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REVIEW ARTICLE

Novel Aspects of Glucocorticoid Actions

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Normal hypothalamic-pituitary-adrenal (HPA) axis activity leading to the rhythmic and episodic release of adrenal glucocorticoids (GCs) is essential for body homeostasis and survival during stress. Acting through specific intracellular receptors in the brain and periphery, GCs regulate behaviour, as well as metabolic, cardiovascular, immune and neuroendocrine activities. By contrast to chronic elevated levels, circadian and acute stress-induced increases in GCs are necessary for hippocampal neuronal survival and memory acquisition and consolidation, as a result of the inhibition of apoptosis, the facilitation of glutamatergic neurotransmission and the formation of excitatory synapses, and the induction of immediate early genes and dendritic spine formation. In addition to metabolic actions leading to increased energy availability, GCs have profound effects on feeding behaviour, mainly via the modulation of orexigenic and anorixegenic neuropeptides. Evidence is also emerging that, in addition to the recognised immune suppressive actions of GCs by counteracting adrenergic pro-inflammatory actions, circadian elevations have priming effects in the immune system, potentiating acute defensive responses. In addition, negative-feedback by GCs involves multiple mechanisms leading to limited HPA axis activation and prevention of the deleterious effects of excessive GC production. Adequate GC secretion to meet body demands is tightly regulated by a complex neural circuitry controlling hypothalamic corticotrophin-releasing hormone (CRH) and vasopressin secretion, which are the main regulators of pituitary adrenocorticotrophic hormone (ACTH). Rapid feedback mechanisms, likely involving nongenomic actions of GCs, mediate the immediate inhibition of hypothalamic CRH and ACTH secretion, whereas intermediate and delayed mechanisms mediated by genomic actions involve the modulation of limbic circuitry and peripheral metabolic messengers. Consistent with their key adaptive roles, HPA axis components are evolutionarily conserved, being present in the earliest vertebrates. An understanding of these basic mechanisms may lead to novel approaches for the development of diagnostic and therapeutic tools for disorders related to stress and alterations of GC secretion.

Key words: glucocorticoids, feedback, HPA axis, neuroplasticity, immune system, food intake

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Living organisms are constantly faced with external and internal challenges or stressors threatening the internal environment equilibrium or homeostasis (1). Adaptation to this changing environment requires the coordinated activation of multiple neuroendocrine responses, prominently including activation of the hypothalamic pituitary adrenal (HPA) axis (2). Control of HPA axis activity is complex, involving sensors conveying signals to corticotrophin-releasing hormone (CRH) neurones in the hypothalamic paraventricular nucleus (PVN), either through direct neural connections, or indirectly through pathways relaying on limbic structures such as the amygdala, frontal cortex, bed nucleus of the stria terminalis and hippocampus (2). CRH and vasopressin (AVP),

released from parvocellular neurones of the PVN into the pituitary portal circulation, increase the secretion of adrenocorticotrophic hormone (ACTH) from pituitary corticotrophs, which in turn stimulates glucocorticoid (GC) secretion from the adrenal. The HPA axis is highly conserved through evolution, with its components being present in early vertebrates. The secretion of GCs (i.e. cortisol in humans, corticosterone in rats and mice) is episodic, following circadian (daily) and ultradian (hourly) rhythms, and shows marked but transient increases after exposure to stressors. This episodic nature of GC secretion is critical for the transcriptional activity of the steroid (3).

Acting through intracellular receptors of the nuclear receptor family, GCs are essential for stress adaptation by controlling energy supply through the stimulation of glycolysis, gluconeogenesis and lipolysis, as well as inducing proteolysis to supply amino acids as substrates for gluconeogenesis. In addition, GCs modulate the immune system, as well as the synthesis and action of a number of hormones. By acting directly in neurones or by modulating the synthesis, secretion or/and activity of neuropeptides and neurotransmitters, GCs affect memory acquisition and consolidation, and impact upon the function of other neuroendocrine systems, such as the central control of metabolic activity, feeding behaviour and reproductive activity. Given these wide ranging effects of GCs, either GC deficiency or a failure to limit HPA axis activation will have profound consequences on the well-being of an organism. An important mechanism for maintaining episodic HPA axis activation and for limiting HPA axis activity comprises negative-feedback by GCs, resulting in the inhibition of expression and secretion of hypothalamic CRH and AVP, as well as pituitary ACTH.

This review covers novel aspects concerning the regulation and function of GCs, including the critical importance of adequate GC regulation on synaptic remodelling, interactions between the HPA axis and inflammatory processes, and other neuroendocrine systems, such as the control of appetite, as well as molecular mechanisms, brain and peripheral circuitries involved in HPA axis regulation and GC feedback.

GC receptors and mineralocorticoid receptors (MRs)

The action of GCs involves binding evolutionary conserved intracellular receptors belonging to the nuclear receptor family, GC receptors type 1 or MR, and type 2 or GC receptor (GR) (4). The genes of MRs and GRs are considered to have arisen from a common ancestor, the corticoid receptor, before the evolution of aldosterone (5). These receptors are ligand activated transcription factors, which, upon ligand binding, translocate to the nucleus and interact with responsive elements in gene promoters or interact with other transcription factors, inducing transcriptional activation or repression. Under basal conditions, GRs and MRs are located in the cytoplasm and are associated with the chaperone proteins, heat shock proteins (HSP) 90 and 70, and co-chaperones such as the immunophilin, FK506 binding protein 5. HSP90 and other chaperone and co-chaperone proteins are part of the mechanism of receptor activation and translocation, as well as the subsequent GR transactivation effects (6).

In addition to these genomic actions regulating gene transcription, there is clear evidence that GRs and MRs also mediate nongenomic responses of their ligands. Some biological actions of GCs are too fast to be mediated by genomic actions (which require protein synthesis) and they are assumed to depend on membrane receptors (7,8). As indicated above, GCs are critical for body homeostasis and act through GRs and MRs; thus, the well-adjusted activation of these receptors is crucial for maintaining homeostasis.

GC receptors are widely expressed in the central nervous system, including the hypothalamus and hippocampus. Under normal conditions, GRs are essentially ligand-free at the nadir of the circadian rhythm and largely occupied by peak plasma levels of GCs. Similarly, GRs are occupied in situations of stress, mediating negative-feedback, which regulates HPA responsiveness (9).

MRs are expressed in the kidney, where they mediate sodium reabsorption, as well as other epithelial and non-epithelial tissues, in which the function of MRs remains to be clearly determined (10). Although MRs have equally high affinity to GCs and mineralocorticoids, the presence of 11-β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in mineralocorticoid target tissues protects MR from the much higher circulating levels of GCs, by converting GCs to inactive 11-keto steroids (10). MRs are also expressed in the central nervous system (CNS), mostly in limbic sites. The hippocampus is the main limbic structure that expresses MRs and, in conjunction with other regions of the CNS, expresses the type 1 isozyme of 11β-HSD, which regenerates active GCs from the circulating inert 11-keto steroids. Consequently, at this site, MRs bind to GCs with a 10-fold higher affinity than GRs. Because of the high affinity for GCs, and the higher circulating concentrations of GCs compared to mineralocorticoids, MR in the hippocampus are occupied by basal and low concentrations of GCs. MRs of the hippocampus plays an important role in the GC-mediated feedback control of the HPA axis and it is assumed that they are involved in the maintenance of the basal HPA activity, mainly at the nadir of the circadian rhythm when hippocampal MRs are significantly occupied (11). The type 2 isozyme of 11β-HSD is not detectable at limbic sites, and co-localisation of 11β-HSD2 and MRs has been identified only in the nucleus of the solitary tract (NTS), an area related to cardiovascular regulation and sodium appetite (12). Thus, although the NTS appears to be the only mineralocorticoid dependent site in the brain, activation of MRs in other brain areas are mediated by GCs and not mineralocorticoids.

