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Stress and the neuroendocrine-immune axis: the pivotal role of glucocorticoids and lipocortin 1

J.C. Buckingham

Department of Pharmacology, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF

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Introduction

Widespread evidence now supports the view that reciprocal communication between the brain-endocrine system and the immune system is fundamental to host defence processes in conditions of health and disease. Importantly, it provides a means whereby the central nervous system can detect alterations in immune status (e.g. infections) and initiate a co-ordinated series of responses (immunological, metabolic, physical, behavioural) which is designed specfically to protect the host and thus to restore homeostasis. Several paths of communication between the two systems have been identified. These include firstly neural links provided by the afferent/efferent autonomic fibres (predominantly sympathetic) which connect the lymphoid organs and tissue with the brain and secondly humoral transmission which is effected by hormones released from the pituitary gland and peripheral endocrine organs and by mediators (termed immunokines) released from activated immune/inflammatory cells. In addition, since immune insults initiate the synthesis of immunokines in the neuroendocrine system while causing lymphoid cells/tissues to produce 'neuroendocrine peptides' such as corticotrophin (ACTH), there is opportunity for a degree of local interplay between the two systems. Many peptide and steroid hormones have been implicated in the processes of neuroendocrine-immune communication. The bulk of attention however has focused on the hypothalamo-pituitary-adrenocortical (HPA) axis largely because its end products, the glucocorticoids, have a well established role in the control of immune/inflammatory cell function (reviewed in Munck *et al.*, 1984; Munck & Naray-Fejes-Tóth 1994; Munck & Gyre, 1991).

The HPA axis provides an essential interface between the internal and external environment and enables the organism to adapt to diverse noxious stimuli, whether they be cognitive (e.g. emotional or physical trauma) or non-cognitive (e.g. immune insults). It is promptly activated in conditions of stress and the substantial quantities of glucocorticoids released into the systemic circulation serve to restore homeostasis through multiple mechanisms which include modulation of immune/inflammatory responses. Failure to mount an appropriate adrenocortical response in conditions of stress is potentially hazardous and indeed disturbances in HPA function are now considered to be a significant contributory factor in the aetiology of a variety of disease processes. For example, excessive glucocorticoid secretion (which may arise from a primary disorder of the HPA axis or from causes as diverse as e.g. ectopic ACTH producing tumours, depression, stress, alcoholism, anorexia) causes diverse metabolic and behavioural disturbances together with immunosuppression which, in turn, may predispose the individual to other disease processes, e.g. infections, cancer. Conversely, adrenocortical insufficiency (which may reflect primary or secondary adrenal failure or steroid resistance in the target tissues) precipitates a vulnerability to stress and is increasingly implicated in the pathogenesis of autoimmune, inflammatory and allergic disorders (e.g. rheumatoid arthritis, multiple sclerosis, systemic lupus erythematous, reviewed in Derjik & Sternberg, 1994).

In healthy individuals, the serum glucocorticoid concentrations are maintained within narrow limits with overt excursions occurring only in accord with the well established carcadian rhythm and in response to stressful stimuli. Critical to this tight control is a complex servo mechanism through which the glucocorticoids negatively regulate their own secretion and thus effectively contain the adrenocortical responses to incoming stimuli (e.g. stress) within appropriate limits. This article, which is dedicated to the memory of the late J.H. Gaddum, will discuss the molecular mechanisms underlying the pivotal feedback actions of the glucocorticoids within the HPA axis, focusing on our recent studies which identify a fundamental role for lipocortin 1 (LC1) as a mediator of glucocorticoid action in the neuroendocrine system. A brief review of the mechanisms by which stressors activate the HPA axis precedes this discussion.

Hypothalamo-pituitary-adrenocortical responses to stress

The increases in glucocorticoid secretion which occur in stress are triggered primarily by stimuli which converge upon the hypothalamus and precipitate the release of the two major corticotrophin releasing hormones, a 41-amino acid peptide termed CRH-41 (Vale *et al.*, 1981) and arginine vasopressin (AVP, Buckingham, 1981; Gillies *et al.*, 1982). These peptides, which are synthesized by parvocellular neurones which originate in the paraventricular nucleus (PVN) and project to the external zone of the median eminence (Sawchenko *et al.*, 1984), are released into the hypothalamo-hypophyseal portal blood vessels and transported to the anterior pituitary gland where they act synergistically to initiate the release of ACTH (Buckingham, 1981; 1985, Gillies *et al.*, 1982). ACTH in turn acts on cells in the zona fasciculata of the adrenal cortex to stimulate the synthesis and hence the release of the glucocorticoids, corticosterone and/or corticosterone according to the species.

The intra-hypothalamic CRH-41/AVP neurones are well positioned to integrate and respond to physical and emotional stimuli which threaten homeostasis (Buckingham *et al.*, 1996a). Importantly, they receive inputs from many ascending and descending nervous pathways. In addition, they are sensitive to a variety of blood-borne substances (e.g. glucose, immunokines, steroids) and to local factors released from glial cells and/or interneurones. Anatomical, electrophysiological, biochemical and pharmacological studies have identified major roles for the ascending noradrenergic tracts and for pathways

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originating in the raphe nuclei, the hippocampus and the amygdala but other tracts, arising for example elsewhere in the limbic system and in the cortex, probably also fulfil significant roles. Not surprisingly the complement of neural pathways, humoral substances and local mediators which orchestrates the release of CRH-41/AVP in stress depends on the nature of the stimulus. Thus, for example, the HPA responses to hypotension (caused by haemorrhage or by injection of sodium nitroprusside) depend on the integrity of the noradrenergic pathways which project from the brain stem to the PVN. On the other hand the increases in glucocorticoid secretion provoked by certain emotional trauma require inputs from the limbic system while those to cold depend on connections between peripheral C-fibres and the PVN.

Amongst the most severe of threats to homeostasis are those posed by diseases which challenge and potentially threaten the body's defence mechanisms and it is not therefore surprising that such insults trigger a pronounced activation of the HPA axis (reviewed in Besedovsky & del Ray, 1992). Early evidence to this effect emerged from reports that innocuous antigens (non-infective, non-self replicating, non-neoplastic) produce significant increases in the serum corticosterone concentration in rodents (Besedovsky *et al.*, 1975). Similar responses occur in animals subjected to viral or bacterial infections (Besedovsky *et al.*, 1985a; Dunn, 1988) and in those bearing transplanted tumours (Besedovsky *et al.*, 1985b). In most instances, the magnitude of the adrenocortical response observed varies according to the intensity of the immune insult (Shek & Sabiston, 1983) although, paradoxically, some antigens have been reported to trigger a robust antibody response at doses below those required to activate the HPA axis (Stenzel-Poole *et al.*, 1993). Not surprisingly, the data in man are less exhaustive but, nonetheless, there is substantial evidence for increased cortisol secretion in conditions of e.g. septicaemia and inflammation (Wolff, 1973; Geelhoed & Chernow, 1985; Imura *et al.*, 1991). Indeed, until the advent of more specific means of assessment, injection of bacterial lipopolysaccharide (LPS) was used clinically as a test of pituitary ACTH reserve.

The mechanisms by which immune insults activate the HPA axis have been avidly researched in the last decade (for detailed reviews see Buckingham *et al.*, 1996a, b). Data from innumerable studies argue strongly that the primary driving force is provided by the battery of mediators (immunokines) released by activated immune/inflammatory cells (Figure 1). Many of these substances have been shown to be potent activators of the HPA axis; these include certain interleukins (e.g. IL-1α, IL-1β, IL-6 and TNFα), interferons, phospholipid metabolites (notably eicosanoids and PAF), peptides (e.g. bradykinin, angiotensin II, thymic peptides) and amines (e.g. histamine, 5-HT) together with enzymes such as phospholipase A_2 (PLA_2) which are released into the systemic circulation in conditions such as septicaemia (Vadas & Hay, 1982). Some of these agents (e.g. IL-1α, IL-1β, IL-6, PLA_2, eicosanoids) appear to target the HPA axis directly, acting within the central nervous system (notably at the hypothalamus), the anterior pituitary gland and the adrenal cortex to trigger hormone release (see Buckingham *et al.*, 1996a, b). Others (e.g. prostanoids) however may act locally at the site of the inflammatory lesion to stimulate the primary nociceptive afferents and thereby activate central pathways which precipitate CRH-41/AVP release (Buckingham *et al.*, 1996b). In addition, many of the mediators provoke widespread pathological effects in the body (e.g. hypotension, hypoglycaemia) which are perceived as stressful by the organism and may therefore activate the HPA axis by other means. A further potential mechanism for communication between the immune and HPA system lies in the propensity of the two systems to produce and use common chemical mediators and receptors in conditions such as infection and inflammation (Blalock, 1989). For example, in rodents a peripheral endotoxin challenge stimulates the synthesis of cytokines such as IL-1 and IL-6 in the hypothalamus and other areas of the CNS (Hagan *et al.*, 1993; Muramani *et al.*, 1993; De Simoni *et al.*, 1995), possibly via mechanisms dependent on the generation of prostanoids at the level of the blood brain barrier (Cunningham & de Souza, 1992) and/or on the integrity of vagal afferents (Lalé *et al.*, 1995). Furthermore,

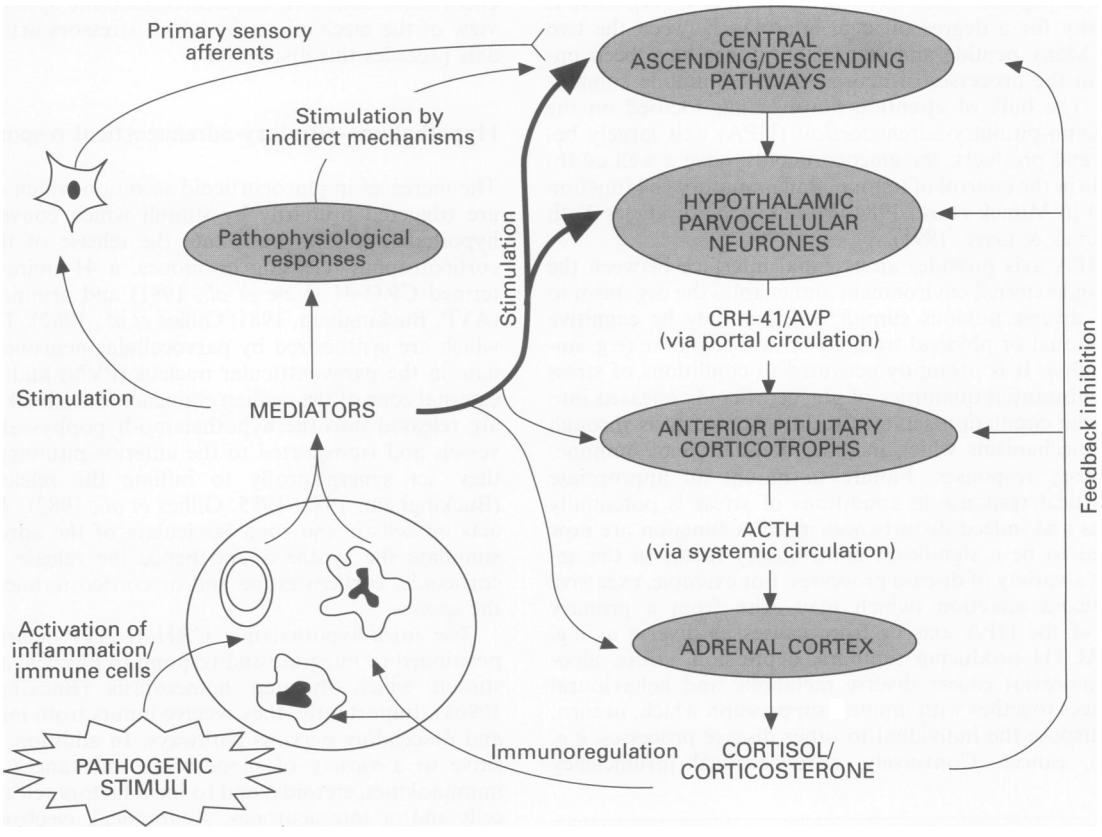

Figure 1 Major paths of communication between the immune system and the hypothalamo-pituitary-adrenocortical axis.

activated immune/inflammatory cells produce ACTH, β-endorphin and other products of pro-opiomelanocortin (POMC) together with CRH- and AVP-like peptides (Blalock, 1989; Angioni *et al.*, 1993). Teleological arguments favour a local regulatory role (autocrine, paracrine or intracrine) for these agents, a view supported by the wealth of evidence that IL-6 is a potent activator of the HPA axis at the level of the hypothalamus and that ACTH, β-endorphin, CRH and AVP each exhibit significant immunoregulatory properties. However, increasing evidence that plasma binding proteins facilitate the transportation and delivery of peptides such as CRH to distant cells/tissues (Lowry, 1995) suggests that some of these 'local mediators' may also fulfil a significant 'endocrine' brief within the periphery. Furthermore, since migratory cells of immunological lineage are found in abundance in both the anterior pituitary gland and the adrenal cortex, it is possible that the peptides they release target the endocrine cells directly and thereby provoke the release of ACTH and the glucocorticoids. The activation of the HPA axis by immune insults is thus a complex event potentially requiring the integration of a broad spectrum of stimuli, the complement of which will reflect the nature and intensity of the insult.

