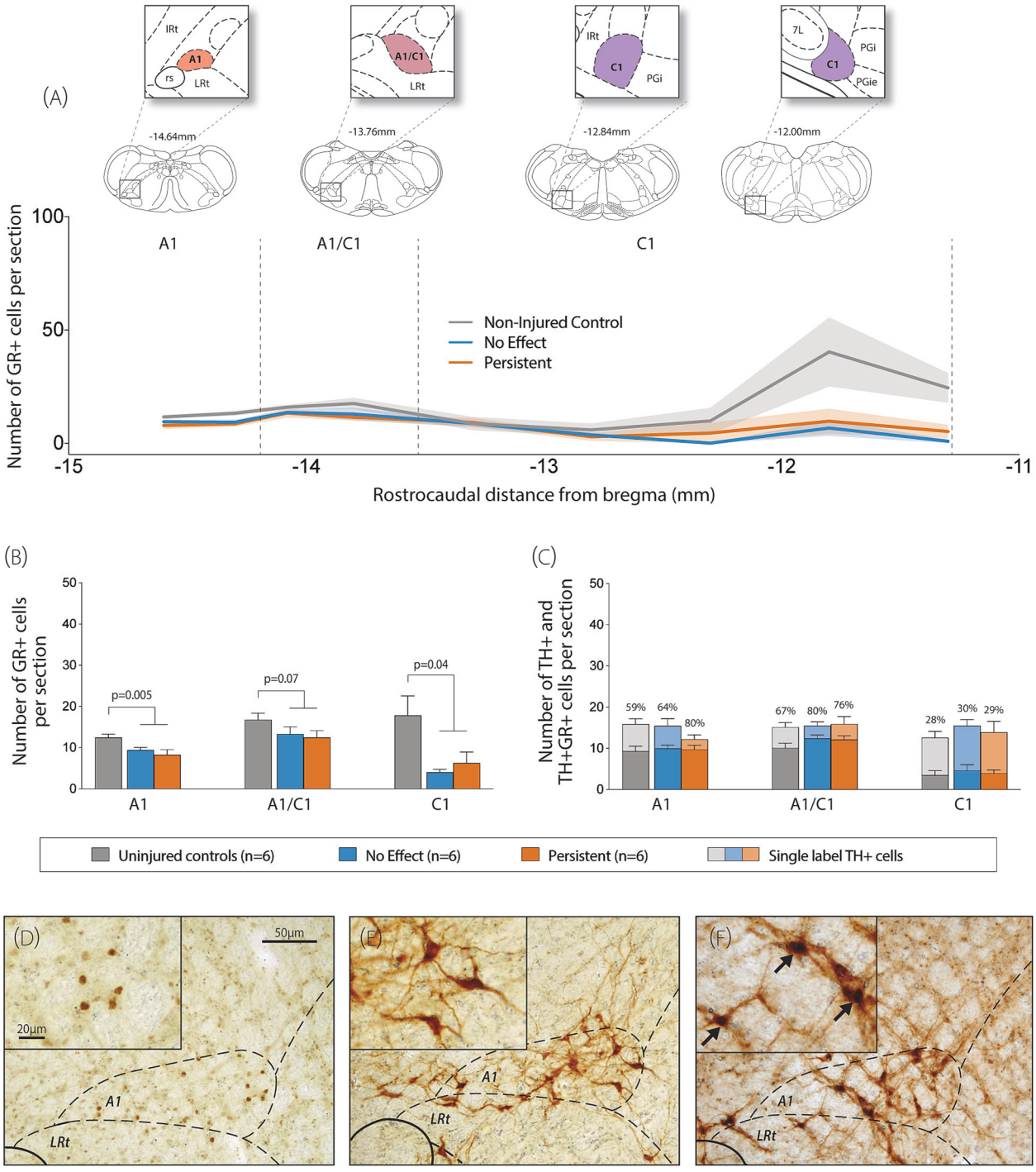

Fig. 1. Glucocorticoid receptor (GR) and tyrosine hydroxylase (TH) immunoreactivity in the A1, A1/C1 and C1 cell groups of the ventrolateral medulla. **(A)** Numbers of GR immunoreactive cells per section across the rostrocaudal axis of the ventrolateral medulla. Example coronal schematic diagrams are shown, with pop-outs depicting the A1, A1/C1 and C1 cell groups (red to purple). Schematics modified from the stereotaxis rat atlas of Paxinos and Watson (2006). *rs*, rubrospinal tract, *IRt*, intermediate reticular nucleus, *LRt*, lateral reticular nucleus, *PGie*, paragigantocellularis lateralis internus/externus, *7L*, facial nucleus (lateral subdivision) **(B)** Total numbers of GR + cells in the A1, A1/C1 and C1 regions in uninjured control rats (grey), No Effect rats (blue) and Persistent Effect rats (Orange). Compared to the uninjured controls, CCI reduced the numbers of GR + cells in these regions, but no significant differences were seen between behavioural subgroups; Welch's unpaired t-tests. **(C)** Numbers of TH+ (light shading) and double labelled TH + GR + cells (dark shading) in the A1, A1/C1 and C1 cell groups of the ventrolateral medulla. The average percentages of TH cells that were double labelled with GR are shown above each bar. No significant differences were detected between uninjured controls and CCI rats, nor between behavioural subgroups; Welch's unpaired t-tests. **(D-F)** Example photomicrographs of GR immunoreactivity (D), tyrosine hydroxylase immunoreactivity (E) and double labelled GR + TH + immunoreactivity (F) in the ventrolateral medulla.

Table 1
Statistical results of Welch's unpaired t-tests. Effect sizes were calculated using Hedges' *g* for comparisons with unequal sample sizes, or Cohen's *d* for comparisons with equal sample sizes.

