

ANTI-STRESS HORMONES

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We are all familiar with stress. But we must make a distinction between acute stress, and the fight or flight response, and chronic pathological stress exemplified by Addison's disease.

		Acute	Chronic
Brain	<i>Alertness/Attention</i>	UP	?
	<i>Sleep</i>	DOWN	Disrupted
	<i>Memory</i>	UP	Disrupted
Heart	<i>Rate</i>	UP	?
Respiration	<i>Rate</i>	UP	?
Thermogenesis		UP	?
Consumption/Obesity		DOWN	UP
Gastric		DOWN	Disrupted
Pigmentation		-	UP
Psychiatric	<i>Depression</i>	-	UP
	<i>Anxiety</i>	-	UP
	<i>Pain sensitivity</i>	DOWN	UP

Stress and antistress hormones

We know a little about how the stress response is mediated; we know rather less about how the response is turned off. Many hormones are involved.

Stress	Hormones and stress
	Antistress
CRF, urocortin	<u><i>Stresscopin/urocopsins2 and 3?</i></u>
Corticotropin / ACTH	<i>MSH analogs? Agouti at the MC4 receptor?</i>
Corticosterone	<u><i>DHEA?</i></u>
Adrenalin	<i>?</i>
	<i>angiotensin?</i>
	<i>melatonin</i>
	<i>neuropeptide Y</i>
	<i>orexin</i>
	<i>orphanin FQ/nociceptin</i>
	<i>progesterone</i>
	<i>prolactin</i>
	<i>substance P</i>

How is stress measured?

Is anxiety the same as chronic stress? Do commonly used tests, for instance the elevated plus maze, or the tail flick test, really measure either stress or anxiety? How does stress, anxiety or depression affect performance in a 'cognitive' tests such as the watermaze?

Focus on two case studies

Here we will discuss emerging evidence for two new anti-stress mechanisms. First, potential antagonism between CRF and stresscopins/urocortins; second, the seeming antagonism of the stress response by DHEA. For keen students, the antagonism of the melanocortin receptor type 4 (MC4) by *agouti* may be of interest (for a good starter review see **1,2**).

Case-study 1. Antistress activity at the hypothalamus -> pituitary junction.

Conventional wisdom suggests that CRF (=CRH) released from the hypothalamus reaches the pituitary where it directs the secretion of ACTH. CRF targets two receptors, CRF-R1 and CRF-R2, with different distributions. Both are expressed widely in the brain but with highest expression in the pituitary. CRF-R1 is the predominant receptor in human brain with CRH-R2 predominating in peripheral organs. CRF predominantly binds to CRF-R1, and interference with CRF-R1 has pronounced anxiolytic effects, suggesting that CRF principally exerts its pro-stress activity via CRF-R1.

However, as discussed by **Reul and Holsboer (3)**, ligands targeting CRF-R2 can have anxiolytic OR anxiogenic properties depending on how the test is done. If CRF principally targets CRF-R1, then CRF-R2 no doubt plays a modulatory role.

This interpretation is bolstered by the discovery that there are several ligands for both CRF-R1 and R2.

Urocortin. This was discovered in 1995 by the Salk group, and was found to activate both CRF-R1 and CRF-R2, efficiently releasing ACTH. It is expressed widely in the brain and peripheral tissues, but in rodents most highly in the pituitary, suggesting that it is involved in autocrine control. In human, some studies have argued that it is not expressed significantly in either the hypothalamus or pituitary,

Sheau Hsu and Aaron Hsueh (4); Stanford. They describe an EST/genomic database search for sequence motifs based on the CRH-urocortin family, including fish and amphibian sequences. This yielded many sequences, that were then screened for proteolytic cleavage sites flanking the primary sequence. On these criteria they sequences that they dubbed stresscopin (SCP) and stresscopin-related protein (SRP) . Full-length sequences were found to encode a prepro-hormones of 120-161 amino acids, with mature proteins of 40-43 amino acids (like CRF itself, 41 amino acids). In situ hybridization showed that both are expressed widely as well as in the brain.

Pharmacologic activity revealed, in cell culture, that SCP and SRP are potent agonists of CRF-R2, promoting cAMP production, but are ineffective at CRF-R1. In

vivo, SCP and SRP failed to promote ACTH secretion under conditions where both CRF and its relative, urocortin, were effective. More refined analysis revealed that SCP and SRP have significant effects on indirect effects of stress - including oedema in response to heat, anorexia and food intake, etc. But the pharmacology is complex, and short and long exposures may have different effects.

Vale and Sawchenko (5,6), Salk Institute. These authors report similar findings, calling their peptides urocortin-II and urocortin-III. They confirm that these peptides selectively bind to the CRF-R2 receptor. Ie, they do not activate ACTH release.

Hormone	Alternative name	Receptor	Action
CRF		CRF-R1	ACTH release
urocortin		CRF-R1 and CRF-R2	ACTH release
urocortin II	stresscopin-related (SRP)	CRF-R2	no ACTH release; switch off?
urocortin III	stresscopin (SCP)	CRF-R2	no ACTH release; switch off?

What do they do? Overall, we must conclude that the urocortins modulate "stress-related autonomic, neuroendocrine, and behavioural function". Hsu argues that these are anti-stress or 'stress-coping' hormones, mediating the recovery phase of the stress response. Or, put differently, "we must learn to imitate the body's own autopharmacologic efforts to combat the stress factor in disease".

	CRF	Urc	Urc2/SRP	Urc3/SCP
ACTH release	+	+		
Enhanced activity	+			
Anti-oedema	+	+	+	+
Feeding suppression	+	+	+	+
Gastric emptying suppression	+	+	+	+

Case study II: Is DHEA really an antiglucocorticoid?

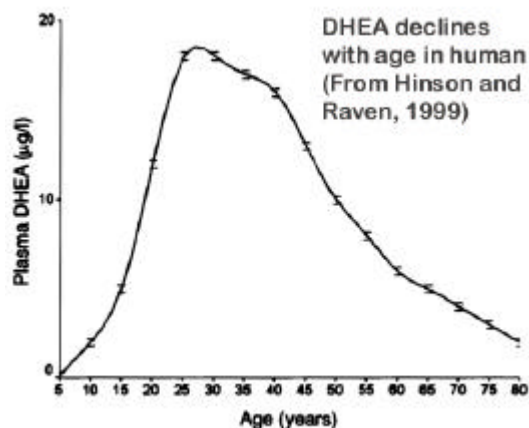
There have been many suggestions that DHEA can have antiglucocorticoid activity. Analysis of this contention requires an understanding at many levels.

1. Antigluocorticoid action of DHEA. Several studies have shown, in the brain and in the immune system, that DHEA can counteract the effects of glucocorticoids.

PARAMETER	GLUCOCORTICOID	DHEA
Learning/memory	High doses inhibit	Promotes
Synaptic potentiation	High doses inhibit	Promotes
Contextual fear conditioning	Promotes	Inhibits
TH induction in brain	Sound stress increases	Reversed
Neurotoxicity	Promotes	Protective
Serum IGF1	Suppressed	Promotes
IL2 synthesis	Inhibits	Promotes
Immunity	Suppression	Restores
Astrocyte proliferation	Inhibits	Inhibition reversed
Hepatic lipid release	Promotes	Reversed

Nevertheless, the effects of DHEA are not always identical to those of a conventional glucocorticoid antagonist. For instance, while both DHEA and RU486 were able to reverse CORT-induced immunosuppression, it was also able to induce immunosuppression brought about by burn stress, while RU486 was ineffective (7). This could suggest that DHEA is acting at receptors that RU486 fails to target.

2. DHEA, CORT and ageing. As individuals age, there is a progressive increase in CORT levels, and a decline in DHEA. While CORT upregulation is not massive, DHEA plummets (8).



Similar declines are seen in other primates, asymptotic with maximum lifetime [*the situation in rodents is confused, but the absence of CYP17 expression in the rodent adrenal may explain why pregnenolone, and not DHEA, is the major blood steroid.*] Lifetime changes are not known. Although there are reasons to suppose that DHEA deficiency is causally linked to cognitive and immune decline in the elderly, replacement trials have mostly had negative results, although significant positive benefits on mood and well-being were seen in patients with adrenal dysfunction. Ie., DHEA does something.

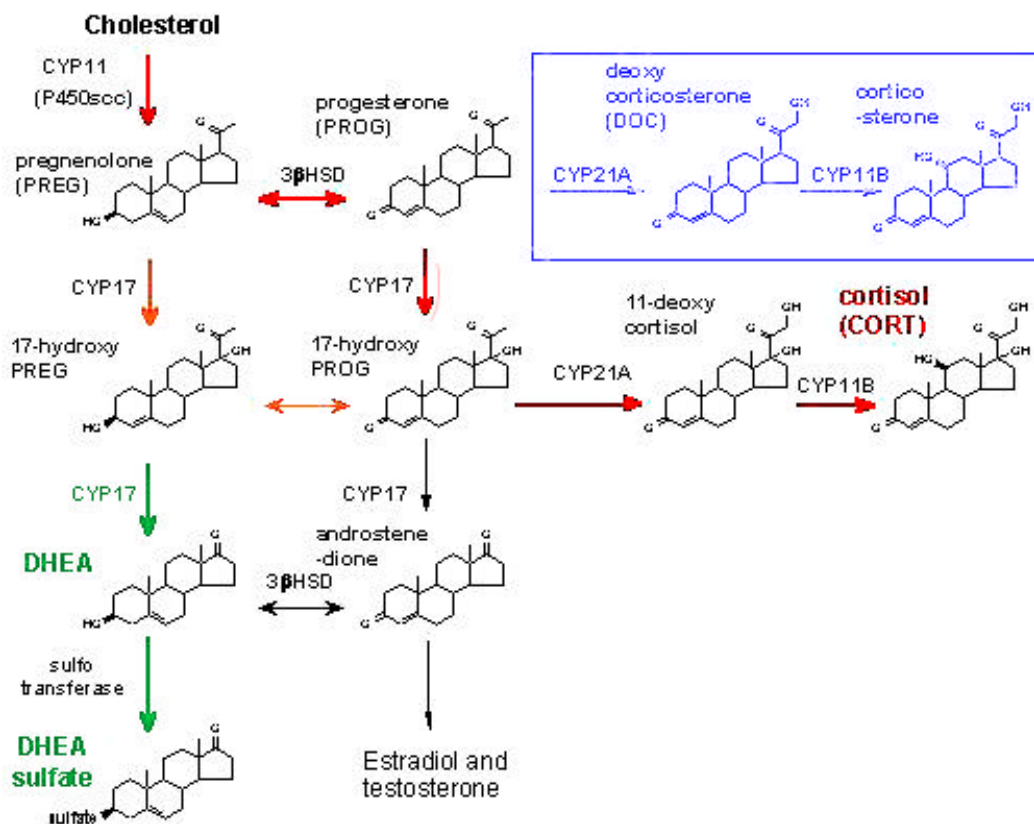
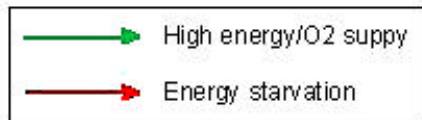
3. Mechanism of DHEA decline. DHEA, like glucocorticoids, are produced in response to ACTH stimulation. However, the steroidogenic capacity of the adrenal varies throughout life (reviewed in 8).