Modulation of the HPA responses to immune insults by glucocorticoids

Glucocorticoids act on specific receptors in the anterior pituitary gland, the hypothalamus and elsewhere in the central nervous system (in particular the hyppocampus) to quench the release of ACTH and its hypothalamic releasing factors and thereby contain the adrenocortical responses to stress and other stimuli within appropriate limits (reviewed in Keller-Wood & Dallman, 1984; Buckingham *et al.*, 1992). Thus, the reductions in circulating corticosteroids evident in e.g. Addison's disease or following adrenalectomy are accompanied by a sustained hypersecretion of ACTH and an exaggeration of the pituitary adrenocorticotrophic responses to stress, both of which are readily corrected by replacement therapy with glucocorticoids. Conversely, the increases in steroid level effected by e.g. adrenocortical tumours or exogenous steroids effectively suppress the resting and stress-induced release of ACTH. Perhaps not surprisingly, the HPA responses to immune insults are highly sensitive to alterations in glucocorticoid status. This is due in part to the powerful ability of glucocorticoids (endogenous or exogenous) to suppress the release and pathophysiological actions of the cytokines and other inflammatory mediators (e.g. eicosanoids) which normally trigger the HPA response (Derjik & Sternberg, 1994; Munck & Naray-Fejes-Tóth, 1994). However, there is also

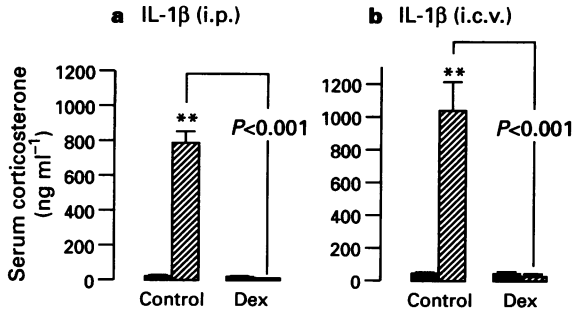

Figure 2 Effects of pretreatment (2 h) with dexamethasone (Dex, 100 μg kg^{-1}, i.p.) on the ability of human recombinant interleukin 1β (IL-1β) given (a) peripherally (3 μg kg^{-1}, i.p.) or (b) centrally (75 ng kg^{-1}, i.c.v.) to increase the serum corticosterone concentrations in conscious rats. Controls received corresponding volumes of the sterile saline vehicle. ■ = Vehicle; ▨ = IL-1β. Values represent the mean ± SEM (n = 5–8). **P < 0.01 vs. vehicle control (ANOVA plus Scheffé's test). Redrawn from Buckingham *et al.* (1994), with permission of Pharmacological Research.

substantial evidence that the actions of the various immunokines on the HPA axis itself are also subject to glucocorticoid regulation. This has been amply illustrated by our demonstrations that dexamethasone effectively suppresses the rises in ACTH and corticosterone secretion induced by central or peripheral injections of IL-1β, IL-6 or histamine (Figure 2, Buckingham *et al.*, 1994). Similarly, the increases in CRH-41 and AVP release from the rat hypothalamus provoked *in vitro* by IL-1α, IL-1β, IL-6, IL-8 or PLA_2 are greatly exaggerated in tissues taken from adrenalectomized rats and suppressed by inclusion of dexamethasone in the incubation medium (Figure 3, Loxley *et al.*, 1993a). The biochemical mechanisms mediating the complex inhibitory actions of the glucocorticoids within the HPA axis are far from fully understood although it is apparent that, in addition to acting at multiple sites within the brain and pituitary gland, the steroids use at least three distinct molecular mechanisms which are effective over different time domains; these are termed respectively the 'rapid', 'early delayed' and 'late delayed' phases of feedback inhibition (Keller-Wood & Dallman, 1984; Buckingham *et al.*, 1992).

Rapid feedback operates within minutes of a rise in circulating glucocorticoids; it is of only short duration (< 15 min) and is followed by a 'silent period' during which the ACTH response to stress is intact even though the plasma steroid level may remain elevated (Figure 4). The second or 'early delayed' phase of feedback inhibition develops approximately 0.5–2 h after an elevation in plasma steroid levels; it is normally maximal within 2–4 h (Figure 4) and, dependent on circumstances, it may persist for up to 24 h. Late delayed feedback has a latency of about 24 h and unlike the other phases of feedback inhibition, normally develops only after a very substantial rise in steroid levels. It is frequently a consequence of repeated or continuous systemic administration of high doses of corticosteroids and may be sustained for days or weeks after the treatment is withdrawn, thereby giving rise to the potentially life threatening hazard of HPA suppression which is invariably associated with such therapy.

Rapid feedback is reputed to be sensitive to the rate of change rather than the absolute concentration of steroid in the blood and may thus provide an important means whereby the stress response can be blunted if the circulating steroid level is already rising as consequence of a previous acute stress. These fast, atypical steroid actions appear to be exerted primarily at the hypothalamic level; weaker actions may also occur at the pituitary level but the contribution, if any, of extra-hypothalamic sites within the CNS is poorly defined (Buckingham *et al.*, 1992). Little is known of the molecular mechanisms responsible although there are suggestions that they may involve a novel 'non-classical' steroid receptor located close to or within cell membranes which serves to inhibit exocytosis by processes which are independent of genomic function and which may involve blockade of Ca^{2+} influx (Jones & Gillham, 1988; Liu *et al.*, 1995).

The mechanisms underlying the early and late delayed phases of feedback differ markedly from those responsible for the rapid phase. Thus, the degree of inhibition of ACTH secretion observed is proportional to the absolute concentration of steroid attained in the blood. Futhermore, both phases are effected via classical intracellular corticosteroid receptors of which there are two main types (viz. the glucocorticoid receptor, GR, and the mineralocorticoid receptor, MR) which differ in their affinity, steroid specificity, distribution and functional roles (for review see de Kloet, 1991). Although highly specific, GRs are of relatively low affinity (K_d cortisol/corticosterone ~ 10–20 nM) and are therefore extensively occupied only when the serum glucocorticoid concentration is raised for example by stress, disease or administration of exogenous steroids; these receptors (located primarily in the anterior pituitary gland, the hypothalamus and elsewhere in the CNS, notably the hippocampus) thus detect physiological and pharmacological elevations in steroid levels and serve to restore them to the normal 'resting' range (Ruel *et al.*, 1987; de Kloet, 1991). By contrast, mineralocorticoid receptors lack

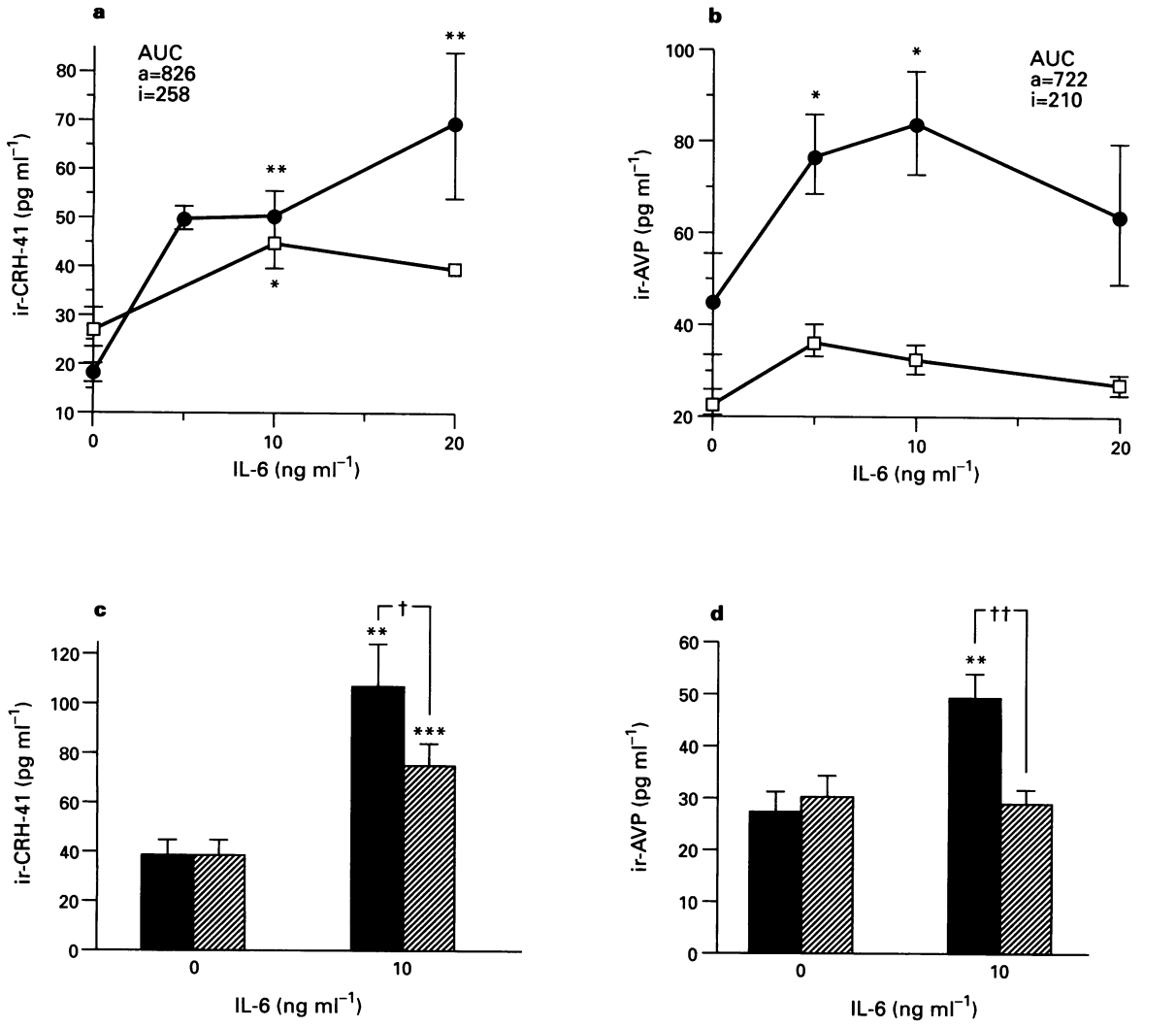

Figure 3 Effects of adrenalectomy (a+b) and dexamethasone (c+d) on the ability of human recombinant interleukin-6 (IL-6) to stimulate the release *in vitro* of immunoreactive-(ir-) CRH-41 (a+c) and ir-AVP (b+d) from rat isolated hypothalami. -□- = tissue from intact rats; -●- = tissue from rats adrenalectomized 10-14 days prior to autopsy; ■ = control; ▨ = dexamethasone (0.1 μM for 90 min prior to cytokine stimulation). Values represent the mean ± SEM (n = 4-6). *P < 0.01 vs. bars indicated on the figure (ANOVA plus Scheffé's test). AUC = area under the curve (arbitrary units squared); a = adrenalectomized; i = intact controls. Redrawn from Loxley *et al.* (1993a), with permission of S.Karger AG, Basel.

specificity in that they do not distinguish between aldosterone and the endogenous glucocorticoids, cortisol/corticosterone, for which they have high affinity ($K_d \sim 1-2$ nM). In the majority of cells/tissues these receptors are effectively protected from cortisol/corticosterone by the enzyme 11β-hydroxy-steroid dehydrogenase (reviewed in Funder, 1993). However, a small population of MRs in the hippocampus appears not to be protected in this way; these receptors are thus normally almost fully occupied by endogenous glucocorticoids even at the nadir of the circadian rhythm and are therefore well positioned to play a significant role in maintaining the tonic inhibitory influence of the steroids on HPA activity (Ruel *et al.*, 1987; de Kloet, 1991).

Many studies have shown that early delayed feedback is dependent on the *de novo* generation of protein second messengers which inhibit the release rather than the synthesis of ACTH and its hypothalamic releasing factors (Arimura *et al.*, 1969; Dayanithi & Antoni, 1989; Buckingham *et al.*, 1992). Recent data from our laboratory (discussed below) point to a major role for lipocortin 1 (LC1), a Ca^{2+} and phospholipid binding protein, in this regard but other proteins (as yet unidentified) are almost certainly also involved. *De novo* induction of second messenger proteins does not however account for the late delayed phase of feedback inhibition which is characterized by inhibition of not only the release but also the synthesis of CRH-41/AVP and ACTH in the hypothalamus and anterior pituitary gland respectively (Sawchenko *et al.*, 1987; Roche *et al.*, 1988; Jingami *et al.*, 1985). The pronounced inhibition of synthesis is in effect an exaggeration of the tonic, inhibitory influence of endogenous glucocorticoids on the genes encoding the three peptides. The concomitant inhibition of peptide release cannot be explained in this way as peptide stores in the hypothalamus and pituitary gland are rarely fully depleted. Reductions in the peptide pools available for release may be important in this regard but the underlying molecular mechanism await explanation.

Lipocortin 1

Lipocortin 1 (LC1, also called annexin 1) is a 37 kDa member of a family of Ca^{2+} and phospholipid binding proteins known collectively as the 'lipocortins' or 'annexins'. The primary structure of the protein is well defined in the rat, the mouse and man and interspecies differences in the amino acid sequence of the protein are detailed in the literature (Horlick *et al.*, 1991; Kovacic *et al.*, 1991). In line with other members of the annexin family, the C-terminal of LC1 comprises a core of four repeated 70 amino acid domains (Crompton *et al.*, 1988); within each repeat is a 17 amino acid motif (termed the con-

sensus sequence) which is highly conserved across the annexin family (Geisow *et al.*, 1986) and which is reported to confer the Ca^{2+} and phospholipid binding properties of the proteins (Figure 5). The N-terminal is unique to LC1 and thereby confers the structural and functional properties which are specific to the protein and which may determine both its location and function in a given cell. This region included potential sites for phosphorylation, glycosylation and peptidase action. Interestingly, although unique within the annexin family, the N-terminal shares a degree of sequence homology with sauvagine, a peptide derived from frog skin which is structurally closely related to and which mimics the biological activity of CRH-41 in the anterior pituitary gland and other tissues. The genes encoding LC1 in the rat, the mouse and man have been cloned (Wallner *et al.*, 1986; Horlick *et al.*, 1991; Kovacic *et al.*, 1991). Each comprises 13 exons, the first of which is non-coding (Figure 5). Much remains to be learnt about the promoter regions of the genes in all three species. However, TATA and CCAAT motifs which are critical for transcription have been identified together with glucocorticoid response elements (Horlick *et al.*, 1991; Kovacic *et al.*, 1991); moreover, potential response elements for AP-1, Oct-1, cyclic AMP and serum have been mapped on the mouse promoter (Horlick *et al.*, 1991).