	CCI + RI testing vs. uninjured	Persistent vs. No Effect	Figure
GR and TH expression in ventrolateral medulla			
GR in A1	$T_{(12.4)} = 3.42$, p = 0.005**, g = 1.58	$T_{(7.4)} = 0.82$, p = 0.44	Fig. 1B
GR in A1/C1	$T_{(10.4)} = 1.95$, p = 0.07	$T_{(10.0)} = 0.34$, p = 0.74	Fig. 1B
GR in C1	$T_{(5.8)} = 2.58$, p = 0.04*, g = 1.68	$T_{(5.7)} = 0.83$, p = 0.44	Fig. 1B
TH in A1	$T_{(15.4)} = 0.50$, p = 0.62	$T_{(8.8)} = 1.76$, p = 0.11	Fig. 1C
TH in A1/C1	$T_{(11.8)} = 0.85$, p = 0.41	$T_{(5.6)} = 0.22$, p = 0.83	Fig. 1C
TH in C1	$T_{(15.1)} = 1.53$, p = 0.15	$T_{(6.4)} = 0.50$, p = 0.63	Fig. 1C
GR + TH in A1	$T_{(8.1)} = 0.39$, p = 0.71	$T_{(9.7)} = 0.16$, p = 0.88	Fig. 1C
GR + TH in A1/C1	$T_{(7.8)} = 1.64$, p = 0.14	$T_{(9.9)} = 0.23$, p = 0.82	Fig. 1C
GR + TH in C1	$T_{(10.2)} = 0.56$, p = 0.59	$T_{(7.9)} = 0.39$, p = 0.71	Fig. 1C
GR and TH expression in dorsomedial medulla			
GR in A2	$T_{(7.1)} = 1.71$, p = 0.13	$T_{(10.0)} = 0.01$, p = 0.99	Fig. 2B
GR in A2/C2	$T_{(6.7)} = 2.40$, p = 0.049*, g = 1.44	$T_{(7.2)} = 0.49$, p = 0.64	Fig. 2B
GR in C2	$T_{(7.1)} = 1.66$, p = 0.14	$T_{(9.2)} = 0.74$, p = 0.48	Fig. 2B
TH in A2	$T_{(14.2)} = 2.36$, p = 0.03* g = 1.03	$T_{(9.3)} = 0.03$, p = 0.98	Fig. 2C
TH in A2/C2	$T_{(6.4)} = 0.11$, p = 0.92	$T_{(9.9)} = 0.84$, p = 0.42	Fig. 2C
TH in C2	$T_{(10.9)} = 0.35$, p = 0.73	$T_{(10.0)} = 0.46$, p = 0.66	Fig. 2C
GR + TH in A2	$T_{(14.5)} = 0.27$, p = 0.79	$T_{(5.98)} = 0.99$, p = 0.36	Fig. 2C
GR + TH in A2/C2	$T_{(12.2)} = 1.60$, p = 0.14	$T_{(7.4)} = 0.99$, p = 0.35	Fig. 2C
GR + TH in C2	$T_{(14.7)} = 1.34$, p = 0.20	$T_{(7.4)} = 0.28$, p = 0.79	Fig. 2C
GR and PNMT expression in ventrolateral medulla			
PNMT in A1	n/a	n/a	Fig. 3A
PNMT in A1/C1	$T_{(10.7)} = 0.64$, p = 0.53	$T_{(9.6)} = 0.57$, p = 0.58	Fig. 3A
PNMT in C1	$T_{(13.2)} = 0.67$, p = 0.52	$T_{(9.9)} = 2.26$, p = 0.047* d = 1.31	Fig. 3A
GR + PNMT in A1	$T_{(16.0)} = 1.08$, p = 0.30	$T_{(9.5)} = 0.62$, p = 0.55	Fig. 3A
GR + PNMT in A1/C1	$T_{(10.4)} = 1.80$, p = 0.10	$T_{(9.9)} = 0.44$, p = 0.67	Fig. 3A
GR + PNMT in C1	$T_{(11.9)} = 1.06$, p = 0.31	$T_{(10.0)} = 1.37$, p = 0.20	Fig. 3A
GR and PNMT expression in dorsomedial medulla			
PNMT in A2	n/a	n/a	Fig. 3B
PNMT in A2/C2	$T_{(15.3)} = 0.53$, p = 0.61	$T_{(6.7)} = 0.10$, p = 0.92	Fig. 3B
PNMT in C2	$T_{(9.4)} = 0.29$, p = 0.78	$T_{(8.9)} = 0.54$, p = 0.60	Fig. 3B
GR + PNMT in A2	$T_{(13.3)} = 0.62$, p = 0.54	$T_{(5.0)} = 1.00$, p = 0.36	Fig. 3B
GR + PNMT in A2/C2	$T_{(9.0)} = 0.00$, p = 0.99	$T_{(8.0)} = 0.00$, p = 0.99	Fig. 3B
GR + PNMT in C2	$T_{(8.8)} = 0.36$, p = 0.73	$T_{(9.7)} = 0.16$, p = 0.87	Fig. 3B
mRNA expression in the caudal medulla			
GR vs. GAPDH	$T_{(5.7)} = 0.03$, p = 0.98	$T_{(7.9)} = 0.85$, p = 0.42	Fig. 4
TH vs. GAPDH	$T_{(4.8)} = 0.82$, p = 0.45	$T_{(4.8)} = 1.06$, p = 0.34	Fig. 4
GR vs. 18S	$T_{(5.2)} = 0.26$, p = 0.81	$T_{(8.0)} = 0.25$, p = 0.81	Fig. 4
TH vs. 18S	$T_{(6.0)} = 0.13$, p = 0.90	$T_{(7.8)} = 0.56$, p = 0.59	Fig. 4
mRNA expression in the rostral medulla			
GR vs. GAPDH	$T_{(4.1)} = 0.68$, p = 0.53	$T_{(7.9)} = 0.25$, p = 0.81	Fig. 4
TH vs. GAPDH	$T_{(6.6)} = 0.15$, p = 0.88	$T_{(7.6)} = 1.32$, p = 0.22	Fig. 4
GR vs. 18S	$T_{(4.2)} = 0.06$, p = 0.95	$T_{(7.1)} = 0.27$, p = 0.79	Fig. 4
TH vs. 18S	$T_{(13.0)} = 0.71$, p = 0.49	$T_{(8.0)} = 0.27$, p = 0.79	Fig. 4

caudal medulla (Fig. 4, top panel) or rostral medulla (Fig. 4, bottom panel) 7 days after surgery. Data were normalised to both GAPDH mRNA and 18S mRNA. Additionally, there were no differences between Persistent Effect rats and No Effect rats for any of these comparisons (see Statistical Results Table 1).

3. Discussion

We have recently shown that rats who demonstrate persistent changes to their resident-intruder behaviours after sciatic nerve CCI have increased numbers (~2×) of CRF expressing neurons in the paraventricular hypothalamus (PVH) (Sosa et al., 2022). The experiments presented here sought to determine whether this anatomically specific change might be driven by differences in the ascending afferent catecholaminergic pathways from the medulla, specifically the A1, C1, A2 and C2 cell populations. If so, such an effect could either be due to differences (pre-existing or experimentally-induced) in the numbers of noradrenergic/adrenergic cells in these regions, or differential glucocorticoid sensitivity of these cell populations. Using immunohistochemistry, we therefore quantified the numbers of phenylethanolamine N-methyltransferase positive (PNMT+, i.e., adrenergic) cells and tyrosine hydroxylase positive (TH+, i.e., estimated noradrenergic) cells in these medullary regions. We also assessed the regional glucocorticoid receptor (GR) expression in the ventrolateral and dorsomedial medulla, and specifically in the catecholaminergic neurons using double labelling.