The fetal adrenal produces large amounts of DHEA and its sulfate, but no cortisol, because the key enzyme 3βHSD is absent. Sulfation of DHEA also diverts the hormone towards export and away from CORT production. Fetal-derived DHEA/S is an important precursor for material estradiol synthesis. Reduced DHEA/S levels on delivery are associated with fetal distress, growth retardation etc.

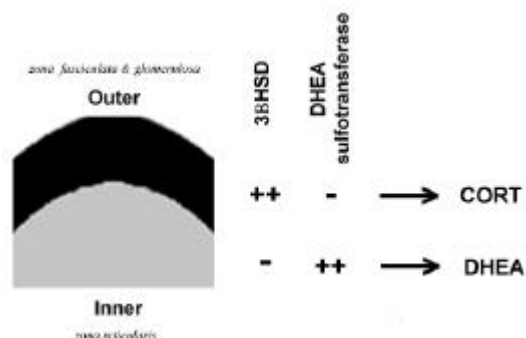
In the infant there is a dramatic decline in DHEA levels, due to major remodelling of the adrenal.

The cortical layers of the adrenal adopt two (possibly three) differentiation fates. Inner layers express the sulfotransferase, but not 3βHSD, while the outer layers express 3βHSD but not sulfotransferase. Thus outer layers make CORT, while inner layers divert precursors to DHEA/S export.

Why do DHEA levels decline with age? There are no clear morphological or enzymatic correlates. Suggestions have included (a) increased 3βHSD activity (b) change in CYP17 function (c) general reduction in steroidogenic capacity of the outer



layers. It is interesting that excess expression of cytochrome b5 in adrenal adenomas was found to increase CYP17 activity and DHEA production. Perhaps energy and oxygen supply to the adrenal dictates which pathway is followed? DHEA synthesis requires oxidative and energy-dependent CYP17 activity; the pathway to CORT synthesis involves oxidoreductase (3β-HSD) activity. Could changes in cofactor requirements be responsible? Energy stress promoting CORT?



4. Uncoupling and the DHEA-CORT switch. It has been asserted that the ratio of DHEA to CORT is more critical than the absolute levels of either. Notably, the DHEA/CORT ratio is not constant (Box).

Oberbeck et al. (Hannover Medical School) 1998. Endocrine mechanisms of stress-induced DHEA-secretion. *J Endocrinol Invest* 1998 Mar;21(3):148-53

Acute psychological stress of a first time parachute jump stimulated DHEA and cortisol secretion in healthy volunteers. A significant shift from cortisol to DHEA occurred during this stress exposure. This effect was more pronounced in subjects receiving the beta-adrenoceptor antagonist propranolol prior to the jump. In contrast, infusion of epinephrine (0.10 microgram/kg/min) or norepinephrine (0.15 microgram/kg/min) for 20 min neither affected DHEA plasma levels nor the DHEA/cortisol ratio. However, pretreatment with propranolol resulted in a significant increase of the DHEA/cortisol ratio upon infusion of the beta-adrenoceptor agonist epinephrine. These data demonstrate that during acute psychological stress stimulation of adrenal steroid release is accompanied by a shift towards DHEA. Augmentation of this effect by beta-adrenoceptor blockade indicates a beta-adrenoceptor-dependent mechanism affecting DHEA release.

In elderly subjects, while DHEA remains responsive to ACTH, the DHEA response is significantly dampened while CORT production is unaffected or increased. In normal adults, CORT production resumes fast after adrenal suppression, while DHEA production is slow to resume. Stress in adults causes a drop in DHEA but an increase in CORT.

5. Is DHEA metabolism required? DHEA is metabolised, in diverse tissues including brain, to 17betaHSD and 7alphaOH derivatives - androstenediol and 7alpha-hydroxyDHEA (7HD). The latter predominates, but the role of 7HD product is not known. It has been suggested that 7-oxygenation of DHEA activates the molecule, and memory, immune and metabolic effects are increased. The active molecule has not been identified (7alpha, 7oxo, 7beta or downstream derivatives) but it is of note that the enzyme responsible, CYP7B, is most abundantly expressed in the dentate gyrus of the hippocampus.



6. Does DHEA counter CORT action or mediate feedback downregulation of CORT production? Administration of DHEA produces a reduction in cortisol levels, both in healthy volunteers and in rodents. The mechanism is not known, but because DHEA can also act at central GABA and sigma sites, it could feed back to the brain to control adrenal output. In support, the central GABA_A agonist alprazolam decreased plasma cortisol but increased DHEA (box), systemic sigma agents also affect adrenal output.

Kroboth et al (Pittsburgh) 1999. Alprazolam increases dehydroepiandrosterone concentrations. *J. Clin Psychopharmacol* 19:114-124.

The gamma-aminobutyric acid (GABA) agonist alprazolam is known to decrease adrenocorticotrophic hormone and cortisol concentrations. Dehydroepiandrosterone (DHEA) is secreted synchronously with cortisol by the adrenal glands and demonstrates diurnal variation. The major objective of this study was to determine whether alprazolam affects concentrations of DHEA and DHEA -S, the sulfated metabolite. In vitro studies have demonstrated that DHEA -S, and perhaps DHEA, have GABA antagonistic activity. Another objective was to determine whether DHEA -S and/or DHEA concentrations are related to psychomotor impairment after alprazolam. Thirty-eight healthy volunteers (25 young men, aged 22-35, and 13 elderly men, aged 65-75) received a single intravenous dose of alprazolam 2 mg/2 min (part 1). Fifteen young and 13 elderly men responded to alprazolam and agreed to participate in part 2 of the study, which was a crossover of placebo and alprazolam infusion to plateau for 9 hours. Plasma samples at 0, 1, 4, and 7 hours were assayed for steroid concentrations. Alprazolam produced (1) significant increases in DHEA concentrations at 7 hours in both young and elderly men; (2) significant decreases in cortisol concentrations; and (3) no change in DHEA -S concentrations. The relationship between psychomotor decrement and DHEA concentrations at 7 hours after alprazolam 2 mg/2 min was described by a u-shaped curve (p

< 0.0047). Both the linear and quadratic components of the equations for the tests were significant ($p < 0.002$). These results suggest that alprazolam modulates peripheral concentrations of DHEA and that DHEA and/or DHEA-S may have an in vivo role in modulating GABA receptor-mediated responses.

7. Is DHEA decline involved in other disorders?

Ferrando et al. (Cornell, New York) 1999. Dehydroepiandrosterone sulfate (DHEAS) and testosterone: relation to HIV illness stage and progression over one year. *J Acquir Immune Defic Syndr* 22:146-154. This study explored associations between serum dehydroepiandrosterone sulfate (DHEAS), free and total testosterone levels, and HIV illness markers, including viral load, and the behavioral problems of fatigue and depressed mood. Subjects were 169 HIV-positive men evaluated at baseline, 6, and 12 months for levels of DHEAS, total and free testosterone, HIV RNA, CD4, HIV symptoms, opportunistic illnesses, fatigue, and depression. Men with AIDS ($N = 105$), compared with men with less advanced illness, had lower mean levels of DHEAS. Baseline DHEAS was positively correlated with CD4 count, HIV symptom severity, and was inversely correlated with HIV RNA. Baseline DHEAS below the laboratory reference range (96 microg/dl) was associated with history of opportunistic infections and malignancies (adjusted odds ratio [OR], 4.4; 95% confidence interval [CI], 1.9-10.4) and with incidence of these complications or death over 1 year (adjusted OR, 2.6; 95% CI, 1-7.2). Initiating protease inhibitor combination therapy was associated with an increase in DHEAS over 6 months. Free testosterone was inversely correlated with HIV RNA, but there were no other significant associations between testosterone and HIV illness markers. No hormone was related to fatigue or depression. This study confirms that low serum DHEAS is associated with HIV illness markers, including viral load, and carries negative prognostic value. Further, protease inhibitor therapy may result in increased circulating DHEAS.

Remarks: Central control of behaviour.

All hormones affect all measurable behaviours. Therefore, all hormones modulate stress, anxiety and depression, but the effects they have will depend on where, when, how long, what concentration, etc.

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Steroid and sterol 7-hydroxylation: Ancient pathways

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Abbreviations: OH; hydroxy
OOH; hydroperoxy
A/enediol; Androstane-3 β ,17 β -diol
A/enediol; Androstene-3 β ,17 β -diol
DHEA; dehydroepiandrosterone
HSD; hydroxysteroid dehydrogenase
oxo; equivalent to keto

Key Words: cholesterol, cytochrome P450, CYP7B, DHEA, evolution, receptor, steroid, hypothesis, review

Abstract

B-ring hydroxylation is a major metabolic pathway for cholesterol and some steroids. In liver, 7α -hydroxylation of cholesterol, mediated by CYP7A and CYP39A1, is the rate limiting step of bile acid synthesis and metabolic elimination. In brain and other tissues, both sterols and some steroids including dehydroepiandrosterone (DHEA) are prominently 7α -hydroxylated by CYP7B. The function of extra-hepatic steroid and sterol 7-hydroxylation is unknown. Nevertheless, 7-oxygenated cholesterol are potent regulators of cell proliferation and apoptosis; 7-oxygenated derivatives of DHEA, pregnenolone and androstenediol can have major effects in the brain and in the immune system. The receptor targets involved remain obscure. It is argued that B-ring modification predated steroid evolution: non-enzymatic oxidation of membrane sterols primarily results in 7-oxygenation. Such molecules may have provided early growth and stress signals; a relic may be found in hydroxylation at the symmetrical 11-position of glucocorticoids. Early receptor targets probably included intracellular sterol sites, some modern steroids may continue to act at these targets. 7-hydroxylation of DHEA may reflect conservation of an early signalling pathway.

1. 7-oxygenated steroids and sterols

Steroid and sterol oxidoreduction governs biological activity and metabolic fate. Oxidative loss of the cholesterol side-chain generates steroids; oxidoreduction of the steroid nucleus (3, 11 and 17 positions in particular) dictates activity and specificity. Recent work now points to an important role for B ring (6 and 7 position) modification.

7-oxygenated steroids and sterols are widespread in mammals, birds, fish and plants. Sterol processing in liver provides the best example of B-ring oxygenation. Hepatic 7α -hydroxylation of cholesterol is the rate-limiting step for bile acid synthesis and elimination ([Figure 1](#)). B-ring hydroxylation of sex steroids in liver may also represent metabolic elimination.