Lipocortin 1 was first identified in extracts of conditioned media from glucocorticoid-stimulated peritoneal macrophages (Blackwell *et al.*, 1980). It was shown subsequently to possess anti-inflammatory and other glucocorticoid-like properties and was thus heralded as a potential second messenger protein for these steroids (Blackwell *et al.*, 1980; 1982). Its relative importance in this regard has since been a subject of some debate for glucocorticoids also influence directly or indirectly the synthesis of many other proteins as well as producing diverse effects in the body which are independent of genomic events (reviewed in Buckingham *et al.*, 1996a). Nonetheless, there is substantial evidence to support a role for LC1 in the signal transduction mechanisms effecting the anti-inflammatory (Cirino *et al.*, 1989; Perretti & Flower, 1993; Perretti *et al.*, 1993), anti-pyretic (Carey *et al.*, 1990) and anti-proliferative (Violette *et al.*, 1990; Croxtall & Flower, 1992) actions of the glucocorticoids (for review see Ahluwalia *et al.*, 1996). Furthermore, as described below, we have identified a fundamental role for LC1 as a mediator of glucocorticoid action in the neuroendocrine system. The experiments which led us to this conclusion were designed specifically to address three main questions, viz. (i) is lipocortin 1 expressed in the steroid sensitive regions of the HPA axis and, if so, is its expression regulated by glucocorticoids, (ii) are the regulatory actions of the glucocorticoids on the secretion of ACTH and its hypothalamic releasing factors (CRH-41 and AVP) observed *in vitro* and *in vivo* mimicked by LC1 and/or attenuated by neutralising anti-LC1 antisera and (iii) does LC1 contribute to the significant regulatory actions of the glucocorticoids on the secretion of pituitary hormones other then ACTH?

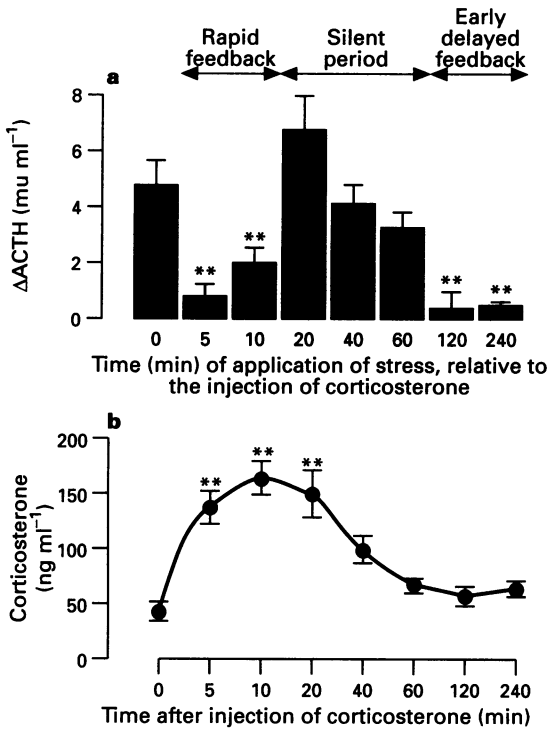

Figure 4 The rapid and early delayed phases of feedback inhibition of HPA function. (a) Increments in plasma ACTH concentration induced by stress (histamine, $6.0\,\mu g\,kg^{-1}$, i.p.) applied at various times after an injection of corticosterone ($100\,\mu g\,kg^{-1}$, i.p.) and (b) serum corticosterone concentrations at the time of stress. Values represent the mean ± SEM ($n=6$). $^{**}P<0.01$ vs. values at $t=0$ min (ANOVA plus Duncan's test).

Expression of lipocortin 1 in the brain and anterior pituitary gland and its modulation by glucocorticoids

In the last decade the distribution of LC1 in the brain and pituitary gland has been examined by a variety of techniques in freshly exised tissues derived from rats, man, sheep and pigs

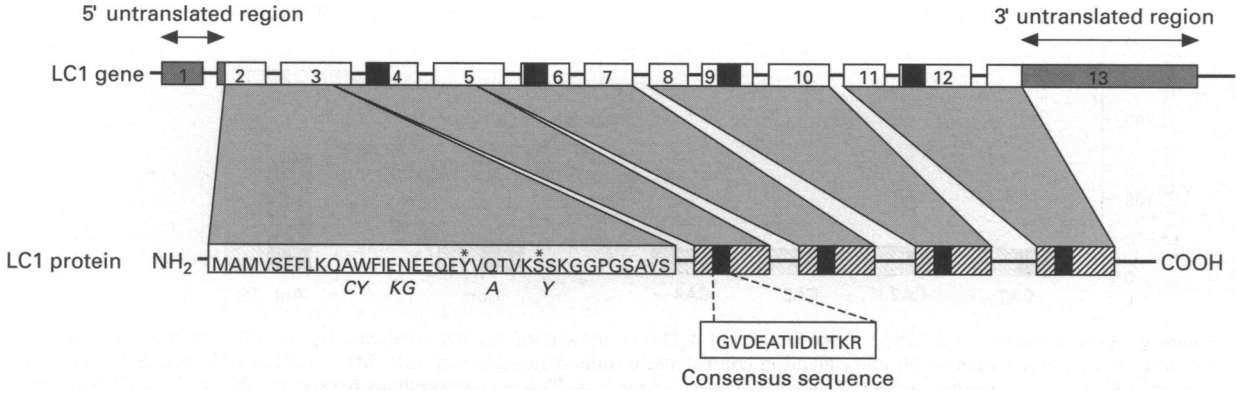

Figure 5 Schematic diagram of the structure of lipocortin 1 and its encoding gene. The gene comprises 13 exons (see numbered blocks) with untranslated elements (shaded areas) at the 5′ and 3′ ends. The N-terminal (human amino acid sequence in full) and four repeater units (hatched blocks, ~70 amino acids each in length) of the protein are shown. Black bars represent the position of the 'consensus sequence' in each of the repeater units and in the encoding DNA. * = potential phosphorylation sites. Differences in N-terminal sequence of rat LC1 are indicated in italics.

and in cultured cells/tissues of murine and rat origin. In studies based on SDS-PAGE and Western blot analysis (Loxley *et al.*, 1993a; Smith *et al.*, 1993; Taylor *et al.*, 1993; Philip *et al.*, 1996) we have repeatedly demonstrated the 37 kDa species of LC1 in extracts of rat anterior pituitary gland, posterior pituitary gland, hypothalamus, hippocampus, striatum and cortex using a panel of polyclonal antisera raised against purified human LC1, human recombinant LC1 (hrLC11-346) or a human synthetic N-terminal LC1 fragment (LC12-26). In many instances we also observed higher molecular weight species (60–96 kDa) in rat neural tissues (Loxley *et al.*, 1993a; Smith *et al.*, 1993) although not in the anterior pituitary gland or other peripher tissues (Smith *et al.*, 1993); these may reflect asymmetrically clipped multimers of the protein or possibly cross reactivity of the antibodies with high molecular weight species of LC2. In addition, we noted low molecular weight breakdown products of LC1 in the brain (32/33 kDa) and in the anterior pituitary gland (22 kDa, Smith *et al.*, 1993). Densitometric analysis of our blots showed that in the adult male rat the absolute amounts of 37 kDa LC1 vary significantly between regions such that, in decreasing order or abundance, anterior pituitary gland = posterior pituitary gland > hypothalamus > striatum = hippocampus = cerebral cortex (Smith *et al.*, 1993). We obtained similar data (Smith *et al.*, 1993) using a sensitive specific sandwich ELISA (Smith *et al.*, 1990) which, unlike Western blot analysis, permits precise quantification of the protein. In addition, by exploiting the ELISA to detect and quantify LC1 in discrete brain nuclei obtained *post mortem* by micropunching, we revealed a distinct regional distribution of the protein in the hypothalamus although not in the hippocampus (Smith *et al.*, 1993). The rank order of concentration of LC1 in hypothalamic nuclei was median eminence > supra-optic nucleus > > parvocellular and magnocellular regions of the paraventricluar nucleus = ventromedial nucleus = periventricular nucleus > > anterior hypothalamus = pre-optic suprachiasmatic nucleus = medial preoptic nucleus. Only very small amounts of LC1 were evident in the suprachiasmatic nucleus which equated with those observed in the CA1, CA2, CA3 & CA4 hippocampal nuclei and the cerebral cortex (Smith *et al.*, 1993). The abundance of LC1 in the median eminence, which has also been observed by immunostaining (Strijbos *et al.*, 1991), was particularly interesting since this is the zone from which the hypothalamic neurohormones are released in the portal vessels for transportation to the pituitary gland. These data are shown in Figure 6.

In accord with our data, several other groups have identified LC1 (37 kDa and higher molecular weight species) by Western blot analysis in various regions of the brain and spinal cord of rats (Bolton *et al.*, 1990; Strijbos *et al.*, 1991; Elderfield *et al.*, 1992) and pigs (Regnouf *et al.*, 1991). However, attempts to identify the cell types expressing the protein in the brain and pituitary gland by immunocytochemistry have generated discordant data, possibly because of problems with the specificity of the antisera, failure of the fixation processes to immobilise LC1 effectively (McKanna, 1993a, b) and, with human studies, the unavoidable delay in autopsy *post-mortem* (Johnson *et al.*, 1989b). In a study on primary cultures of rat hypothalamic cells we observed specific LC1 staining in both nerve and glial cells (Gillies, Lynch & Davidson & Buckingham, unpublished observations). Similarly, Stirjbos *et al.* (1991) reported dense LC1 staining in both neural and non-neural cells in sections of rat brain. Others by contrast have claimed that LC1 is expressed only by non-neuronal cells, in particular by activated glia at sites of tissue damage but also by cells lining the ventricles and epithelial cells of the choroid plexus (Johnson *et al.*, 1989b; Go *et al.*, 1993). In accord with this premise, dense LC1 staining has been described in human gliomas (Johnson *et al.*, 1989a), primary cultures of astrocytes (Gebicke-Haerter *et al.*, 1991; McLeod & Bolton, 1995) and in a glioma cell line

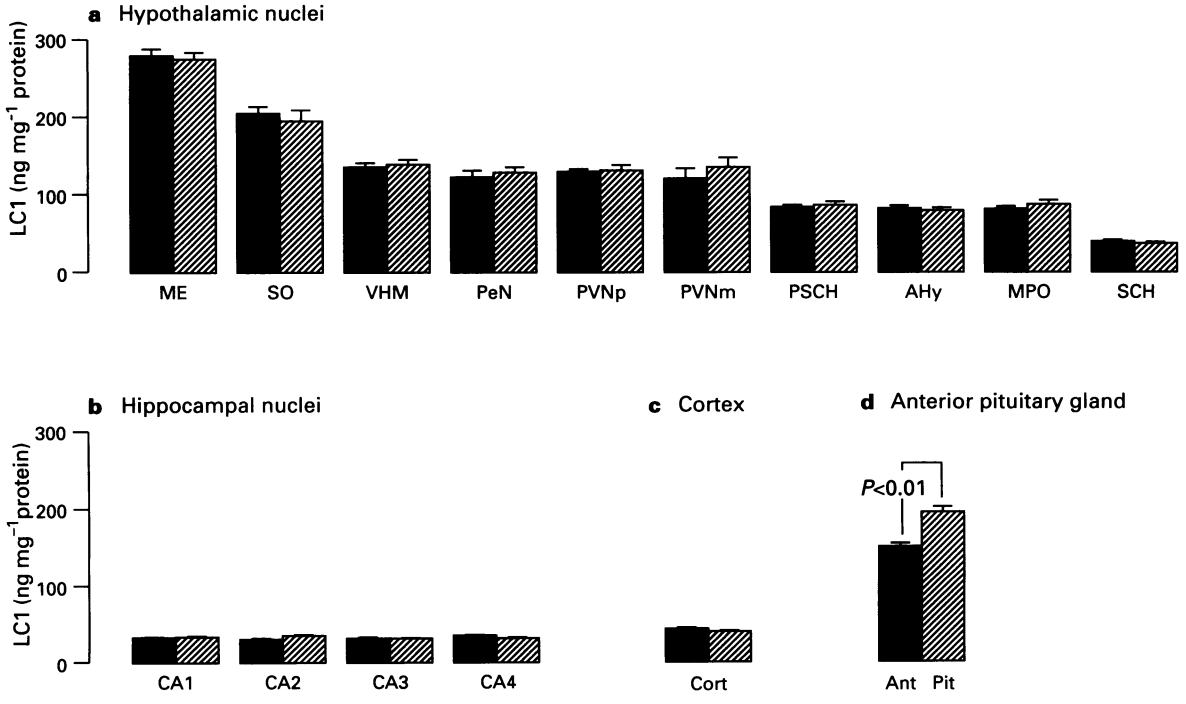

Figure 6 Determination of ELISA of the lipocortin 1 (LC1) content of (a) hypothalamic nuclei, (b) hippocampal nuclei, (c) cerebral cortex and (d) anterior pituitary gland in control and dexamethasone-treated rats. ME = median eminence; SO = supraoptic nucleus; VHM = ventro-medial nucleus; PeN = periventricular nucleus; PVNp = parvocellular paraventricular nucleus; PVNm = magnocellular paraventricular nucleus; PSCH = pre-optic suprachiasmic nucleus; AHy = anterior hypothalamus; MPO = medial pre-optic nucleus; SCH = suprachiasmatic nucleus; CAI-4 = cornu ammonis 1-4; Cort = cerebral cortex; ant. pit. = anterior pituitary gland. ■ = saline (5.0 ml kg^{-1}, i.p.); ▨ = dexamethasone (80 μg kg^{-1}, i.p. 4 h prior to autopsy). Values represent the mean ± SEM ($n = 5$). With the exception of the anterior pituitary gland, there were significant differences between the values in control and steroid-treated groups ($P > 0.05$, ANOVA plus Duncan's test). Similar data were obtained 2 h post-injection and with a higher steroid dose (25 mg kg^{-1}, i.p.). Redrawn from Smith *et al.* (1993), with permission.