The numbers of noradrenergic and adrenergic cells in the A1, A2, C1 and C2 groups that we report here are consistent with previous studies (Harfstrand et al., 1986; Fuxe et al., 1987; Harfstrand et al., 1989; Sawchenko & Bohn, 1989). Comparisons between Persistent Effect and No Effect groups revealed that there were no significant differences in the numbers of PNMT + or TH + cells in any of the adrenergic or noradrenergic populations investigated. Additionally, these behavioural groups had nearly identical numbers of GR expressing neurons in each medullary region and within each catecholaminergic cell population. Therefore, contrary to the hypothesis that emerged from the discussion of our results of Sosa et al. (2022), these findings suggest strongly that the increased CRF expression seen in the PVH of Persistent Effect rats is unlikely due to either pre-existing or experimentally-induced differences in GR expression modulating adrenergic and noradrenergic projections into the PVH. This presents yet another example that the rebalancing that occurs during chronic conditions is not simply a prolongation of acute response mechanisms.

Furthermore, unexpectedly, comparisons between the Persistent Effect and No Effect groups to uninjured animals revealed that our experimental protocol reduced regional GR expression across the rostral-caudal extent of the ventrolateral and dorsomedial regions of the medulla, but this downregulation of GR was not within the catecholaminergic cell groups. Taken together, these data raise two key questions: Firstly, what caused the regional GR downregulation and why were the catecholaminergic cell groups unaffected? Secondly, what are the likely physiological and behavioural consequences of these brain GR expression patterns?

3.1. *What caused the downregulation in GR expression in the medulla of both the Persistent and No Effect rats?*

Our data demonstrate that the two behavioural phenotypes are remarkably similar in terms of (1) the numbers and distribution of GR positive cells, (2) the numbers of catecholaminergic cells, and (3) the numbers of double-labelled cells in both the ventrolateral and dorsomedial medulla. Additionally, these two groups of rats also had identical numbers of adrenergic and noradrenergic cells as seen in the uninjured control rats. Interestingly, the decreases in GR expression seen in both the Persistent and No Effect rats appears to be anatomically localized for both the ventrolateral and dorsomedial medulla, with the most robust decreases seen in the rostral portion of the C1 region, and in the A2/C2 transition zone. These results indicate that this is not simply a generalized or global change in GR expression.

We know that GR expression downregulation can either occur (1) in response to increased levels of circulating glucocorticoids (Sapolsky et al., 1984; Sapolsky & McEwen, 1985; Reul et al., 1987; Dong et al., 1988), or (2) via neural regulation (see Herman (1993) for review). Since the uninjured control rats did not undergo Resident-Intruder testing, we are unable to resolve the effects of CCI and the effects of the Resident-Intruder testing on this GR expression in the medulla. However, our previous study (Sosa et al., 2022), and others, have