Nevertheless, prominent B-ring hydroxylation is also seen in diverse extra-hepatic tissues. This could argue against simple substrate inactivation. The major 3β -hydroxysteroids including dehydroepiandrosterone (DHEA), pregnenolone, and androstane- $3\beta,17\beta$ -diol (A/enediol) are efficiently 7α -hydroxylated in diverse tissues including brain [1-16] ([Figure 2](#)), with some 6α and 7β modification depending on the substrate conformation. Other steroids are modified: testosterone is 7α -hydroxylated in testis [17,18] while 5α - 3α steroids give rise to 6α -OH derivatives in prostate and lymphocytes [7,13,19].

With the exception of hepatic bile acid formation via 7α -hydroxylation, almost nothing is known of the biological role of B-ring oxygenated sterols and steroids. One insight is provided by studies on the enzymes that catalyze their synthesis.

2. B-ring hydroxylated molecules: enzymes mediating their formation

2.1. Sterol 7-hydroxylation

At least three enzymes mediate sterol B-ring hydroxylation in liver. (1) CYP7A, whose expression is restricted to liver, hydroxylates cholesterol at the 7α position [20,21]; the enzyme has not been reported to metabolize steroids. (2) Studies on mice lacking CYP7A revealed an alternative pathway for bile-acid synthesis via a related enzyme, CYP7B [22,23], expressed in liver and multiple other tissues (below). (3) A hepatic 7α -hydroxylase specific for 24(S)-hydroxycholesterol, CYP39A1, has also been described [24]. In brain, but not in other tissues, 24(S) hydroxylation of cholesterol is a major export pathway [25-27]; brain-derived 24(S)-hydroxycholesterol is further metabolized in liver by CYP39A1.

2.2. Steroid 7-hydroxylation

In brain, several different B-ring hydroxylase enzymes were suspected. Although DHEA is primarily 7α -hydroxylated, inhibitor studies pointed to a second enzyme with activity at 7β [12, 14-16]. A/enediol is principally 6α -hydroxylated in brain and prostate [6,7,9,28], suggestive of a further enzyme.

We reported molecular cloning of an enzyme from rodent hippocampus, CYP7B, with sequence similarity to CYP7A [29]. The enzyme differs from CYP7A in a number of significant respects. First, in addition to catalyzing the 7α -hydroxylation of sterols (25- and 26-hydroxycholesterols; [30-32]), it is robustly active against steroids including DHEA, pregnenolone, and androstane- $3\beta,17\beta$ -diol (A/enediol); 17β -estradiol was also modified at a lower rate [31]. Second, CYP7B is not restricted to liver, and is expressed widely in the brain and other tissues [29,33]. In addition to affecting sterol 7α -hydroxylation in liver [34], CYP7B gene disruption abolished steroid and sterol hydroxylation in diverse tissues including brain, spleen, thymus, heart, lung (in male), prostate, uterus and mammary gland [33]. Third, CYP7B may modify other positions including 6α and 7β : knockout mice fail to modify A/enediol (that is normally 6α -hydroxylated) while recombinant CYP7B enzyme expressed in vaccinia or in yeast generates minor secondary metabolites including 7β -hydroxysteroids [33, Vico et al., in preparation]; 7β metabolites are also abolished in CYP7B knockout mice [33] though onwards metabolism from 7α was not excluded [see Lardy, this volume].

2.3. Other B-ring hydroxylases

Hepatic steroid metabolism includes hydroxylation at the 6β position by members of the CYP3A family [35,36]. Outwith liver, CYP7B appears to be the primary steroid and sterol hydroxylase, though a distinct testosterone 7α -hydroxylase has been described in testis (CYP2A9/15 [17,18]) and in human (but not rodent) prostate, 6α -hydroxylation of 5α - 3α steroids is performed by a non-P450 enzyme [7,13]. Other enzymes may exist.

3. Signalling by B-ring oxygenated sterols

Sterol hydroxylation at the 7 α -position is central to bile acid synthesis in liver; the role steroid and sterol B-ring hydroxylation in other tissues is unknown. There is evidence that they may play regulatory and/or signalling roles, exemplified (below) by effects on cholesterol regulation and apoptosis; other diverse effects of B-ring modified sterols have been noted (not reviewed).

3.1 Cholesterol homeostasis

Excess cholesterol represses its own synthesis and upregulates hepatic CYP7A expression to promote elimination. Conversely, bile acid excess can repress expression of CYP7A. Complex transcriptional regulation acts via a series of nuclear hormone receptors including LXR α , LXR β , FXR and LRH-1 [37-39] that mediate responses to oxygenated sterols and bile acids. B-ring modification does not appear to be essential for this regulation. While 6 α -hydroxycholesterol may activate LXR [40] and 6 α -epoxycholesterols and 7-oxocholesterol can inhibit [41], LXR is most efficiently activated by 22(R)- and 24(S)-hydroxycholesterols [42,43]. Though 6- or 7-hydroxylated sterols could contribute to this regulation, B-ring hydroxylated sterols may exert effects via other pathways.

3.2 Apoptosis control

One pathway links cholesterol supply to cell proliferation and/or programmed cell death. Oxysterols are inhibitors of cell activation and proliferation, and can induce cell death, particularly in lymphocytes [44-47]. 7 β -OH and 7-oxocholesterols are neurotoxic [48]. The most potent apoptosis-inducing activity found in oxidized LDL was identified as 7 β -hydroperoxycholesterol (7 β -OOH-cholesterol) [49,50]. Death in these models can be via classical apoptotic pathways [51,52].

Cell death may be a consequence of cholesterol biosynthesis inhibition; however, the specific pathways by which sterols can induce apoptosis remain to be elucidated.

4. Signalling by B-ring hydroxylated steroids

4.1 Brain function

The major metabolic route for dehydroepiandrosterone (DHEA) in extra-hepatic tissues is via 7 α -hydroxylation [31-33]. The metabolism of DHEA is of some interest. DHEA (and pregnenolone) promote synaptic plasticity and memory function in experimental animals [53-59]. Further, blood DHEA levels fall markedly with age in primates [61-65]. Cognitive decline in old age could be causally linked to DHEA decline [66-69]. However, oral DHEA replacement has not brought the hoped-for improvements in cognitive function [66,70,71] although beneficial effects are reported in adrenal dysfunction [72].

DHEA may require metabolism in target tissues. It is of note that the DHEA metabolizing enzyme CYP7B is particularly well-expressed in the hippocampus [29,33], a brain region centrally involved in memory formation. Lardy and colleagues suggested that 7 α -hydroxylation of DHEA is on a metabolic pathway to more potent derivatives [73] and recently reported that 7-oxoDHEA (that may interconvert with 7-OH derivatives) is more active in promoting brain function than DHEA [74]. We have observed that 7 α -OH DHEA is more active than DHEA in preventing hypoxic cell death of neurones in vitro (Martin, Lathe, Seckl and Wulfert, unpublished data). In the brain, therefore, 7-oxygenation seems to be associated with activation of DHEA.

4.2. The immune system

DHEA and its metabolites promote the immune response in experimental animals [75-84]; however, attempts to boost immune-responsiveness in the elderly by DHEA replacement have not been entirely promising [85].

As in brain, DHEA may require metabolism for bioactivity. CYP7B is expressed in thymus and in lymphocytes [33; our unpublished data]. There is debate about the stereoconfiguration of the active metabolite. 7 α -OH DHEA is a major immunity-promoting derivative of DHEA [86,87] others have argued that 7 β -OH derivatives of A/enediol are most effective [78,82,88,89; Loria, this volume].

4.3 Origins of 7 β -hydroxylated molecules

Both 7 α and 7 β modified molecules have biological activity, particularly in the immune system, but the origin of 7 β -OH molecules is enigmatic (see Lardy, this volume). Several routes are possible (1) *Enzymatic hydroxylation*. Trace 7 β -modified molecules are seen in CYP7B reactions [31]; allosteric modulation could favor 7 β modification (see [12]). (2) *Epimerization*. 7 α -hydroperoxycholesterol (7 α -OOH cholesterol) and 7 α -OH cholesterol may spontaneously epimerize to their 7 β counterparts [88,89]; a 7-epimerase similar to the 3-epimerase enzyme [90] could contribute. (3) *Dehydrogenation and reduction*. 11 β HSD activity against 7 α -hydroxycholesterol [91] generates 7-oxo molecules that could in turn generate 7 β -OH derivatives. All three are consistent with abolition of both 7 α and 7 β derivatives by disruption of the CYP7B [33].

5. Did signalling by B-ring hydroxylated molecules predate conventional steroid signalling?

5.1. Dearth of conventional receptor targets

No dedicated conventional (nuclear) receptor has been identified for 7-OH steroids. These could then act through via gating (ligand inactivation) of typical nuclear receptors, through the modulation of cell-surface ion channels (particularly in brain), or at atypical receptors.

A/andiol and DHEA are modest agonists of the estrogen and androgen receptors (ER and AR) [92,93]. 7-oxygenation reduces activity of both molecules [1,94]. Clearly hydroxylation can gate nuclear receptor access, but the significance *in vivo* is unclear.

Hydroxylation of steroids (and possibly sterols) may modulate activity at cell-surface ion channels. Diverse channels respond to steroids [95], but the GABA_A receptor has received most attention. DHEA and related steroids are antagonists of GABA_A, promoting neuronal activity (while 3 α -5 α steroids are agonists with potent anaesthetic properties). B-ring hydroxylation of DHEA and related steroids could gate access to these receptors.

Gating of either sex steroid receptors or ion channels such as GABA_A does not easily explain the apoptotic regulatory action and brain/immune system effects of these molecules. For instance, GABA agonists can inhibit apoptosis, but steroids are orders of magnitude more effective than the classic GABA agonist, muscimol [96]. This implies that they are binding to other receptors. This could make sense if these targets predated both the development of ion-channel sensitivity to steroids and the radiation of the steroid hormone receptor superfamily.

5.2. Late emergence of steroid signalling

Traditional wisdom depicts the evolution of intercellular steroid signalling from intracellular sterol signalling by an evolutionary breakthrough - the oxidative removal of the long hydrophobic side-chain of cholesterol via the action of the P450_{scc} (side-chain cleavage; SCC) enzyme, CYP11B. This interpretation may be incomplete.

Steroid signalling proper emerged late in eukaryotic evolution. The genome of the yeast, *Saccharomyces cerevisiae*, contains no homolog to the vertebrate steroid hormone receptor family. Steroid signalling proper has been placed with primitive fishes during the massive radiations taking place in the Cambrian period [97-100]. Ion-channel sensitivity to steroids only appears late in chordate evolution [101]. Therefore, the full spectrum of growth, differentiation, and reproduction was achieved, in precursors to the vertebrate lineage, without conventional steroid signalling at either nuclear or ion channel receptors. These processes might have been subserved by sterols (rather than steroids) acting at atypical receptors.