Overall, in the context of the regulation of the HPA axis, the available evidence indicates that MRs are involved in the feedback of GCs during the phase of the nadir of the circadian rhythm, whereas increasing levels of GCs recruit GRs during the reactive feedback and during stressful episodes.

Molecular mechanisms of GC feedback

Inhibitory feedback by GCs at the central and pituitary levels plays a major role in reducing HPA axis responses to stress. As discussed below, an important mechanism for GC feedback involves the modulation of direct and indirect circuitry controlling CRH neurone activity. GCs inhibit CRH expression (13) and secretion (14) in the

PVN, and inhibit both ACTH output (15,16) and the transcription of the precursor protein pro-opiomelanocortin (POMC) (17,18) in the anterior pituitary corticotroph. Concerning transcriptional regulation, it is clear that inhibition of POMC transcription by GCs is a genomic effect dependent on the interaction of GR with the POMC promoter (17,18). However, the mechanism by which GCs inhibit CRH transcription is less obvious (19). In addition, there is evidence that GCs may influence translational activity and mRNA stability of both POMC and CRH mRNA (20,21).

In vitro studies performed in primary cultures of rat anterior pituitary or hypothalamic neurones have shown marked differences between the effects of GCs on CRH and POMC transcription. In these experiments, transcription rates were assessed by measuring levels of primary transcript or heteronuclear RNA (hnRNA). As shown in Fig. 1(A), preincubation of primary cultures of rat anterior pituitary cells with 100 nM corticosterone for 30 min before the addition of 1 nM CRH completely prevented the stimulatory effect of CRH on POMC hnRNA. Full inhibition of CRH-stimulated POMC transcription persisted 18 h after addition of corticosterone. By contrast, exposure of primary cultures of foetal rat hypothalamic neurones to corticosterone had only minor effects of cyclic AMP-stimulated CRH hnRNA production. In these experiments, 7-day neuronal cultures maintained for 48 h in steroid-free culture medium were exposed to 100 nM corticosterone before incubation with the adenylate cyclase stimulator, forskolin, for an additional 45 min. As seen in Fig. 1(B), corticosterone tended to inhibit forskolin-stimulated CRH hnRNA in cells preincubated with corticosterone for 30 min, an inhibition that was statistically significant only after log transformation of the data (22). Similarly, the administration of corticosterone doses that increased the plasma concentration to 100-fold stress levels in adrenalectomised rats did not affect the magnitude or duration of the increase in CRH hnRNA in the PVN in response to a mild stress (22,23) (Fig. 2A). In the same rats, the injection of corticosterone markedly attenuated stress-induced AVP hnRNA levels in parvocellular neurones (Fig. 2B) (23). Similarly, Kovacs and Sawchenko (24) showed that the injection of corticosterone 10 min before ether stress in rats failed to inhibit stress induced increases in CRH hnRNA.

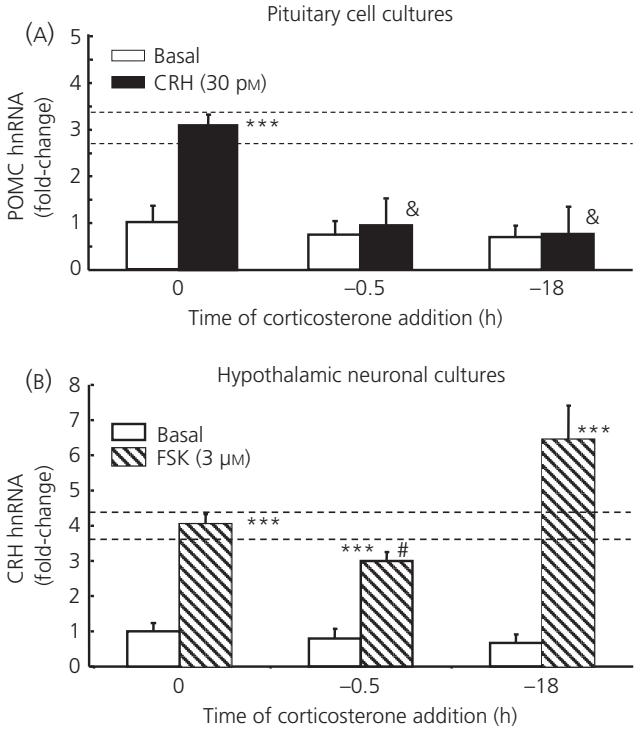

Fig. 1. Effect of corticosterone on corticotrophin-releasing hormone (CRH)-stimulated pro-opiomelanocortin (POMC) heteronuclear RNA (hnRNA) in primary cultures of rat anterior pituitary cells (A) and forskolin-stimulated CRH hnRNA in primary cultures of hypothalamic neurones (B). Three-day cultured trypsin-dispersed anterior pituitary cells, maintained in stripped serum for 36 h were exposed to 100 nM corticosterone for 18 or 30 min before the addition of CRH 30 pM for an additional 30 min. Bars represent the mean and SE of POMC hnRNA levels determined by a quantitative reverse trnascriptase-polymerase chain reaction in three cell preparations. In (B), 10-day cultured foetal rat hypothalamic neuronal cultures were exposed to 100 nM corticosterone for 18 h or 30 min before addition of forskolin (FSK) for an additional 45 min before RNA preparation. Data points are the mean and SE of CRH hnRNA levels, normalised to GAPDH mRNA in four experiments. ***$P < 0.001$ compared to basal; #$P < 0.05$ lower than Fsk at 0 min after log transformation of the data. &$P < 0.001$ versus CRH at time 0. The horizontal dashed lined represent the SE of maximal stimulated values in the absence of corticosterone.

Although the above studies suggest that GCs have little effect on CRH transcription, GRs are present in the CRH neurone (25,26) and there is clear evidence that GCs negatively regulate CRH mRNA levels. For example, removal of endogenous GCs by adrenalectomy markedly increased CRH mRNA and peptide content in the PVN (27,28), and also potentiated the stimulatory effect of stress on CRH transcription (29). GC administration, systemic or directly, in the PVN region had the converse effect (13,30). Also, it is clear from *in situ* hybridisation studies that chronic GC administration inhibits basal and stress-stimulated CRH transcription (30). However, from the above evidence, it is not clear whether GCs repress CRH transcription directly through interaction of GR with the CRH promoter.