(Johnson *et al.*, 1989a; McLeod *et al.*, 1995). Furthermore, we observed a pronounced increase in LC1 expression in primary hypothalamic cultures treated with the kainic acid which causes neuronal destruction and glial proliferation; by contrast, blockade of glial proliferation with cytosine β-arabinofuranoside substantially reduced the amounts of LC1 detected (Philip *et al.*, 1995). McKanna (1993b) has argued vehemently that LC1 expression is confined to the microglia. By contrast, others advocate that the LC1 generated in areas surrounding an infarct in both experimental animals and in man arises from a variety of cell types which, in addition to microglia, includes astrocytes and infiltrating macrophages (Bolton *et al.*, 1990; Johnson *et al.*, 1989b; Relton *et al.*, 1991; Mullens *et al.*, 1994).

Several lines of evidence support the view that alterations in the glucocorticoid milieu such as those caused by adrenalectomy or administration of the steroids themselves modulate the expression of LC1 in peripheral immune/inflammatory cells. Our initial attempts to demonstrate analogous glucocorticoid regulation of LC1 expression in the brain were largely unsuccessful. Thus, the LC1 contents (as determined by Western blot analysis or by ELISA) of gross brain areas (hypothalamus, striatum, hippocampus, cerebral cortex) and of individual hypothalamic or hippocampal nuclei collected by micropunching (Figure 6, Smith *et al.*, 1993) were unaffected by the pronounced changes in glucocorticoid tone produced either by adrenalectomy (14 days prior to autopsy) or by treatment of intact or adrenalectomized rats with dexamethasone (25–80 $\mu g\ kg^{-1}$, i.p.) 1, 2, 3, 4 or 24 h prior to tissue collection. Similarly, the acute increase in endogenous corticosterone secretion induced by a single injection of endotoxin (25 $\mu g\ kg^{-1}$, i.p.) had no discernible effect on the LC1 content of any of the brain areas studied (Figure 7, Smith *et al.*, 1991). Adrenalectomy also failed to influence the LC1 content of the anterior pituitary gland. However, dexamethasone induced a small but significant time-dependent rise in anterior pituitary LC1 content when given *in vivo* (80 $\mu g\ kg^{-1}$, i.p., Figure 6) or when applied to pituitary tissue *in vitro* (Smith *et al.*, 1993). Furthermore, injection of endotoxin (25 $\mu g\ kg^{-1}$, i.p.) increased LC1 expression in the pituitary glands of intact but not adrenalectomized rats, thus suggesting a role for endogenous glucocorticoids (Figure 7, Smith *et al.*, 1991). Taken together, these findings indicated that glucocorticoids augment the expression of LC1 in the anterior pituitary gland but not in the brain and thus raised the possibility that the steroid regulation of LC1 gene expression is tissue-specific. Similar conclusions were drawn from complementary studies on extracts of rat brain tissue (Bolton *et al.*, 1990), cultured astrocytes (Gebicke-Haerter *et al.*, 1991) and mouse corticotroph tumour cells (AtT20, Sudlow *et al.*, 1993). However, other workers challenged this view and provided evidence based on immunocytochemistry that corticosteroids exert discrete effects on LC1 expression in hippocampal neurones (Strijbos *et al.*, 1991) and glial cells (Go *et al.*, 1994).

An important concern about any study measuring the tissue content of a chemical messenger is that significant changes in turnover of the substance may go undetected. Since earlier studies on macrophages indicated that, in addition to inducing LC1 synthesis, glucocorticoids promote the exportation of LC1 from intracellular stores to pericellular sites (Browning *et al.*, 1990), we embarked on a further series of experiments designed to investigate whether a similar phenomenon operates in the brain and pituitary gland. Initial examination of explants of anterior pituitary and hypothalamic tissue from untreated control rats showed clearly that a small proportion of cellular LC1 is attached to the outer surface of the cells by a Ca^{2+}-dependent mechanism (Figure 8). This 'pericellular' pool of LC1 could be readily detected by washing the cells with EDTA (Ca^{2+}-chelating agent which causes LC1 to detach from the membrane) and harvesting the protein from the wash. Subsequent experiments showed clearly that exposure of anterior pituitary or brain (hypothalamus, hippocampus, striatum, cerebral cortex) tissues *in vitro* to dexamethasone (1–100 mM) results in a concentration-dependent increase in the amount of

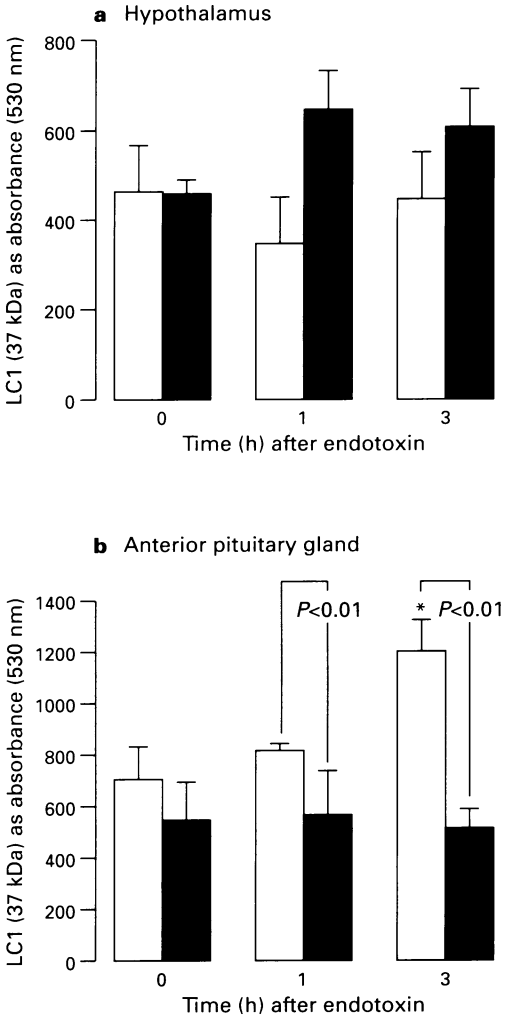

Figure 7 Effects of a single injection of endotoxin (bacterial lipopolysaccharide, 250 $\mu g\ kg^{-1}$ i.p.) on the expression of lipocortin 1 (LC1 37 kDa) in (a) hypothalamus and (b) anterior pituitary gland in rats subjected to adrenalectomy or a corresponding sham operation 14 days prior to autopsy. Measurements were made by Western blot analysis using an antibody raised in rabbit (coded 842) against purified human LC1. □ = sham-operated; ■ = adrenalectomized. Values represent the mean ± SEM ($n = 4-5$). $^{**}P < 0.01$ vs. value at $t = 0$ h (ANOVA plus Scheffé's test). Data from Smith *et al.* (1993), with kind permission of my co-authors, T. Smith, S.F. Smith and R.J. Flower.

LC1 attached to the outer cell surface and concomitant fall in the residual intracellular pool (Figure 8b, Loxley *et al.*, 1993a; Taylor *et al.*, 1993; Philip *et al.*, 1996). Similar responses were observed in all tissues when the steroid was injected (0.2 mg kg^{-1}, i.p.) 24 h prior to autopsy and tissue collection (Philip *et al.*, 1996). Further studies, focused largely on the anterior pituitary gland and hypothalamus, showed that the responses to dexamethasone are time-dependent and corticosteroid-specific. They are thus evident within 30 min and maximal within 90 min of steroid contact (Figure 8a and b; Taylor *et al.*, 1993). In addition, they are mimicked readily by corticosterone (100 nM) while aldosterone and progesterone (100 nM) are only very weakly active and oestradiol, tri-iodothyronine and thyroxine (100 nM) are without effect (Taylor *et al.*, 1995a, b). In accord with these data, dexamethasone has recently been shown to increase the expression of cell surface LC1 in primary astrocyte cultures (McLeod & Bolton, 1995) and in a glioma cell line (McLeod *et al.*, 1995).

The molecular mechanisms by which glucocorticoids promote the exportation of LC1 by cells have not yet been elucidated. Data from a recent study on human blood monocytes and rat peripheral leukocytes in which [^{32}S]-methionine was

used as a tracer indicate that the exported protein is newly synthesized (Coméra & Russo Marie, 1995). In agreement with these findings we have reported that the dexamethasone-induced externalisation of LC1 by pituitary cells is abolished by cycloheximide (1.0 $\mu g\ ml^{-1}$), an inhibitor of mRNA translation (Figure 9a, Taylor *et al.*, 1993). Surprisingly, however, actinomycin D (0.5 $\mu g\ ml^{-1}$), an inhibitor of transcription, proved to be ineffective in this regard (Figure 9a) suggesting that the steroid action is effected via novel non-genomic mechanism (Taylor *et al.*, 1993). Our attempts to verify this potentially important observation by molecular techniques have met with limited success for, while we can readily demonstrate LC1 mRNA in pituitary and brain tissues by the reverse transcriptase polymerase chain reaction (RT-PCR), detection and quantification by Northern blot analysis and *in situ* hybridization have been hampered by an apparent low abundance of the message (Cover, Rattery and Buckingham, unpublished). This may reflect instability of the ribonucleotide for examination of the 3′ untranslated region of LC1 mRNA reveals an abundance of adenylate/uridylate elements which, as reviewed by Chen & Shyu (1995), have been identified as important factors in the regulation of mRNA stability. The processes by which LC1 crosses the cell membrane remain to be determined. LC1 differs from classical peptide hormones but resembles certain cytokines (e.g. IL-6) in that it lacks a signal sequence and is therefore unlikely to access secretory vesicles for release by the conventional process of exocytosis. Since LC1 binds readily to membranes both within the cell and on the cell surface, one obvious possibility is a transport mechanism but, to date, no such system has been positively identified.

While much remains to be learnt about the underlying mechanisms, the studies described above provided novel evidence that glucocorticoids specifically increase the turnover

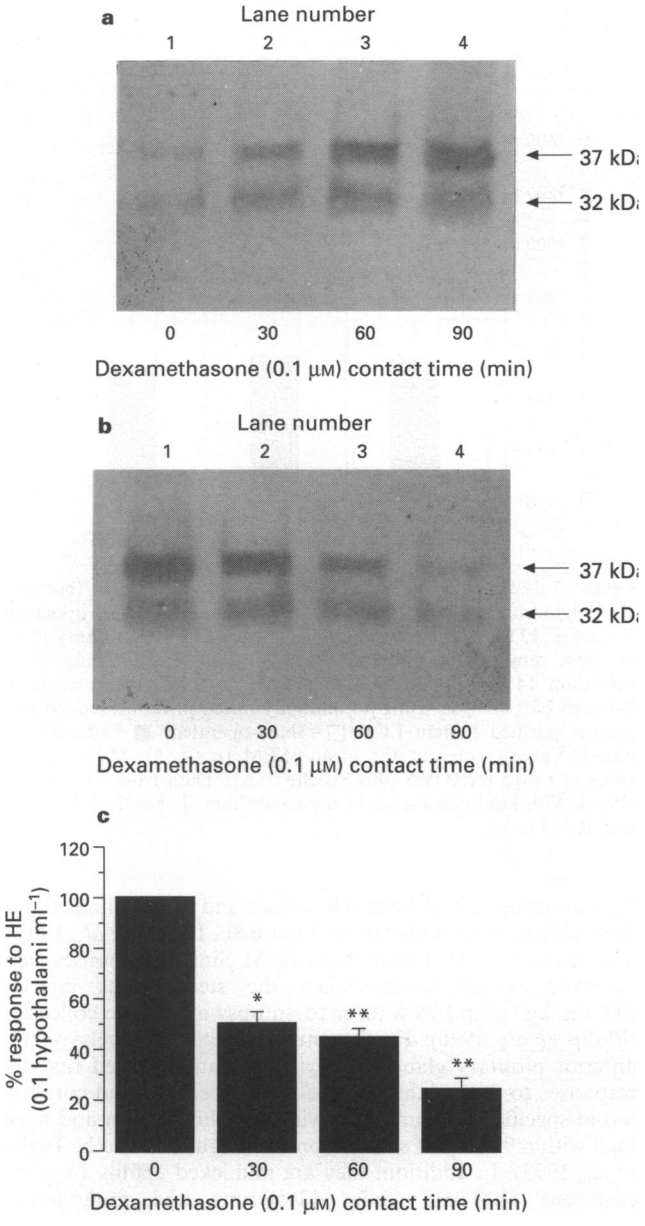

Figure 8 Time-dependent effects of dexamethasone (0.1 μM) *in vitro* on (a) the expression of lipocortin 1 on the outer surface of the pituitary cells (i.e. in the EDTA washes), (b) the expression of lipocortin 1 in extracts of the remaining pituitary tissue and (c) the secretion of ir-ACTH initiated by hypothalamic extracts (0.1 HE ml^{-1}) (a) & (b) represent western blot analysis; in (c) each column represents the mean ± SEM ($n = 5-6$) $^*P < 0.05$; $^{**}P < 0.01$, vs. steroid free control (ANOVA plus Duncan's test). The data are typical of those from 4 experiments. From Taylor *et al.* (1993), with permission of S.Karger AG, Basel.