5.3. Emergence of sterol-derived messengers: B-ring modified derivatives can be generated non-enzymatically

Membrane sterols probably arose from terpenoids including the hopanoids of bacteria [102,103] with which they share axial and longitudinal dimensions required for membrane stabilization (but not the 3 β -hydroxy group of steroids/sterols). Sterols of modern eukaryotes generally contain the 3 β -hydroxy group, including ergosterol and lanosterol of fission and budding yeasts (*S. cerevisiae*, *S. pombe*), plant phytosterols such as stigmasterol, and the insect (and crab) hormone ecdysone (Figure 4).

Signalling molecules can arise from abundant cell components. Membrane sterols are relatively insoluble; chemical oxidation of cholesterol primarily generates the more soluble 7 α -OH, 7 β -OH and 7oxo

derivatives (Figure 5). Lower amounts of 6 α -OH molecules, 5 α -6 α epoxides, and 7 α and β -hydroperoxides are also produced, as are side-chain oxidized cholesterols. Oxygenation at the 5 β unsaturated bond (perhaps facilitated by the 3 β -hydroxy group) may produce 5-6 epoxides that convert to 7-hydroperoxides, followed by thermal degradation to produce 7 α -OH, β -OH and 7 α -cholesterols [88,89,104-107]. Products of other membrane sterols may be similar [108]. B-ring oxidation is promoted by horseradish peroxidase, lipoxygenases, gamma irradiation and metal ions (most particularly copper ion) and reduced in the presence of metal chelating agents (reviewed by Schroepfer [109]).

5.4. What do we know about the earliest sterol messengers?

Through increased solubility, and non-enzymatic production, 7oxygenated sterols have considerable signalling potential. First, oxidized cholesterols are toxic, can bind to DNA and have mutagenic activity [110-112] possibly providing an early driving force for inducible elimination (a relic of which may be found in the CYP7A export pathway). Second, they have the potential to signal both sterol abundance (growth) or sterol oxygenation (oxidative stress), suggestive of early growth and stress signals.

This latter idea finds some support in the structure of modern steroid receptors. The earliest steroid-type nuclear receptor most resembled the present estrogen receptor; this primordial receptor subsequently diverged to generate the estradiol/growth (ER α , ER β , ERR) and glucocorticoid/stress families (GR, MR, PR and later AR) [106,123-125].

Early ligands could have included 7-modified molecules. (1) *Estrogen receptors*. These respond to diverse β -hydroxylated steroids [124,117]; ligand binding to modern ER α is promoted by small α substitutions that fit into an unoccupied cavity in the receptor [117]. (2) *Glucocorticoid receptors*. These are activated by 11 β -hydroxylated steroids. Crucially, the 11 β and 7 α positions are rotationally symmetrical Figure 6: emphasized by an 11 β -hydroxysteroid dehydrogenase with dual 11 β - and 7 α -dehydrogenase activity [91] and promotion of ligand binding to ER α by 11 β -substitutions [117]. Early 11 β modifications may have exploited receptor targets binding 7 α molecules. Thus, existing 7modified molecules (produced non-enzymatically) could have been early ligands for the joint precursor to ER and GR.

6. Early receptors may have included intracellular sterol sites

If signalling by sterols, possibly 7modified sterols, predated steroid signalling proper, what were the earliest targets for regulatory sterols? Molecular cloning experiments have begun to reveal a class of intracellular sterol-responsive targets (see Moebius, this volume; reviewed in [118]). These include the emopamil binding protein (EBP), the sigma site, and the peripheral benzodiazepine receptor (PBR). (1) EBP encodes a sterol C8-C7 isomerase that catalyzes the penultimate step in the synthesis of cholesterol [119-123]. (2) Sigma-1 shares significant homology with yeast ERG2 (ergosterol synthesis; C8-C7 sterol isomerase) enzyme [122,124,125] but its catalytic activity has not yet been elucidated; related sigma-2 and -3 receptors have been discussed. (3) The peripheral benzodiazepine receptor (PBR) participates in translocating cholesterol from the outer to the inner mitochondrial membrane [126-128]. These sites emerged early in evolution. Sigma finds a strict equivalent in the *S. cerevisiae* ERG2 gene product. PBR has only distant relatives in *S. cerevisiae* but a close counterpart in the fission yeast *Schizosaccharomyces pombe* (SPBC725.10); EBP has no obvious match in either yeast but the EBP-related protein EBRP is highly homologous to a *S. cerevisiae* gene product, YDL222C, of unknown function.

These and other enzymes and transporters accompanied the evolutionary switch to sterol-rich membranes, and are contenders for the early regulatory targets for oxygenated sterols. However, it is not known which, if any, were modulated by B-ring oxygenated molecules.

Some major drugs target intracellular sterol sites. Ligands have marked effects on apoptosis and the immune system. An anti-estrogen (tamoxifen) used in hormone-responsive breast cancer may act via sterol sites; important brain-active drugs, including anti-epileptics (diazepam), anti-ischemics (emopamil) and neuroleptics (haloperidol) are ligands for sterol sites. Sterols modulate the risk of Alzheimer's disease. An understanding of these primitive pathways is vital.

6.1. Did the first steroids act at sterol sites? - the oxysterol hypothesis

The first steroids, sterols lacking the hydrophobic side-chain of cholesterol, may have targeted existing sterol sites. In support, steroid action at oxysterol targets has been demonstrated. Some sterol sites have

significant affinity for natural steroids including glucocorticoids, estrogens (and antiestrogens) and DHEA [129-137]. Different steroids can have markedly different downstream effects at the same sterol site. Functional overlap between sterols and steroids is emphasized by present-day enzymes (CYP7B, 11 β -HSD) that can modify both types of molecule.

Modern systemic steroids (including DHEA, estradiol and glucocorticoids and their metabolites) continue to target sterol sites, acting in concert or in competition with endogenous sterols. By this means steroids could, and can, control cell life and death at a systemic level. 7-oxygenation of 3 β -hydroxysteroids including DHEA may reflect conservation of early signalling pathways. In the search for targets for B-ring modified steroids, intracellular sterol sites may deserve some attention.

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Figures

Fig. 1. Co-action of CYP7A and CYP7B on cholesterols to form bile acids.

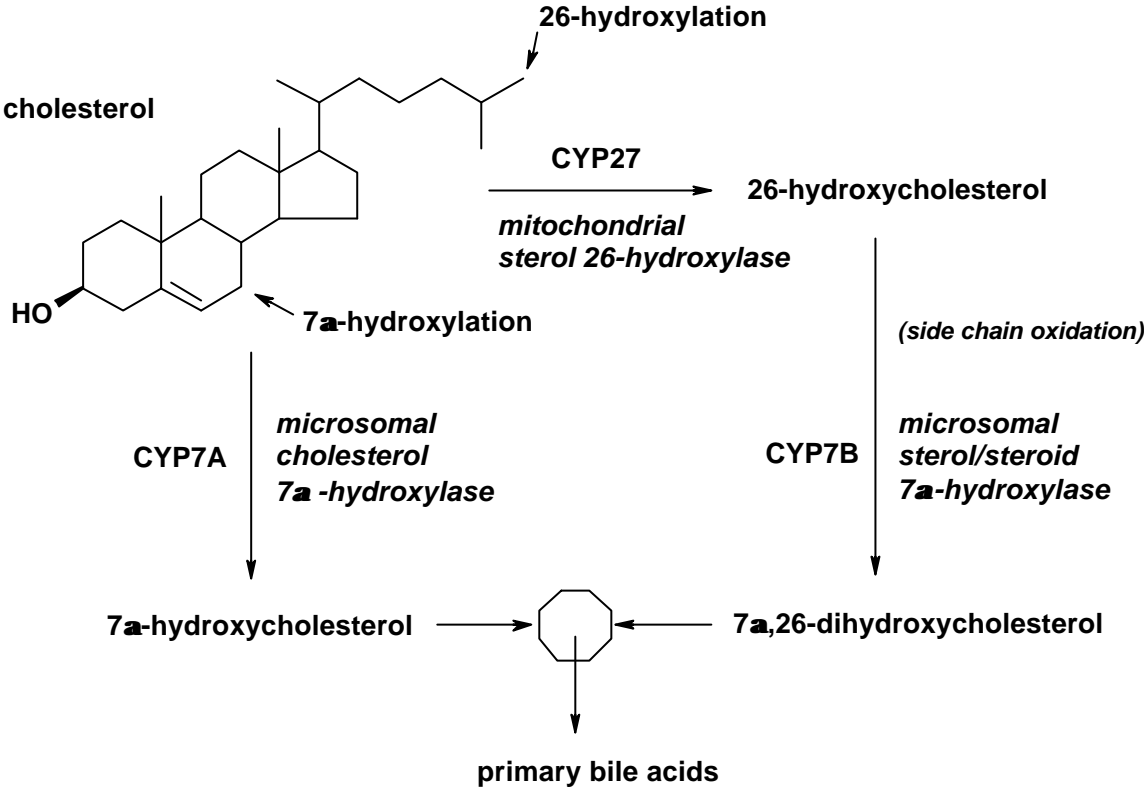


Figure redrawn from Schwarz et al. [30].

Fig. 2. 7-hydroxylation of hydroxycholesterol and 3β-hydroxysteroids.