Several experiments have been performed to clarify the mechanisms underlying GC suppression of CRH transcription. Indeed, no classical GC response element in the CRH promoter has been reported in the literature but, by using *in vitro* systems (e.g. reporter gene assays, gel shift assays), Malkoski and Dorin (31,32) characterised a conserved sequence located closely upstream of the essential cyclic AMP response element (CRE) of the CRH promoter, capable of mediating repression of promoter activity by GCs. However, other studies have not confirmed the functional activity of this site and show that the repressor effect of GCs requires the CRH promoter CRE (33), suggesting that the effect is mediated by protein–protein interactions. Because the interpretation of data based on reporter gene assays or gel shift analyses can be questionable as a result of the lack of context of construct DNA with the chromatin landscape, studies were also conducted aiming to examine the interaction of GR with the proximal CRH promoter during physiological changes in circulating GCs (22). Restraint stress in rats causes marked increases in circulating corticosterone (Fig. 3A), which is associated with transient increases in CRH hnRNA in the PVN (29,34–36) (Fig. 3B), suggesting that the declining phase

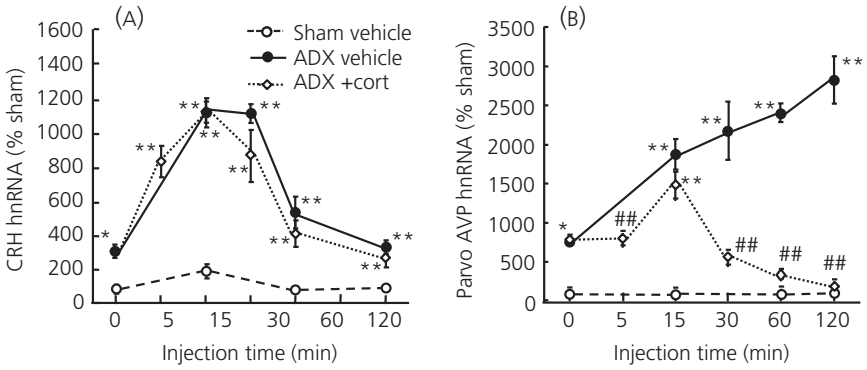

Fig. 2. Time course of the changes in corticotrophin-releasing hormone (CRH) heteronuclear RNA (hnRNA) (A) and vasopressin (AVP) hnRNA (B) after injection of corticosterone (2.8 mg/100 g body weight, i.p.) or vehicle in 48-h adrenalectomised (ADX) or sham-operated rats. Note that vehicle injection caused marked increases in CRH hnRNA in ADX but not in intact rats. Data points are the mean ± SE of the optical density values obtained from *in situ* hybridisation film autoradiograms in six rats per experimental group. *P < 0.01 versus sham; **P < 0.001 versus time 0 and sham; ##P < 0.01 lower than ADX vehicle. From Ma and Aguilera (23).

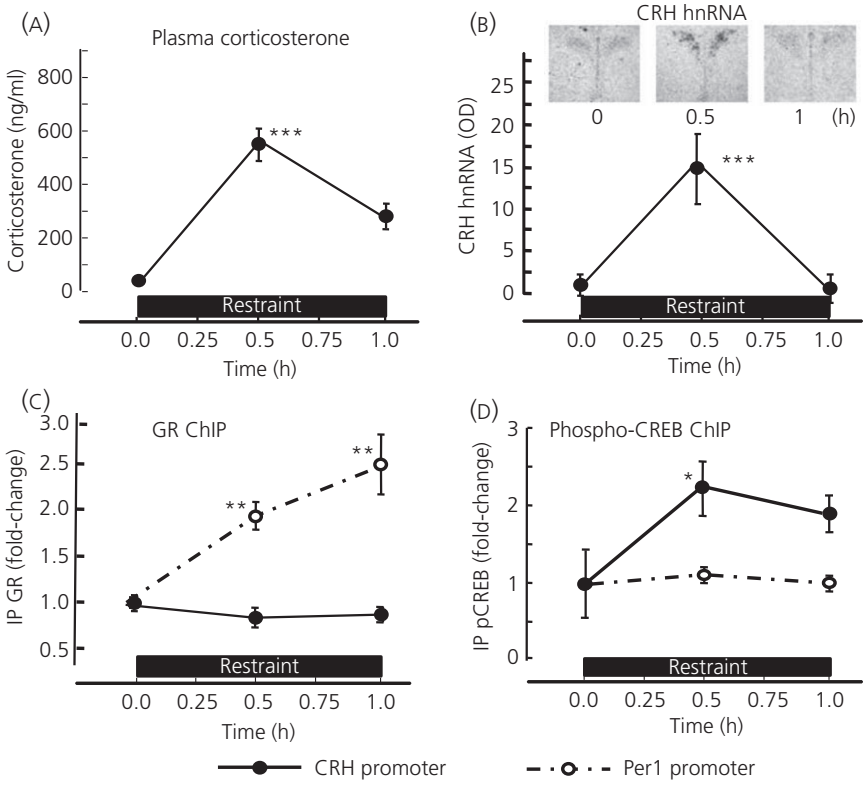

Fig. 3. Effect of restraint stress on plasma corticosterone, corticotrophin-releasing hormone (CRH) heteronuclear RNA (hnRNA) levels in the paraventricular nucleus (PVN) (B) and GC receptor recruitment by the CRH and Per1 promoters in intact male rats. Restraint stress caused marked increases in plasma corticosterone (A), associated with transient increases in CRH hnRNA in the PVN (B). CRH hnRNA was measured by *in situ* hybridisation expressed as optical density (OD) of film autoradiographs (representative images are displayed at the top of data points showing the pooled values in six rats). Chromatin immunoprecipitation (ChIP) of microdissected hypothalamic PVN region using anti-glucocorticoid receptor (GR) antibody (IP GR) shows no association of GR with the CRH promoter (solid line and circles) but marked immunoprecipitation of Per1 promoter (dashed lines open circles) by GR antibodies (C). Immunoprecipitation with phospho-CREB antibody (IP pCREB) yielded high CRH but not Per1 promoter (D). Measurements were performed under basal conditions (time 0), 0.5 and 1 h during stress. Data points are the mean ± SE of the results of three experiments (using pooled hypothalamic tissue from three rats per experimental group). The dashed lines correspond to the Per1 promoter, and solid lines show different regions of the CRH promoter. The restraint stress period is shown by the horizontal boxes above the x-axis. ***P < 0.001 versus respective basal; **P < 0.01 versus respective basal; *P < 0.05 versus respective basal.

of transcription is a result of repression by elevated GCs. However, chromatin immunoprecipitation assays failed to identify changes in CRH promoter in chromatin pulled down by a GR antibody cocktail, at the same time as detecting marked increases in period 1 (Per1) promoter, a recognised GC dependent gene (22) (Fig. 3c). By contrast, immunoprecipitation with phospho-CREB antibody yielded the expected increases in CRH promoter at 30 min during restraint stress (22) (Fig. 3d). A similar lack of change in CRH promoter immunoprecipitation was observed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) primers targeting regions up to 2 kb upstream from the transcription start site (22). In the same study, immunoprecipitation of hypothalamic chromatin from intact or adrenalectomised rats subjected to corticosterone injections showed minor association of the proximal CRH promoter with GR compared to phospho-CREB (22). The above data suggest that repression of CRH transcription by GCs is not mediated by interaction of GR with the proximal CRH promoter. However, a possible interaction of GR with another protein in the transcriptional complex cannot be ruled out because a short-arm cross-linker, formaldehyde, was used to stabilise GR–DNA interactions before sonication and immunoprecipitation. Also, interaction of GR with sites upstream of the 2000 bp that was scanned could lead to changes in chromatin configuration and interaction with the CRE region in the proximal promoter, as has been described for a number of genes (37–39).