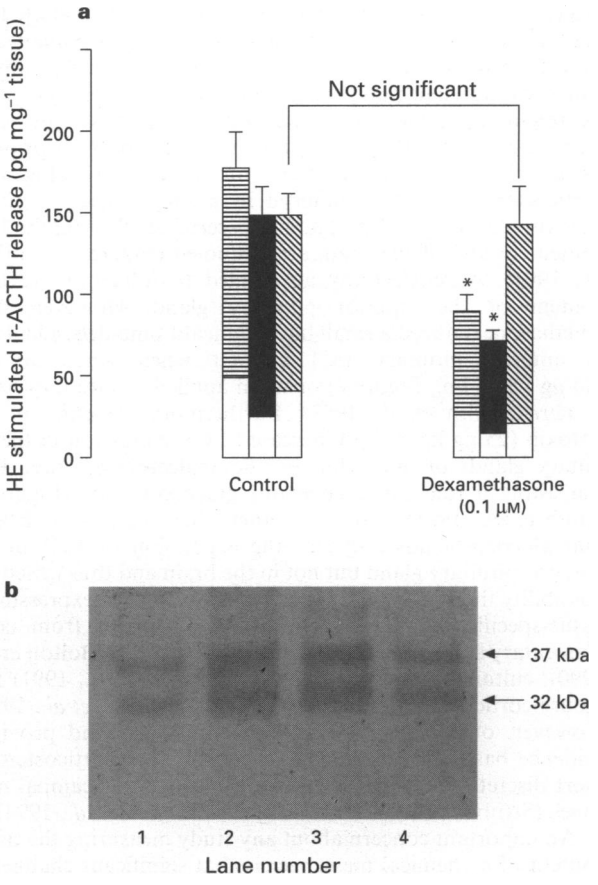

Figure 9 Effects of actinomycin-D (0.5 $\mu g\ ml^{-1}$) and cycloheximide (0.1 $\mu g\ ml^{-1}$) on the ability of dexamethasone (0.1 μM) (a) to inhibit the hypothalamic extract-stimulated (0.1 HE ml^{-1}) release of ACTH from rat anterior segments *in vitro* and (b) to increase the expression of lipocortin 1 on the outer surface of the pituitary cells as assessed by western blot analysis. In (i) ▤ = 0.1 HE ml^{-1}; ■ = 0.1 HE ml^{-1} + actinomycin D (0.5 $\mu g\ ml^{-1}$); ▨ = 0.1 HE ml^{-1} + cycloheximide (1.0 $\mu g\ ml^{-1}$). The open areas at the base of each column represent the corresponding hypothalamic extract free (i.e. basal) controls. In (b) lane 1 = control; lane 2 = dexamethasone (0.1 μM) alone; lane 3 = dexamethasone (0.1 μM) + actinomycin D (0.5 $\mu g\ ml^{-1}$); lane 4 = dexamethasone (0.1 μM) + cycloheximide (1.0 $\mu g\ ml^{-1}$). In the absence of dexamethasone, neither actinomycin D nor cycloheximide influenced the expression of LC1 on the outer surface of the cells (data not shown). Each value represents a mean ± SEM ($n = 5-6$); $^*P < 0.05$, vs. corresponding dexamethasone free group (ANOVA plus Duncan's test). The results are typical of those from 6 experiments. From Taylor *et al.* (1993), with permission of S.Karger AG, Basel.

and exportation of LC1 target cells in the brain and pituitary gland. Our functional studies suggest that these processes are critical to the early delayed feedback actions of the steroids on the secretion of CRH-41/AVP and ACTH. They also advocate a role for LC1 in other aspects of glucocorticoid action in the neuroendocrine system and have led us to hypothesize that LC1 may be pertinent to at least some of the diverse actions of the steroids elsewhere in the brain.

Modulation of the secretion of ACTH and its hypothalamic releasing factors in vitro and in vivo by glucocorticoids, lipocortin 1 and anti-lipocortin 1 antisera

In vitro *experiments*

Our *in vitro* studies were based primarily on the use of well established preparations which involve static incubation of anterior pituitary (Buckingham & Hodges, 1977a) and hypothalamic (Buckingham & Hodges, 1977b) tissue removed from rats immediately *post mortem*. Since their development, these preparations have been used widely in this (Loxley *et al.*, 1993a, b; Taylor *et al.*, 1993, 1995c) and other (e.g. Tsagarakis *et al.*, 1989; Yasin *et al.*, 1994) laboratories in studies on the mechanisms controlling the release of ACTH and its hypothalamic releasing hormones. Their advantages and disadvantages versus *in vitro* systems based on the use of perifusion systems and/or enzymatically dispersed cells, primary cell cultures or cell lines have recently been reviewed (Buckingham & Gillies, 1992; Gillies & Buckingham, 1995a, b). Importantly, the tissue pieces retain the three dimensional structure and cell-cell connections inherent to the tissue *in vivo* and respond reproducibly to physiological/pharmacological secretagogues (e.g. anterior pituitary gland; CRH-41, AVP, the adenylyl cyclase activator, forskolin, and the L-Ca^{2+}-channel opener, BAY K 8644; hypothalamus: acetylcholine, noradrenaline, 5-hydroxytryptamine, histamine, IL-1, IL-2, IL-6 and IL-8) with specific, time- and concentration-dependent increases in peptide release which can be readily detected by conventional assay techniques. Furthermore, exposure of the tissues to low concentrations of glucocorticoids causes a biphasic suppression of peptide release analogous to the 'rapid' and the 'early delayed' phases of steroid feedback observed *in vivo* (Buckingham, 1979). The preparations thus provide valuable models in which to examine the molecular mechanisms underlying these two distinct phases of feedback inhibition.

With regard to the pituitary preparation, an 'early delayed' feedback signal effected by pre-incubation of the tissue with dexamethasone (0.1 nM – 100 nM) causes a concentration dependent inhibition of ACTH release initiated by sub-maximal concentrations of hypothalamic extract (HE, a cocktail of hypothalamic peptides, 0.1 HE ml^{-1}) CRH-41 (1 nM), forskolin (1 mM), or BAY K 8644 (1 nM). Similar inhibitory effects are produced by the endogenous steroids, corticosterone

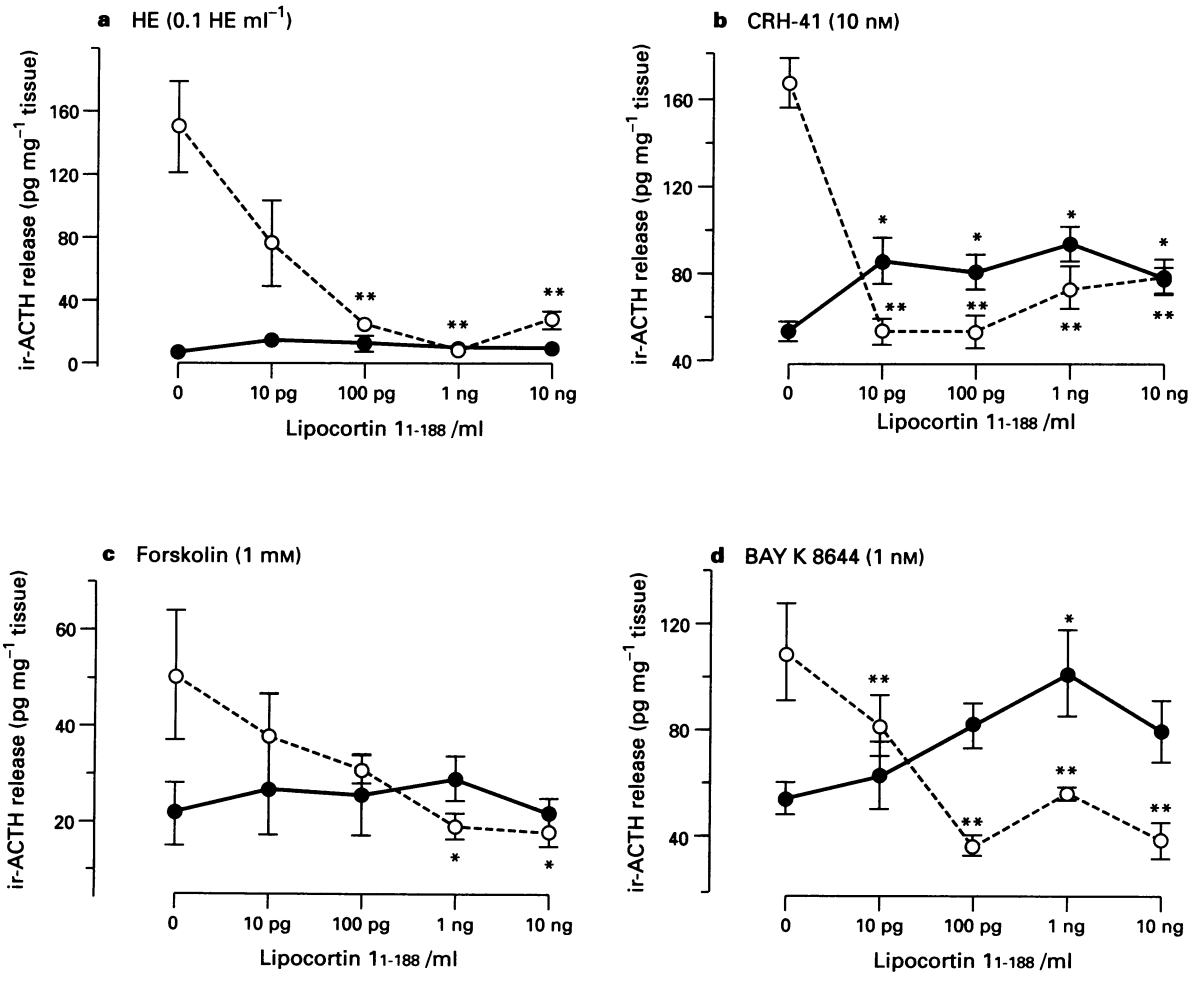

Figure 10 The effects of graded concentrations of lipocortin 1 1-188 on the spontaneous release of ir-ACTH by rat anterior pituitary segments *in vitro* (–●–, a-d) and the secretion evoked by (a) hypothalamic extracts (0.1 HE ml^{-1}), (b) corticotrophin releasing factor-41 (CRF-41 1 nM), (c) forskolin (1 mM) and (d) BAY K8644 (1 nM). NB drug contact times were 30 min in (a) + (c) and 60 min in (b) and (d). In all four graphs the evoked release is represented by (–○–). Each point represents the mean ± SEM ($n = 5-6$); $P < 0.05$, $^{**}P < 0.01$, vs. corresponding LC11-188-free control (ANOVA plus Duncan's test). The data are typical of those from three experiments. From Taylor *et al.* (1993), with permission of S.Karger AG, Basel.

and cortisol, and by a variety of semi-synthetic glucocorticoids e.g. betamethasone (Buckingham & Hodges, 1977c; Buckingham, 1979); by contrast, non-glucocorticoid steroids (e.g. oestradiol, testosterone) are ineffective in this regard (Buckingham, 1982). On a temporal basis the onset of the early delayed inhibitory action of dexamethasone on ACTH release parallels closely the steroid-induced appearance of lipocortin 1 on the outer surface of the pituitary cells, emerging within 30 min of steroid contact and reaching a maximum by 90 min (Figure 8c). Furthermore, like the exportation of LC1 from the cells, the inhibition of peptide release evoked by the steroid is blocked by cycloheximine (1.0 $\mu g\ ml^{-1}$) but not by actinomycin D (0.5 $\mu g\ ml^{-1}$), suggesting it too depends on the translation but not the transcription of new protein (Figure 9b, Taylor *et al.*, 1993).

In a series of experiments designed to investigate the potential role of LC1 in glucocorticoid action in the pituitary gland, we found that the powerful inhibitory actions of dexamethasone on secretagogue-induced ACTH release were mimicked consistently by the inclusion of a stable N-terminal LC1 fragment (LC1 1-188) in the medium. Thus, LC1 1-188 (10 $pg\ ml^{-1}$ – 10 $ng\ ml^{-1}$) produced significant ($P<0.01$, Duncan's test) concentration-dependent reductions in the overt increases in peptide release evoked by sub-maximal concentrations of HE (0.1 HE ml^{-1}), CRH-41 (10 nM), forskolin (1 mM) or BAY K 8644 (1 nM). These results are illustrated in Figure 10. Qualitatively similar effects were effected by the less stable full length recombinant molecule (hu-r-LC11-346, Taylor *et al.*, 1993). Conversely, a specific monoclonal anti-LC1 antibody (anti-LC1 mAb, Zymed, clone ZO13, diluted 1 : 15000) substantially reversed the inhibitory actions of dexamethasone on ACTH secretion evoked by each of these secretagogues while a corresponding dilution of an isotype matched (IgG1) control mAb (anti spectrin α and β) was without effect (Figure 11). A parallel but less exhaustive series of experiments performed on static incubates of enzymatically dispersed pituitary cells generated a similar pattern of results (Christian *et al.*, 1996). Thus, pre-incubation of the cells with dexamethasone (1 – 100 nM) suppressed CRH-41 (1 nM) stimulated ACTH release and promoted the translocation of LC1 from its intracellular stores to the cell surface. Furthermore, the inhibitory actions of the steroid on peptide release were readily reversed by anti-LC1 mAb (diluted 1 : 15000) but not by the control mAb (anti spectrin α and β).

