NONGENOMIC ACTIONS OF STEROID HORMONES

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Steroid hormones modulate many physiological processes. The effects of steroids that are mediated by the modulation of gene expression are known to occur with a time lag of hours or even days. Research that has been carried out mainly in the past decade has identified other responses to steroids that are much more rapid and take place in seconds or minutes. These responses follow nongenomic pathways, and they are not rare.

ERYTHROCYTE
A mature red blood cell, which lacks a nucleus and mitochondria. It contains haemoglobin and functions in the transport of oxygen.

ACROSOME REACTION
On appropriate stimulation, the outer acrosomal membrane at the front of the sperm head ruptures and liberates its contents (mainly enzymes and actin). An early event in the reaction is a rapid increase in the concentration of intracellular calcium that can be measured easily.

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In 1942, Hans Selye, in a study on steroid hormones (hereafter referred to as steroids), noted that some steroids induced effects (such as anaesthesia) only minutes after their application, even in the absence of 'main' hormonal activity, whereas the main hormone actions that were known at that time — corticoid, folliculoid, luteoid and testoid — were only visible hours or days after application of the steroid[1]. According to contemporary knowledge, but without the strict dogmatic theories, which emerged later on, to explain the action of steroids, this was yet to become an acceptable observation. In the light of present knowledge, Selye's paper was actually the first to detail the nongenomic action of steroids (BOX 1); this discovery also led to the development of steroidal anaesthetics that were routinely used in human medicine and are still used in animals.

Two decades later, acute cardiovascular effects of aldosterone that occurred within five minutes of its administration were reported in humans[2]. Here, again, the short time frame was indicative of a nongenomic action. *In vitro* studies on the modulation of sodium ion (Na^+) exchange by aldosterone in dog ERYTHROCYTES[3] contributed more evidence of a nongenomic action of steroids, because these cells do not contain a nucleus. As transcription requires DNA, which is located in the nucleus, the absence of the nucleus therefore excludes the possibility of transcription and, so, genomic action. However, when these studies were carried out, no comprehensive theory to explain the action of hormones had been established. Therefore, the peculiarity of these particular findings, and possibly the results of some other studies, is only recognized retrospectively.

Genomic versus nongenomic action

According to the classical model of steroid action[4], steroid molecules enter the cell — either passively by diffusion through the lipid membrane or assisted by transporter proteins — and are bound to 'classical' steroid receptors (BOX 2) that are located mainly in the cytosol. Ligand binding induces a conformational change in the receptor protein, which is accompanied by the dissociation of accessory proteins, thereby exposing the DNA-binding domain. In the nucleus, the receptor–ligand complex binds to DNA and modulates gene transcription. Eventually, after it has been transported and modified by independent mechanisms, the protein that is made from the newly synthesized messenger RNA elicits the genomic response. The length of time between steroid entry and the accumulation of significant amounts of newly formed protein is in the order of hours, and the whole pathway is sensitive to particular inhibitors, such as actinomycin D or cycloheximide.

As outlined above, some steroid responses do not fit this classical genomic model of steroid action. Many steroid-induced phenomena occur rapidly — for example, the ACROSOME REACTION that is induced by progesterone takes place seconds after sperm come into contact with this steroid. Although it is often, but not always, straightforward to distinguish between genomic and nongenomic action, considerable controversy still exists as to the identity of the receptors that initiate nongenomic phenomena. Nongenomic action often involves the generation of intracellular second messengers, and various signal-transduction

Box 1 | **Criteria for defining nongenomic action**

A nongenomic action defines any action that does not directly and initially influence gene expression, as do the classical steroid receptors, but rather drives more rapid effects such as the activation of signalling cascades. There are several criteria that indicate nongenomic action:

- The absence of a 'normal, functional' nucleus in some cell types, such as in erythrocytes, PLATELETS and spermatozoa, excludes genomic action. Another example is chondrocyte matrix vesicles, although these are not real cells.
- Effects that are not blunted by inhibitors of transcription, such as actinomycin D, cannot involve gene expression. In a similar way, insensitivity to cycloheximide, a protein synthesis inhibitor, provides evidence of a nongenomic mechanism.
- A short time frame — in the seconds to minutes range — is one of the strongest pieces of evidence. However, many nongenomic effects might also occur over a timescale that might include genomic action.
- The observation of effects that are elicited by steroid hormone analogues that do not access the cell interior excludes the classical mechanism of translocation of the liganded receptor to the nucleus. However, direct experimental proof is difficult to obtain.

The term 'membrane-initiated steroid signalling' (MISS) has been proposed as an alternative to 'nongenomic' action; however, it is possible that there are nongenomic effects that do not necessarily originate at the membrane.

cascades, such as ion fluxes (often calcium), cyclic AMP modulation and protein kinase pathways, have been shown to be involved.

Receptors that mediate nongenomic action

Although it has been discovered recently that classical steroid receptors can initiate second messenger production or interact with other cellular signalling systems, their most reported property is still their transcriptional activity. In addition, the pharmacological agonist and antagonist profiles for the genomic and nongenomic actions often differ markedly. First, in many cases, antagonists that strongly inhibit the genomic effects that are mediated by the classical receptors are virtually inactive towards the nongenomic effects (see the examples below); unfortunately, there are only a few selective inhibitors for nongenomic steroid action that are known at present. Second, rapid, nongenomic responses have been shown in cells in which the respective classical receptor was thought not to be expressed; such responses can often also be elicited by steroids that are coupled covalently to a polymer, which presumably is unable to enter the cell. Although the latter approach has some drawbacks (for a detailed discussion, see REF. 5), it is often used to show that receptors are present at the cell membrane. Such nonclassical receptors are not yet well characterized, and only limited information about their identities is available. Antibodies have been used to prove either the presence or absence of certain steroid receptors, or even their involvement in given effects by showing functional inhibition. However, this approach is sometimes misleading, owing to antibody crossreactivity or the low abundance of the antibody substrate.