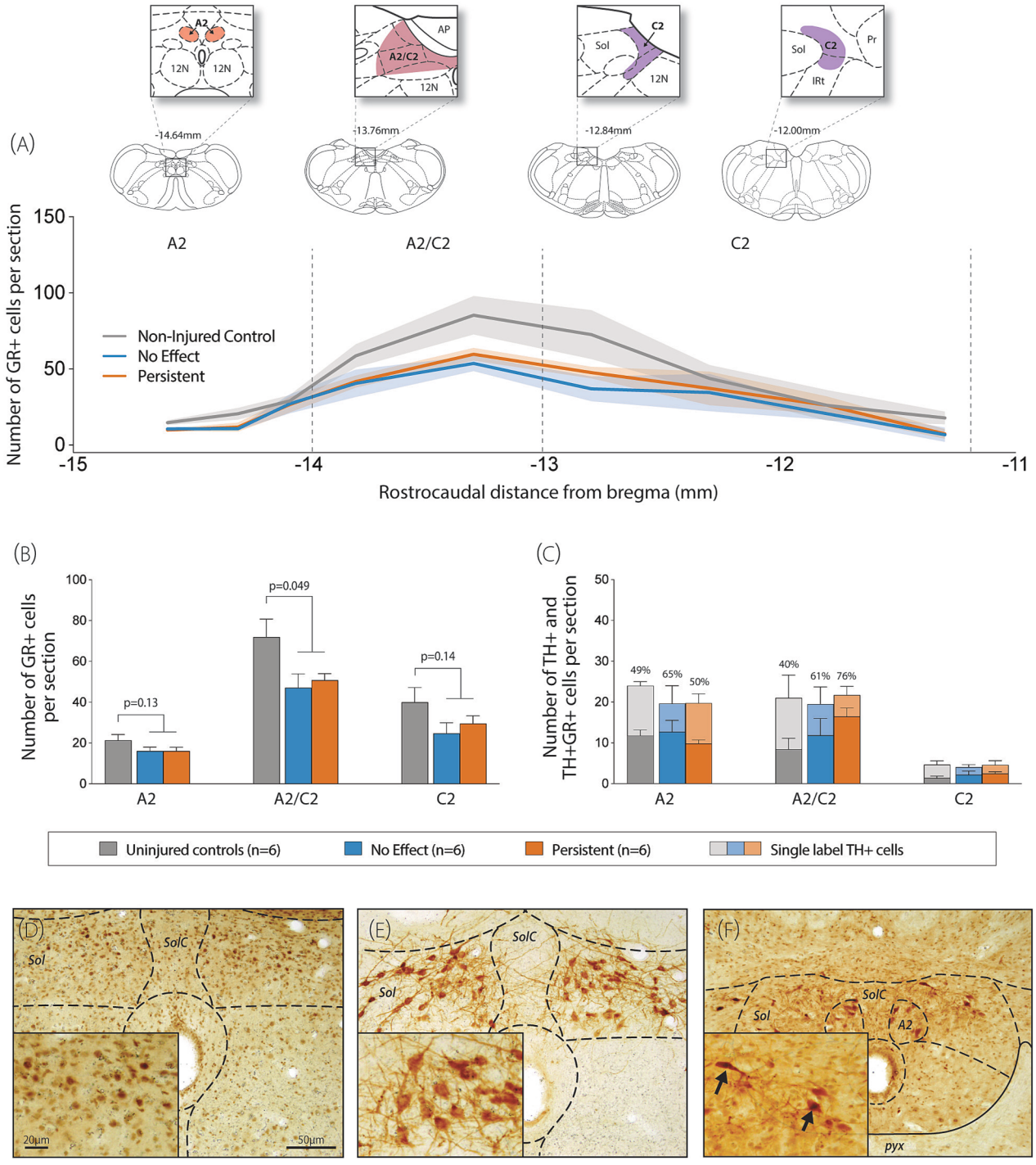

Fig. 2. Glucocorticoid receptor (GR) and tyrosine hydroxylase (TH) immunoreactivity in the A2, A2/C2 and C2 cell groups of the ventrolateral medulla. **(A)** Numbers of GR immunoreactive cells per section across the rostrocaudal axis of the dorsomedial medulla. Example coronal schematic diagrams are shown, with pop-outs depicting the A2, A2/C2 and C2 cell groups (red to purple). Schematics have been modified from the stereotaxis rat atlas of Paxinos and Watson (2006). *12 N*, hypoglossal nucleus, *AP*, area postrema, *Sol*, nuclei of the solitary tract, *IRt*, intermediate reticular nucleus, *Pr*, prepositus nucleus. **(B)** Total numbers of GR + cells in the A2, A2/C2 and C2 regions in uninjured control rats (grey), No Effect rats (blue) and Persistent Effect rats (Orange). Compared to the uninjured controls, CCI tended to reduce the numbers of GR + cells in these regions, but no differences were seen between behavioural subgroups; Welch's unpaired t-tests. **(C)** Numbers of TH+ (light shading) and double labelled TH + GR + cells (dark shading) in the A2, A2/C2 and C2 cell groups of the dorsomedial medulla. The average percentages of TH cells that were double labelled with GR are shown above each bar. No significant differences were detected between uninjured controls and CCI rats, nor between behavioural subgroups; Welch's unpaired t-tests. **(D-F)** Example photomicrographs of GR immunoreactivity (D), tyrosine hydroxylase immunoreactivity (E) and double labelled GR + TH + immunoreactivity (F) in the dorsomedial medulla.

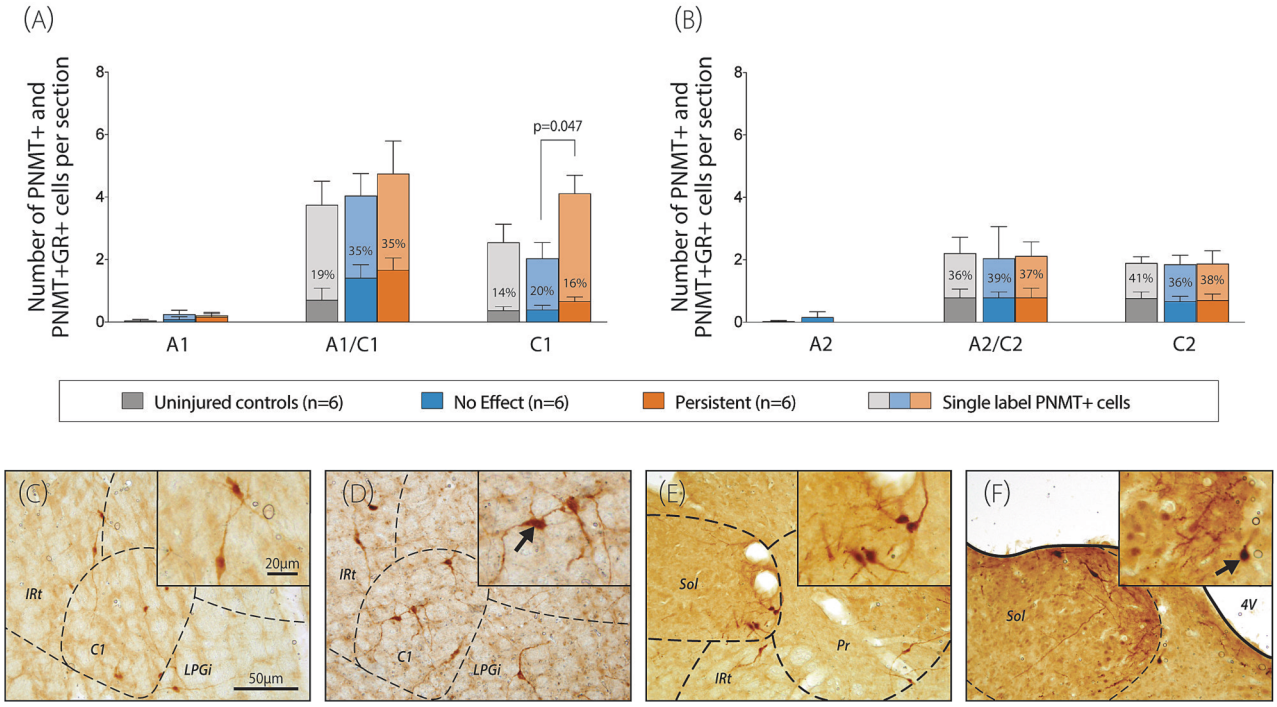

Fig. 3. Phenylethanolamine N-methyltransferase (PNMT) immunoreactivity and double labelled PNMT and glucocorticoid receptor (GR) immunoreactivity in the ventrolateral and dorsomedial medulla. Total numbers of PNMT+ (light shading) and double labelled PNMT + GR + cells (dark shading) in **(A)** the A1, A1/C1 and C1 cell groups of the ventrolateral medulla and **(B)** the A2, A2,C2 and C2 cell groups of the dorsomedial medulla. The average percentages of PNMT + cells that were double labelled with GR are shown above each bar. No significant differences were detected between uninjured controls and CCI rats (Welch's unpaired t-tests). Persistent Effect rats had significantly greater numbers of PNMT + cells in the C1 cell group compared to the No Effect rats, but there we no significant differences between these behavioural subgroups in any other cell group, nor in the numbers of double labelled cells (Welch's unpaired t-tests). **(C-F)** Example photomicrographs of single label PNMT immunoreactivity in the ventrolateral medulla corresponding to the C1 cell group (C), the dorsomedial medulla corresponding to the C2 cell group (E), and double labelled GR + PNMT + immunoreactivity in the same ventrolateral (D) and dorsomedial (F) regions.

demonstrated that a CCI injury has no significant effect on basal corticosterone levels (Bomholt et al., 2005; Ulrich-Lai et al., 2006; Le Coz et al., 2014a; Le Coz et al., 2014b). Additionally, given that the CCI is a unilateral injury and that spinal cord inputs to the both the RVLM and CVLM each terminate predominantly ipsilaterally, our observation that GR and TH label was identical on both sides of the brainstem make it unlikely that the unilateral injury is driving these changes.