CRH transcription depends upon activation and binding to the CRH promoter CRE of phospho-CREB and its co-activator, transducer of regulated CREB activity (TORC) (40,41), and it is also possible that GCs interfere with the activation and binding of these proteins to the CRH promoter. In this regard, GC administration to rats or mice abolishes stress-induced increases in phospho-CREB immunoreactivity in the PVN and prevents nuclear translocation of TORC (42). However, this effect could reflect the inhibition of afferent pathways to the PVN, rather than direct effects on the CRH neurone, because several studies show that GCs do not inhibit nuclear accumulation of phospho-CREB *in vitro* (22,43,44). A recent study reported that corticosterone has no effect on the activation and nuclear translocation of TORC in the hypothalamic cell line 4B (22), suggesting that the inhibition of TORC translocation by GCs reported in mice depends on modulation of afferent inputs to the PVN neurone.

Although it is generally assumed that GCs repress CRH transcription by interacting with the putative negative GC response element in the proximal promoter, accumulating evidence suggests that such an interaction does not occur during physiological elevations of the natural GC, corticosterone. The minor effects of GCs on CRH transcription compared to the marked transcriptional repressor activity on AVP in parvocellular neurones and pituitary POMC strongly suggest that the major mechanism by which GCs repress CRH transcription is indirect, as a result of the modulation of neural pathways controlling CRH neurone function.

Novel mechanisms of GC feedback signalling

As already noted, the HPA axis is reliably activated by psychogenic or systemic stressors, and the largely catabolic actions of high GC levels mandate mechanisms to limit overexposure. This is accomplished by negative-feedback inhibition of hormone release, which is regulated in large part by the GR (as a result of its binding capacity). Whereas the genomic effects of GCs contribute to long-term inhibition of ACTH release (so-called 'delayed feedback'), the minute-to-minute control needed to constrain HPA axis function is likely to require nongenomic mechanisms. Consequently, GC feedback appears to be modulated by multiple processes that converge to limit activation of the HPA axis by inhibition of PVN neurones driving ACTH release. Here, we discuss three recently-delineated feedback processes that highlight the richness of the biology of GC signalling, including rapid PVN feedback mediated by retrograde messengers, a brainstem feedback pathway that appears to involve modulation of RNA stability, and peripheral mechanisms that capitalise on the interaction of GCs with metabolic effector pathways (Fig. 4).

GC fast feedback

Rapid effects of GCs were observed as early as the 1960s, primarily in the form of rapid feedback inhibition of GC release following stress (45). Rapid GC feedback inhibition is nongenomic in nature, and occurs at time delays consistent with possible membrane actions. Work by Tasker and colleagues has demonstrated that GCs rapidly inhibit PVN by way of a membrane-associated receptor (46,47). The mechanism of rapid inhibitory action is accomplished by postsynaptic G-protein-mediated release of endocannabinoids (46). Anandamide and 2-arachidonoylglycerol are rapidly synthesised following GC exposure or acute stress in the PVN, and act as

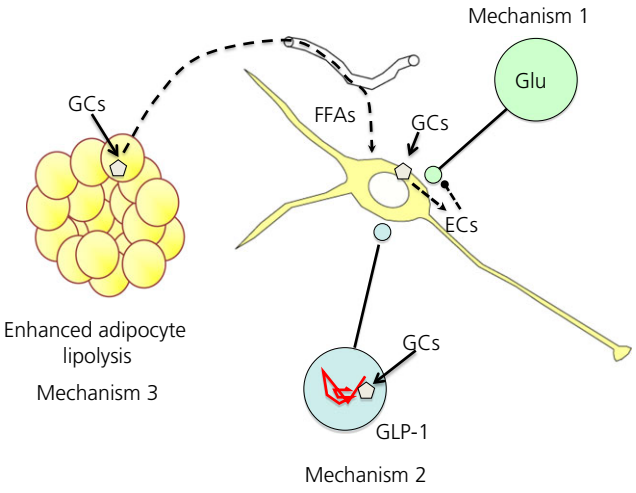

Fig. 4. Proposed 'nontraditional' glucocorticoid (GC) feedback mechanisms. Mechanism 1: GCs act via nongenomic mechanisms to inhibit paraventricular nucleus corticotrophin-releasing hormone (CRH) neurones, acting via membrane GC receptors (pentagon) to mobilise endocannabinoids (ECs), which bind to CB1 receptors and inhibit presaynaptic glutamate release. Mechanism 2: GCs act to destabilise preproglucagon mRNA, reducing the magnitude of glucacon-like peptide 1 (GLP-1) excitation of CRH neurones. Mechanism 3: GCs act in the periphery (possibly at adipocytes) to generate inhibitory messengers, such as free fatty acids (FFAs), which can inhibit hypothalamic-pituitary-adrenal (HPA) axis activation secondarily.

retrograde messengers by binding to presynaptic CB1 receptors (46). A role for endocannabinoids in GC inhibition has been demonstrated *in vitro* and *in vivo* because CB1 antagonism or knockout (KO) leads to elevated CRH expression in the PVN and increased plasma ACTH and corticosterone (48–51). Furthermore, bilateral PVN injections of dexamethasone and a CB1 inverse agonist block the suppression of HPA axis responses to acute stress seen following dexamethasone administration alone (52).

Although the receptor mechanism mediating GC signals at the membrane has not been completely worked out, most of the evidence suggests that the effects are associated with the 'classical' GR molecule. Pre-administration of the GR antagonist mifepristone is not sufficient to completely block the inhibitory effects of dexamethasone *in vitro* (46). However, rapid GC feedback is blocked in slices from mice with targeted deletion of the GR in PVN neurones (generated by breeding transgenic mice with cre recombinase expression driven by the *simple-minded-1* promoter with mice engineered to have exon 2 of the GR gene flanked by loxP sequences) (53). Moreover, GR can be localised to neuronal postsynaptic membranes, supporting membrane localisation of the so-called 'nuclear' receptor (54–57). Finally, PVN administration of dexamethasone-bovine serum albumin conjugate, which should not cross the cell membrane, inhibits stress-induced HPA axis activation *in vivo* (52).

Intermediate time-frame: rapid GC inhibition of ascending stress effector pathways

The medial parvocellular PVN receives synaptic innervation from ascending stress-regulatory neurones in the caudal medulla and locus coeruleus (58,59). Most of the innervation of the CRH-containing subregion of the PVN comes from the NTS (58,59). There is a strong body of evidence suggesting that NTS norepinephrine–epinephrine neurones are involved in excitation of PVN neurones controlling HPA axis responses to stress. Stress causes norepinephrine release in the PVN (60) and local norepinephrine administration is sufficient to cause ACTH release and enhance the expression of *crh* gene transcription (61,62). Moreover, local α-adrenergic receptor blockade reduces stress-induced HPA axis activation (61). Recent data suggest that HPA axis activation is also mediated by noncatecholaminergic projections from the NTS, emanating from neurones expressing glucagon-like peptide 1 (GLP-1). These neurones send GLP-1ergic projections to CRH neurones (63,64). Blockade of GLP-1 receptors reduces ACTH and corticosterone responses to acute physiological or psychogenic stressors, and local infusion of GLP-1 into the PVN causes the release of corticosterone (65), emphasising the need for GLP-1 in acute stress reactivity. Moreover, central infusion of a GLP-1 receptor antagonist reduces the impact of chronic stress exposure on HPA axis end points (66). Taken together, the data suggest a prominent role for GLP-1 in stress excitation at the level of the PVN.