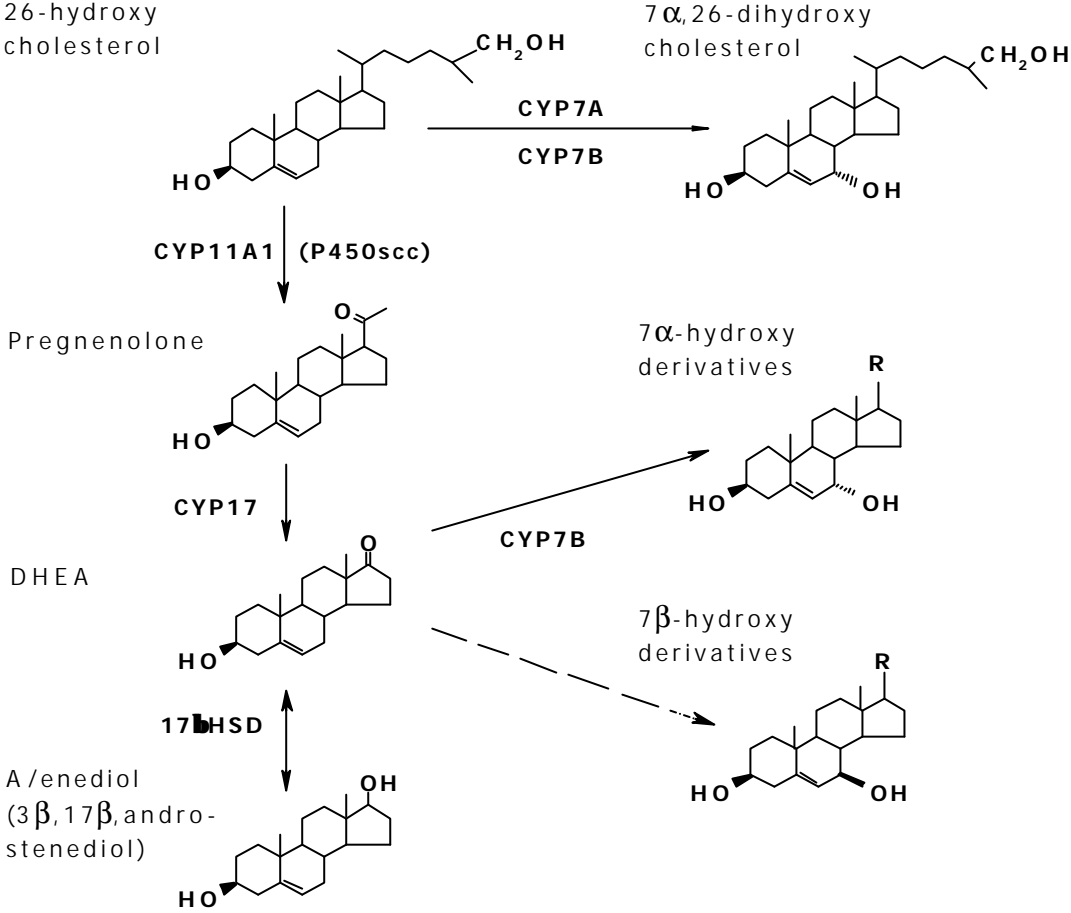
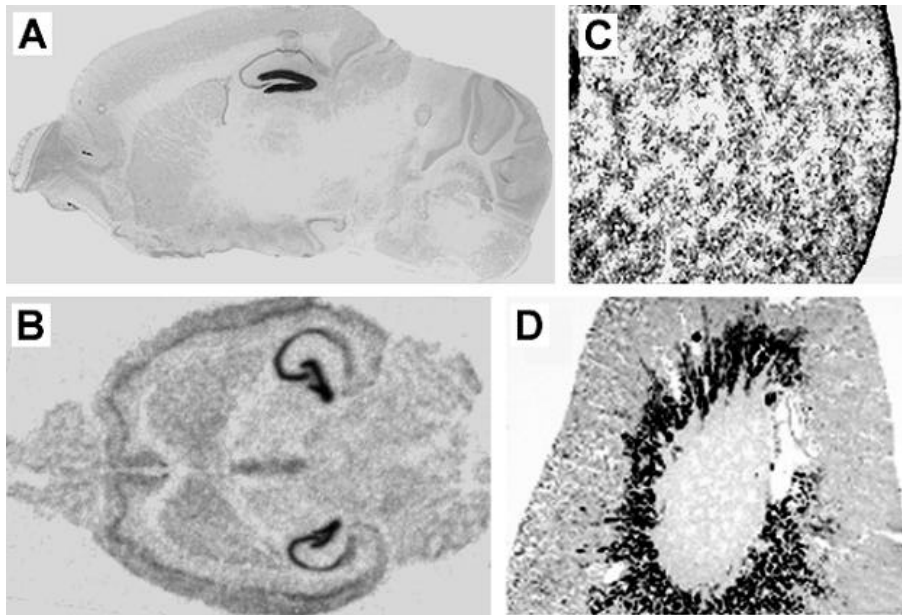
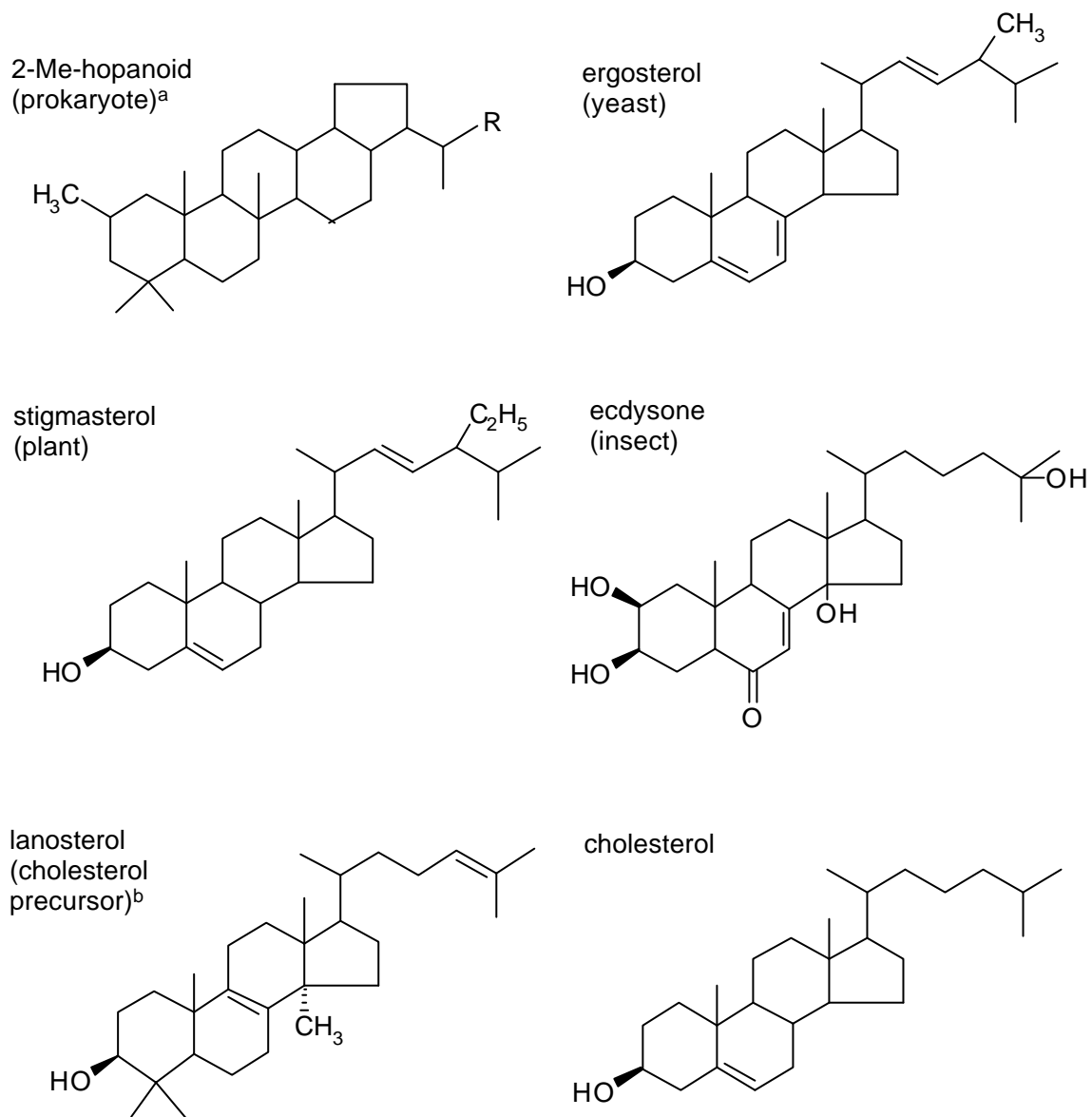


Fig. 3. CYP7B expression in brain and liver and kidney.



Panels A, C and D depict reporter gene expression (dark coloration), in transgenic mice, under control of CYP7B regulatory elements (A, brain, sagittal section, C, liver; D, kidney); Panel B shows in situ hybridization (dark/grey coloration) using a CYP7B probe (brain, horizontal section) [33]. Expression in neonates is very much more widespread and abundant [138].

Fig. 4. Sterols/steroids from different organisms.



Notes: ^a2 (and 3-) methyl-bacteriohopanoids are commonly substituted (R in the figure) with a long side-chain (C8 outwith the hopanoid nucleus) bearing clustered OH groups (eg. bacteriohopantetrol [103];
^bcycloartenol rather than lanosterol is the sterol precursor in plants.

Fig. 5. Non-enzymatic oxidation products of cholesterol.

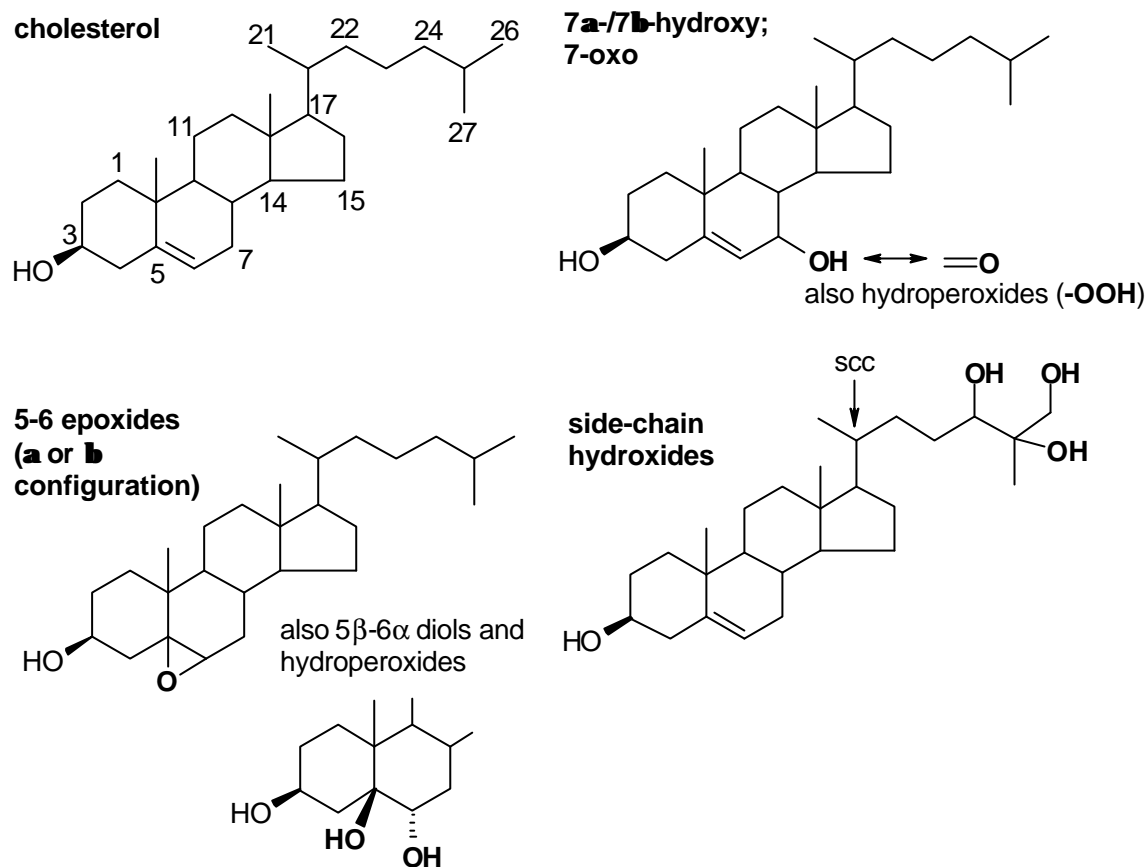
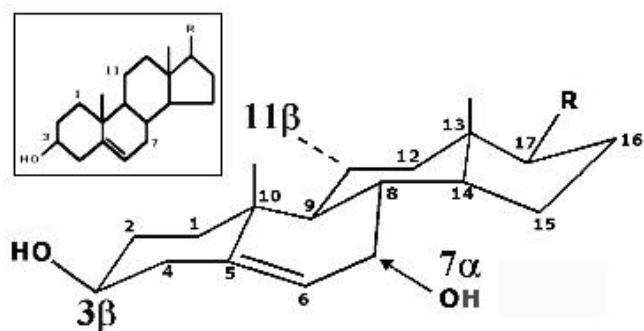


Fig. 6. Rotational symmetry between the 7 α and 11 β positions of the steroid nucleus. Some binding sites for 7 α -modified molecules may accept 11 β -modified equivalents.



