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Dexamethasone increases production of C-type natriuretic peptide in the sheep brain

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Abstract

Although C-type natriuretic peptide (CNP) has high abundance in brain tissues and cerebrospinal fluid (CSF), the source and possible factors regulating its secretion within the central nervous system (CNS) are unknown. Here we report the dynamic effects of a single IV bolus of dexamethasone or saline solution on plasma, CSF, CNS and pituitary tissue content of CNP products in adult sheep, along with changes in CNP gene expression in selected tissues. Both CNP and NTproCNP (the amino-terminal product of proCNP) in plasma and CSF showed dose responsive increases lasting 12-16 h after dexamethasone whereas other natriuretic peptides were unaffected. CNS tissue concentrations of CNP and NTproCNP were increased by dexamethasone in all of the 12 regions examined. Abundance was highest in limbic tissues, pons and medulla oblongata. Relative to controls, CNP gene expression (*NPPC*) was upregulated by dexamethasone in 5 of 7 brain tissues examined. Patterns of responses differed in pituitary tissue. Whereas the abundance of CNP in both lobes of the pituitary gland greatly exceeded that of brain tissues, neither CNP nor NTproCNP concentration was affected by dexamethasone — despite an increase in *NPPC* expression. This is the first report of enhanced production and secretion of CNP in brain tissues in response to a corticosteroid. Activation of CNP secretion within CNS tissues by dexamethasone, not exhibited by other natriuretic peptides, suggests an important role for CNP in settings of acute stress. Differential findings in pituitary tissues likely relate to altered processing of proCNP storage and secretion.

Introduction

C-type natriuretic peptide (CNP), a paracrine growth factor which regulates cell proliferation and maturation, is widely expressed along with its receptor (NPR-2) throughout the brain and spinal cord in mammals (Komatsu *et al.* 1991, Langub *et al.* 1995, Herman *et al.* 1996) including primates (Abdelalim & Tooyama 2011). In contrast to other natriuretic peptides, concentrations of products of CNP gene expression (CNP and amino-terminal proCNP, NTproCNP) in cerebrospinal fluid (CSF) greatly exceed those in the systemic circulation (Schouten *et al.* 2011, Wilson *et al.* 2015) and presumably reflect the high CNP abundance relative to other natriuretic peptides identified in brain tissues (Pemberton *et al.* 2002). Although the functional role of CNP in nervous tissues is unclear, *in vitro* evidence shows that CNP stimulates neural growth and connectivity (Zhao & Ma 2009) and neuroplasticity in hippocampal tissues (Decker *et al.* 2010). Changes in CNP gene expression in the perinatal and later periods of brain maturation (Müller *et al.* 2009) further suggest that CNP in brain tissues is subject to regulation but putative secretagogues have yet to be identified.

In the course of study of the regulation of CNP products in CSF in conscious sheep, we determined that dexamethasone — which suppresses plasma levels of CNP and NTproCNP when chronically administered to growing lambs (Prickett *et al.* 2009) or children (Prickett *et al.* 2012a) — conversely, abruptly increases concentrations of these peptides in CSF and plasma after an IV bolus injection. This novel observation has initiated a series of studies aimed to determine the temporal sequence and dose responsivity of CNP peptides in plasma and CSF to stimulation by dexamethasone and to identify the sites in the central nervous system (CNS) targeted by this glucocorticoid. Mindful of the well-recognised and profound effects of glucocorticoids on brain function (Wolkowitz *et al.* 2009), the focus of

these studies has been the brain and nearby organs. Based on the initial observations of CNP responses in CSF, we hypothesised that i) the increases in CSF concentrations of CNP would be dose dependent and differ from the dynamic changes exhibited in plasma and ii) the increases in CSF concentration would be associated with corresponding increases in brain tissue abundance of CNP as well as increased CNP gene expression. Because concentrations of CNP in CNS tissues greatly exceed those of other natriuretic peptides in several species – including human (Minamino *et al.* 1991) porcine (Ueda *et al.* 1991) and ovine tissues (Pemberton *et al.* 2002) – we further postulated that among the family of natriuretic peptides, the response would be specific to CNP products.

Materials and Methods

Animal procedures

All procedures involving animals were conducted at Lincoln University and carried out in accordance with the Animal Welfare Act 1999 (New Zealand) and were approved by the Lincoln University Animal Ethics Committee.

Responses of CNP peptides in plasma and CSF to graded doses of dexamethasone. (Study 1).

We first determined the dose response of peripheral venous plasma and cisternal CSF concentrations of CNP peptides to dexamethasone in chronically cannulated conscious sheep (Study 1). Eight healthy yearling Coopworth ewes (average live weight 42 kg, 9–14 months old) were housed indoors for 1 week prior to study and fed concentrated lucerne pellets (SealesWinslow, Ashburton, New Zealand) and lucerne chaff at 0900 h every day at maintenance nutritional level, with water provided *ad libitum*. This feeding regime was

continued for the duration of the study. One day before cannulation, sheep were fasted for 24 h and water withheld overnight. Initially CSF samples were collected from 2 of the sheep using cannulae that were placed into the cervical epidural space whilst the sheep were anaesthetised. Thereafter, with the need for improved cannula patency, all CSF samples from other sheep were collected from the cisterna magna via an indwelling cannula (Wilson & Barrell 2015). For samples collected from the cisterna magna, 0.5 mL of CSF — which occupied the dead space in the cannula — was withdrawn under aseptic conditions using a 3 mL disposable syringe and discarded. At each sampling time point, 1.0–1.2 mL of CSF was withdrawn and transferred immediately to a polycarbonate tube on ice, then stored at -20°C until assayed. Blood samples were obtained as described previously (Wilson *et al.* 2015) and were collected on the same occasions as the CSF samples. Dose response studies commenced at least 2 days after cannulation, and continued over a study period of 6 days. Dosing comprised a single IV bolus injection of dexamethasone sodium phosphate in aqueous solution (Dexa 0.2, PhoenixPharm Distributors Ltd, Auckland, New Zealand) at 0.025, 0.063, 0.125, and 0.25 mg dexamethasone/kg live weight, or saline solution (0.9% w/v) that was delivered according to a balanced incomplete block design. This ensured that 4 different individuals were allocated to each dose of dexamethasone. The sampling was conducted immediately prior to administration of dexamethasone or saline solution, and at 4, 8, 12, and 16 h post administration for measurement of CNP and NTproCNP. To assess the glucocorticoid activity of these treatments, plasma glucose concentration was measured before and at 8 h post injection (dexamethasone or saline) as shown in Supplemental Figure 1. Plasma glucose concentration was measured by the hexokinase method using an automated analyser (Abbott c8000 Clinical Chemistry Analyzer, Abbott Diagnostics Inc., IL, USA) by Canterbury Health Laboratories, Christchurch, New Zealand.