Interestingly, although in the pituitary segment preparation LC1 1-188 (10 pg – 10 $ng\ ml^{-1}$) had no significant effect on the basal release of ACTH over a 30 min period (Figure 10a and c), when the contact time was extended to 60 min, a small but

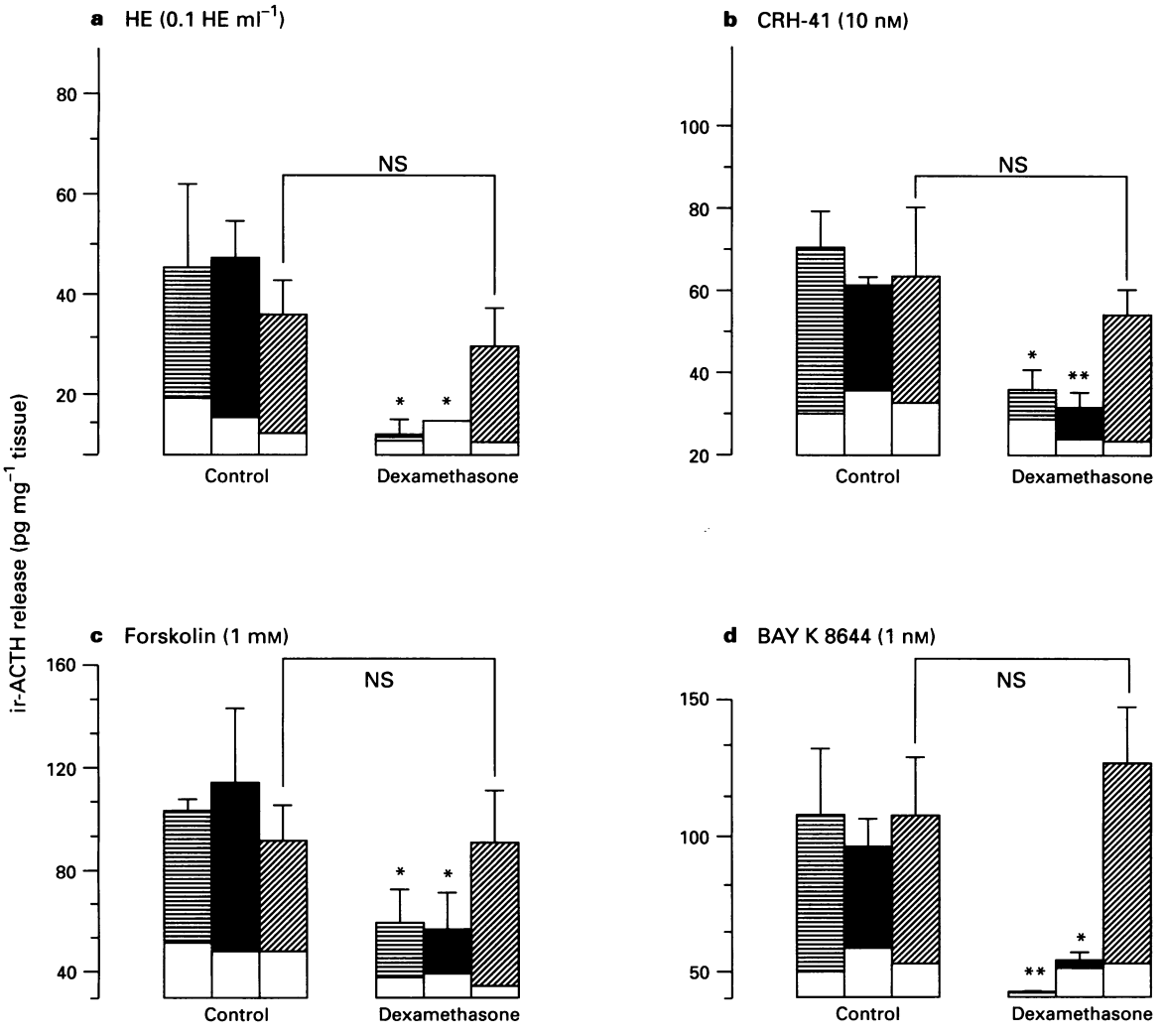

Figure 11 Neutralization by an anti-lipocortin 1 monoclonal antibody (anti-LC1 mAb) but not by a corresponding dilution (1 : 15000) of an isotyped matched control antibody (anti-spectrin $\alpha+\beta$) of the inhibitory effects of dexamethasone (0.1 μM) on the release of immunoreactive ACTH (ir-ACTH) from rat anterior pituitary segments induced by (a) hypothalamic extracts (HE, 0.1 HE ml^{-1}); CRH-41 (1 nM); forskolin (mM) and (d) Bay K9644 (1 nM). ▤ = secretagogue alone; ■ = secretagogue + control antibody; ▨ = secretagogue + anti-LC1 mAb. The open area at the base of each column represents basal ir-ACTH release in the abscence of secretagognes. Values represent the mean ± SEM ($n=5-6$); $*P<0.05$, $**P<0.01$, vs. corresponding dexamethasone-free group. NS = not significant. (ANOVA plus Duncan's test). These data are typical of those from 2–4 experiments. From Taylor *et al.* (1993), with permission of S.Karger AG, Basel.

significant ($P<0.05$, Duncan's test) increase in basal peptide release was consistently observed (Figure 10b and d); this we believe must be explained by time-dependent degradation of the exogenous protein to prosecretory peptide(s), a view supported by our studies based on Western blot analysis (Smith *et al.*, 1993) and by our observations (Taylor, Flower & Buckingham, unpublished) that ACTH release *in vitro* is promoted by a number of short (<24 amino acid residues) synthetic N-terminal LC1 fragments which share sequence homology with sauvagine, itself an ACTH secretagogue.

Complementary experiments on isolated hypothalamic tissue yielded a profile of data which resembled closely that obtained with pituitary tissue. Thus, dexamethasone (10 nM–1 μM) readily inhibited the release of CRH-41 and AVP induced *in vitro* by sub-maximal concentrations of a number of cytokines, viz. IL-1α, IL-1β, IL-6 and IL-8 (Figure 3). The concentration and time-dependent responses to the steroid coincided with the appearence of LC1 on the outer surface of the hypothalamic cells. Moreover, the inhibitory actions of the steroid on the cytokine stimulated the release of CRH-41 evoked by each of the cytokines tested were mimicked by hu-r LC1 1-346 (10 ng ml^{-1}) and LC1 1-188 (10 ng ml^{-1}) and specifically reversed by anti-LC1 mAb (Figures 12 and 13, Loxley *et al.*, 1993a, b; Taylor *et al.*, 1995c). The data obtained with AVP were less consistent in this regard in that hu-r-LC1 1-346 (10 ng ml^{-1}) and LC1 1-188 (10 ng ml^{-1}) either failed to influence or potentiated the AVP responses to cytokine stimulation *in vitro* (Loxley *et al.*, 1993a, b). Furthermore, the ability of anti-LC1 mAb to reverse specifically the inhibitory actions of dexamethasone on AVP release appeared to depend on the steroid status of the tissue at autopsy. Thus, in hypothalamic tissues removed from rats adrenalectomized 14 days prior to autopsy, anti-LC1 mAb readily reversed the steroid-inhibition of the increases in AVP evoked by IL-1α (0.2 ng ml^{-1}), IL-1β (0.5 ng ml^{-1}), IL-6 (10 ng ml^{-1}) and IL-8 (1 ng ml^{-1}). In stark contrast, in tissue removed from intact rats the antibody failed to modify the significant inhibitory actions of the steroid on the responses to these cytokines (Taylor *et al.*, 1995c). The overt differences between the effects of LC1 on the release of CRH-41 and AVP in our *in vitro* preparation may reflect differences in the cellular origin of the peptides. Certainly AVP is contained within the parvocellular CRH-41 neurones which project from the PVN to the median eminence where its expression is increased markedly by adrenalectomy (Sawchenko *et al.*, 1984). However, the bulk of AVP in the hypothalamus is located in the magnocellular neurones which project from the supraoptic and paraventricular nuclei to the posterior pituitary gland.

In vivo *experiments*

These experiments further examined the potential role of LC1 in the feedback regulation of HPA function by testing the influence of hu-r-LC1 1-346 and anti-LC1 antisera, given centrally or peripherally, on the secretion *in vivo* of ACTH and corticosterone in control and steroid-treated rats. In accord with the wealth of data in the literature (reviewed in Kellar-Wood & Dallman, 1984; Buckingham *et al.*, 1992), pretreat-

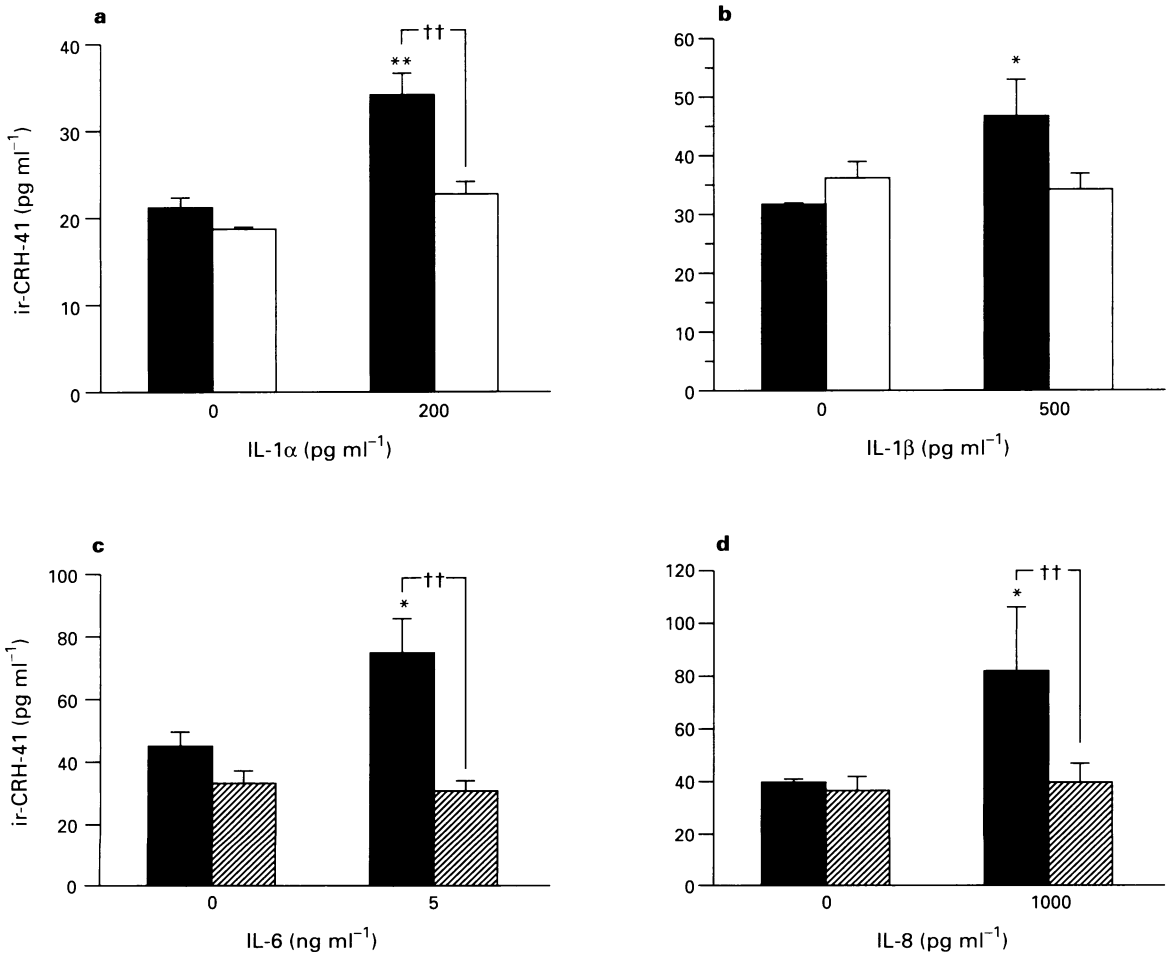

Figure 12 Effects of human recombinant lipocortin 1 (hu-r-LC1, a + b) and the N-terminal LC1 fragment, LC1 1-188 (c + d) on the increases in the release *in vitro* of immunoreactive (ir-) CRH-41 induced by (a) hu-r-interleukin-1α (hu-r-IL-1α), (b) hu-r-IL-1β, (c) hu-r-IL-6 and (d) hu-r-IL-8. ■ = control; □ = hu-r-LC1 (10 ng ml^{-1}); ▨ = LC11-188 (10 ng ml^{-1}). Values represent the mean ± SEM ($n=4-6$) *$P<0.05$; **$P<0.01$, vs. corresponding basal; †† $P<0.01$ vs. IL-1β alone (ANOVA plus Scheffé's test). The data are typical of those from 3 experiments. From Loxley *et al.* (1993a), with permission of S.Karger AG, Basel.

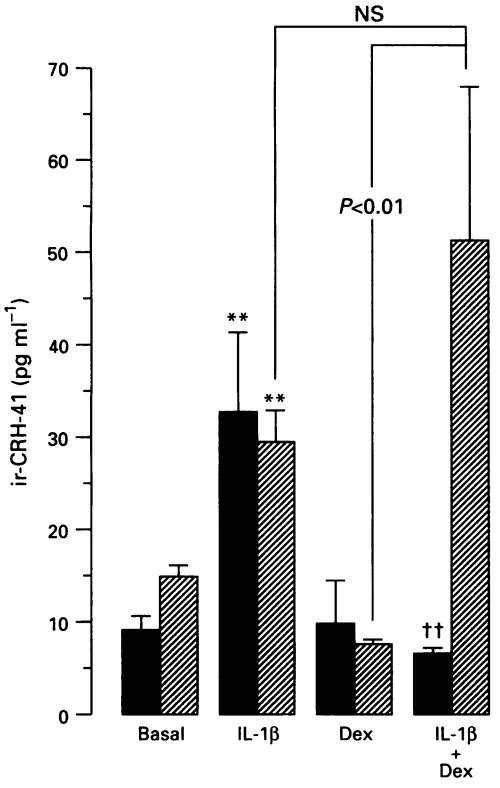

Figure 13 Neutralization by an anti-lipocortin monoclonal antibody (anti-LC1 mAb) but not by a corresponding dilution (1:15000) an isotype matched control antibody (anti-spectrin $\alpha+\beta$) of the inhibitory effects of dexamethasone (Dex, 0.1 μM) on the release *in vitro* of immunoreactive (ir-) CRH-41 induced *in vitro* by human recombinant interleukin-1β (IL-1β, 0.5 ng ml^{-1}). ■ = control Ab; ▨ = anti-LC1 mAb. Values represent the mean ± SEM (n = 4–6). **P<0.01 vs. corresponding basal; †† P<0.01 vs. IL-1β alone (ANOVA plus Scheffé's test). The data are typical of those from 3 experiments. From Taylor *et al.* (1995c), with permission of S.Karger AG, Basel.