In contrast, agonistic social interactions have been shown to elevate corticosterone levels (Haller et al., 1996; Sgoifo et al., 1996), including Resident-Intruder testing, which produces substantial increases in corticosterone that persist for at least 1 h after testing (De Miguel et al., 2011). We therefore suggest that the bilateral downregulation in GR expression seen in the Persistent and No Effect rats most likely resulted from their repeated Resident-Intruder tests over the previous 11 days.

3.2. *What are the likely physiological and/or behavioural consequences of GR downregulation in these regions?*

The action of glucocorticoids on GRs to induce both short-term and long-term cellular changes via transcriptional regulation is well characterised (Weikum et al., 2017). However, the binding of glucocorticoids to GRs has also been shown to have direct and immediate effects on the firing rate of neurons. For instance, Rong et al. (1999) demonstrated using electrophysiology that direct application of corticosterone onto barosensitive cardiovascular neurons of the RVLM caused increased activity in the majority of neurons tested. Additionally, microinjections of corticosterone or aldosterone into the RVLM have immediate cardiovascular effects (Zhu et al., 1997; Wang et al., 2005). Similarly, inhibiting the action of glucocorticoids on GRs via pharmacological antagonism in the dorsomedial medulla exacerbates the cardiosympathetic aspect of the stress response. These studies indicate that cardiosympathetic and respiratory reflexes deployed in response to a range of stressors can be differentially regulated by medullary GRs. Therefore, a decrease in GR expression in these regions, such as we observed in both the Persistent Effect and No Effect animals, would modify these reflexes to stress-induced glucocorticoid release (such as during the Resident-Intruder test).

Despite there being a bilateral decrease in regional GR expression in both the ventrolateral and dorsomedial medulla, GR downregulation was not in the adrenergic or noradrenergic cell populations. Therefore, these cells retain their direct sensitivity to circulating glucocorticoids. As such, the catecholaminergic drive onto the PVH neurons during stress responses is likely similar in each of these behavioural phenotypes. However, it has been shown that corticosterone binding to the GR receptor in activated neurons can dampen down noradrenaline-induced excitation in the hippocampus (Joels & de Kloet, 1989). Therefore, since we have shown that the CRF expressing neurons in the PVH show a strong reduction in the GR expression of the Persistent Effect animals, it is possible that identical levels of noradrenergic and adrenergic drive on the PVH could have different cellular outcomes for each of these behavioural subpopulations.

3.3. *Extending the Sosa et al. (2022) model*

Unfortunately, the data we report here do not provide us with evidence for differences in the size, or glucocorticoid sensitivity, of the catecholaminergic afferents known to modulate the HPA-axis as we hypothesized in Sosa et al. (2022). Instead, it suggests that the increased numbers of CRF expressing neurons in the PVH of Persistent Effect animals is much more likely to be driven by either supra-medullary inputs or differential glucocorticoid sensitivity of the CRF neurons themselves. Fig. 5 expands the model first presented in Sosa et al. (2022) to

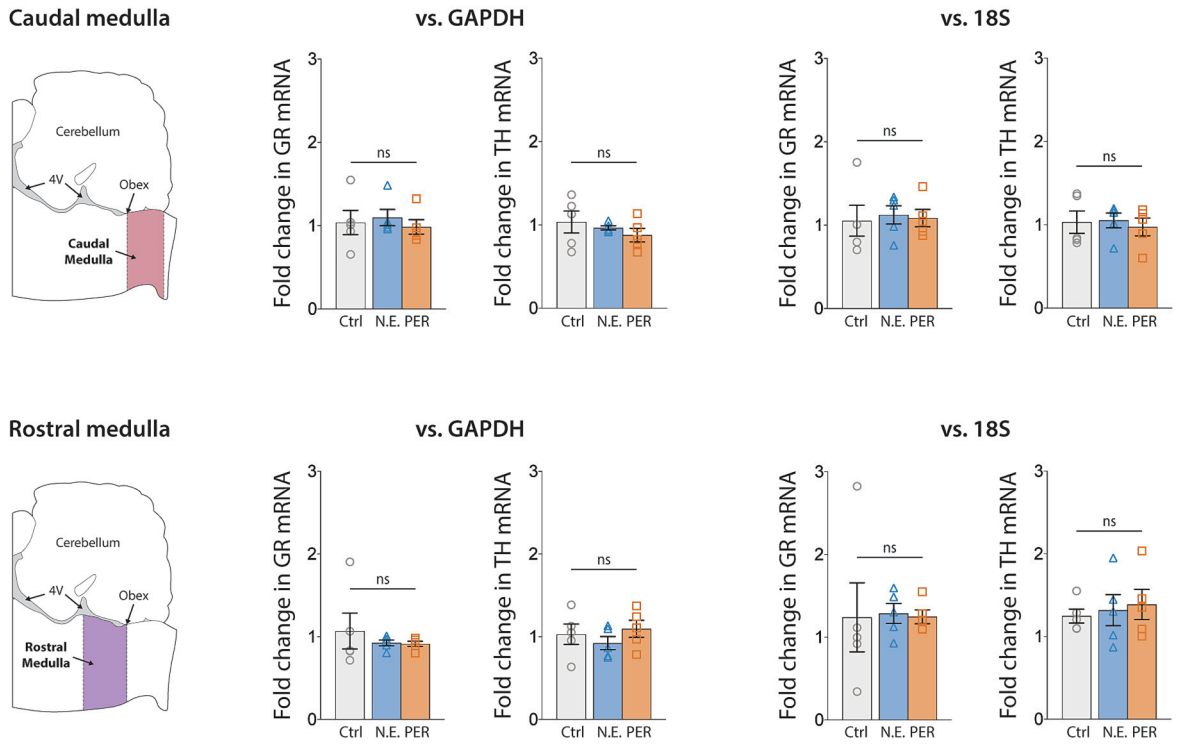

Fig. 4. Glucocorticoid receptor (GR) and tyrosine hydroxylase (TH) mRNA expression in the caudal and rostral medulla. Relative to uninjured control rats, CCI rats had no changes to their GR or TH mRNA expression in either the caudal medulla (top row) or rostral medulla (bottom row) 7 days after surgery, when normalised to either GAPDH mRNA expression or 18S mRNA expression (Welch's unpaired t-tests). Additionally, there were no differences between Persistent Effect rats (PER) and No Effect rats (N.E.) for any of these comparisons (Welch's unpaired t-tests). *4 V*, fourth ventricle.

accommodate these observations and speculations.