Recent data suggest that the GLP-1 system is also a target for GC feedback. Experiments were performed to assess the regulation of NTS stress-excitatory pathways following exposure to acute stress regimens. Surprisingly, a very rapid depletion of preproglucagon (PPG; the GLP-1 precursor protein) mRNA is observed following acute stress, with mRNA levels falling to less than 50% of basal values within 20–30 min of stress onset (67). This rapid down-regulation is mimicked by exogenous corticosterone administration. Moreover, stress-induced down-regulation is blocked in adrenalectomised animals clamped at basal circulating corticosterone levels (67), indicating that PPG down-regulation was mediated by stress-related GC release. Importantly, PPG hnRNA was substantially increased by acute stress exposure, suggesting that changes in PPG mRNA were not a result of decreased transcription. Reductions in PPG mRNA are relevant at the level of the synapse because stress causes a rapid decrement in PVN GLP-1 immunoreactivity (67), perhaps as a consequence of synaptic release associated with the stressor. Given the rapid timing of the stress-induced PPG decrement, we hypothesise that the effects of stress are mediated by rapid, presumably nongenomic effects of GCs on PPG mRNA stability. GCs are known to function in this capacity in cell-based systems (68,69) and, in combination with the data reported above, suggest that GC-mediated modulation of mRNA stability may participate in feedback regulation of the HPA axis function at the level of the NTS.

The observed time-frame of gene turn off and loss of GLP-1 immunoreactivity implies a translational 'pause' that will not affect the immediate release of neuropeptide, although it reduces PVN excitability subsequently. Consequently, the excitatory impact of GLP-1 on HPA axis activation will be reduced as a consequence of down-regulation during the period of depletion of peptide in terminals, approximately 30–60 min after stress (Fig. 5), predicting that the HPA axis may be less 'excitable' during the immediate post-stress period. This is indeed the case because numerous studies report that imposition of a second stress 5–60 min after an initial stressor causes a marked reduction in HPA axis responsiveness. The stress-refractory period can also be observed if the initial stressor is replaced with a bolus injection of GCs, suggesting that the inhibition is GC-driven [referred to as 'intermediate feedback' by Keller-Wood

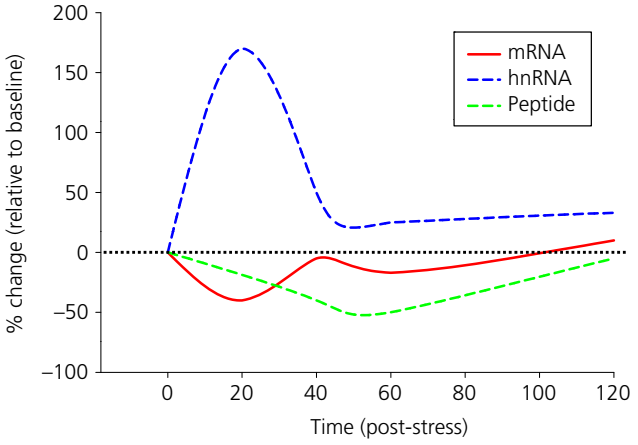

Fig. 5. Schematic comparing the time course of preproglucagon (PPG) mRNA degradation (PPG mRNA) with PPG transcription (PPG mRNA) and loss of paraventricular nucleus GLP-1 immunoreactive terminals (peptide). Note that transcriptional effects do not correspond with a loss of mRNA, suggesting the mRNA and peptide loss are likely linked to mRNA degradation or turnover. hnRNA, heteronuclear RNA.

and Dallman (70)]. Our data suggest that at least part of the intermediate feedback effect may be mediated by temporary destabilisation of PPG mRNA by stress-induced GC release.

Peripheral GC signalling and stress regulation

In addition to the actions of GCs in brain, it is important to consider that GC receptors are abundantly expressed in the periphery, and may exert indirect effects on the HPA axis via ascending vagal afferents or hormonal messengers. Work by Laugero *et al.* (71) has demonstrated that the pronounced HPA axis activation seen following adrenalectomy (e.g. increased PVN CRH synthesis) could be reversed by allowing the animals to drink sucrose, suggesting that GCs may inhibit the HPA axis by modulating peripheral metabolic signals. These data are supported by studies showing that central sucrose administration is not able to reduce adrenalectomy-induced activation of the HPA axis, implying a peripheral mechanism of action (72). Conversely, increasing energy stores (in the form of fat depots) by voluntary intake of a high-fat diet reduces HPA axis responses to stress, accompanied by elevated insulin secretion (73). The data suggest that GCs act in the periphery to promote glucose and insulin release, which is relayed to the hypothalamus to inhibit PVN activation.

Recent studies suggest the involvement of adipose-derived signals. Specific deletion of the GR in adipocytes enhances GC responses to stress and attenuates GC feedback inhibition of the HPA axis (de Kloet *et al.*, unpublished observations). GCs increase adipocyte lipolysis by activation of hormone sensitive lipase, which increases circulating free fatty acids (74). Depletion of free fatty acids, in particular palmitic acid (75,76), causes elevated plasma ACTH and corticosterone, suggesting a role with respect to constraining the HPA axis. One of the major sensors of free fatty acids and lipid messengers, peroxisome-proliferator activated receptor gamma (PPARγ), is localised to PVN neurones (77). Treatment with a PPARγ agonist rosiglitazone inhibits stress-induced PVN Fos induction and corticosterone release (78), whereas direct infusion of the PPARγ antagonist GW9662 into the PVN increases ACTH release. These data suggest that FFAs may provide a blood-borne inhibitory signal at the level of the PVN, and are consistent with GC-mediated increases in lipolysis in adipocytes.

GC feedback: a distributed process

The data reported above highlight the richness of the GC feedback process, identifying a number of check-points whereby secretion can be limited. These studies complement a rich literature citing trans-synaptic feedback inhibition from neural sources, such as the prefrontal cortex and hippocampus (79); genomic and nongenomic actions at the level of the pituitary (80); and rich interactions with the autonomic nervous system that can alter secretory profile at the level of the adrenal (81).

GCs, stress and neuroplasticity

GCs in conjunction with a myriad of chemical mediators released during stress response modify several aspects of brain function, including learning and memory formation (82). Major brain areas targeted by GCs and other stress hormones include the hippocampal formation (mediating declarative memory), amygdala (mediating fear response) and prefrontal cortex (mediating working memory) (83). Morphological and biochemical studies have found that normal circadian or stress-induced variations in circulating GCs promote neuroplasticity in these structures, especially in the hippocampus, with modifications of neurone morphology and changes in neuronal excitability and synaptic efficacy (83).