Various steroids have been shown to bind to many biological membranes, but the characterization of the proteins to which they bind has mostly been limited to the determination of their molecular weight or their tentative identification by antibodies. A progesterone-binding protein from liver MICROSOMES was isolated by our group[6], then sequenced and cloned. When expressed recombinantly, it binds progesterone, and antibodies raised against it have been shown to suppress the rapid progesterone action in sperm. However, it must be considered that showing the existence of a binding site does not necessarily indicate that the receptor confers properties in terms of cellular signalling, unless other evidence is available.

In several cases, however, nongenomic steroid actions can be attributed to classical steroid receptors, or modified classical receptors. This is particularly true for some of the effects of oestrogen, and the classical progesterone receptor has also been shown recently to interact with signalling components (see below). The Mannheim classification[7] has been proposed to put the existing distinct forms of nongenomic steroid action into an order.

Progesterone

Consistent with its role as a gonadal steroid, most of the nongenomic effects of progesterone have been described in germ cells such as oocytes or sperm. *Xenopus laevis* oocytes that are arrested in the G2 phase undergo maturation when progesterone is added. This phenomenon is insensitive to actinomycin D and even occurs in enucleated cells[8], which indicates that it is the result of nongenomic action. The initial response to progesterone involves the inhibition of adenylate cyclase and, consequently, a decrease in the levels of cAMP[9,10]. Interestingly, progesterone is more effective when applied outside the cell than when microinjected into the cytoplasm[11], a finding that is consistent with the membrane localization of the respective receptor. Recent findings indicate that an isoform of the *Xenopus* homologue of the classical progesterone receptor — which is known as XPR — is present in small amounts in the cell membrane[12]. Furthermore, XPR seems to be associated with phosphatidylinositol 3-kinase (PI3K) and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), both of which are activated on the addition of progesterone. However, other steroids such as hydrocortisone, deoxycorticosterone and the classical progesterone receptor antagonist RU486, which can all elicit oocyte maturation, do not trigger this mechanism. This indicates that there are other, potentially nonclassical, receptors and signalling mechanisms, or modified classical receptors.

PLATELETS
The smallest blood cells, which are important in haemostasis and blood coagulation.

MICROSOME
A small, heterogeneous vesicular particle, 50–150-nm wide, that is the product of homogenization of eukaryotic cells. Rough microsomes, which have ribosomes attached to their surface, are derived from the rough endoplasmic reticulum, whereas smooth microsomes lack ribosomes and might be derived from the smooth endoplasmic reticulum or the plasma membrane.

RU486
(Mifepristone). A steroidal progesterone receptor- and glucocorticoid receptor-antagonist that prevents implantation of a fertilized ovum in the uterus.

Box 2 | Classical steroid receptors

Classical, or nuclear, steroid receptors are proteins that are located in the cytosol (or sometimes in the nucleus). On binding their ligands, they undergo a conformational change that allows them to interact with DNA. The receptors consist of a carboxy-terminal domain that contains the ligand-binding pocket; a conserved cysteine-rich central domain that is probably responsible for DNA-binding activity; and a variable amino-terminal region that might modulate transactivation. Nuclear steroid receptors show considerable homology to each other, and form a protein superfamily together with the receptors for vitamin D_3 and the thyroid hormones. More than 60 different receptor proteins have been identified in vertebrates. In some cases, their ligands have not been identified, and in this case they are described as 'orphan receptors'.

GRANULOSA CELL
A cell that makes up a layer that surrounds the cavity of mature Graafian and secondary follicles. It catalyses the conversion of androgens to oestrogen.

Sperm cells respond to progesterone by initiating the acrosome reaction[13,14], which is often monitored by determining the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This response is undoubtedly nongenomic, and there is evidence for the involvement of a nonclassical receptor. Although the question as to whether the classical progesterone receptor is present in spermatozoa has yet to be settled[15–18], the steroid specificity for the induction of the acrosome reaction differs too much to be compatible with the selectivity pattern of the classical progesterone receptor[19]; RU486 has been reported to antagonize the progesterone-induced Ca^{2+} influx[20,21] only minimally, and other studies have reported a partial antagonism that was thought to be nonspecific[22]. Using fluorescent progesterone–albumin conjugates, the membrane localization of receptors has been studied[23]. Fluorescence microscopy has shown that progesterone-binding proteins are localized on the outer surface of the head of the sperm cell. Furthermore, it has been shown that the progesterone effect can be reduced by antibodies against the progesterone membrane-binding protein (mPR)[24], which was isolated previously from liver[6]. This protein shows specific, high-affinity binding of progesterone and is one of the few candidate nonclassical receptors that has been sequenced and cloned[25]. Interestingly, higher levels of the rat homologue of this protein, which is known as 25Dx, have been reported in the brains of female mice in which the progesterone receptor has been genetically knocked out than in their wild-type littermates[26], which might indicate that there is some kind of compensatory regulatory mechanism.

The classical progesterone receptor includes an amino-terminal proline-rich motif that has been shown, for the human protein, to interact with Src-homology-3 (SH3) domains of various proteins in response to progesterone[27]. One such SH3-containing protein is Src, and the interaction of progesterone with Src activates the Ras and/or Raf1 and ERK/MAPK cascades. Mutagenesis studies have shown this activation to be independent of the transactivation properties of the progesterone receptor (FIG. 1). This indicates that progesterone might have a dual role, through both genomic and nongenomic pathways.

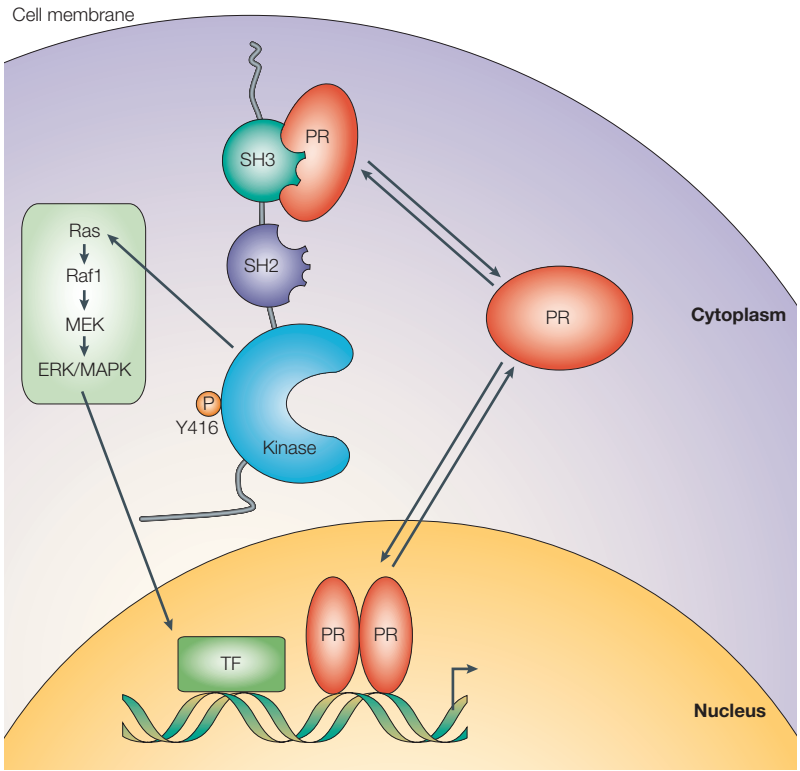


Figure 1 | **Nongenomic actions of the progesterone receptor.** This schematic shows the interaction of the classical progesterone receptor (PR) with Src-homology-3 (SH3) domains and the initiation of the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. The amino-terminal proline-rich domain of PR interacts with the SH3 domain of Src. This activates the Ras–Raf–ERK/MAPK pathway, which then influences the activity of transcription factors (TFs) in the nucleus. The more conventional (genomic) action of the PR is depicted on the right of the figure; PR functions as a dimer in the nucleus to mediate transactivation and subsequent transcriptional changes. SH2, Src-homology-2. Modified with permission from REF. 27 © (2001) Cell Press.

Oestradiol

Similar to progesterone, the gonadal steroid oestradiol has been reported to give rise to nongenomic effects in cells of the reproductive system. Oestradiol-induced (sometimes at subnanomolar concentration) rapid increases in the $[Ca^{2+}]_i$ have been observed in cultured endometrial cells[28,29], maturing oocytes[30,31] and GRANULOSA CELLS. Although there seems to be a pronounced preference for oestradiol and related compounds, neither the classical oestrogen receptor (ER) inhibitor tamoxifen nor actinomycin D or cycloheximide inhibited the calcium response in granulosa cells[30]. In endometrial cells, oestrogen binds to specific sites on the cell surface, which indicates that ERs might be present at the cell surface[32].

In addition to the effects that are related to the reproductive system, oestradiol has a direct action on the vascular system. As shown in several species, both 17α- and 17β-oestradiol induce significant relaxation in coronary arteries and vessels in the aorta[33,34]. In endothelial cells, 17α-oestradiol activates endothelial nitric oxide synthase (eNOS); this results in increased levels of nitric oxide (NO)[35], which acts as a vasodilator. In this case, classical ER antagonists such as ICI-182780 (REF. 36) blunt the response, whereas overexpression of ERα augmented the effect[37]. The PI3K–Akt pathway has been shown to be involved in this response[38], which leads

Figure 2 | **Rapid changes in nuclear pore structure induced by aldosterone.** Atomic-force microscopy was used to visualize the structural changes in the nuclear envelope that occur minutes after addition of the steroid. Before aldosterone stimulation, pore complexes have smooth surfaces, but granular structures (arrows) appear 2 min after the addition of aldosterone. The latter have disappeared 19 min after steroid stimulation and plugs (arrows) in the central channels become visible instead. Reproduced with permission from REF. 123 © (2000) S. Karger AG, Basel.

ultimately to the phosphorylation of eNOS. PERTUSSIS TOXIN inhibits oestradiol-induced activation of eNOS[39], which indicates that a G-protein-coupled receptor might be involved. This has indeed been confirmed by immunoprecipitation studies in cells that are cotransfected with ERα and various Gα proteins. So far, the evidence points to the presence of a receptor that is closely related to ERα, is located at the membrane[40] and interacts with $G_{i\alpha}$ (REF. 39). The similarity of ERα and membrane ERs has been supported in recent studies, in which ER was shown to co-localize with caveolin 1 (REF. 41). Furthermore, overexpression of caveolin 1 modulates the activation of ERK/MAPK by oestradiol. The organization of eNOS and ERα into a signalling module in CAVEOLAE has been described previously[42].

There are, however, nongenomic effects of oestrogen that do not require ERα. The rapid activation of ERK/MAPKs by oestradiol also occurs in ERα-knockout mice[43]. This points to the involvement of either ERβ, which has also been shown to initiate nongenomic steroid effects[44], or a nonclassical receptor. To clarify this issue, a double knockout (ERα and ERβ) is now being studied. However, recent studies have indicated that a nonphysiological splice variant of ERα is present in these double knockouts[45], so it is difficult to draw a definite conclusion at present.

Interestingly, recent studies have identified a large number of genes that are upregulated by oestradiol through a pathway that is sensitive to inhibitors of PI3K. Some of these transcripts have been shown to be relevant to cell function[46].

Aldosterone

As mentioned previously, the nongenomic action of aldosterone was shown both *in vitro* and *in vivo* some decades ago. Subsequent studies then reported the presence of specific aldosterone binding sites[42], and showed that aldosterone induced various responses in mononuclear leukocytes[47]. These responses included activation of the Na^+/H^+ antiporter[48], which leads to alkalinization of the cytosol and subsequent changes in cell volume[49]. Although inhibitors of the classical mineralocorticoid receptors (MRs) spironolactone and canrenone cannot inhibit this response, the timescale (15 min) does not completely rule out a genomic mechanism. Ion fluxes have been shown in this and many other systems, such as vascular smooth muscle cells[50] or arterial endothelial cells (Online Fig. 1; for a review, see REF. 51), which might underline a role for aldosterone in the regulation of vascular function.

An interesting finding that provides evidence for the involvement of receptors that are unrelated to the classical MRs is the persistence of the Ca^{2+} and cAMP responses to aldosterone in mice that lack these receptors[52].

Recently, aldosterone-induced alkalinization has been studied in human arteries[53]. Although cortisol is not able to induce this effect on its own, it becomes as potent as aldosterone when the hydroxysteroid dehydrogenase inhibitor carbenoxolone is present[53]. This finding (which so far remains unconfirmed) is compatible with the involvement of classical MRs, however, the sensitivity to inhibitors is not. In particular, the potent classical MR antagonist spironolactone is ineffective, whereas the open-ring compound RU28318 strongly inhibits this response. According to binding studies, the closed-ring lactone moiety that is present, for example, in spironolactone, is required for high-affinity binding to MR, whereas open-ring compounds have binding affinities that are orders of magnitude lower[54]. This reversed behaviour points to the participation of a nonclassical receptor with different ligand selectivity.

A rapid, transient Ca^{2+} increase that is maximal after 2 min, followed by a slower, more sustained rise, is produced by aldosterone in skeletal muscle[55]. At the single-cell level, the rapid Ca^{2+} effect occurs as a sequence of rapid oscillations. In this, and many of the systems that are described above, the Ca^{2+} signal is preceded by a rise in inositol trisphosphate ($InsP_3$), which liberates Ca^{2+} from certain intracellular stores. Furthermore, Ca^{2+} can also enter from the extracellular space. The contribution of these two mechanisms varies between cells, and protein kinase C (PKC) often seems to be involved in the Ca^{2+} response, as its inhibition abolishes the Ca^{2+} effect[56].

Using ATOMIC-FORCE MICROSCOPY, rapid contraction of nuclear pore complexes in response to a Ca^{2+} signal — evoked by aldosterone — has been seen in canine kidney cells[57] (FIG. 2). In addition, aldosterone causes nuclei to shrink within minutes of its administration and produces different patterns of response. This shrinking of

PERTUSSIS TOXIN
A mixture of proteins that is produced by *Bordetella pertussis*. It activates G_i proteins by catalysing the ADP ribosylation of the α-subunit.

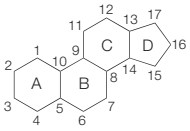
CAVEOLAE
Specialized rafts that contain the protein caveolin and form a flask-shaped, cholesterol-rich invagination of the plasma membrane. Caveolae might mediate the uptake of some extracellular materials, and are probably involved in cell signalling.

ATOMIC-FORCE MICROSCOPY
A microscope that nondestructively measures the forces (at the atomic level) between a sharp probing tip (which is attached to a cantilever spring) and a sample surface. The microscope views structures at the resolution of individual atoms.

Box 3 | **Steroids**

Hormonally active molecules have a core that is formed from four fused rings (A–D) as a common chemical structure.

The figure shows the steroid skeleton with the rings named and the positions numbered according to commonly used nomenclature. In vitamin D derivatives, the carbon–carbon bond between positions 9 and 10 is severed, which is denoted by the prefix *seco*. For a detailed summary of steroid nomenclature, see the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology joint commission on biochemical nomenclature: The Nomenclature of Steroids (see Online links box for further information).



the nuclei was interpreted as occurring during the import of the liganded receptor, and is followed by swelling of the nuclei. Early aldosterone-induced transcripts might then be exported, with a concomitant shrinking of the nuclei back to normal.

An effect that could have great physiological relevance has been described in the isolated rat heart. Here, aldosterone has a rapid (within minutes) and positive INOTROPIC effect. Spironolactone does not antagonize this response, but has an autonomous inotropic action that is additive to the aldosterone response[58].

Glucocorticoids

For many decades, glucocorticoids have been used routinely to treat many clinical conditions, and they have contributed to therapeutic success. However, the nongenomic effects of glucocorticoids have not been studied as thoroughly as those of other steroids. As glucocorticoids occur naturally at high levels and often are applied at rather high doses, nonspecific action at the membrane level must be considered. By their very nature, steroids are highly lipophilic substances that tend to accumulate in lipid membranes. Here, they might alter membrane fluidity and possibly influence the function of embedded proteins, such as ion channels or receptor proteins. However, these phenomena are assumed to occur mainly at concentrations above 10 μM, and there are many nongenomic glucocorticoid effects that occur well below this concentration range[59].

Glucocorticoids are assumed to be involved in stress-mediated responses, many of which occur rapidly and therefore presumably in a nongenomic manner. Comprehensive studies have been carried out on the behaviour of the roughskin newt *Taricha granulosa*, in which corticosterone administration can mimic the rapid stress reaction of courtship behaviour[59,60]. In this system, a membrane receptor that mediates glucocorticoid action in the amphibian brain has been tentatively assigned, and it seems to share similarities with OPIOID κ-RECEPTORS[61]. Biochemical studies indicate that this membrane glucocorticoid receptor has properties that are inconsistent with classical glucocorticoid receptors[62].

At the cellular level, high levels of DEXAMETHASONE have been reported to rapidly (in under 10 min) stabilize lysosomal membranes[63]. A similar effect could be detected after 24 h, but this was sensitive to the glucocorticoid receptor antagonist RU486, whereas the rapid effect was insensitive. This indicates that a dual action occurs through classical and nonclassical receptors.

The membrane-receptor-mediated mechanisms of glucocorticoid action, which involve disruption of mitochondrial function[64], are assumed to participate in cellular processes that lead to apoptosis[65–67]. This underlines the, as yet, incompletely recognized physiological and therapeutic importance of the additional mechanisms of glucocorticoid action. This is also reflected in differing hierarchies for the nongenomic high-dose glucocorticoid effects when compared with the classical genomic responses[68], as the potency ranking for various glucocorticoids is altered.

Glucocorticoids have been shown to activate eNOS in a nongenomic manner that is mediated by PI3K and Akt phosphorylation. This leads ultimately to vasorelaxation, which might explain some of the cardioprotective effects of glucocorticoids[69].

Vitamin D

Vitamin D — or its active form 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) — is an unusually flexible molecule, with regard to its conformation. Unlike 'real' steroids (BOX 3), its A-ring might rotate relative to the fused C- and D-rings, which generates different conformers (FIG. 3). Analogues of these conformers that have bonds that are 'locked' in the *cis* or *trans* conformation, and other isomers, have been synthesized. These compounds can elicit specifically nongenomic and/or genomic responses to $1\alpha,25(OH)_2D_3$. This property is unique among the steroids, and supports the involvement of nonclassical receptors.

The most prominent rapid effect of $1\alpha,25(OH)_2D_3$, at subnanomolar concentrations, is the stimulation of intestinal uptake of Ca^{2+} (REF. 70), which is known as transcaltachia. The DIASTEREOMER $1\beta,25(OH)_2D_3$ potently inhibits this nongenomic effect, but it cannot block the genomic action[71,72]. Several studies clearly show that *cis*-locked analogues activate rapid pathways, whereas they are only weak agonists for genomic responses[73,74] and

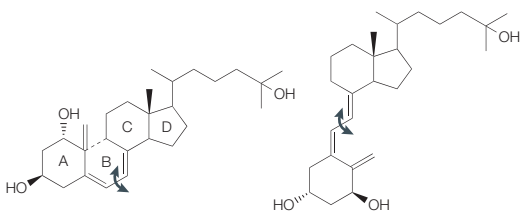


Figure 3 | **Flexibility of vitamin D.** Owing to a missing bond (shown as a dotted line) in the sterane scaffold, the molecule undergoes rotation along the bond between ring A and rings C and D (arrow). This rotation is not possible in 'normal' steroids and gives rise to different conformers that are interconverted easily. Structural analogues have been synthesized that have a 'locked' conformation, and these molecules elicit well-defined subsets of the vitamin D response.

INOTROPIC
Influencing the contractility of muscles.

OPIOID RECEPTORS
These seven transmembrane receptors are produced at high levels in the nervous system and are important for modulating pain responses. The κ-type inhibits a G-protein-modulated calcium channel.

DEXAMETHASONE
A synthetic glucocorticoid that has actions similar to the adrenal corticosteroids. It has negligible mineralocorticoid activity.

DIASTEREOMER
Any stereoisomer of a given molecule that does not represent its exact mirror image.

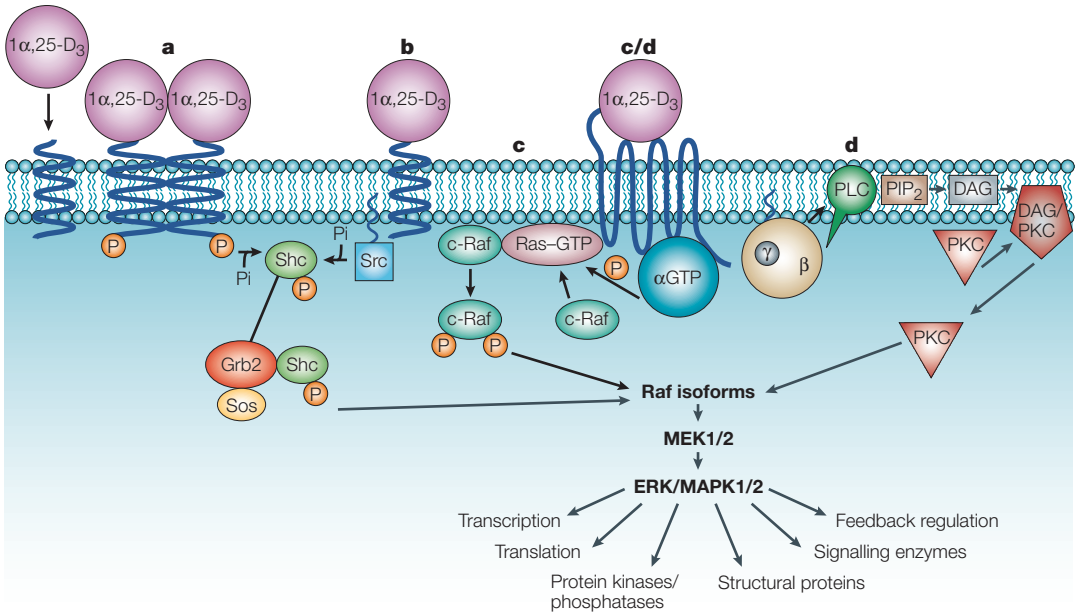


Figure 4 | **Activation pathways of the MAPK cascade in response to vitamin D.** Activation might occur through **a** | membrane receptor proteins with tyrosine kinase activity, which interact with Shc/Grb/Sos, finally targeting Raf; **b** | membrane receptors without tyrosine kinase activity, using Src to phosphorylate Shc, ultimately activating Raf; **c** | formation of membrane-associated Ras–GTP, which phosphorylates Raf; or **d** | activation of either phosphatidylinositol 3-kinase (PI3K) or phospholipase C (PLC), leading to activation of protein kinase C (PKC) and finally activation of Raf. Activated Raf, with several intermediate steps, activates extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), which, in turn, regulates several cellular processes. Shc, Src-homology-2-containing; Grb, growth-factor-receptor bound; Sos, son-of-sevenless; DAG, diacylglycerol; PIP_2, phosphatidylinositol bisphosphate; MEK, MAPK and ERK kinase. Modified with permission from REF. 74 © (2001) Elsevier Science.

hardly bind to classical receptors. In a strikingly different way, *trans*-locked analogues do not promote transcaltachia even at elevated concentrations. The modified analogues of $1\alpha,25(OH)_2D_3$ affect proliferation rate, PROTEOGLYCAN production and PKC activity in CHONDROCYTES[75,76], but they only bind to the classic vitamin D receptor with 0.1% of the effectiveness of $1\alpha,25(OH)_2D_3$.

Subnanomolar levels of $1\alpha,25(OH)_2D_3$ have been shown to increase the $[Ca^{2+}]_i$ within many different cells[77–79]— even those that lack the vitamin D receptor — in minutes[80]. Although a Ca^{2+} influx from the extracellular space through voltage-dependent channels seems to be involved[77–79], $1\alpha,25(OH)_2D_3$ also initiates membrane-lipid hydrolysis, which causes the production of second messengers such as $InsP_3$ that, in turn, release Ca^{2+} from intracellular stores[81]. In addition, diacylglycerol (DAG) is formed, and this probably activates PKC[82,83]. Again, analogues of $1\alpha,25(OH)_2D_3$ that hardly bind to the vitamin D receptor elicit significant PKC activation[76]. As is often observed, the latter is followed by the rapid activation of Raf and ERK/MAPK[84] (FIG. 4).

The nongenomic action of $1\alpha,25(OH)_2D_3$ also involves the cAMP–protein kinase A (PKA) pathway. Inhibitors of adenylate cyclase or PKA block the rapid Ca^{2+} response in various cells[85,86]. Conversely, $1\alpha,25(OH)_2D_3$ increases cAMP levels within minutes in vitamin-D-deficient avian muscle[87] and other cells[88,89].

PROTEOGLYCAN
A class of acidic glycoproteins that contain more carbohydrate than protein.

CHONDROCYTE
A differentiated cell of cartilage tissue.

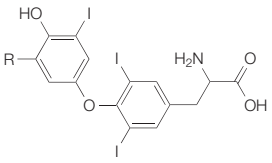
STATS
A family of cytoplasmic transcription factors (signal transducers and activators of transcription) that dimerize on phosphorylation and translocate to the nucleus to activate transcription of target genes.

Thyroid hormones

Although the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are chemically different from steroids (FIG. 5), their classical receptors belong to the steroid-receptor superfamily. Similar to 'true' steroids, the thyroid hormones have also been shown to have nongenomic effects.

Rapid increases in the uptake of 2-deoxyglucose caused by low nanomolar ranges of T_3 have been described in various cells and organs[90,91]. Similarly, and often associated with it, rapid rises in intracellular Ca^{2+} occur[92]. In addition, T_3 and T_4 stimulate plasma-membrane and sarcoplasmic-/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) activity[93,94], which results in Ca^{2+} efflux from the cytosol. This effect is probably mediated by PKC[95]. PKC is itself activated by thyroid hormones[96], although another study reported that PKC activation was dependent on phospholipase C[97]. T_4 has been shown to initiate a dual diacylglycerol-liberating pathway. This involves phospholipase C, which is activated directly by T_4, and then PKC-stimulated phospholipase D[98].

It has been reported that the ERK/MAPK signalling cascade is driven by T_4, and is possibly mediated by a putative G-protein-coupled receptor[99] that has yet to be identified. Numerous phenomena then occur, such as phosphorylation of signal transducers and activators of transcription (STATS)[100] and p53. Furthermore, activated ERK/MAPK has been shown to phosphorylate thyroid receptor-β1, which causes the dissociation



Triiodothyronine: R = H
Thyroxin: R = I

Figure 5 | **The thyroid hormones.** Thyroxin and triiodothyronine, together with other, related compounds, are referred to as thyroid hormones. Their structure is different from steroids, but the classical thyroid hormone receptor belongs to the superfamily of nuclear (steroid) receptors.

of co-repressor proteins[101]. Although these events lead ultimately to a modulation of gene expression, it is exerted by a nonclassical, probably nongenomic, pathway.

The activation of some enzymes, such as pyruvate kinase[102] and cytochrome *c* oxidase[103], seems to be modulated directly by iodothyronines. Iodothyronines abolish the sensitivity of cytochrome *c* oxidase activity to ATP inhibition, and this lack of feedback inhibition might explain the rapid effect of thyroid hormones on mitochondrial respiration.

Neurosteroids

Many steroids act on the nervous system, modulating mainly neuron excitability, but according to the definition of Baulieu[104], true 'neurosteroids' not only act on, but also are synthesized in, the brain. Neuroactive steroids can influence sleep, the reaction to stress, mood, memory and some other functions. As these effects often occur with a considerable time lag, it is not always easy to distinguish between genomic and nongenomic actions.

Steroids that modulate GABAergic transmission (where GABA is γ-aminobutyric acid) through $GABA_A$ receptors are often used in clinical studies. Dehydroepiandrosterone (DHEA) and its sulphate metabolite DHEA-S, which are known allosteric modulators of $GABA_A$ receptors, influence ELECTROENCEPHALOGRAMS and change sleep pattern[105]. However, the doses administered were comparatively high and the results have been puzzling. The $GABA_A$ receptor is the site of action of some 3α,5α-tetrahydrosteroids, which are potent BARBITURATE-like ligands[106,107]. The clinically used anaesthetics alphaxolone and alphadolone also belong to this group of modulators and act on the $GABA_A$ receptor. The GABA receptor also seems to be the ultimate site of action of progesterone in the VENTRAL TEGMENTAL AREA in rodents. Again, the tetrahydrosteroids are the active molecules. Interestingly, in the VENTROMEDIAL HYPOTHALAMUS, progesterone action seems to be dependent on an intracellular progesterone receptor[108].

The NMDA (*N*-methyl-D-aspartate) receptor is essential for proper cognitive functions and nervous-system development. Its allosteric modulation by PREGNENOLONE has been linked to enhanced memory performance in rodents[109]. As memory involves a complex interaction of different mechanisms, it is not surprising that it can also be influenced positively by the $GABA_A$ ligand DHEA as well as σ1-receptor ligands. The σ1-opioid receptors are putative multifunctional proteins that regulate ion-channel function and have an unusually diverse ligand selectivity[110].

Antagonists of the NMDA receptor, such as some preganolone derivatives, prevent cell death in neuronal cell cultures, as does 17β-oestradiol[111]. Furthermore, dementia has been correlated with decreased amounts of neurosteroids.

The anxiolytic activity that is seen with several steroids, mainly A-ring reduced compounds[112,113], might also be correlated with positive allosteric modulation of $GABA_A$ receptors. Although there might be broad clinical applications for steroids in the treatment of conditions such as depression and stress, very few studies have so far been conducted in humans.

Another receptor that is modulated by steroids is the glycine receptor — for example, progesterone[114] and pregnenolone sulphate[115] inhibit glycine-induced currents, although different mechanisms seem to be involved. The modulation of the 5-hydroxytryptamine (5-HT) receptor type 3 by both oestradiol and progesterone has been described[116]; as this receptor is involved in the onset of nausea, this might be a mechanism that is involved in gestational nausea.

Although neurosteroids might have considerable clinical potential, owing to their extensive metabolism, at present, it is not always possible to identify the active substance that is responsible for a particular response, or to limit the steroid action to certain desired effects.

Interaction of distinct pathways for steroid action

Rapid, nongenomic effects of steroids — that is, the generation of second messengers and activation of signalling cascades — might influence the genome through several mechanisms. Many rapid signalling messengers, although they do not act primarily on DNA, indirectly modulate gene expression by acting on transcription factors. They might also influence genes that are targeted by the same steroid in a synergistic, immediate and delayed manner. An example of this is the response of a nerve cell line to oestrogen: a two-pulse stimulation schedule was used to show that the early membrane-mediated oestrogen effect was required for full subsequent induction of genes[117]. Both PKA and PKC seem to be involved in the rapid action.

PKA, which is usually activated by cAMP, is a mediator that is frequently involved in nongenomic steroid pathways. It phosphorylates cAMP response element binding protein (CREB) directly[118], as has been shown for aldosterone[119], and is thought to activate the ERK/MAPK pathway, which eventually leads to the phosphorylation of the steroid receptor co-activator 1 (SRC1)[120]. Both phosphorylated CREB and phosphorylated SRC1 affect the co-activation of some steroid receptors[120].

Other modes of interaction have been described for XPR[12]. Its membrane form, when stimulated by steroids, activates the ERK/MAPK pathway, which, in turn, phosphorylates a different form of XPR, which might indicate that there is a functional regulation of

ELECTROENCEPHALOGRAM
A recording of the electrical activity of different parts of the brain.

BARBITURATES
Pharmacologically active molecules with a potent depressor effect in the central nervous system.

VENTRAL TEGMENTAL AREA
A nucleus of the midbrain. The main supplier of dopamine to the cortex.

VENTROMEDIAL HYPOTHALAMUS
An area of the brain that is found in the middle region of the hypothalamus. It is important for the regulation of appetite and other consummatory behaviours.

PREGNENOLONE
A key intermediate in the biosynthetic pathway from cholesterol to progesterone.

CREB
Cyclic AMP response-element-binding protein. A transcription factor that functions in glucose homeostasis and growth-factor-dependent cell survival, and has also been implicated in learning and memory.

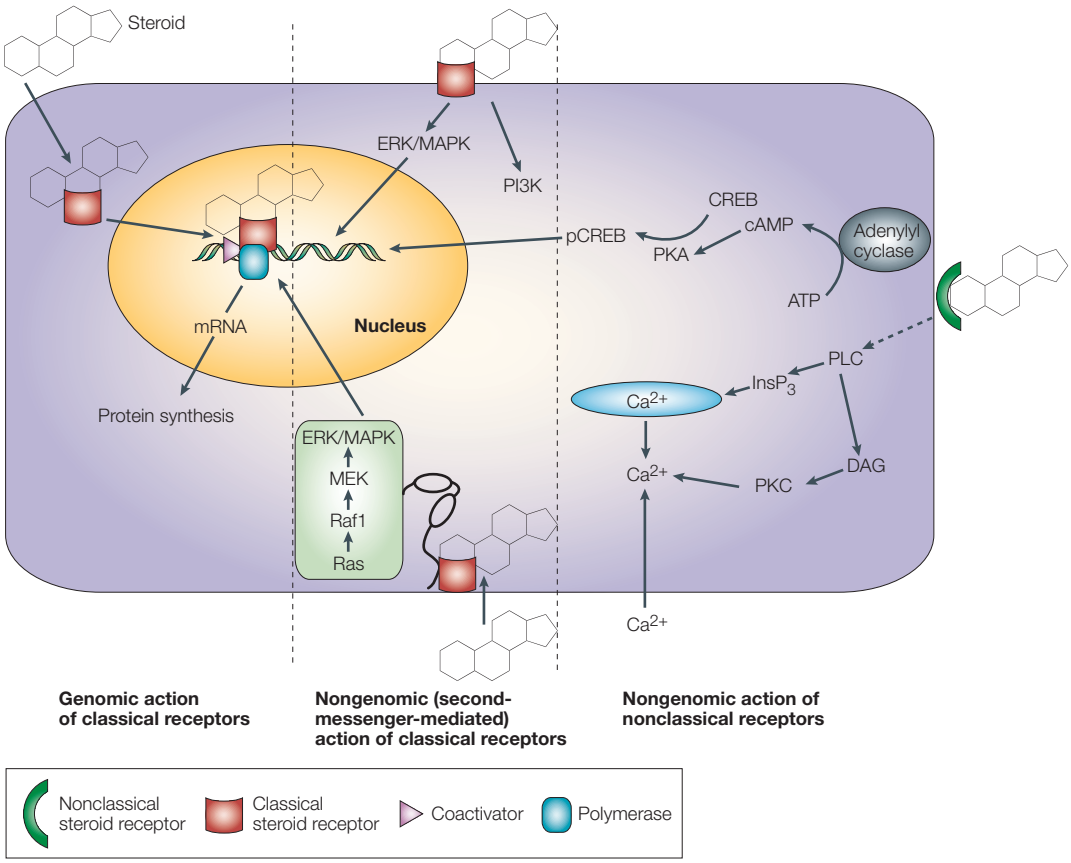


Figure 6 | **Scheme for the numerous actions of steroids by different pathways.** This schematic representation summarizes the mechanisms of nongenomic action that can occur within a generic cell — any one cell type has not been shown to display all these effects. The pathways of action comprise direct transcriptional activation by classical receptors (left), kinase pathways driven by classical receptors (middle part), as well as cyclic AMP, lipase and kinase pathways, including ion fluxes, which are driven by nonclassical receptors (right). Some signalling pathways eventually lead to (indirect) modulation of gene expression by modification of transcription factors. CREB, cyclic AMP response-element-binding protein; DAG, diacylglycerol; ERK/MAPK, extracellular-signal-regulated kinase/mitogen-activated protein kinase; $InsP_3$, inositol trisphosphate; MEK, MAPK and ERK kinase; pCREB, phosphorylated CREB; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

the delayed response. As mentioned above, the human progesterone receptor has been shown to interact in a ligand-dependent way with the SH3 domains of various proteins. So, stimulation of the progesterone receptor drives the ERK/MAPK pathway[27], and this eventually affects transcription factors in the nucleus.

The possible modes of interaction between nongenomic and genomic processes are complex. A multistep model for steroid action — that is, an extension of the crosstalk model that was proposed originally for aldosterone — aims to assemble and inter-relate some of the more prominent mechanisms of steroid action (FIG. 6).

Physiology, conclusions and perspectives

The short time frame of many nongenomic responses is essential; such rapid effects are always required when similarly rapid and also transient stimuli are present. The changes in plasma aldosterone levels that occur minutes after posture changes — that is, the increased amount of aldosterone in the plasma that is observed shortly after rising from a recumbent to an upright position — are an explicit example of a rapidly regulated signal. If only the slower genomic responses occurred, the instant regulation of plasma aldosterone levels would be ineffective. Such rapid responses might affect the cardiovascular system, aiding circulatory homeostasis[2,58,121] or the recovery from physical exercise in muscle[122]. Those actions indicate that aldosterone might be a rapidly acting stress hormone that — in contrast to most peptide hormones — might diffuse freely through membranes, possibly preparing cells for the action of other hormones or for the slower genomic action of aldosterone itself. The physiological relevance of the rapid retardation of sodium exchange in erythrocytes[3], however, is unclear.

A similar example is the high local concentration (micromolar) of progesterone that is present in the region that surrounds the oocytes. Spermatozoa are, therefore, subjected to a rapid increase in progesterone concentration as they enter this area, and this, in turn, helps to trigger the acrosome reaction.

Box 4 | **Evidence needed for the assessment of physiological relevance**

To attribute physiological relevance to laboratory findings, effects should be detectable in living animals, or in humans, at hormone levels that are either within the physiological range or that are reasonably achieved by pharmacological intervention. This excludes many nonspecific effects that are due to a high concentration of steroid.

Animal models offer the advantage of working with classical receptor knockout systems. However, as general knockouts often have severely affected physiology and signalling pathways, they are probably less well suited for such studies. Conditional, tissue-specific, knockouts, or even inducible ones, will be the option of preference. Even in cases in which the classical receptors themselves mediate nongenomic action, genetically modified animals in which constituents of the signalling pathway, such as kinases, are knocked out might be helpful for the elucidation of physiological relevance. Furthermore, inhibitors, which cannot be used in humans for obvious reasons, can be readily used in animal models.

Glucocorticoids, which are secreted in response to stress, might also contribute to the adaptation to difficult conditions, as they rapidly induce vasorelaxation and therefore ameliorate the supply of blood to the heart in addition to reducing the inflammatory response to ischaemia[69].

Although the mechanisms that underlie the rapid responses to steroids at the cellular and molecular levels are diverse, common pathways that are engaged by several classes of steroids have been identified. The ERK/MAPK pathway is frequently involved, although the upstream signalling might be different. Similarly, PKC often participates in the rapid steroid effects. Ion fluxes, mostly calcium, have been shown for virtually all steroids, and second messengers such as cAMP and $InsP_3$ have also been frequently reported to be involved. However, even in one steroid class, the pathway used depends very much on the cell type that is studied.

To assess the role of nongenomic steroid action in physiology, clinical trials and animal models are needed (BOX 4). However, clinical trials that involve humans are hampered by the fact that it is not possible to use inhibitors of the genomic pathway, such as actinomycin D or cycloheximide. So, the primary criteria of these trials must be the time frame and the efficacy of classical-receptor inhibitors. Despite these obstacles, some studies provide convincing evidence of the importance of rapid nongenomic effects in mammalian, or more specifically, human physiology. It is hoped that further investigation of such phenomena at the level of the whole organism, which will hopefully incorporate specific 'nonclassical' inhibitors, will help to identify targets for therapeutic intervention that were hitherto unknown. So far, the importance of nongenomic steroid actions has been underestimated, despite the early reports of this phenomenon.

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25Dx | ENOS | ERα | ERβ | progesterone-binding protein | vitamin D receptor | XPR

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