Responses of brain, pituitary and spinal cord levels of CNP peptides to dexamethasone. (Study 2). In this study, 14 healthy Texel-Romney wethers (average live weight 77 kg, 2 years old) were sampled as described above except that CSF samples were obtained under light anaesthesia – as previously described (Wilson *et al.* 2015) – and blood samples were collected (Wilson *et al.* 2015) immediately prior to a single IV bolus of dexamethasone containing 0.25 mg/kg live weight (n = 7), or saline (control, n = 7), and again at 8 h post injection. At that point, sheep were individually euthanised by captive bolt and exsanguinated. The brain and pituitary gland were rapidly removed and approximately 0.5 g of selected tissues from 14 sites were excised and instantly placed in liquid nitrogen. Specific zones sampled were the anterior pituitary gland, posterior pituitary gland, olfactory bulb, septum, thalamus, hypothalamus, mammillary body, hippocampus, occipital cortex, pineal gland, cerebellum, pons, medulla oblongata, and cervical spinal cord. The frozen samples were stored on dry ice until they were transferred to a -80°C freezer within 2–3 h.

Measurement of peptide concentration and gene expression

Samples of frozen brain and pituitary tissue (mean 70 ± 10 mg) were finely diced on a chilled melamine chopping board, weighed and gently boiled for 5 minutes in 10 mL distilled water containing 10 µL Triton X-100. After boiling, the tissue suspension was cooled on ice and 610 µL of glacial acetic acid was added. The tissue suspension was homogenised (3 x 20 second bursts at 400 Hz) using an Ultra-Turrax homogeniser (IKA-Labortechnik, Staufen, Germany). The tissue homogenates were then centrifuged (3000 *g*, 4°C, 30 minutes), and processed thereafter in an identical manner to the CSF and plasma samples.

Hormone assays. Hormones levels in CSF, plasma, brain and pituitary tissue were measured by radioimmunoassay after extraction using solid phase cartridges (Sep Pak, Waters Corp.,

Milford, MA, USA). All samples from an individual animal were processed in duplicate in a single assay. CNP and NTproCNP were assayed as previously described (Wilson *et al.* 2015) and tissue concentrations were calculated from wet weight of tissue homogenised, assay buffer reconstitution volume and radioimmunoassay result. The ratio of NTproCNP to CNP (NTproCNP:CNP) was calculated from molar concentrations of the respective peptides in each sample. Atrial natriuretic peptide (ANP) concentration was measured as previously reported (Yandle *et al.* 1986) except: 50 µL standard/sample was pre-incubated with 50 µL of primary rabbit antiserum diluted to 1:12500 for 24 h at 4 ^{o}C, to which 50 µL of iodinated ANP was added (2500 cpm/50 µL). Following a second incubation period, bound and free-labelled antigen were separated by addition of 500 µL of solid phase secondary antibody (5 % v/v Donkey anti-Rabbit Sac-cell (IDS Ltd, UK) diluted in assay buffer containing 2 % polyethylene glycol. After 30 minutes incubation at room temperature, tubes were centrifuged for 10 minutes, and radioactivity of the pellet was counted following aspiration of the supernatant. B-type natriuretic peptide (BNP) concentration was measured as previously described (Pemberton *et al.* 1997) except: 50 µL standard/sample was pre-incubated for 24 h at 4 ^{o}C with 50 µL of primary rabbit antiserum, to which 50 µL of iodinated BNP was added (5000 cpm/50 µL). After a second incubation period, bound and free-labelled antigen were separated in a similar manner as the ANP protocol, except the assay was incubated for 30 minutes in an ice bath. The detection limit (pmol/L) for each assay was: 7.3 for ANP, 4.9 for BNP, 0.6 for CNP and 1.9 for NTproCNP. Intra- and inter-assay coefficients of variation respectively were 7.8% (21-100 pmol/L) and 10.4% (at 86 pmol/L) for ANP, 9.9% (4-20 pmol/L) and 15% (at 23.6 pmol/L) for BNP, 6.3 and 7.9% at 9 pmol/L for CNP and 7.4 and 11.4% at 64 pmol/L for NTproCNP.

Quantitative real-time PCR. Total RNA was extracted from approximately 40 mg of tissue using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA), according to the manufacturer's instructions. RNA purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, Wilmington, DE, USA). cDNA was synthesised from 1 µg of RNA template using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real-time PCR was performed using iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA) and specific primers for ovine *NPPA* (Forward: CCT CCG AGA TCT GTC CTC CT; Reverse: CTT CGA TAC CGG AAG CTG TTG), *NPPB* (Forward: GCT GCT AGG ATG TCG TTC CC; Reverse: TCC AAC AGC TCC TGT AAC CCA), *NPPC* (Forward: GGT CAG AAG GGC GAC AAG A; Reverse: TGT ATT TGC GCG CGT TGG G), *NPR1* (Forward: CCC TAT CAG CAG AGA GCA CG; Reverse: CAC CGA TGG TCT CCA CCT TG), *NPR2* (Forward: TGC CCT CTA TGC CAA GAA GC; Reverse: GTA GAA AGG CCC ACT GCG AA), and *NPR3* (Forward: CAC CCA GGA GGT TAT TGG TGA; Reverse: AAG GAG AGC TGT TCG TGT GCT) on a Stratagene MX3000p thermal cycler (Agilent Technologies, Santa Clara, CA, USA). Gene expression was normalised to cDNA concentration, quantified using a Quant-iT™ OliGreen® ssDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). To determine relative gene expression, mean Ct values were power transformed from their logarithmic format, and divided by sample cDNA concentration.