4. Conclusions

Rats that show persistent changes to their social interactions following nerve injury (Persistent Effect) show increased hypothalamic CRF and GR expression compared to rats that show no changes to their social interactions (No Effect). Hypothalamic CRF is regulated by inputs from brainstem medullary noradrenergic and adrenergic cell populations, which are modulated by circulating glucocorticoids via GR activation. In this study, we show that the numbers of noradrenergic and adrenergic neurons in both the ventrolateral and dorsomedial medulla are unaffected by CCI and Resident-Intruder testing, and do not differ between the behavioural subpopulations. We also found an overall reduction in GR expression in these regions in these behavioural groups, likely due to the repeated Resident-Intruder testing, but interestingly, not in the catecholaminergic cell populations. As such, these cells populations would retain their direct sensitivity to circulating glucocorticoids, presumably enabling them to maintain their role in both spinal (sympathetic preganglionic) and hypothalamic (paraventricular nucleus) regulation. Taken together, these data suggest strongly that brainstem mechanisms are unlikely to play a key role in the rebalancing of the HPA-axis triggered by a neuropathic injury.

5. Experimental procedures

5.1. Experimental design

Experiments and procedures were designed and performed in accordance with the Australian National Health and Medical Research Council's guidelines outlined in the 'Australian code for the care and use of animals for scientific purposes' 8th 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' (Zimmermann, 1983). The data presented in this study are available from the corresponding author upon reasonable request.

Resident-Intruder social interactions testing following a sciatic nerve chronic constriction injury (CCI) was conducted to categorize rats into either Persistent Effect or No Effect phenotypes (Monassi et al., 2003). The 22 Resident-Intruder tested animals from which the data were collected for this study are a subset of a larger cohort reported in an earlier study (Sosa et al., 2022). Fig. 6 shows the Resident-Intruder behaviours of these 22 rats specifically. Comparisons between Persistent Effect rats and No Effect rats were made to investigate whether these behavioural phenotypes had distinct patterns of GR, TH and PNMT expression in the ventrolateral and dorsomedial medulla. Comparisons between behaviourally tested CCI rats (i.e., pooled data from Persistent Effect and No Effect groups) and behaviourally untested, uninjured control rats were made to investigate the effects of CCI and Resident-Intruder testing on patterns of GR, TH and PNMT expression in the ventrolateral and dorsomedial medulla.

5.2. Animals and housing

Outbred, male Sprague-Dawley rats (ARC, Perth, Australia), weighing between 250 g and 350 g at the time of CCI surgery were used in these experiments. Upon arrival to the laboratory, rats were arbitrarily assigned as either 'Resident' or 'Intruder' rats. Residents were housed individually in clear Perspex cages, while Intruders were group-housed (4–6 per cage) in polycarbonate open-top cages in a separate room. All rats had *ad libitum* access to water and food (standard laboratory chow). Housing rooms were on a reversed 12:12hr dark-light cycle (lights off at 0800 h), and all procedures and behavioural testing was performed in the dark phase. Uninjured control rats were age- and sex- matched, and grouped housed in the same fashion as the Intruder rats.