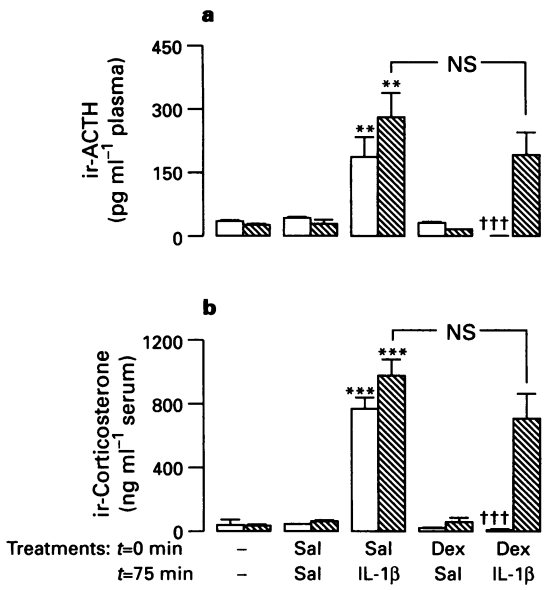

Figure 14 Reversal by a purified polyclonal anti-lipocortin 1 antibody (anti-LC1 pAb, 1 ml/day for 2 days) but not by non-immune sheep serum (NSS, 1 ml day^{-1} for 2 days) of the ability of dexamethasone (Dex, 100 μg kg^{-1}, i.p.) to inhibit the rises in (a) plasma immunoreactive- (ir-) ACTH and (b) serum ir-corticosterone concentrations induced in intact rats by injection of rat-recombinant interleukin 1β (2 μg kg^{-1}, i.p.). □ = NSS: ▨ = anti-LC1 pAb. Values represent mean ± SEM (n = 6–8). **P<0.01; ***P<0.001 vs. corresponding saline- or dexamethasone-treated groups; †††P<0.001 vs. dexamethasone-free IL-1β-stimulated group (ANOVA plus Scheffé's test). The data are typical of those from 3 experiments. From Taylor *et al.* (1995c), with permission of S.Karger AG, Basel.

ment of rats with dexamethasone (10–100 μg kg^{-1}, i.p.) reproducibly inhibited (P<0.01 *vs* vehicle control) the pituitary adrenocorticotrophic responses to a variety of stressors including administration of IL-1β (3 μg kg^{-1}, i.p. or 10 ng per rat, i.c.v.) or IL-6 (30 ng per rat, i.c.v.), surgical trauma (laparotomy under ether anaesthesia) or histamine injection (6.0 mg kg^{-1}, i.p.). Similarly, pretreatment of rats with of hu-r-LC1 1-346 (0.3–1.2 μg per rat, i.c.v.) produced a significant (P<0.01 vs saline vehicle) dose-dependent inhibition of the pituitary adrenocortical responses to IL-1β (10 ng per rat, i.c.v.) or IL-6 (30 ng per rat, i.c.v.) although not histamine (6.0 mg kg^{-1}, i.p., Loxley *et al.*, 1993a, b). Furthermore, passive immunisation of rats against LC1 with a well characterized, purified polyclonal anti-LC1 antiserum (1 mg kg^{-1}, s.c. on two successive days) effectively quenched the ability of dexamethasone (100 μg kg^{-1}, i.p. on day 3) to inhibit the ACTH and corticosterone responses to a systemic injection of rat recombinant IL-1β (3 μg kg^{-1}, i.p.) while treatment with the non-immune control serum (NSS) was without effect (Figure 14, Taylor *et al.*, 1995c).

Taken together, the data from our *in vitro* and *in vivo* experiments provide substantial evidence that LC1 is an important mediator of the early delayed feedback actions of the glucocorticoids at the levels of both the anterior pituitary gland and the hypothalamus. The molecular mechanisms through which LC1 effects its inhibitory actions are obscure. LC1 has been implicated the intracellular processes of membrane fusion (Francis *et al.*, 1992). However, several lines of evidence suggest that the inhibition of peptide release in the anterior pituitary gland and hypothalamus is effected via cell surface receptors. Firstly, the steroid-induced exportation of LC1 from intracellular to pericellular 'sites', which on a temporal basis parallels the onset of the feedback response, provides opportunity for the protein to interact with membrane bound 'receptors'. Secondly, while we cannot exclude the possibility that our neutralising monoclonal and polyclonal anti LC1 antisera traverse cell membranes to sequester LC1, the antibodies would be expected to associate more readily with the protein at an extracellular site and thereby quench its biological activity. Similarly, neither LC1 1-188 nor hu-r-LC1 1-346 would be expected to penetrate cell membranes readily although, interestingly, the small N-terminal LC1 fragments which release ACTH in our system enter neutrophils (M. Perretti, personal communication). Our attempts to identify LC1 binding sites in the brain and pituitary gland by conventional autoradiographic techniques were not successful due to problems with the iodinated ligand (Taylor, Croxtall, Flower & Buckingham, unpublished). However, by means of computerized fluorescent activated cell cytometry/sorting (FACS) we have recently demonstrated high affinity (K_d ~ 1 nM), Ca^{2+}- and trypsin-sensitive LC1 binding sites on the surface of anterior pituitary cells (Christian *et al.*, 1995). These sites closely resemble those deemed essential for LC1 action in certain peripheral cell types (e.g. monocytes and polymorphonuclear leukocytes, Goulding *et al.*, 1992; Goulding & Guyre, 1993). Electron microscope analysis of the pituitary cells sorted by FACS suggests that the LC1 binding sites are expressed on multiple secretory cell types, viz. corticotrophs, thyrotrophs, lactotrophs, gonadotrophs and somatotrophs (Christian *et al.*, 1996).

The nature of the signal transduction membrane used by the LC1 'receptors' is a focus of current research in our laboratory. Our finding (Figures 10d and 11d) that LC1 1-188 and the anti-LC1 mAb exhibit particularly striking abilities of mimic and abolish respectively the steroid-induced inhibition of ACTH release initiated by the L-Ca^{2+} channel opener, BAY K 8644,

led us to propose that LC1 may, directly or indirectly, block Ca^{2+}-influx. However, our Ca^{2+}-imaging studies (done in collaboration with Dr Ian Davidson, IAPGR, Babraham, Cambridge, U.K.) failed to demonstrate any effects of either LC11-188 or dexamethasone on BAY K-stimulated Ca^{2+} entry in dispersed anterior pituitary cells. A further putative mechanism is inhibition of type IV (cystolic) phospholipase A_2 ($cPLA_2$) a process which is strongly implicated in LC1 action in other tissues (Kim *et al.*, 1994). Such an action would be expected to suppress the release of ACTH and CRH-41/AVP since arachidonic acid metabolites generated by the cytochrome P450 pathway are critical to ACTH release from the corticotrophs (Cowell *et al.*, 1991) and prostanoids are strongly implicated in the processes underlying cytokine evoked CRH-41/AVP release from the hypothalamus (Navarra *et al.*, 1991; Yasin *et al.*, 1994). Furthermore, since in several systems $cPLA_2$ appears to be a substrate for protein kinases (Glaser *et al.*, 1993), blockade of its actions may provide opportunity for interruption of the adenyl cyclase and phospholipase C driven cascades which operate in both the corticotrophs and the intrahypothalamic CRH-41/AVP neurones.

Our finding that passive immunisation of rats against LC1 effectively overcomes the ability of dexamethasone to block the HPA responses to an immune insult (systemic administration of IL-1β) is particularly important as it demonstrates for the first time a role for endogenous LC1 in the regulation of HPA function *in vivo*. Confidence in the effectiveness and specificity of the immunisation protocol was enhanced by our observations that the antibody titres attained at autopsy in serum and in several tissues (anterior pituitary gland, hypothalamus and cerebral cortex) were sufficient to quench the activity of endogenous LC1 and that purified, non-immune control sheep serum was inert in all of our experiments (Taylor *et al.*, 1995c). Furthermore, the secretion of three other pituitary hormones (lutenising hormone, thyrotrophin and prolactin) in cytokine and/or dexamethasone-treated rats was unaffected by the pretreatment with either the anti-LC1 antiserum or the control, non-immune serum (Taylor *et al.*, 1995c). From our present data it is not possible to identify the site(s) at which the antibody is acting but our observation that substantial amounts of the antibody reach both the anterior pituitary gland and the hypothalamus points to actions in these tissues as also do the results of our *in vitro* studies and our observation that central administration LC1 suppresses the HPA responses to cytokine stimulation (Loxley *et al.*, 1993a). It is also possible that the antibody acts in part at the level of the blood-brain-barrier to facilitate the access of IL-1β to its hypothalamic receptors by attenuating the ability of dexamethasone either to reduce the permeability of the barrier (Long & Holaday, 1985) or to quench the local generation of eicosanoids, a mechanism reputed to be important in effecting the local synthesis of IL-1β in the hypothalamus (see section 'Modulation of the HPA responses to immune insults by glucocorticoids'). By the same token, removal of the steroid brake on the cytokine-induced generation of prostanoids in the periphery may be expected to promote the vagus-dependent induction of cytokine synthesis in the CNS recently described by Lalé *et al.* (1995) and, thereby, augment HPA activity.

An interesting paradox lies in the fact that our immunisation process failed to neutralize the steroid-induced blockade of the HPA response to surgical trauma (laparotomy under ether anaestheisa, Taylor *et al.*, 1995c). It may be that laparotomy is more sensitive to the powerful negative feedback actions of dexamethasone and that the antibody would have proved effective had a lower, sub-maximal dose of the steroid been employed. Nonetheless, this observation raises the possibility that multiple molecular mechanisms contribute to the early delayed feedback effects of the glucocorticoids on HPA function and that the mechanism adopted may depend on the nature of the challenge and hence on the complement of neural and humoral factors recruited to drive the release of CRH-41/AVP.

Role of lipocortin 1 in the regulation of the secretion of thyrotrophin and prolactin by the anterior pituitary gland

Glucocorticoids exert widespread regulatory actions in the neuroendocrine system and, in addition to suppressing the activity of the HPA axis, they also serve to modulate the secretion of several other hormones including thyrotrophin (TSH), prolactin, growth hormone and in some instances, the gonadotrophins (Pamenter & Hedge, 1980; Lamberts & MacLeod, 1990; Frohman *et al.*, 1992; Samuels *et al.*, 1994). The inhibitory influence of the steroids on the secretion of TSH and prolactin is particularly well documented in both experimental animals and in man. With regard to TSH, numerous studies have demonstrated the ability of exogenous glucocorticoids to depress the serum TSH concentration (Brabant *et al.*, 1987; Vinje *et al.*, 1993; Samuels *et al.*, 1994), the circadian excursion in TSH release (Brabant *et al.*, 1987) and the TSH responses to thyrotrophin releasing hormone (TRH, Brabant *et al.*, 1987, Nicoloff & Spencer, 1992). Endogenous glucocorticoids are also effective in this regard. Thus, the elevations in serum corticosterone/cortisol evident in stress (Zaloga *et al.*, 1985), Cushing's syndrome (Benker *et al.*, 1990; Rubello *et al.*, 1992) and depression (Maes *et al.*, 1990; 1994) are accompanied by reciprocal decreases in serum the TSH concentration as also are those in aging subjects (Iovino *et al.*, 1990). Conversely, TSH secretion is enhanced in conditions of glucocorticoid deficiency resulting from adrenalectomy (Mitsuma & Nogimori, 1982), primary adrenocortical insufficiency (Ismail *et al.*, 1989) or metyrapone treatment (Re *et al.*, 1976). In the same vein, prolactin secretion is highly sensitive to alterations in glucocorticoid tone. Thus, in the rat administration of glucocorticoids attenuates the rises in prolactin release induced by psychological trauma (Yelvington *et al.*, 1984) and by physical stresses such as hypoglycaemia (Copinischi *et al.*, 1975), footshock (Rossier *et al.*, 1980), ether anaesthesia (Piroli *et al.*, 1993) and restraint (Lopez-Calderon *et al.*, 1989). Moreover, pharmacological blockade of glucocorticoid receptors potentiates prolactin release (Nishino *et al.*, 1992; Caron *et al.*, 1994) while surgical adrenalectomy produces glucocorticoid-reversible increases in the basal and stress-induced release of prolactin (Watanobe, 1990).

The powerful inhibitory actions of the glucocorticoids on the secretion of TSH and prolactin have been attributed to actions on the anterior pituitary gland (Iovino *et al.*, 1991; Nicoloff & Spencer, 1992; Houben & Denef, 1992; Robberecht *et al.*, 1992) and the central nervous system notably the hypothalamus (Brown & Hedge, 1974; Kakucska & Lochan, 1991; Bartha *et al.*, 1991; Briski & Sylvester, 1991). Our demonstration that LC1 plays a key part in the molecular mechanisms effecting the steroid-induced suppression of ACTH release from the corticotrophs led us to exploit our *in vitro* model to investigate whether the protein fulfils an analogous role in the regulation of TSH and prolactin secretion.