The CRF peptide family and their receptors: yet more partners discovered

Frank M. Dautzenberg and Richard L. Hauger

Abnormal signaling at corticotropin-releasing factor CRF₁ and CRF₂ receptors might contribute to the pathophysiology of stress-related disorders such as anxiety, depression and eating disorders, in addition to cardiac and inflammatory disorders. Recently, molecular characterization of CRF₁ and CRF₂ receptors and the cloning of novel ligands – urocortin, stresscopin-related peptide/urocortin II, and stresscopin/urocortin III – have revealed a far-reaching physiological importance for the family of CRF peptides. Although the physiological roles of the CRF₂ receptor remain to be defined, the preclinical and clinical development of specific small-molecule antagonists of the CRF₁ receptor opens new avenues for the treatment of psychiatric and neurological disorders.

For more than a century, the ability of the body to adapt to stressful stimuli and the role of stress maladaptation in human diseases have been intensively investigated. However, in 1981 the isolation and characterization of corticotropin-releasing factor (CRF) [1] forged a major breakthrough in understanding the human stress response. Today, a considerable body of evidence suggests that CRF peptides play biologically diverse roles in generating the stress response by acting at CRF₁ and CRF₂ receptors that are differentially expressed on brain neurons located in neocortical, limbic and brainstem regions of the CNS and on pituitary corticotropes. In addition to their neurotransmitter and neuroendocrine roles [2–4], CRF ligands acting at their peripheral receptor sites might regulate cardiovascular and inflammatory processes, among others. The CRF binding protein (CRF-BP) might also modulate the stress response by limiting CRF receptor activation [5]. However, with the recent identification of two new members of the CRF peptide family, stresscopin-related peptide/urocortin II and stresscopin/urocortin III from human, mouse and fish, it is not surprising that we are still deciphering the regulatory intricacies of the body's stress system that are essential for homeostasis and survival. In this article, recent findings regarding the molecular properties, distribution and regulation of CRF ligands and their receptors will be discussed.

Structure and tissue distribution of CRF and urocortin CRF exists as a 41-amino-acid polypeptide in a large variety of mammalian species and is generated by cleavage of the C-terminus of pre-proCRF, the 196-amino-acid precursor [2]. Human and rat CRF

(h/rCRF) are identical to one another but differ from ovine CRF (oCRF) by seven amino acids (Fig. 1). Two non-mammalian CRF-like peptides, the 40-amino-acid amphibian peptide sauvagine (SVG) and the 41-amino-acid fish peptide urotensin I (URO), share ~50% sequence identity with h/rCRF (Fig. 1). Although SVG and URO were originally considered to be CRF homologs in fish and amphibians, the cloning of CRF from fish and frogs [2] and mammalian urocortin (UCN), a 40-amino-acid CRF peptide [6], established that vertebrates possess additional members of the CRF peptide family. To date, UCN has been isolated from human, rat, mouse and sheep [6–8]. UCN is more highly conserved than is CRF across species and closely resembles fish URO (53–63% amino acid identity) but diverges from CRF (43–45% amino acid identity) and SVG (35% amino acid identity). The CRF and UCN precursor genes both contain two exons with the second exon encoding the entire precursor protein [7]. No additional bioactive peptides appear to be encoded by their precursor genes.

CRF mRNA and protein are abundantly distributed in the CNS with major sites of expression in the paraventricular nucleus of the hypothalamus, cerebral cortex, cerebellum and the amygdalar–hippocampal complex, an area important for stress adaptation, learning and memory [9]. In the periphery, CRF is expressed in the adrenal gland, testis, placenta, gut, spleen, thymus and skin [2]. In the brain, expression of UCN mRNA and protein is restricted to the Edinger–Westphal locus, the hypothalamic area and a small population of neurons in the forebrain [10]. In the periphery, UCN is broadly expressed, particularly in the pituitary, gastrointestinal tract, testis, cardiac myocytes, thymus, spleen and kidney [11].

Identification of two novel members of the CRF family Because the CRF₂ receptor binds UCN with >100-fold greater affinity than it binds CRF, UCN was hypothesized to be the endogenous ligand of the CRF₂ receptor [2,4]. However, the limited overlap between the expression of UCN-containing neurons and those neurons expressing CRF₂ receptors in the rat CNS suggested that additional endogenous CRF receptor ligands existed in mammals [10]. Recently, UCN-like

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Peptide	Sequence	Length	Identity (%)
hCRF	SEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLM E I	41	100
oCRF	SQEPPI SLDLTFHLLREVLEMTKADQLABQ AHSNRKLLDIA	41	83
URO	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRYLDEV	41	54
hUCN	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	40	43
SVG	ZGPPISIDL SLELLRKMIEIEKQEKEKQQAANNRLLD TI	40	48
hSRP	I VLSLDVPIGLLQILLEQARARAAREQATTNARILARV	38	34
mUCNII	VILSLDVPIGLLRILLEQARYKAARNQATNAQILAHV	38	34
hSCP	F T L S L D V P T N I M N L L F N I A K A K N L R A Q A A A N A H L M A Q I	38	32
mUCNIII	F T L S L D V P T N I M N I L F N I D K A K N L R A K A A A N A Q L M A Q I	38	26

TRENDS in Pharmacological Sciences

Fig. 1. Alignment of members of the corticotropin-releasing factor (CRF) peptide family. The amino acids that are homologous between the CRF peptides are boxed. Human stresscopin-related peptide (SRP) and human urocortin II (UCNII) (originally designated human urocortin-related peptide [14]) are identical. Human stresscopin (SCP) and human UCNIII also have identical amino acid sequences. Abbreviations: h, human; m, mouse; o, ovine; SVG, sauvagine; URO, urotensin I.

Box 1. CRF receptor nomenclature

To facilitate scientific investigation, a standardized nomenclature of receptors and their endogenous ligands is a prerequisite. After the corticotropin-releasing factor CRF₂ receptor was cloned, the following system for classifying CRF receptors was adopted: (1) the two receptors were numerically ordered (CRF₁ and CRF₂) [a]; and (2) a capital letter was used to designate splice variants (CRF_{1A-D} and CRF_{2A-C}). Because the CRF₂ receptor is highly selective for urocortin (UCN) compared with the CRF₁ receptor, renaming the CRF₂ receptor as the 'urocortin' receptor is being considered. However, this nomenclature could fail to distinguish the biology of both receptors because UCN binds equally well to both CRF₁ and CRF₂ receptors [b–e]. The isolation of stresscopin (SCP)/UCNIII and stresscopin-related peptide (SRP)/UCNII [f–h] further complicates the issue because both ligands show similarly low homologies with UCN and CRF. Finally, the possibility of a third CRF receptor has been raised. Therefore, we encourage the research laboratories that isolated SRP/UCNII and SCP/UCNIII to agree on common names for these two novel ligands. The IUPHAR receptor nomenclature committee should then consider revising the current CRF receptor classification system.

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genes were identified in the pufferfish genome [12]. Although URO is highly conserved among fish species (>93% identity), UCN-like peptides sequenced in pufferfish were only ~20% homologous with fish URO and mammalian UCN. The search for novel members of the CRF family culminated with the cloning of stresscopin (SCP) and stresscopin-related peptide (SRP) from human cDNA [13] and urocortin II (UCNII) and urocortin III (UCNIII) from mouse cDNA [14,15]. To simplify the terminology for these new members of the CRF peptide family (Box 1), the species homologs will be abbreviated as SRP/UCNII and SCP/UCNIII.

Although the SCP/UCNIII and SRP/UCNII precursors predict 38-amino-acid mature peptides [14,15], isolating and sequencing SCP/UCNIII and SRP/UCNII peptides will be required to establish their native sequences. Although amino acid sequences of human and mouse SCP/UCNIII are ~90% identical, only 76% sequence conservation has been reported for SRP/UCNII homologs. SRP/UCNII shows moderate homology to h/rCRF (34%), hUCN (43%), SCP/UCNIII (37–40%) and URO (34%) but minimal homology to SVG (<25%) (Fig. 1). SCP/UCNIII is more distant from other members of the CRF family (18–32% identity) (Fig. 1). CRF peptides only share four amino acids with each other (Fig. 1) and secondary structure, rather than linear sequence, appears to determine biological activity. Because human SRP/UCNII lacks the standard consensus site required for proteolytic cleavage and C-terminal amidation [13] (i.e. a glycine residue followed by a lysine or an arginine residue), this peptide might not be processed to a biologically active form *in vivo* [13,14]. Because the human SRP/UCNII gene encodes a glycine followed by the basic amino acid histidine, an unidentified enzyme might be capable of cleaving SRP/UCNII thereby forming an amidated, biologically active peptide. Cloning of this novel enzyme could resolve whether or not human tissues synthesize only three out of the four CRF-like peptides isolated in rodents.

SRP/UCNII mRNA is highly expressed in the paraventricular, supraoptic and arcuate nuclei of the hypothalamus, the locus coeruleus, and motor nuclei of the brainstem and spinal cord [13,14]. In the periphery, SRP/UCNII mRNA is detected in the heart, adrenal gland and peripheral blood cells [13]. Important brain sites that express SCP/UCNIII mRNA include the rostral perifornical area of the hypothalamus, the posterior part of the bed nucleus of the stria terminalis, the lateral septum and the medial amygdaloid nucleus [13,15]. Although SCP/UCN III mRNA is considerably less abundant than SRP/UCNII mRNA, high levels of SCP/UCNIII mRNA expression have been detected in the gastrointestinal tract, muscle, adrenal gland and skin [13,15].