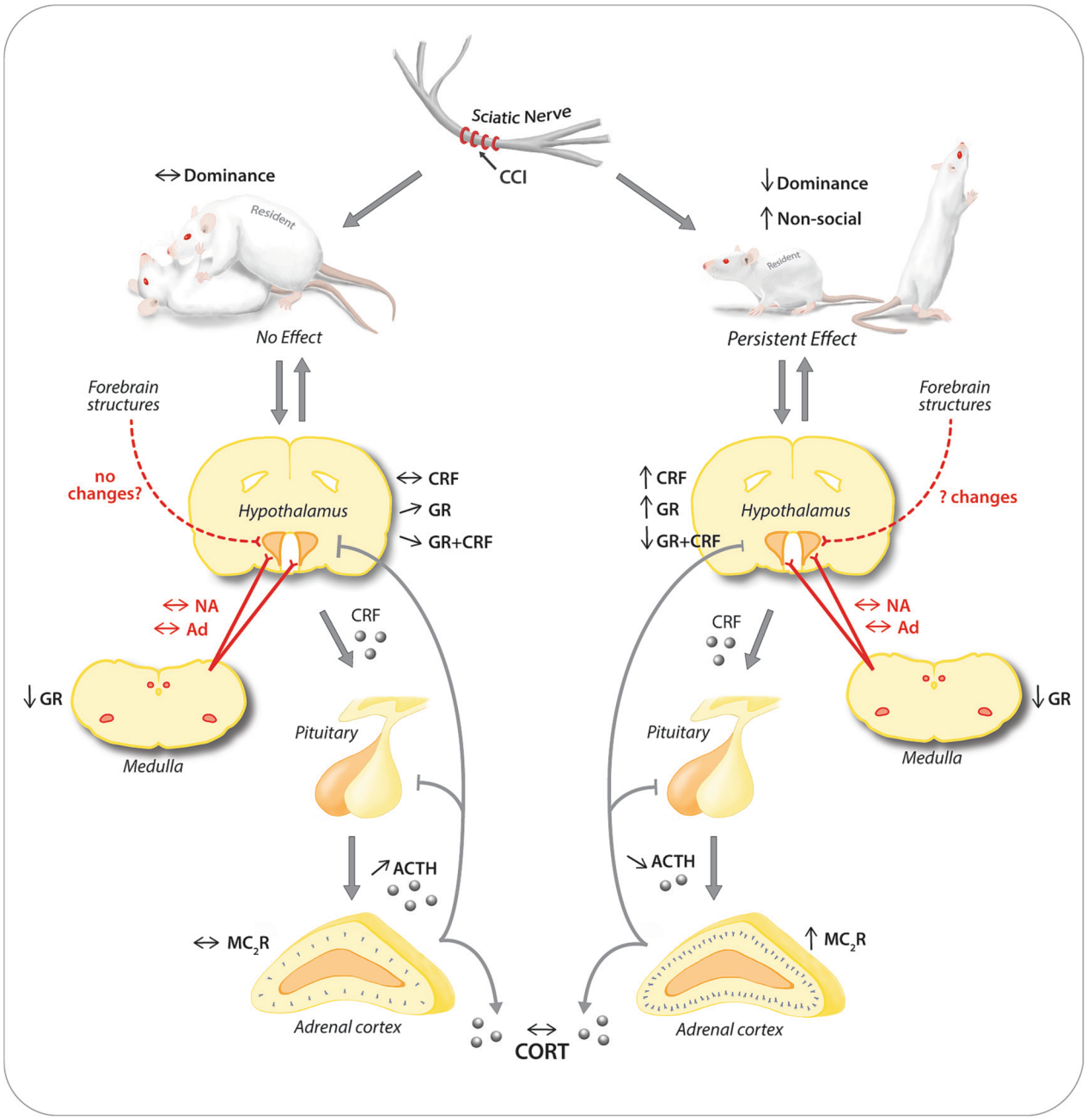

Fig. 5. Updated summary model first presented in Sosa et al. (2022). Sciatic nerve chronic constriction injury (CCI) results in behaviourally distinct subpopulations of rats (Persistent Effect and No Effect), each of which are associated with distinctive differences and/or modifications to their HPA-axis. Both sets of modifications, however, appear to rebalance the HPA-axis so as to result in normal basal corticosterone levels. Most notably, Persistent Effect rats show double the number of CRF neurons in the paraventricular hypothalamus (PVH). Our data suggest that this effect is unlikely to be driven by differences in the ascending catecholaminergic projections (noradrenergic and adrenergic) from the ventrolateral and dorsomedial medulla. We hypothesize that forebrain structures known to project to the PVH might be driving the increase in CRF neuron density. CRF, corticotropin releasing factor, GR, Glucocorticoid receptor, ACTH, Adrenocorticotropic hormone, MC_2R, Melanocortin receptor type-2, Cort, Corticosterone.

5.3. CCI surgery

The details of the CCI surgery performed on these rats has been reported previously (see Sosa et al. (2022)). Briefly, under halothane anaesthesia, rats received a unilateral CCI, in which 4 catgut chrome ligatures (5–0, Johnson & Johnson) were tied around the sciatic nerve, spaced 1 mm apart. These ligatures gently compressed the nerve, yet still permitted epineural circulation. This procedure replicates that originally described by (Bennett & Xie, 1988).

5.4. Resident-Intruder testing

All procedures, experimental timelines and behavioural analysis of Resident-Intruder testing has been described in detail previously (Sosa et al., 2022). Briefly, Resident rats were introduced to an age-, weight-, and sex-matched Intruder rat in their home cage once per day for 11 days. Each encounter lasted six minutes. The behaviour of the Resident rat was classified into 4 mutually exclusive categories: (1) dominance, (2) social, (3) non-social or (4) submissive. After the 5th Resident-Intruder test, all Resident rats underwent CCI surgery. These rats were then classified as either Persistent Effect rats or No Effect rats based on

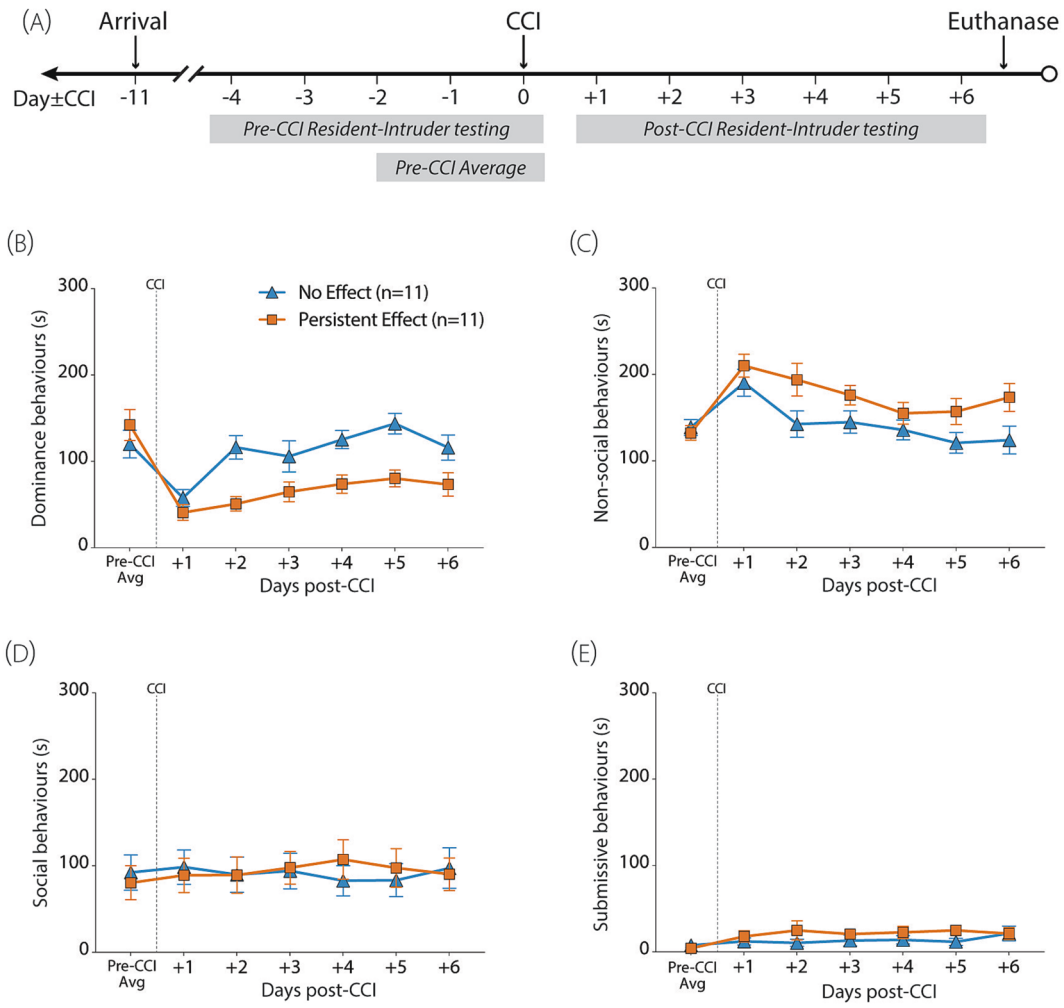

Fig. 6. Experimental timeline and Resident-Intruder behaviours. (A) Experimental timeline. Male Sprague-Dawley rats (n = 22) underwent Resident-Intruder social interactions testing for 11 consecutive days. After the fifth Resident-Intruder test, on the same day, all rats underwent chronic constriction injury surgery. The time spent performing 4 stereotyped, mutually exclusive behaviours was recorded (see methods sections for descriptions of each behaviour): (B) dominance behaviours, (C) non-social behaviours, (D) social behaviours or (E) submissive behaviours. Rats were classified into groups based on whether they showed a persistent reduction in dominance behaviours post-CCI (Persistent Effect), or negligible changes to their dominance behaviours post-CCI (No Effect). Reductions in dominance behaviours were most often accompanied by corresponding increases in non-social behaviours. The experimental timeline shown in panel A has been modified from Fig. 1 in Sosa et al. (2022).

the relative change in their dominance behaviours over the subsequent 6 days. Persistent Effect rats were those who demonstrated more than a 30% reduction in their dominance behaviours compared to their pre-CCI baselines on at least 5 of the 6 days, while No Effect rats had negligible changes to their dominance behaviours across this testing period.