In the hippocampus, GCs regulate neuronal turnover through effects on both cell death, as well as proliferation of neural progenitors. Adrenalectomy induces apoptosis of mature granule cells of the hippocampal dentate gyrus, which is prevented by corticosterone or MR agonist administration (84–88). The trophic influence of adrenal steroids on granular neurone survival appears to be related to the regulation of intrinsic apoptotic signalling pathways (89). Evidence indicates that adrenalectomy increases mRNA expression of the proapoptotic gene *bax* (89) and promotes the activation of cysteine protease caspase-9 (90). This enzyme promotes the proteolytic cleavage of executor caspases that subsequently destroy several proteins, culminating in apoptosis. In addition, adrenalectomy induces an increase in the rate of proliferation of progenitor cells in the subgranular zone of the dentate gyrus, an effect that is prevented by corticosterone administration (91). On the other hand, the increase in GC secretion induced by acute stress can act as a positive or negative modulator of learning, memory and retrieval (92). Several studies have suggested that acute stress is associated with increased excitatory glutamatergic neurotransmission in areas of the forebrain (93–95). Some studies have demonstrated that adrenalectomy attenuates the stress-induced release of glutamate in the hippocampus and prefrontal cortex, suggesting a direct relationship between GCs and glutamate release (96). Moreover, intrahippocampal perfusion of corticosterone by retrodialysis produces a fast and reversible increase in the release of glutamate *in vivo* (97). In addition, electrophysiological studies have shown that application of corticosterone to hippocampal slices increases the frequency of excitatory postsynaptic potentials in the CA1 area of the hippocampus, suggesting a fast action of corticosteroids on glutamate release (98). This fast action of corticosterone is likely to be nongenomic and appears to involve the MR (99).

GC fluctuations can also modulate the learning processes through modifications of the postsynaptic elements that receive the majority of excitatory glutamatergic inputs in the CNS. These postsynaptic elements are named spines, which are small actin-rich protrusions formed by a head that is connected to the dendrite shaft by a neck. Circadian GC peaks induce the formation and development of dendritic spines in the mouse cortex after motor skill learning and the circadian GC troughs are required for the stabilisation of new spines (100). Furthermore, the improved associative learning promoted by acute stress is accompanied by a rise in the spine density of hippocampal neurones (101). In line with these observations, brief exposure of hippocampal slices to dexamethasone, a GR agonist (55), or corticosterone (100–1000 nM) (102) promotes an increase in spine density in pyramidal neurones

of the CA1 hippocampal area. Furthermore, the co-administration of RU486, an antagonist of GR, abolished the effect of corticosterone (102). It has been proposed that spinogenesis is modulated by synaptic GRs and kinases, including protein kinase A, protein kinase C (102) and extracellular signal-regulated protein kinases 1 and 2 (103). Although GRs have been localised to neuronal cell bodies and dendrites, a recent study has shown that GRs are localised to dendritic spine heads and to spine necks of CA1 pyramidal cells (103). Additionally, a recent study in KO mice for fragile X mental retardation protein (FMRP) demonstrated a reduction in GR levels in CA1 dendrites (104). FMRP acts as a protein that transports a subset of neuronal mRNAs from the nucleus into dendrites and spines (104,105). Furthermore, the bulk of FMRP is associated with polyribosomes and represses the translation of various mRNAs (104,105). In accordance with these findings, it has been shown that spine GR levels increase rapidly by metabotropic glutamate receptor (mGluR) activation, an effect not observed in KO mice for FMRP (103). Thus, it has been proposed that synaptic levels of GR in CA1 spines are regulated by local GR mRNA translation involving mGluR activity and a FMRP-dependent mechanism (103).

It appears that GR binding also affects glutamatergic receptor levels. It was shown that GR activation increases the surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor subunit 2 in primary hippocampal cultures (106). This GC-induced increase in GluA2 subunit of AMPA receptors at the hippocampal synaptic sites is involved in the facilitation of learning during acute stress (107). The variation in GluA2 levels is probably related to receptor trafficking mechanisms rather than changes in GluA2 mRNAs levels and/or translation (107).

The mRNA encoding the immediate early gene, activity regulated cytoskeletal-associated protein (*Arc*), is increased by activation of N-methyl-D-aspartate receptor. Arc mRNA traffics to dendrites and its translation is controlled by FMRP and the activation of mGluR (108). Reduction of Arc protein levels in the hippocampus blocks both late-phase long-term potentiation and the consolidation of some forms of memory (109). Furthermore, it has been reported that Arc reduces the surface expression of GluA2/A3 receptors, promoting a decrease in AMPA receptor-mediated synaptic currents (110). These findings suggest that Arc protein stabilises the internal pool of AMPA receptors, which would presumably increase or decrease the levels of these receptors in synaptic sites depending on the initial stimulus (111). Because activation of GRs produce variation in the synaptic levels of GluA2 (107), the effect of acute restraint stress on Arc protein levels was evaluated. Figure 6 shows that 0.5 h of restraint stress promotes an increase of Arc protein levels in the hippocampus, suggesting a fast Arc mRNA translation. Thus, it is plausible that stress-induced corticosterone secretion mediates mGluR activation, increasing Arc mRNA translation, which in turn may regulate the surface expression of AMPA receptors. Overall, these findings suggest that stress and GCs may modulate excitatory neurotransmission via increased glutamate release, modified spine density and AMPA receptor trafficking.

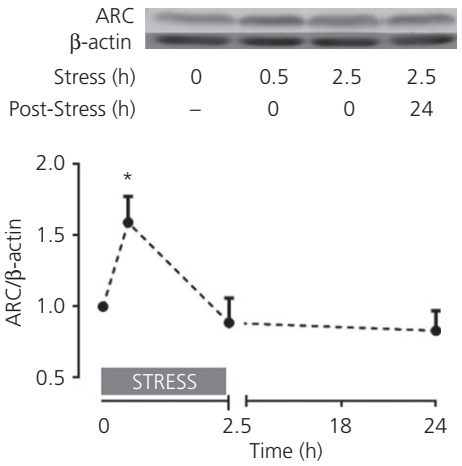

Fig. 6. Effect of acute restraint stress on the Arc (activity-regulated cytoskeleton-associated protein) protein levels in the hippocampus of rats. Acute restraint stress induces an increase of Arc protein levels in rat hippocampus. Sprague–Dawley male rats were restrained for 0.5 h or 2.5 h and immediately sacrificed or restrained for 2.5 h and sacrificed 24 h after the restraint. (A) Representative immunoblots of homogenates from the hippocampus of stressed rats using anti-Arc and β-actin antibodies. (B) Graph shows the relative ratio of Arc levels relative to *β*-actin. Data represent the mean ± SD of n = 4 per experimental condition. *P < 0.05 compared to nonstressed animals (i.e. time = 0).

GCs in the control of food intake

Food intake is a basic behaviour regulated by multiple factors, including the adiposity signals leptin and insulin, and satiety signals, such as mechanical and chemical stimulation of the stomach and small intestine, as well as hormones released during a meal, such as cholecystokinin (112). The adiposity factors are involved in the long-term control of energy balance and act primarily in hypothalamic neurones expressing orexigenic or anorexigenic neuropeptides, which are key mediators in the control of energy homeostasis (112). Neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus of the hypothalamus, and orexins and melanin-concentrating hormone in the lateral hypothalamic area comprise important hypothalamic orexigenic neuropeptides (113–115). On the other hand, POMC and cocaine- and amphetamine-regulated transcript (CART) in the arcuate nucleus of the hypothalamus, as well as CRH and oxytocin in the PVN, represent the main hypothalamic mediators involved in the inhibition of food intake (112,115). The satiety signals, in turn, are implicated in the short-term control of food intake and have their actions mediated by brainstem areas, mainly the NTS, controlling the size of a meal (116).