Statistical analyses

Changes in CNP and NTproCNP concentration in CSF, plasma and CNS tissue in response to saline or dexamethasone administration were analysed separately. In Study 1, a repeated measures ANOVA was used to compare area under the curve for the logged ($\log_{10}$ here and in all cases thereafter) data and logged glucose concentration using Genstat Version 16 (VSN

194 International Ltd., Hemel Hempstead, UK). Data from one sheep were excluded when
195 pregnancy became apparent at the time of sample collection. In Study 2, a repeated
196 measures ANOVA was used to compare logged concentrations of the various peptides in
197 CSF, plasma, and CNS tissues in response to dexamethasone or saline solution, as well as the
198 ratio of CNP:NTproCNP concentration in CNS tissues which was calculated for each sheep.
199 Least significant differences were used to identify differences in CNP and NTproCNP
200 concentration between treatment groups in specific CNS tissues. The relation between
201 peptide concentration in tissue (logged) and the percentage difference in peptide
202 concentration after dexamethasone was determined using linear regression analysis in
203 GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, CA, USA,
204 www.graphpad.com). Gene expression levels of *NPPA, NPPB, NPPC*, *NPR1, NPR2* and *NPR3*
205 were compared separately using a repeated measures ANOVA in Genstat Version 16 (VSN
206 International Ltd., Hemel Hempstead, UK) using logged data, and least significant
207 differences were used to identify differences between saline- and dexamethasone-treated
208 sheep for each tissue.

209

210 **Results**

211 All studies were accomplished as planned, and data collection was complete for Study 1. In
212 Study 2, CSF samples were collected from 13 of 14 sheep at baseline, and from 10 of 14
213 sheep following treatment. No adverse events were noted in any animal during either study,
214 nor were any signs of infection evident in cannulated animals.

215 <u>Dose responses to dexamethasone (Study 1).</u>

On the control day (saline injection), plasma and CSF concentration of both CNP and NTproCNP were stable during the 16 h period of sampling. As expected, mean concentrations of CNP and NTproCNP in CSF were higher than in plasma (3.32 ± 0.14 vs 0.94 ± 0.02 pmol/L; 817 ± 24 vs 47.7 ± 1.3 pmol/L respectively, $P < 0.001$ for both). There was no significant association between time matched plasma and CSF concentrations for either peptide. Serial changes in CSF and plasma levels evoked by a range of doses of dexamethasone are shown in Figure 1. Dose dependent increases in both CNP peptides were observed within 4 h in plasma and somewhat later in CSF, although the magnitude and duration of responses differed in the two circulations. Overall, relative responses of plasma CNP and NTproCNP concentration to the highest dose of dexamethasone (8-fold and 6-fold increase respectively) exceeded those in CSF (3.3-fold and 1.5-fold respectively). In CSF both the onset and the offset of responses were delayed relative to those observed in plasma. Further, time to peak CSF concentrations of NTproCNP (Cmax, 16 h) was delayed compared with that of CNP (Cmax 8-12 h) and the NTproCNP concentration in CSF remained above pre injection levels 16 h after dexamethasone administration in 3 of the 4 studies. Whereas a significant increase above control levels of plasma CNP and NTproCNP concentration was generated by the two lowest doses of dexamethasone (0.025 and 0.063 mg/kg, $P < 0.05$), these doses failed to raise levels of these peptides in CSF. All doses of dexamethasone used here elevated glucose levels in plasma sampled 8 h after administration ($P < 0.05$, Supplemental Figure 1) where concentrations had increased from 4.18 ± 0.08 mmol/L (mean plasma glucose concentration of all sheep at baseline, n = 24) to between 5.98 ± 0.11 mmol/L (lowest dose,) and 7.28 ± 0.77 mmol/L (highest dose).

239 CNS and pituitary tissue responses to dexamethasone (Study 2).

240 To ensure that responses in CSF and systemic concentrations of CNP products on the day of
241 tissue collection were comparable to those observed in the dose response study, both
242 peptides were measured in plasma (at 2 hourly intervals over 8 h) and CSF (pre injection and
243 at 8 h after treatment). Concentrations of both peptides prior to injection were similar in
244 saline- and dexamethasone-treated sheep and did not change significantly after saline
245 injection in the 8 h period prior to tissue collection (Figure 2). Again, as in Study 1, significant
246 increases of CNP and NTproCNP concentration in both plasma and CSF were recorded
247 following IV dexamethasone (0.25 mg/kg).

248 Across the wide range of neural tissues examined, both CNP and NTproCNP were detected
249 in all tissue extracts. As shown in Figure 3 A, in control sheep, concentrations of CNP in the
250 brain were highest in limbic tissues (thalamus, hypothalamus, mammillary body), medulla
251 oblongata and pons. Low abundance was observed in the pineal gland, olfactory bulb and
252 occipital cortex. In contrast, CNP concentrations were much higher in the anterior pituitary
253 (28.2 ± 3.5 pmol/g) and posterior pituitary (15.7 ± 4.5 pmol/g) lobes (Figure 3 C). Excepting
254 pituitary tissue, concentrations of NTproCNP (Figure 3 B and D) in tissues from saline
255 injected (control) sheep were almost 10-fold higher (8.3 ± 0.7) than those of CNP.
256 Dexamethasone increased concentrations of both peptides in most tissues examined (Figure
257 3 A and B). Compared with controls, there were significant increments in CNP concentration
258 following dexamethasone in 6 of the 14 selected tissues and those for NTproCNP were
259 significant in 11 of these tissues. The relative abundance of both peptides across brain
260 tissues from controls was largely preserved in tissues extracted from dexamethasone-
261 treated animals. Again, responses in pituitary tissues differed. Neither CNP nor NTproCNP

abundance in the posterior lobe was affected by dexamethasone (Figure 3 C and D). NTproCNP levels in the anterior pituitary lobe were increased after dexamethasone (Figure 3 D), however this was not significant — and in contrast to brain tissues — CNP concentration was unaffected. Considering all 14 tissues, the percentage difference (dexamethasone versus control) in CNP concentration was significantly greater in tissues with relatively low abundance ($r = 0.64$, $P < 0.05$, Supplemental Figure 2). A similar inverse relation was observed for NTproCNP ($r = 0.54$, $P < 0.05$).