5.5. Euthanasia, perfusion and tissue collection

On the day following the final Resident-Intruder test (i.e., 7 days post-CCI) all Resident rats were euthanased and their tissues collected. The rats whose tissues were used for immunohistochemistry were deeply anaesthetized with sodium pentobarbital (Nembutal i.p 120 mg/kg, Boehringer Ingelheim, Australia) and perfused transcardially with 500 mL of heparinised sterile saline (0.9%, 4 °C), followed by fixation with 500 mL of 4% formaldehyde in acetate-borate buffer (pH 9.6, 4 °C). Their brains were immediately extracted and stored in 30% sucrose in 0.1 M phosphate buffered saline until processing. The rats whose tissues were used for RT-PCR were rapidly decapitated, and their brains were extracted immediately and placed into TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA) and frozen in liquid nitrogen to be stored at −80 °C until processing.

5.6. Immunohistochemistry

A block containing the medulla was isolated from the fixed brains (Persistent Effect, n = 6; No Effect, n = 6; uninjured controls, n = 6). Serial coronal sections were cut on a freezing microtome (50 μm at 18 °C) as a 1 in 5 series and collected into PBS. All 5 series were used: (1) single-label GR, (2) single-label TH, (3) single-label PNMT, (4) double label GR + TH, (5) double-label GR + PNMT. Immunohistochemistry was performed on free-floating sections. All washes were in 0.1 M PBS (pH 7.4) for 3x10 minutes unless otherwise stated. To begin, sections were washed in PBS, washed in 50% ethanol (30 min), then washed in 3% H_2O_2 in 50% ethanol (15 min) to quench endogenous peroxidase activity. Sections were washed, then blocked in 10% normal horse serum in PBS (5 min). Sections were then incubated overnight at 4 °C in either rabbit anti-GR (1:5000; RRID:AB_2155786, Santa Cruz Biotechnology, USA), mouse anti-TH (1:1000; RRID:AB_572268, Immunostar, Hudson, USA) or rabbit anti-PNMT (1:1000; Immunostar, Hudson, USA). The following day sections were washed, then incubated for 2 h in biotinylated secondary antibody targeting the species of their primary antibody (1:500 in either anti-rabbit IgG, RRID:AB_2336201 or anti-mouse IgG, RRID:AB_2313581; Vector Laboratories, Burlingame,

USA). Sections were washed and then incubated in ExtrAvidin Peroxidase at 1:1000 in PBS for 2.5 h at room temperature. The bound antibody complex was visualised using 0.05% 3-3′ diaminobenzidine tetrahydrochloride (DAB), following our previously published procedures (Boorman et al., 2019). For double-labelled sections, GR procedures were performed first using nickel-intensified DAB (2% $NiCl_2$), which produced a black precipitate in the nucleus of cells, then TH or PNMT labelling subsequently using non-intensified DAB, which produced a brown precipitate in the cytoplasm. Sections were then mounted onto gelatinised glass slides, dried overnight, dehydrated in ascending alcohols and coverslipped with DPX.

For each animal, single label cell counts for GR, TH and PNMT, and double-label cell counts were performed across the rostro-caudal extent of the ventrolateral medulla (A1, A1/C1 and C1 cell groups) and the dorsomedial medulla (A2, A2/C2 and C2 cell groups) at 9 different levels: −14.60, −14.30, −14.08, −13.80, −13.30, −12.80, −12.30, −11.80, and −11.30 mm from bregma. Single or double labelled cells were verified at a range of magnifications (40x – 200x) and manually counted using a light microscope (Olympus BX-51).

5.7. Quantitative real time RT-PCR

The frozen brains were thawed, and the medulla was isolated: rostrally at the pontomedullary junction just posterior to the entry point of the vestibulocochlear nerve (−11.50 mm from bregma), and caudally at the spinomedullary junction (−15.50 mm from bregma). This block was divided into two portions: the caudal medulla (2 mm caudal to the obex) and the rostral medulla (2 mm rostral to the obex). Quantitative real-time RT-PCR was then performed using the identical procedures to those described in Sosa et al. (2022). We quantified glucocorticoid receptor (GR) mRNA and tyrosine hydroxylase (TH) mRNA relative to the housekeeping genes ribosomal 18S RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The Taqman® probes used in this study were:

GR:	Rn00561369_m1
TH:	Rn00562500_m1
18S:	Hs99999901_s1
GAPDH:	Rn99999916_s1

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 (Pfaffl et al., 2002). Gene expression for each sample is expressed as fold change relative to the average of the control population. This also includes the naïve control samples so that the variance within the control population can be seen.

5.8. Statistical analysis

Since we found no significant differences between left and right sides in either the ventrolateral medulla or the dorsomedial medulla for any of the single-labelling or double-labelling, the cell counts from the two sides were averaged for all analyses. To determine if there were any significant differences between Persistent Effect and No Effect rats in the numbers of immunoreactive cells in each of the catecholaminergic cell populations, separate multiple unpaired t-tests (Welch's correction, two tailed) were performed for each region of interest (i.e., A1, A1/C1, C1, A2, A2/C2, C2) for each label. The increased likelihood of Type-I errors, due to multiple comparisons was corrected using False Discovery Rate (FDR) approach originally described by Benjamini and Hochberg (1995). To determine if experimental rats had differential expression in immunoreactive labelling compared to uninjured control animals, these procedures were replicated with the data from the Persistent Effect and No Effect groups pooled (Experimental group). These procedures were once again replicated to probe differences in RT-PCR quantified mRNA expression between behavioural subpopulations (Persistent Effect vs. No Effect) and injury (CCI vs. uninjured). All statistical tests were performed GraphPad Prism version 9.0.1 (GraphPad Prism Software, San Diego, California, USA), with $\alpha = 0.05$. Graphs were created in GraphPad Prism and then exported into Adobe Illustrator 2021 (v 25.2) to create figurework.

CRediT authorship contribution statement

Maria K.S. Sosa: Investigation, Data curation, Writing – original draft. **Damien C. Boorman:** Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Kevin A. Keay:** Conceptualization, Methodology, Investigation, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing 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 paper.

Data availability

Data will be made available on request.

Acknowledgement and conflicts of interest statement

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