CRF receptor subtypes

cDNAs encoding CRF₁ and CRF₂ receptors have been cloned from vertebrates as evolutionary distant as

fish and humans [4,16]. Both CRF₁ and CRF₂ receptors belong to the class B subfamily of seven-transmembrane receptors that signal by coupling to G proteins. The CRF₂ receptor, a 397–438-amino-acid protein, has three functional splice variants, CRF_{2A-C}. Although their N-terminal sequences and tissue distribution differ, these splice variants show no major pharmacological differences [2,4]. There is one functional variant and several non-functional variants of the CRF₁ receptor, a 415–420-amino-acid polypeptide [2,4]. The CRF₁ and CRF₂ receptors are remarkably homologous (~70% amino acid identity). The lowest degree of homology (<60% amino acid identity) exists in their extracellular domains, particularly in the N-termini (40% identity). By contrast, intracellular and transmembrane domains are highly homologous (80–85% amino acid identity) with third intracellular loops being completely identical between all cloned CRF receptors [4,16]. Because both CRF₁ and CRF₂ receptors signal via cAMP as a second messenger, the stimulatory G protein (G_s) most probably couples to this intracellular loop [17]. A CRF₃ receptor isolated from catfish has been found to be highly homologous with the CRF₁ receptor (~85% amino acid identity) [16]. Although the pituitary gland is the main locus of CRF₁ receptor expression in many species, CRF₃ (but not CRF₁) receptor expression has been detected in the catfish pituitary [16]. Mammalian homologs of the catfish CRF₃ receptor might not exist.

CRF receptor expression in the mammalian CNS and periphery

CRF₁ receptor mRNA is widely expressed in mammalian brain and pituitary. Highest densities of CRF₁ receptor mRNA have been found in the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulbs [18,19]. In primates, but not in rodents, CRF₁ receptor mRNA is found in the hypothalamus and locus coeruleus. In the periphery, a low level of CRF₁ receptor mRNA expression occurs in the testis, ovary and adrenal gland [11,20]. Although peripheral CRF₁ receptors can upregulate during pregnancy [21] and inflammation [22], the CRF₁ receptor appears to be more crucial in regulating brain and pituitary functions whereas the CRF₂ receptor might be more important in the periphery.

CRF₂ receptor mRNA is widely expressed in peripheral tissues, particularly in cardiac myocytes, gastrointestinal tract, lung, ovary and skeletal muscle [23–25]. Interestingly, the CRF_{2B} receptor splice variant is the CRF₂ receptor that is peripherally expressed in rodents whereas the CRF_{2A} receptor is the major splice variant found in the periphery of humans [4]. The CRF_{2A} receptor is the dominant CRF₂ receptor splice variant expressed in the mammalian brain. Central CRF_{2A} receptor mRNA is expressed in a discrete pattern with highest densities in the parvocellular nucleus of the hypothalamus, lateral septum, amygdala, hippocampus and retina

[19,23,25]. The major non-neuronal brain sites for CRF₂ receptor expression are the choroid plexus and arterioles, where only CRF_{2B} receptor mRNA is found [18]. Interestingly, the rat pituitary and cortex express CRF₁ receptor mRNA exclusively whereas in primates and Tupaia these tissues express substantial levels of both CRF₁ and CRF₂ receptor mRNA [19,25]. Thus, the CRF₂ receptor might possess additional functions in primates.

Pharmacology of CRF₁ and CRF₂ receptors and CRF receptor antagonists

Homologs of CRF and CRF-like peptides (e.g. UCN, URO and SVG) bind with high affinities to the mammalian CRF₁ receptor in a nonselective manner [26,27]. Only the amphibian CRF₁ receptor discriminates between CRF, UCN, URO and SVG [4]. UCN, SVG and URO bind to the CRF₂ receptor with affinities considerably higher than that of h/rCRF and oCRF, which are more potent at CRF₁ receptors [6,27]. These findings coupled with recent data showing that SCP/UCNIII and SRP/UCNII fail to bind and activate CRF₁ receptors [13–15] provide evidence that the CRF₁ receptor possesses ligand selectivity. Recently, SCP/UCNIII and SRP/UCNII were observed to bind exclusively to the CRF₂ receptor, although the K_d of SCP/UCNIII is more than tenfold lower than K_d values for UCN and SRP/UCNII [13,15]. Therefore, it is tempting to speculate that a SCP/UCNIII-selective receptor exists in mammals.

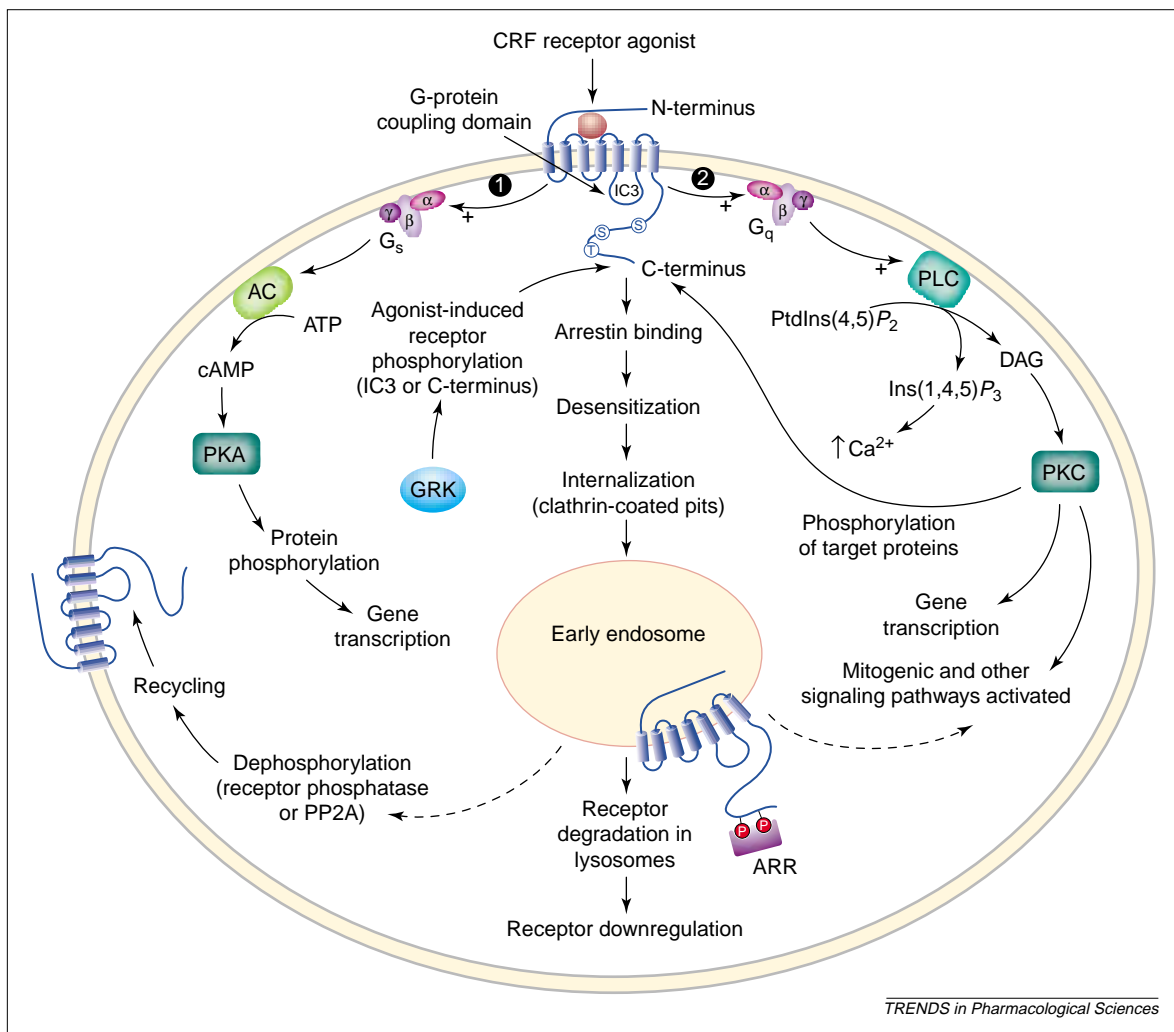
Recently, the N-terminally truncated SVG analog, antisauvagine-30, has been found to display >300-fold selectivity for CRF₂ over CRF₁ receptors [27,28]. This CRF₂-receptor-specific antagonist has become a valuable tool for *in vivo* characterization of CRF₂ receptor function. Several non-peptidic small molecules have been designed to antagonize CRF₁ receptors with >1000-fold selectivity over CRF₂ receptors [29]. In clinical trials, the CRF₁ receptor antagonist R121919 has produced antidepressant properties in patients with major depression [30]. Although one series of oxo-7H-benzo[e]perimidin-4-carboxylic acid derivatives exhibited high-affinity binding at both CRF₁ and CRF₂ receptors [31], its antagonist activity at CRF₂ receptors has not been investigated. Given the high degree of sequence conservation between CRF₁ and CRF₂ receptors, particularly in the predicted pocket where small-molecule antagonists would be expected to bind [32], the paucity of CRF₂-receptor-selective antagonists is surprising. Finally, CRF-BP inhibitors are being studied as potential medications for Alzheimer's disease and obesity [33].

CRF receptor signaling and regulation

G-protein coupling and CRF receptor signaling pathways

It is well established that the binding of CRF receptor agonists to extracellular domains of the CRF₁ or CRF₂ receptor transforms the membrane conformation of these receptors into an active state, thereby

Fig. 2. Corticotropin releasing factor (CRF) receptor signaling pathways and their regulation. CRF₁ and CRF₂ receptors signal mainly through G_s (1), which leads to increased levels of cAMP. In addition, both CRF receptors have been shown to couple to G_q and signal via the phospholipase C (PLC) pathway. G-protein receptor kinase 3 (GRK3) mediation of CRF₁ receptor desensitization has recently been discovered but little information exists regarding molecular mechanisms for CRF receptor desensitization, internalization and recycling, which might differ based on the specific cellular and/or neuronal background. Abbreviations: AC, adenylyl cyclase; ARR, arrestin; DAG, 1,2-diaclyglycerol; IC3, intracellular loop 3; Ins(1,4,5)P₃, inositol (1,4,5)-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; PP2A, phosphatase 2A; PtdIns(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate.



increasing their affinity for the G_s protein (Fig. 2). CRF₁ and CRF₂ receptors signal in a variety of brain-derived and peripheral cell lines (Table 1) by coupling to G_s, leading to the stimulation of adenylyl cyclase and activation of protein kinase A (PKA) and other cAMP pathway events [17,34]. Nevertheless, other second messengers might be implicated in CRF receptor signaling. Multiple G proteins have been observed to couple to CRF₁ receptors endogenously expressed in the placenta and cerebral cortex or overexpressed in HEK293 cells [21,35,36]. In Leydig cells and placenta, CRF₁ receptors might signal exclusively via G_q-mediated stimulation of phospholipase C (PLC) and formation of inositol phosphates [35]. CRF_{2B} receptors endogenously expressed in A431 cells also stimulate the formation of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃] [37]. The observation that CRF increases intracellular Ca²⁺ concentrations in astrocytes and melanoma cells suggests that the undefined CRF receptor subtypes in these settings might also signal via the PLC pathway [38,39]. However, two studies suggest that CRF₁ receptors in cerebral cortex and transfected LLCRK-1 cells can couple to both G_s and G_q proteins thereby activating cAMP and PLC pathways (Fig. 2) [40].