In addition to their important role regulating metabolic activity in the periphery, there are profound interactions between GCs and appetite control (117). Feeding is a major synchroniser of HPA axis rhythmicity (118), and the amount of food ingested is associated with GC secretion (119). Reciprocally, increased food intake and body weight gain in humans are associated with increases in circulating GCs associated with the therapeutic administration of GCs or Cushing's disease (120). Excess GC in these cases is associated with

increased glucose production, decreased glucose transport and utilisation, decreased protein synthesis, increased protein degradation in muscles and body weight gain (121,122). By contrast, chronic GC deficiency as occurs in Addison's disease or primary adrenal insufficiency is characterised by malaise, fatigue, weight loss, joint and back pain, darkening of the skin, hyponatraemia, hypoglycaemia, and hyperkalaemia, with anorexia being a remarkable symptom (123).

Studies in rodents have shown that GC effects on food intake depend on the dose of corticosterone replacement (124). Low doses of corticosterone in adrenalectomised rats, with corticosterone plasma levels ranging from 1 to 2 μg/dl, were demonstrated to activate MR and to have a stimulatory effect on fat intake, body weight gain and fat depots, which occur at the late phase of the feeding period (125–127). On the other hand, GRs are activated by higher doses of circulating corticosterone (2–10 μg/dl) just before or in the first hours of the active feeding period, which induces carbohydrate ingestion and metabolism (125–130). In addition, extremely high corticosterone plasma concentrations (> 10 μg/dl), as a result of stress or food restriction, stimulate fat and protein catabolism, mainly muscle protein catabolism, and consequently body weight loss, to increase the availability of gluconeogenesis substrates and to enhance the glucose plasma concentration as an energy source (127,131). Central actions of GCs on feeding behaviour have been demonstrated by different studies showing that dexamethasone injection into the lateral ventricle not only stimulated food intake, but also enhanced body weight gain in rats, accompanied by hyperleptinaemia and hyperinsulinaemia (132,133).

The central effects of GCs on the regulation of food intake appear to be mediated by the interaction of GCs with hypothalamic neuropeptides involved in the control of energy homeostasis, as demonstrated by the presence of GC receptors in neurones expressing neuropeptides involved in the control of energy homeostasis (134,135). This interaction between GCs and hypothalamic neuropeptides involved in the control of food intake has been evaluated, showing that central GC treatment increases hypothalamic NPY levels and decreases those of CRH (133). In addition, circulating GCs were shown to be required for a feeding-induced decrease in the expression of orexigenic neuropeptides in the arcuate nucleus of the hypothalamus and an increase in the expression of the anorexigenic neuropeptide POMC in this nucleus, as a result of the direct modulation of GCs in these neurones in the arcuate nucleus of the hypothalamus and, indirectly, as a result of changes in leptin and insulin secretion induced by food intake (136).

As observed in humans with Addison's disease, removal of endogenous GCs induced by bilateral adrenalectomy in rodents has been shown to reduce food intake and body weight gain, and these effects are reversed by GC replacement in rats (137–140). Furthermore, adrenalectomy diminishes hyperphagia and obesity in different experimental models, whereas GC replacement reverses these effects (137,141–143). The hypophagic effect induced by the removal of endogenous GCs by adrenalectomy is associated with increased expression of the anorexigenic neuropeptides CRH and oxytocin in the PVN, as well as a reduction in the expression of the orexigenic neuropeptides NPY and AgRP in the arcuate nucleus of the hypothalamus, indicating that changes in the expression of these hypothalamic neuropeptides could account for a reduction in appetite following adrenalectomy (136,139,140). Concerning the anorexigenic neuropeptides of the arcuate nucleus of the hypothalamus, adrenalectomy was shown to reduce POMC and CART, suggesting that the anorexigenic effects in response to the removal of adrenal glands are dissociated from any elevations of these neuropeptides in the arcuate nucleus of the hypothalamus (136,144).

It is well established that the action of GCs on food intake involve their stimulatory drive to eat, and thus adrenalectomy-induced hypophagia involves, at least in part, a reduction in the drive to eat. However, recent studies have highlighted the role of GCs in the short-term control of food intake, indicating that the hypophagic effect in response to adrenal gland removal is associated with increased activation of satiety-related responses through brainstem and hypothalamic pathways (138,139). Accordingly, NTS neurones, as well as CRH and oxytocin neurones in the PVN, were increasingly activated in response to a meal after adrenalectomy (138,139). In addition, CRH and oxytocin neurones in the PVN project to the NTS, and oxytocin axonal projections from the PVN to the NTS are enhanced after adrenalectomy (145). Interestingly, CRH and oxytocin were shown to be involved in the enhanced satiety-related responses after adrenalectomy because both hypophagia and the increased activation of NTS neurones induced by feeding following adrenalectomy were reversed by CRH type 2 receptor and oxytocin receptor antagonists (138,140,145). Accordingly, CRH and oxytocin arise as pivotal mediators of the enhanced satiety-related responses in the NTS, contributing to hypophagia in the primary adrenal insufficiency. Thus, GCs have well-established roles in the regulation of feeding behaviour and energy homeostasis, stimulating the drive to eat, as well as reducing satiety-related responses (Fig. 7).

Interactions between the HPA axis and the immune system

GCs have well recognised effects as immune suppressors, and their release during an immune challenge acts to dampen cytokine

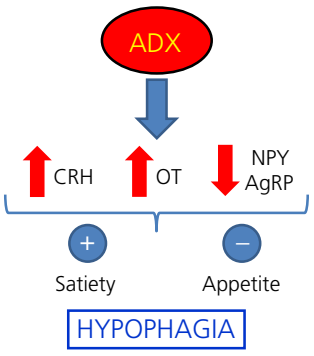

Fig. 7. Schematic showing the hypophagic effect in response to adrenalectomy (ADX) through the inhibition of appetite and the stimulation of satiety pathways, mediated by the increase on the expression of the anorexigenic neuropeptides corticotrophin-releasing hormone (CRH) and oxytocin (OT) and the decrease of the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related protein (AgRP).

production and inflammatory responses. However, the HPA axis and immune system show complex bidirectional interactions not only under immune challenges, but also during exposure to non-immune stressors. A growing body of literature suggests that stress significantly impacts many facets of neuroimmune function. For example, exposure to a variety of acute stress challenges increased the expression of interleukin-1β (IL-1β) in the hypothalamus (146–148), whereas increased prostaglandin activity has been observed throughout the cortex in response to acute stress (149). Converging lines of evidence suggest that these changes in neuroimmune signalling factors (cytokines, chemokines, prostaglandins, etc.) are associated with other tell-tale signs of neuroinflammation, such as priming (150), proliferation (151) and activation (152,153) of microglia. Consistently, injection of the putative microglial inhibitor minocycline blocked cytokine expression evoked by stress (152,154). Thus, a rich constellation of neuroimmune changes occur in response to stress, which raises several critical issues, including: (i) how do hormones classically associated with stress (nor/epinephrine and GCs, in particular) influence cytokine expression and other aspects of neuroimmune function; (ii) to what extent do neuroimmune signalling pathways serve as either moderators or mediators of HPA axis output across natural diurnal rhythms and/or in response to later stress challenges; and (iii) under what circumstances do neuroimmune consequences of stress either compromise or protect against the development of pathological states of the CNS?