Concentrations of the 2 peptides in control sheep were highly correlated in brain ($r = 0.68$, $P < 0.0001$) and pituitary gland ($r = 0.81$, $P < 0.05$), and even more so after dexamethasone: ($r = 0.80$ and 0.88 respectively, $P < 0.0001$ for both). However, as shown in Supplemental Figure 3, across all studies the concentration ratio of NTproCNP to CNP in both pituitary lobes was close to unity (1:1) — much lower than found in brain tissues (5:1 to 10:1).

CNP-related gene expression

NPPC, NPR2, and *NPR3* gene expression in selected tissues from saline- and dexamethasone-treated sheep is shown in Figure 4. *NPPC* expression levels were upregulated by dexamethasone ($P < 0.001$) in 5 of the 7 tissues examined: anterior pituitary, posterior pituitary, hypothalamus, hippocampus and pons. No significant change in *NPPC* expression was found in occipital cortex or olfactory bulb tissue. Except for the downregulation of *NPR2* in hypothalamic tissue following treatment with dexamethasone ($P < 0.05$), changes in *NPR2* and *NPR3* gene expression in the other tissues after dexamethasone were not significant (Figure 4). Despite markedly lower NTCNP:CNP ratios in the anterior and posterior pituitary gland, *NPR3* expression in these tissues did not differ from that in the other regions examined here.

Specificity of CNP responses.

Whether natriuretic peptide responses to dexamethasone are specific to CNP was addressed by measuring the concentration of ANP and BNP in samples obtained in Study 2. As shown in Figure 5, whereas small fluctuations in plasma ANP and BNP concentration were observed after saline or dexamethasone, the mean values for either hormone after dexamethasone (0.25 mg/kg live weight) did not differ from those following the saline treatment. Both ANP and BNP were undetectable in CSF, including samples obtained after dexamethasone. In brain tissue extracts, ANP was undetectable in 29 of 42 samples examined. In 3 tissues where ANP was detectable (olfactory bulb, thalamus and pons) concentrations after dexamethasone (mean 0.84 ± 0.43 pmol/L, n = 6) were low and did not differ from control sheep (mean 0.72 ± 0.30, n = 7). BNP was undetectable in 37 of 39 samples analysed. Gene expression levels of *NPPA*, *NPPB,* and *NPR1* did not differ between dexamethasone- and saline-treated sheep (P = 0.461, 0.702, and 0.22, respectively, n = 6/group) in tissue sampled from the anterior or posterior pituitary gland, hypothalamus, hippocampus, pons, occipital cortex and olfactory bulb (Figure 5).

Discussion

CNP is the most abundant of the natriuretic peptides present in CNS tissues (Ueda *et al.* 1991, Kaneko *et al.* 1993). Whereas its role in the early development and maturation of cerebral neurons (Müller *et al.* 2009), and in axonal branching of sensory neurons entering the CNS (Zhao & Ma 2009) is well-defined, factors regulating CNP production within the brain and related tissues of adults *in vivo* have not been studied. Here we show that a single IV bolus injection of dexamethasone abruptly increases plasma and CSF concentrations of CNP peptides selectively and dose dependently. These changes are associated with marked

increases in peptide concentrations in a wide range of CNS tissues, and upregulation of *NPPC* mRNA expression in 5 of the 7 cranial tissues examined here. Collectively these novel findings suggest that CNP may mediate some of the acute effects of stress on brain function, which now warrants further study.

As part of physiological studies examining fluctuations of CNP peptides in CSF drawn from conscious adult sheep, we found that doses of dexamethasone — at levels that can reduce plasma concentrations of CNP peptides in lambs when administered for several days (Prickett *et al.* 2009) — actually raise concentrations of CNP in CSF during the first 12 h. In more focussed studies we now show that in contrast to relatively unchanged levels that persist after administration of saline, a single IV bolus injection of dexamethasone induces a prompt increase in CNP and NTproCNP concentrations in both plasma and CSF. Peptide concentrations of ANP and BNP were unaffected, and levels of gene expression of *NPPA* and *NPPB* in brain and pituitary tissue did not differ between dexamethasone- and saline-treated animals. As noted in humans (Schouten *et al.* 2011), in the absence of interventions (i.e. saline-treated animals here), concentrations of CNP and NTproCNP were much higher in CSF than in time matched samples of plasma. Lack of any significant correlation between levels in the two circulations suggests independent regulation and little, if any, exchange across the blood-brain barrier (BBB). After dexamethasone, onset of the response in CNP peptide levels occurred earlier in plasma (within 4 h), was dose dependent and of shorter duration than was observed in CSF. Presumably delayed entry of dexamethasone to the CNS (Balis *et al.* 1987), diffusion and bulk flow of CNP peptides from extra-cellular fluid to CSF (Leng & Ludwig 2008) and slower clearance of CNP peptides in CSF (particularly NTproCNP), are the basis of these temporal differences. Sustained elevations of NTproCNP in CSF 16 h after dexamethasone, when plasma levels had returned to baseline, suggests that any

increase in permeability of the BBB by CNP (Bohara *et al.* 2014) is minimal in this experimental setting.

In order to examine likely sources of the response of CNP peptides in CSF, we measured their relative abundance in 12 selected tissues within the CNS 8 h after saline or dexamethasone (0.25 mg/kg live weight) – a dose which strongly stimulated plasma and CSF CNP peptides in these same sheep when compared with the control study. In controls, the relative abundance of CNP peptides in CNS tissues was similar to that previously reported in adult sheep (Pemberton *et al.* 2002), rodents (Jankowski *et al.* 2004) and humans (Komatsu *et al.* 1991, Totsune *et al.* 1994). Highest concentrations were found in tissue sampled from thalamus, hypothalamus, mammillary body and medulla oblongata. Overall, concentrations of the two peptides were highly correlated in brain and pituitary gland of control sheep — with levels of NTproCNP approximately 5–10 fold those of CNP in brain tissue. Of the 12 CNS tissues examined, the concentration of both peptides was higher after dexamethasone when compared with controls — significantly so in 11 and 6 tissues for NTproCNP and CNP respectively. Tissues with lower abundance after saline exhibited proportionately higher levels after dexamethasone (Supplemental Figure 2). This trend was more obvious in the CNP response — possibly reflecting higher rates of secretion (loss to extracellular fluid and CSF) for this peptide, particularly in tissue zones adjacent to cerebral ventricles. Again, after dexamethasone significant associations were observed between CNP and NTproCNP but in 4 tissues (hypothalamus, pineal gland, occipital cortex and medulla oblongata) the ratio of NTproCNP to CNP was significantly increased compared with control values. Presumably higher rates of CNP degradation and/or egress from the neuropil account for these glucocorticoid-induced differences. Viewed in relation to proportionate increases in CSF at 8 h after dexamethasone (0.25 mg/kg live weight) the increase in brain tissue CNP content (2-

4 fold) is commensurate with that observed in CSF (3-4 fold) and lends credibility to the possibility that the changed levels in CSF are a consequence of enhanced secretion from tissues within the CNS. Proof that these changes involve increased synthesis of the peptides is provided by the evidence presented here for upregulation of *NPPC* in the hypothalamus, hippocampus, and pons. In contrast, *NPPC* expression in the occipital cortex and olfactory bulb was not increased by dexamethasone — despite significant increases in both CNP and NTproCNP concentration in these tissues. This discrepancy may relate to differences in mRNA stability (rapid degradation compared with peptide loss) and/or co-production of inhibitory microRNAs.

Whether longer term treatment with dexamethasone could sustain the responses we observed here in CNS tissues is unknown but important to resolve – particularly since plasma levels of CNP peptides are suppressed by prolonged dosing with dexamethasone in growing lambs and children (Prickett *et al.* 2009, Prickett *et al.* 2012a). In the latter studies, contributions of NTproCNP to its plasma concentrations are likely to be sourced from growth plate proliferating chondrocytes (Prickett *et al.* 2005, Prickett *et al.* 2012b) — which are depleted by glucocorticoids (Siebler *et al.* 2002) — so it is not surprising that the temporal responses of CNS tissues to glucocorticoids are likely to vary from those in other body regions. In this context, possible stimulation of CNP by endogenous increases in glucocorticoid secretion also need consideration although previous study showing stable levels of CNP products in both plasma and CSF over periods of 24 h sampling (Wilson *et al.* 2015) make this unlikely.

Although it is not part of the CNS, and is unprotected by the BBB, the pituitary gland in sheep and humans has an unusually high CNP content (Yandle *et al.* 1993, Pemberton *et al.*

2002, Thompson *et al.* 2009), so its potential to contribute to the amount of CNP in plasma or CSF was an important factor in selecting this organ for closer study. Findings in pituitary tissue differ from those in CNS tissues in several respects. First, in control sheep the concentration of CNP is much higher in both anterior and posterior lobes than in any of the regions of the CNS reported here. Second, in both lobes there are equimolar concentrations of CNP and NTproCNP, reducing the ratio of NTproCNP:CNP to unity (1:1) – much lower than that found in CNS tissues (5:1 to 10:1, Supplemental Figure 3). Previous work (Yandle *et al.* 1993) has also shown that the pituitary processing of proCNP is unique in that pituitary tissue contains predominantly CNP-53, contrasting with the presence of equal amounts of CNP-53 and the smaller peptide CNP-22 in hypothalamic extracts. These findings suggest that under physiological conditions different functions are subserved in the pituitary gland — with less degradation and reduced processing being consistent with accumulation and storage. Thirdly, although *NPPC* expression in both lobes is significantly upregulated after dexamethasone, abundance of peptides was not changed. Together these unexpected findings raise the possibility that both peptides are actively secreted from the pituitary gland into the systemic circulation in response to dexamethasone, and thus could make significant contributions to the responses recorded in plasma after IV administered dexamethasone. If so, a disproportionate amount of CNP-53 would be expected to appear in the systemic circulation. More recent studies in our laboratory confirm this and show that the profile of high molecular weight immunoreactive CNP forms in plasma closely reflect the profile found in anterior pituitary tissue extracts (manuscript in preparation). Others have shown that CNP in anterior pituitary tissue appears to be associated with gonadotroph cells (Thompson *et al.* 2009, McArdle *et al.* 1994) as well as other cell lineages in human pituitary adenomas (Thompson *et al.* 2012). Reports that acute stress or glucocorticoid administration stimulate

403 gonadotrophin secretion in some settings (Maeda & Tsukamura 2006) may be relevant in
404 this context. Although we have not found evidence of a CNP arterio-venous concentration
405 gradient across the pituitary gland in samples of plasma drawn from the inferior petrosal
406 vein in human subjects with Cushing's disease (unpublished), further study of the acute
407 effect of glucocorticoids on pituitary secretion of CNP peptides is warranted.

408 Our study was not designed to address either the origins or actions of CNP at the cellular
409 level but these questions become highly relevant in light of the present findings – and of the
410 manifold effects of glucocorticoids on brain function (Wolkowitz *et al.* 2009). Notably, CNP
411 responses in CSF were observed after a dexamethasone dose of 0.125 mg/kg live weight –
412 which corresponds to the therapeutic range for adult humans. The wide array of
413 glucocorticoid-responsive CNS tissues identified here suggests that commonalities – such as
414 capillary networks (Vigne & Frelin 1992) or glial tissues (Parpura & Zorec 2010) – all of which
415 are recognised sites of CNP production – are likely to be involved. One possible action of
416 dexamethasone, increasing the CNP mRNA response to shear stress in brain capillaries
417 (Zhang *et al.* 1999), might account for the present results. In cultured murine cerebral cortex
418 neurons, activation of voltage-sensitive calcium channels during potassium-induced
419 depolarisation strongly upregulates *NPPC* expression over a 6 h period (Kim *et al.* 2010).
420 Since glucocorticoids specifically enhance L-type calcium channel amplitude in a variety of
421 neurons sourced from brain tissues (Joëls & Karst 2012), the glucocorticoid-induced
422 increases in CNP we observed may have resulted from such membrane-level events
423 (Wolkowitz *et al.* 2009). Responses from astrocytes or microglia — ubiquitous in CNS tissues
424 and an important source of natriuretic peptides, including CNP (Deschepper 1998) – may
425 also have contributed to these findings. Of note, dexamethasone elicits a dose dependent
426 CNP response from cells of monocyte/macrophage lineage (Kubo *et al.* 2001) which, within

the BBB, constitute the microglia. To our knowledge, no glucocorticoid response element has been identified in the CNP gene in any species. Interestingly, glucocorticoids act directly and specifically to increase ANP gene transcription in rodent cardiomyocytes (Gardner *et al.* 1988) and other tissues (Gardner *et al.* 1986) yet dexamethasone did not alter *NPPA* (or *NPPB*) expression in any of the central tissues studied here, and we found no evidence that ANP or BNP concentration is affected by dexamethasone in either the systemic circulation or CNS tissues. In further support of a CNP-specific action of dexamethasone in central tissues, the expression of the ANP and BNP receptor, *NPR1*, was also unaffected. Concerning possible actions of CNP in glial tissues, there is a strong body of evidence that cGMP, a downstream mediator of CNP activity and more responsive to CNP than either ANP or BNP (Deschepper & Picard 1994), regulates several crucial intercellular actions including Na^+/H^+ exchangers, neurotransmitter re-uptake, gap junctions, cell pH and brain cell water content (Kim *et al.* 2010). In this context, it is important to note that dexamethasone mitigates cerebral glioma tumour oedema – a cell type highly responsive to CNP (Eguchi *et al.* 1992, Wu *et al.* 2017). Conceivably, this well-described pharmacological action of dexamethasone is mediated at least in part by CNP which could have therapeutic implications now that CNP agonists are available for use in humans.

Declaration of interest

E.A.E. is a consultant for BioMarin Pharmaceutical. The authors have no conflicts of interest to disclose.

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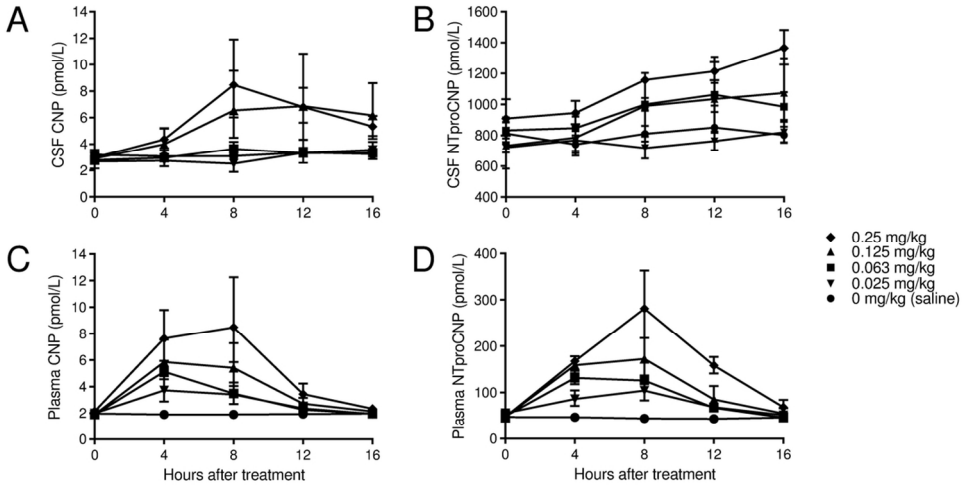

Figure 1. Mean CNP (left) and NTproCNP (right) concentration in CSF (A and B) and plasma (C and D) of sheep following different doses of dexamethasone: 0 (saline), 0.025, 0.063, 0.125, 0.25 mg/kg live weight. Data are presented as geometric means. n = 8 (saline) and n = 4 per dexamethasone dose level.

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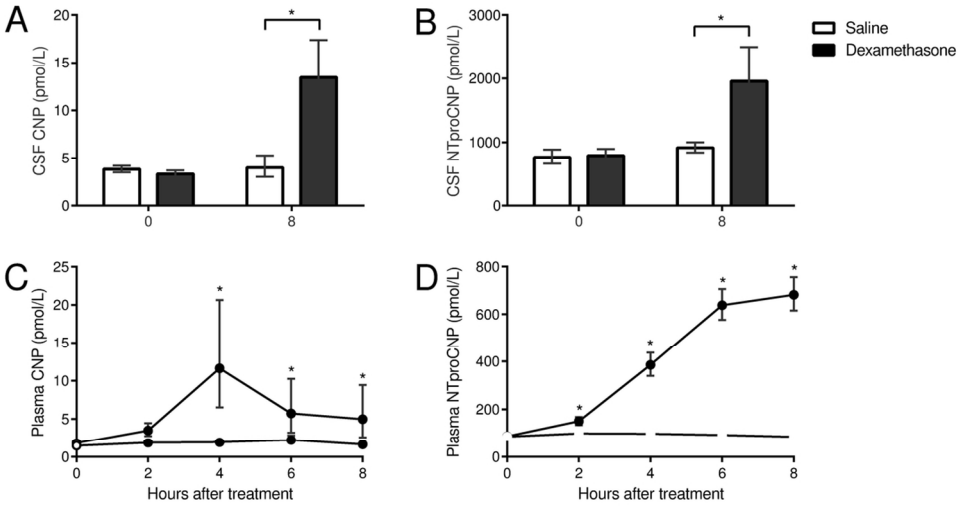

Figure 2. Mean CNP (left) and NTproCNP (right) concentration in CSF (A and B) and plasma (C and D) of sheep treated with saline solution (open bars/circles) or dexamethasone (closed bars/circles). Data are presented as geometric means. In CSF, n = 6 (saline) and n = 7 (dexamethasone) per group at 0 h, n = 5 per group at 8 h. In plasma, n = 7 per group. *significant difference between groups, $P < 0.05$.

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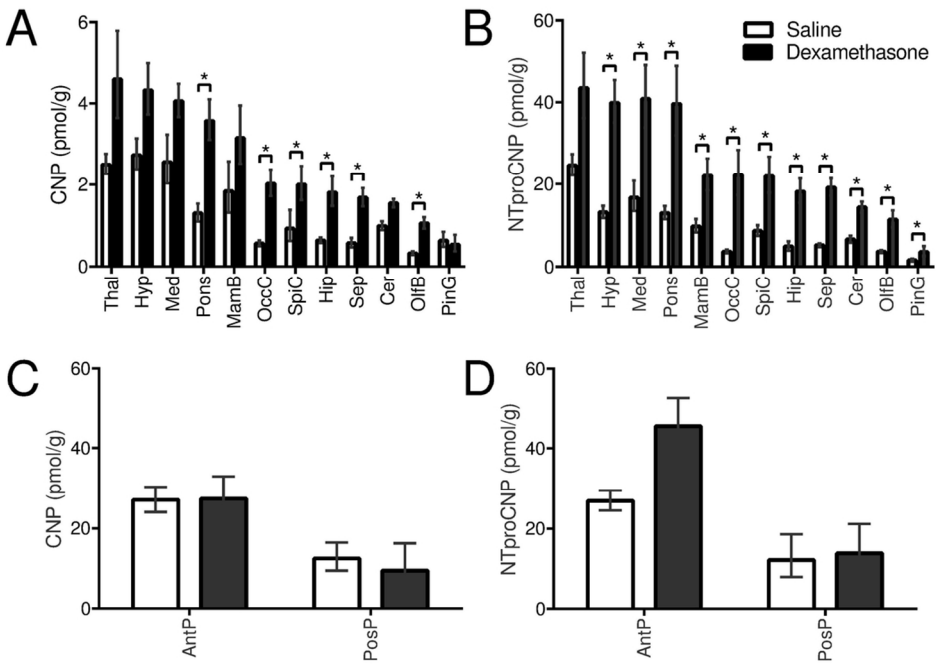

Figure 3. Mean concentration (wet weight basis) of CNP (left panels) and NTCNP (right panels) in brain (A and B) and pituitary gland (C and D) tissues in saline- (open bars) and dexamethasone-treated sheep (filled bars) 8 h following treatment. Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$ Tissue regions are abbreviated to the following: AntP (anterior pituitary gland), PosP (posterior pituitary gland), Thal (thalamus), Hyp (hypothalamus), Med (medulla oblongata), Pons (Pons), MamB (mammillary body), OccC (occipital cortex), SpiC (spinal cord), Hip (hippocampus), Sep (septum), Cer (cerebellum), OlfB (olfactory bulb), PinG (pineal gland).

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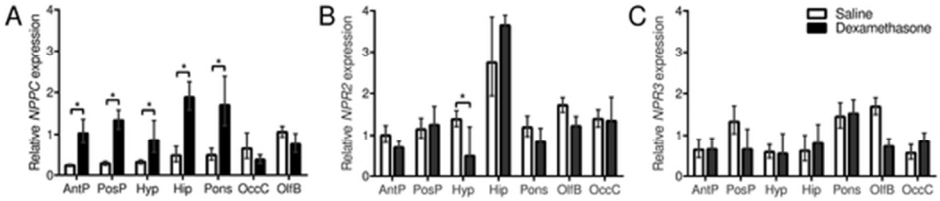

Figure 4. Relative gene expression of A) NPPC B) NPR2 and C) NPR3 in tissues from brain and pituitary gland of sheep obtained at 8 h following treatment with IV saline solution (open bars) or a single IV dose of dexamethasone (closed bars). Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$. See Figure 3 for abbreviations.

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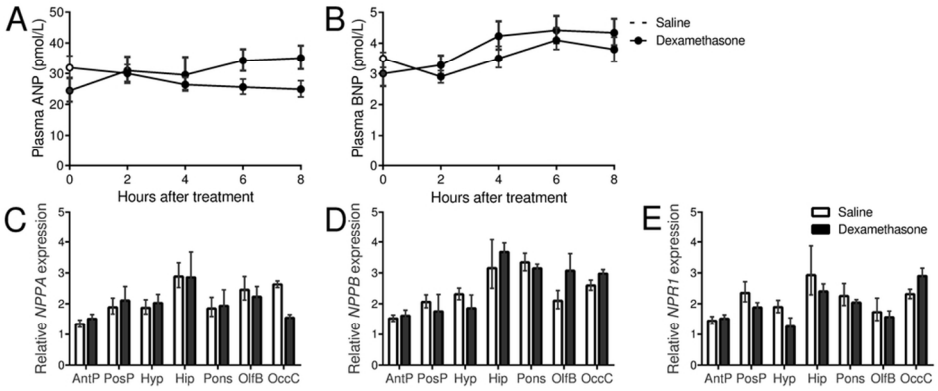

Figure 5. Mean A) plasma ANP concentration, B) plasma BNP concentration and mean relative gene expression of C) NPPA, D) NPPB and E) NPR1 in brain and pituitary tissues in sheep treated with saline solution (open circles/bars) or dexamethasone (filled circles/bars). Data are presented as geometric means, n = 7 per group. See Figure 3 for abbreviations.