In preliminary experiments we confirmed that the TSH/prolactin secretagogues TRH (100 pM – 1 μM), vasoactive intestinal polypeptide (VIP, 100 pM – 1 μM), forskolin (1 μM – 10 mM) and L-Ca^{2+} channel opener, BAY K 8644 (10 nM – 100 μM) each produced concentration-dependent increases in the release of both peptides ($P<0.01$ vs basal, Duncan's test). The secretory responses to a submaximal concentration of BAY K 8644 (10 μM) were readily blocked by nifedipine (1 μM – 100 μM) but not by pre-incubation (2 h) of the tissue with dexamethasone (1 nM – 1 μM). By contrast, dexamethasone (1 nM – 1 μM) effectively blocked the release of both TSH and prolactin evoked by submaximal concentrations of TRH (10 nM), VIP (10 nM) and forskolin (100 μM) by mechanisms dependent on *de novo* protein synthesis: its effects were near maximal (90 – 100%) at a concentration of 0.1 μM (Taylor *et al.*, 1995a, b).

Inclusion of LC1 1-188 (100 pg – 10 ng ml^{-1}) in the incubation medium suppressed in a concentration-dependent manner the release of TSH induced by TRH (10 nM), VIP

(10 nM) and forskolin (100 μM) but, like dexamethasone, it failed to modify the TSH responses to BAY K 8644 (10 μM). Conversely, exposure of the pituitary tissue to the monoclonal anti-LC1 antibody (anti-LC mAb, diluted 1:15000) but not the isotype matched control antibody (anti-spectrin α and β) readily reversed the inhibitory effects of dexamethasone on the TSH responses to these three secretagogues. These findings (Taylor *et al.*, 1993) are illustrated in Figure 15. The inhibitory effects of dexamethasone on the rises in prolactin release stimulated by VIP (10 nM) and forskolin (100 μM) were also mimicked by LC1 1-188 (100 pg ml^{-1} – 10 ng ml^{-1}) and specifically reversed by anti-LC1 mAb (Figure 16, Taylor *et al.*, 1995b). However, in contrast to our results with TSH, the dexamethasone-induced blockade of TRH-induced prolactin secretion was neither mimicked by LC11-188 (100 pg ml^{-1} – 10 ng ml^{-1}) nor quenched by anti-LC1 mAb (Figure 16, Taylor *et al.*, 1995b).

The mechanisms through LC1 which inhibits TSH and prolactin secretion await investigation. For reasons discussed in the section relating to ACTH secretion, it is likely that the protein acts via membrane bound receptors and, in support of this contention, our studies based on a combination of FACS analysis and electron microscopy have identified high affinity LC1 binding sites on both thyrotrophs and lactotrophs. It is also attractive to speculate these 'receptors' are positively coupled to cystolic PLA_2 since arachidonic acid metabolites are also strongly implicated in the signal transduction cascades which trigger the release of both TSH and prolactin (for review see Cowell & Buckingham, 1989). Our finding that the steroid blockade of the prolactin responses to VIP and forskolin (both of which act by raising intracellular cyclic AMP) but not to TRH (which acts via phospholipase C) is dependent on LC1 is particularly interesting as it raises the possibility that LC1 acts selectively within the lactotrophs at a point within the cyclic AMP-dependent signal transduction sequence; in this event LC1 may serve to augment the influence of dopamine (the major hypothalamic factor regulating prolactin release) which inhibits lactotroph activity by depressing cyclic AMP generation. Our observation that LC1 depresses the secretory responses of the thyrotroph but not the lactotrophs to TRH is also intriguing and points to important differences in the biochemical processes controlling peptide release in these two distinct cell populations. The fact that the steroid blockade of the TRH-induced rise in prolactin secretion is overcome by inhibitors of RNA/protein synthesis advocates a role for protein(s) distinct from LC1. The nature of the protein(s) is/are obscure but reports that dexamethasone increases the synthesis and activity of ATP-sensitive K^+ channels in two prolactin secreting cell lines, GH3 and GH4C1, (Levitan *et al.*, 1991) together with evidence that the depolarization caused by TRH is associated with a decrease in membrane K^+ flux (Taraskevich & Douglas, 1978; Schofield, 1983; Israel *et al.*, 1985) raise the possibility that K^+ channels are important in this regard.

We have not yet had the opportunity to investigate the influence of LC1 and anti-LC1 antisera on the secretion of the hypothalamic hormones which regulate the secretion of TSH and prolactin, viz dopamine TRH and VIP. Nor have we investigated to any degree the ability *in vivo* of LC1 to mimic and passive immunisation to quench the ability of glucocorticoids to suppress the rises in TSH and prolactin release induced by provocative stimuli. Nevertheless, we have shown that our immunisation protocol overcomes the steroid blockade of the prolactin responses to surgical trauma, a finding which points to a physiological role for the LC1 mediator of glucocorticoid action in the lactotrophs. Further studies are now necessary to verify these findings and to extend our *in vivo* studies to the thyrotroph.

Conclusions

The studies described here were based on the use of a combination of biochemical and molecular technologies together with established 'bioassay' systems which, in their fundamental, design exploit the principles pioneered by Gaddum some half a century ago. The data accrued demonstrate a major role for lipocortin 1 as a mediator of glucocorticoid action in the neuroendocrine system and they thus represent a significant advance in our understanding of the physiology and pharmacology of this important group of steroid hormones. Our findings suggest that LC1 is released from target cells in the hypothalamus and anterior pituitary gland in response to a glucocorticoid challenge and that it acts locally, possibly via cell surface receptors, to inhibit peptide release and thereby serves as an autocrine or paracrine agent. Many important questions now need to be answered about the molecular mechanisms underlying the production, 'exportation' and actions of LC1 in these tissues. We are currently undertaking histological studies (in collaboration with Drs John Morris and Valerie Traverso, University of Oxford) to determine whether LC1 is produced and externalised by the peptide secreting cells themselves following a steroid challenge and thus has an autocrine role and/or whether it originates from adjacent cells and thereby exerts a paracrine effect. Such a paracrine mechanism may provide a novel and important route of neuroendocrine communication in the pituitary gland and the median eminence in conditions of e.g. inflammatory disease, providing a means whereby not only resident but also migrating/infiltrating steroid sensitive cells (e.g. macrophages in

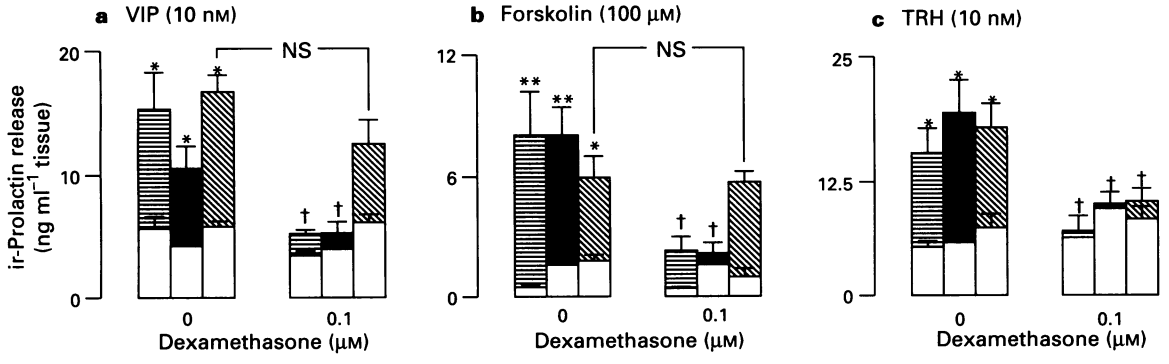

Figure 15 Ability of anti-lipocortin 1 antisera to specifically reverse the dexamethasone induced suppression of the ir-PRL release from rat anterior pituitary segments *in vitro* by (a) VIP (10 nM) and (b) forskolin (100 μM) but not by (c) TRH (10 nM). ▤ = antibody free control; ■ = control monoclonal antibody (anti-spectrin α + β, diluted 1:15000); ▨ = anti-LC1 monoclonal antibody (1:15000); the open areas at the base of each column = basal ir-PRL release. Values represent the mean ± SEM ($n = 6$). *$P < 0.05$; **$P < 0.01$ vs. corresponding secretagogue-free control; †$P < 0.05$, ††$P < 0.01$ vs. corresponding LC1 free control (ANOVA plus Duncan's multiple range test). The data are typical of those from 3–4 experiments. From Taylor *et al.* (1995a), with permission of S.Karger AG, Basel.

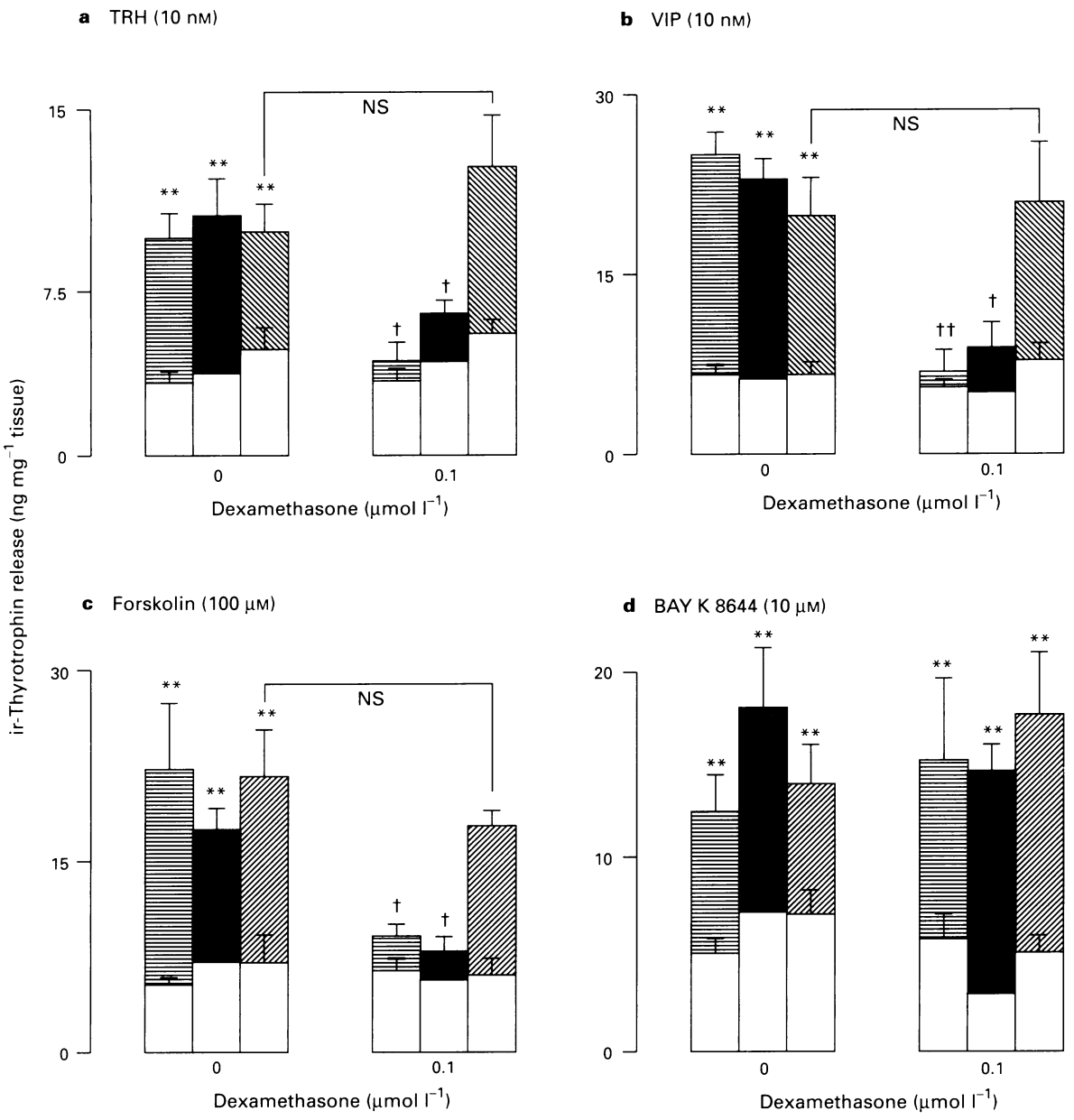

Figure 16 Neutralization by anti-LC1 monoclonal antibody (anti-LC1 mAb) but not by a corresponding dilution (1 : 15000) and isotyped matched control antibody (anti spectrin $\alpha+\beta$) of the inhibitory effects of dexamethasone (0.1 μM) on the release of TSH initiated by submaximal concentrations of (a) TRH (10 nM), (b) VIP (10 nM) and (c) forskolin (100 μM). (d) Failure of dexamethasone or anti-LC1 mAb to influence the TSH response to BAY K8644 (10 μM). ▤ = antibody free controls; ■ = control antibody, ▨ = anti-LC1 mAb. Shaded areas = secretagogue-stimulated ir-TSH release; open areas at the base of each column show basal ir-TSH release in the absence of secretagogues. Values represent a mean of 6. $^{**}P<0.01$ vs. corresponding basal release. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. corresponding dexamethasone free control; NS not significant (ANOVA plus Duncan's test). The data are typical of those from 3–4 experiments. From Taylor *et al.* (1995b), with permission of the Journal of Endocrinology Ltd.

which LC1 expression is abundant) temper peptide release from specific target cells and thus contain the secretion of peptides (CRH-41, ACTH, prolactin, TSH and possibly other pituitary hormones) which themselves, directly or indirectly, exert significant immunoregulatory properties. Parallel ongoing studies, based on the exploitation of a range of molecular and cellular technologies together with anti-sense and other gene targeting strategies, are directed towards elucidating the molecular mechanisms underlying the glucocorticoid-induced exportation of LC1 by cells in the brain and pituitary gland and the subsequent actions of the protein via its putative membrane bound receptors on its various target cells. These studies should provide new insight to our understanding of the glucocorticoid regulation of neuroendocrine function in conditions of health and disease. In addition, we anticipate that the findings will also prove relevant to the diverse physiological, pharmacological and pathological actions of the steroids elsewhere in the brain and in the periphery.

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