Although the precise mechanisms controlling cytokine expression during times of stress have not been fully delineated, several key mechanisms have emerged. Previous work has shown that the administration of β-adrenergic blockers inhibits or severely blunts the expression of interleukin-1 evoked by stress, and the administration of the β-adrenergic agonist isoproterenol both recapitulates and potentiates the expression of IL-1 induced by stress (152,154,155). By contrast to the dependence of central cytokine responses on β-adrenergic receptor activation, plasma cytokine responses to stress appear to be selectively mediated by α1-adrenergic receptors (156). These findings are supported by lesion studies, where complete lesions of central adrenergic systems incurred by injection of the neurotoxin DSP4 completely blocked the IL-1 response produced by stress in several brain structures (155), whereas more targeted lesions of the ventral noradrenergic bundle only partially attenuated the IL-1 response to stress in the PVN (156). These data fit well with strain differences in central cytokine responses to stress demonstrating that hyperadrenergic Fisher 344 rats show much greater increases in central cytokine expression relative to their less reactive Sprague–Dawley comparators (157). Indeed, the potentiation of cytokine responses to stress in hyperadrenergic, Fisher 344 rats is particularly noteworthy given that this strain also evinces a potentiated HPA axis response relative to other strains (158). Thus, norepinephrine appears to be a key driver of stress-induced cytokine expression (Fig. 8). However, it should be noted that other transmitter systems such as glutamate (151) and other intermediaries such as danger associated molecular patterns (159) may participate in various features of the neuroimmune response to stressor exposure as well.

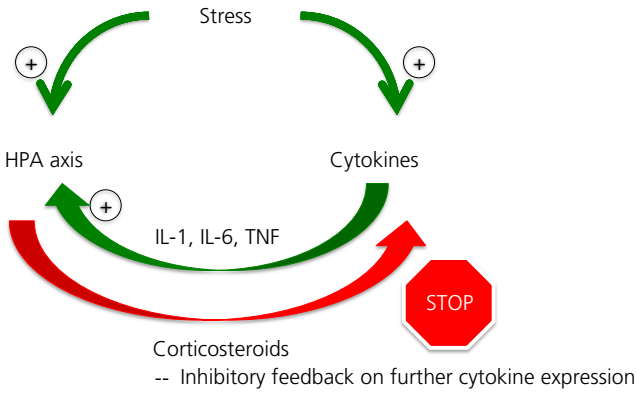

Fig. 8. Schematic showing the interaction between corticosteroids and cytokine expression. Stress exposure directly induces the release of hypothalamic-pituitary-adrenal (HPA) axis hormones and increases the expression of cytokines. Although there is emerging evidence to suggest that corticosteroids may enhance cytokine expression under certain circumstances, a primary effect of corticosteroids is the suppression of cytokine expression. Cytokines, on the other hand, directly stimulate activation of the HPA axis via actions that are both intrinsic and extrinsic to the axis, and appear to augment HPA axis sensitivity to later stress challenges. IL, interleukin; TNF, tumour necrosis factor.

GCs, on the other hand, powerfully constrain the expression of cytokines during times of stress. Evidence to support this is provided by studies showing that the removal of endogenous GCs via adrenalectomy (160) and the administration of GC receptor antagonists (161) or the GC synthesis inhibitor metyrapone (152) massively potentiates the expression of cytokines evoked by stress. These findings fit well with a range of molecular interactions through which corticosteroid receptors squelch inflammatory signalling pathways (162–164). Very recently, however, a few studies have begun to reveal effects that indicate priming-like actions of GCs as well (165,166). However, it should be noted that these non-canonical actions may be (i) unique to specific tissue types within the body; (ii) occur at only low-dose or low-physiological concentrations of circulating GCs; or (iii) reflect a short-term, early response to GC signalling. These intriguing findings suggest that the relationship between corticosteroid signalling and neuroimmune consequences of stress will require detailed consideration of numerous key parameters (tissue, dose, timing, etc.). Nevertheless, it is clear that in the absence of effective GC signalling, neuroimmune consequences of stress are quite severe.

Moving beyond mechanisms, neuroimmune consequences of stress (particularly increased cytokine expression) may play an important role as moderators of HPA axis activation. For example, IL-1 receptor 1 KO mice show blunted HPA axis response to mild but not intense stress challenges (167). Previously stressed rats showed a faster HPA axis response to later lipopolysaccharide injection, an effect that was reversed by IL-1 receptor antagonist (168). Furthermore, it has been noted that dissociations between ACTH and corticosterone are often observed under inflammatory conditions (169,170), including after immune activation by lipopolysaccharide (171,172), as well as in inflammatory disease states (173,174). Thus, cytokines and other immune-related signalling

factors have the capacity to induce (through actions extrinsic to the axis) or augment (through intrinsic actions) HPA axis responses to later stress challenges.

The impact of neuroimmune consequences of stress is not restricted to the HPA axis. For example, the neuroimmune consequences of stress appear to control certain behavioural consequences of stress exposure (175), as well as impairments in cognitive function precipitated by stress (176,177), and may be involved in certain depressive-like consequences of stress such as learned helplessness (178). Indeed, stress-related cytokines have been argued as a potentially critical mechanistic bridge between stressful experiences and the development of stress-related pathology (179–181). Thus, with such far-reaching implications of stress-related changes in neuroimmune function, the interaction between classic stress-responsive systems (HPA axis, sympathetic nervous system) and neuroimmune function provides an important starting block for gaining a better understanding of the long-term, cumulative impact of stress on animals and humans (180).

Concluding remarks

Important progress has been made during the last few years in understanding the physiological actions of GCs and the mechanisms of GC feedback regulation of the HPA axis. GCs have pleotropic actions regulating metabolism and brain function and modulating the function of most cells in the body. Growing evidence indicates that normal GC-dependent regulation depends upon the prevailing levels of the steroid, secretion pattern, as well as interaction of GCs with neurotransmitters and neuropeptides. This is partly illustrated by the concentration-dependent effects of GCs in hippocampal neuroplasticity and immune function discussed above. It is also clear that not all the effects of GCs are direct, with a number of actions depending of changes in other regulatory factors. For example, the profound modulatory effects of GCs on food intake largely depend on the opposing effects of GCs on orexigenic and anorexigenic neuropeptides.

Negative GC feedback is essential for the fine control of HPA axis activity to avoid deleterious consequences of excessive CRH and GC production. New evidence indicates that GC feedback inhibits HPA axis activity at a number of anatomical and molecular targets, rapidly shutting-off hypothalamic and pituitary responses at the cell membrane level, controlling the intensity and duration of stress responses at limbic sites, and inducing long-term inhibition by modulating transcription and mRNA stability at the central and pituitary levels.

Despite recent advances in the field of HPA axis regulation and GC actions, a number of challenges still remain. This includes the identification of peripheral metabolic signals and central sensors impacting on the sensitivity of GC feedback and HPA axis activity, and the exact identity of the membrane GC receptor and its signalling mechanisms. Understanding the functions of GCs and mechanisms of feedback regulation of HPA axis activity will contribute to the development of new diagnostic and therapeutic tools for disorders related to stress and alterations of GC secretion.

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