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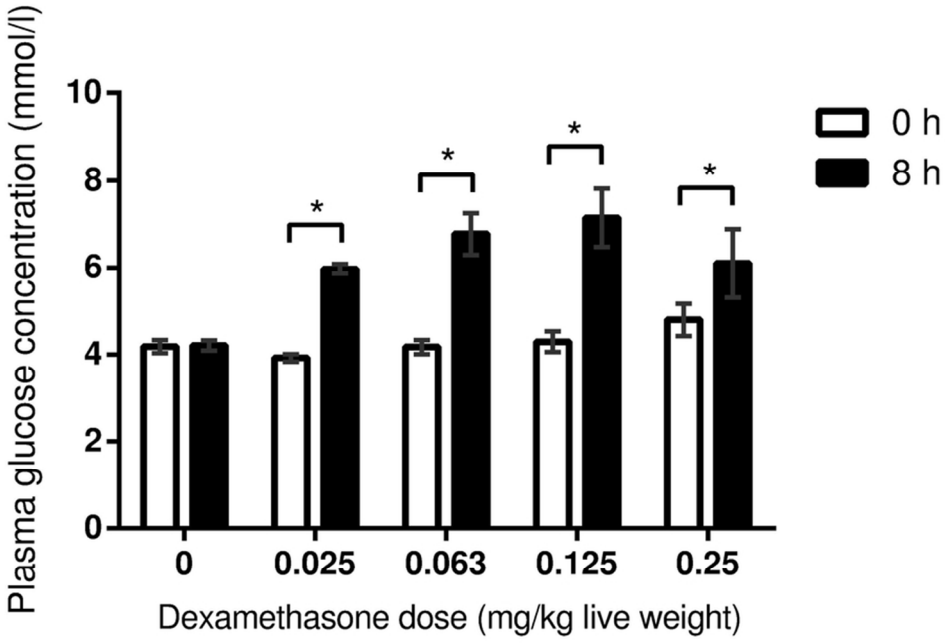

Supplemental Figure 1. Mean glucose concentration (± s.e.) in plasma from sheep immediately before (open bars) and 8 h after (closed bars) i.v. injection with saline solution (n = 8 per group) or dexamethasone (n = 4 per group). *significant difference between groups, $P < 0.05$.

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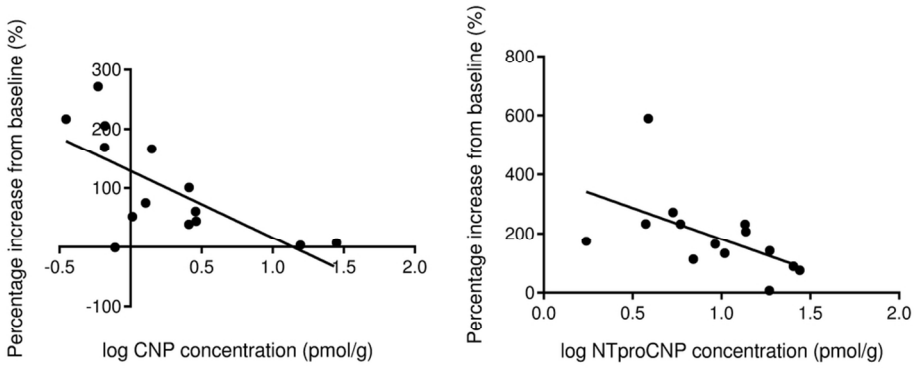

Supplemental Figure 2. Relationship of the percentage increase in mean log10 CNP (left) and NTproCNP (right) concentration following dexamethasone (n = 7) above that of saline-treated sheep (n = 7) for 14 cranial tissues.

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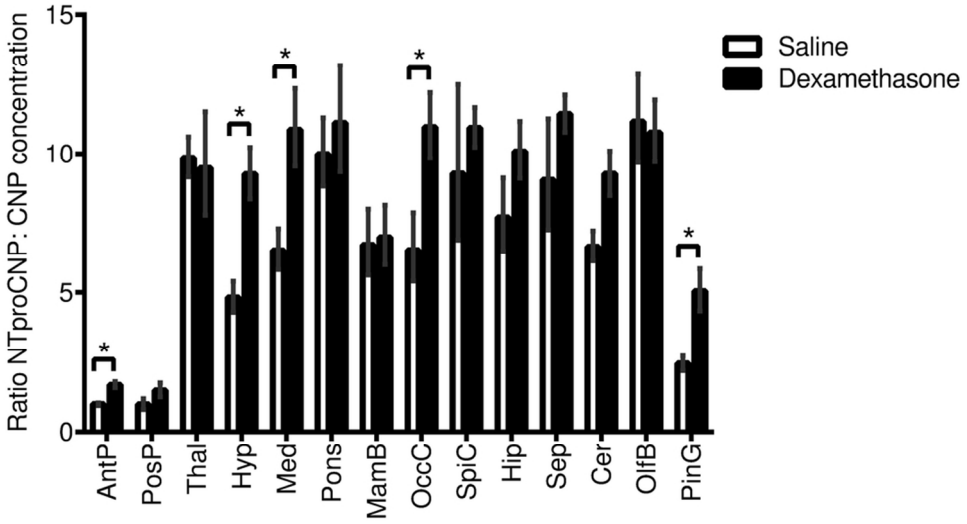

Supplemental Figure 3. Mean ratio of NTproCNP:CNP in 14 cranial tissues from saline- (open bars) and dexamethasone-treated (filled bars) sheep obtained at 8 h following treatment. Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$. See Figure 3 for abbreviations.

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