SVG and UCN (but not h/rCRF) can stimulate phosphorylation of cAMP response element-binding protein (CREB) and mitogen-activated protein kinase (MAPK) in cells that overexpress CRF₁ or CRF₂ receptors [35,41]. The PKA inhibitor H89 blocked formation of phosphorylated CREB but not phosphorylated p44/42 MAPK [41], which suggests that SVG-stimulated MAPK phosphorylation does not require PKA activation. MAPK becomes phosphorylated and activated in third-trimester human myometrial cells exposed to UCN possibly via G_q-mediated Ins(1,4,5)P₃ formation and PKC activation [35]. Because CRF₁ receptor activation promotes neurite growth in CATH.a cells that can be blocked by PKA and MAPK kinase inhibitors [42], these two downstream signaling events might have important roles in neuronal function. Signaling pathways that become selectively activated when endogenous ligands bind to CRF₁ or CRF₂ receptors might mediate physiological responses in the brain, pituitary and peripheral tissues.

G-protein receptor kinases and CRF₁ receptor desensitization

During stress adaptation, sustained exposure of the anterior pituitary to high concentrations of CRF

Table 1. Cell lines that express endogenous CRF receptors^a

Cell line	CRF receptor	Species and origin	Signaling	Refs
AtT20	CRF ₁	Mouse anterior pituitary	cAMP (G _s)	[58]
CATH.a	CRF ₁	Mouse brain	cAMP (G _s)	[42]
NCI-H82	CRF ₁	Human small lung cell carcinoma	cAMP (G _s)	[59]
Y 79	CRF ₁	Human retinoblastoma	cAMP (G _s)	[44]
IMR-32	CRF ₁	Human neuroblastoma	cAMP (G _s)	[46]
HEK293	CRF ₁	Human embryonic kidney (not all subclones express the receptor)	cAMP (G _s)	[34]
SH-SY5Y	CRF ₁	Human neuroblastoma	cAMP (G _s)	[60]
HSC-2 and HaCaT	CRF ₁	Human keratinocyte	cAMP (G _s)	[61,62]
SK-MEL188	CRF ₁	Human melanoma	Ca ²⁺	[62]
A7R5	CRF _{2B}	Rat aortic smooth muscle	cAMP (G _s)	[11,13]
A431	CRF _{2B}	Human epidermoid	Ca ²⁺	[37]
H5V	CRF ₁ and CRF ₂	Mouse endothelioma	iNOS inhibition	[63]
HUVEC	CRF ₂	Human umbilical vein endothelial	iNOS stimulation	[63]
AR-5	CRF _{2A}	Rat amygdala	cAMP (G _s)	[64]

^aAbbreviations: CRF, corticotropin-releasing factor; iNOS, inducible nitric oxide synthase.

produces desensitization of CRF-stimulated cAMP accumulation and decreased adrenocorticotrophic hormone (ACTH) release in corticotrope cells [17,43]. Likewise, when anterior pituitary or AtT20 cells are exposed to increased concentrations of CRF, large reductions in CRF-stimulated cAMP accumulation and ACTH release and downregulation of CRF₁ receptors also develop [17]. In a setting favoring strong G-protein receptor kinase (GRK) action (i.e. high agonist concentration), CRF-stimulated cAMP accumulation was markedly decreased in human retinoblastoma Y79 and neuroblastoma IMR-32 cells exposed to CRF [44–46]. An increasing degree of homologous CRF₁ receptor desensitization occurs in brain-derived cells exposed to physiological concentrations of CRF without changes in CRF₁ receptor mRNA levels [43,46]. However, CRF₁ receptor mRNA expression decreases in anterior pituitary cells exposed to CRF for several hours [47]. Homologous CRF₁ receptor desensitization can be markedly inhibited in Y79 cells after a large reduction in GRK3 expression is induced by uptake of a GRK3 antisense oligonucleotide or transfection of a GRK3 antisense cDNA construct [45]. These findings suggest that GRK3-mediated phosphorylation contributes importantly to the homologous desensitization of brain CRF₁ receptors. However, a high degree of CRF₁ receptor phosphorylation was detected in COS cells expressing an epitope-tagged CRF₁ receptor after exposure to CRF [48]. Because COS cells express GRK2, but not GRK3 [49], GRK2 appears to be capable of desensitizing CRF₁ receptors in this cell system. However, homologous desensitization of CRF₁ receptors was significantly less in transfected HEK293 and Ltk cells compared with native cells endogenously expressing CRF₁ receptors [40,44], suggesting that GRK2 might be less effective than GRK3 in desensitizing CRF₁ receptors. Nevertheless, the importance of cell type and structural motifs in determining GRK CRF₁-receptor specificity is not presently known.

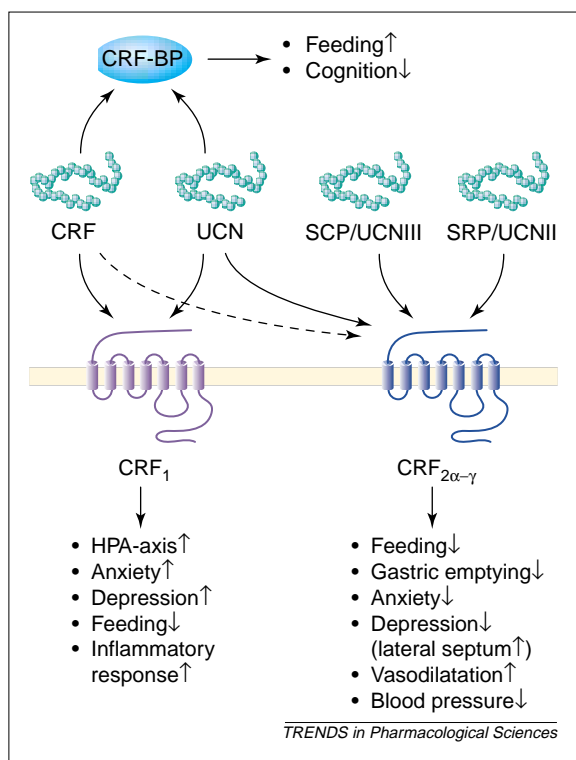
Regulation of CRF₂ receptors

Stress and left ventricular hypertrophy can increase UCN mRNA expression and downregulate CRF₂ receptor mRNA expression in the heart [11,50,51]. When aortic smooth muscle A7R5 cells or ventricular myocytes are exposed to UCN or h/rCRF, a dose-dependent reduction in CRF₂ receptor mRNA expression develops [11,50]. However, the homologous desensitization of CRF₂ receptors has not been investigated to date.

A possible pathophysiological role of CRF receptors in anxiety and depression

Many studies implicate alteration of CRF₁ and/or CRF₂ receptor functioning in the etiology of human stress disorders, particularly anxiety and depression [17,52–55]. Severe anxiety and depression has been hypothesized to result from exaggerated neurotransmission in one or more of the following CRF-regulated pathways mediating the stress response: the hypothalamic–pituitary–adrenal (HPA) axis, the locus coeruleus (LC) noradrenaline-mediated system, and the 5-HT-mediated dorsal raphe nucleus system. Patients with severe melancholic depression exhibit behavioral and physiological symptoms that are associated with overly active CRF-driven stress systems, including comorbid anxiety and agitation and HPA axis dysregulation [52–55]. Post-mortem measurement of very high concentrations of CRF in the cerebrospinal fluid of severely depressed suicide victims has provided additional support for the hypothesis that chronic hypersecretion of CRF plays a leading role in the etiology of major depression [52,53]. Although one post-mortem study of depressed suicide victims reported downregulation of CRF₁ receptors in the frontal cortex, a subsequent study found no evidence for brain CRF receptor downregulation in depressed patients who committed suicide [53,56]. In addition, mRNA levels for CRF₁ and CRF₂ receptors were not different in anterior pituitaries collected from depressed suicide victims and controls [57].

Fig. 3. Interactions between mammalian corticotropin releasing factor (CRF)-like peptides, their receptors and the CRF binding protein (CRF-BP). Binding to the CRF-BP or activation of CRF₁ or CRF₂ receptors stimulates or decreases a wide range of physiological responses. Abbreviations: SCP, stresscopin; UCN, urocortin.



Because CRF receptors normally undergo rapid desensitization, internalization and downregulation during continued agonist presence [17,43–48], it is difficult to understand how chronic CRF hypersecretion alone could lead to overly active CRF-driven stress systems. Alteration of cellular mechanisms that regulate the sensitivity, magnitude and duration of CRF receptor action rather than brain CRF hypersecretion *per se* might instead underlie pathologically excessive CRF neurotransmission. An inherited or acquired deficit in the expression of GRK3 or other signaling molecules that regulate

desensitization of CRF₁ receptors might trigger long-term psychopathology by increasing the sensitivity of and/or prolonging agonist-stimulated CRF₁ receptor signaling in brain pathways that mediate the expression of fear/anxiety responses. Alternatively, curtailment of CRF₂ receptor signaling resulting from more efficient desensitization and internalization of agonist-activated receptors could impair the putative 'anxiolytic' action of this receptor, thereby increasing the 'anxiogenic' effects of CRF₁ receptor signaling.

Concluding remarks

To date, four CRF-like peptides exist in vertebrates, two of which (CRF and UCN) bind with high affinity, and two of which (SCP/UCNIII and SRP/UCNII) have no affinity for the CRF₁ receptor (Fig. 3). Conversely, CRF has only low affinity for the CRF₂ receptor whereas UCN binds to both receptors and SCP/UCNIII and SRP/UCNII are exclusive CRF₂ receptor ligands. On the basis of *in vitro* data, it is likely that CRF and UCN represent the natural agonists for the CRF₁ receptor, and UCN, SCP/UCNIII and SRP/UCNII are likely to be the natural ligands for the CRF₂ receptor. However, as a result of the lower agonist potency of SCP/UCNIII at the CRF₂ receptor and its inactivity at the CRF₁ receptor, it cannot be ruled out that a yet undiscovered SCP/UCNIII receptor exists.

Although genetic models have provided first information on the contribution of each CRF receptor in the progression of several psychiatric and peripheral disorders, the *in vivo* functions of both receptors are unknown. Promising clinical trial data has revealed an antidepressant action for small-molecule CRF₁-receptor-specific antagonists. The synthesis of highly selective, brain-penetrating CRF₂ receptor antagonists should help to elucidate the role of the CRF₂ receptor in the regulation of physiological processes and pathophysiological states.

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Chemical name

H89: N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide