REVIEW

Non-canonical dimerization of the androgen receptor and other nuclear receptors: implications for human disease

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Abstract

Nuclear receptors are transcription factors that play critical roles in development, homeostasis and metabolism in all multicellular organisms. An important family of nuclear receptors comprises those members that respond to steroid hormones, and which is subdivided in turn into estrogen receptor (ER) isoforms α and β (NR3A1 and A2, respectively), and a second subfamily of so-called oxosteroid receptors. The latter includes the androgen receptor (AR/NR3C4), the glucocorticoid receptor (GR/NR3C1), the mineralocorticoid receptor (MR/NR3C2) and the progesterone receptor (PR/NR3C3). Here we review recent advances in our understanding of the structure-and-function relationship of steroid nuclear receptors and discuss their implications for the etiology of human diseases. We focus in particular on the role played by AR dysregulation in both prostate cancer (PCa) and androgen insensitivity syndromes (AIS), but also discuss conditions linked to mutations of the GR gene as well as those in a non-steroidal receptor, the thyroid hormone receptor (TR). Finally, we explore how these recent results might be exploited for the development of novel and selective therapeutic strategies.

Introduction

Nuclear receptors (NRs) form a superfamily of related transcription factors that play essential roles in multicellular organisms through coordination of pivotal signaling networks (Evans & Mangelsdorf 2014). One important family of NRs groups together those members that respond to steroid hormones, accordingly termed steroid family. In vertebrates, the steroid NRs include estrogen receptor (ER) isoforms ERα (NR3A1) and ERβ (NR3A2) together with the androgen receptor (AR/NR3C4), the glucocorticoid receptor (GR/NR3C1), the mineralocorticoid receptor (MR/NR3C2) and the progesterone receptor (PR/NR3C3). Phylogenetic studies show that AR, GR, MR and PR comprise a subfamily of so-called oxosteroid NRs, which markedly differ from both ER isoforms (Bledsoe et al. 2002, Evans & Mangelsdorf 2014, Gallastegui et al. 2015, Zennaro & Fernandes-Rosa 2017, Katzenellenbogen et al. 2018). This phylogenetic separation is also reflected at the level of tertiary and quaternary structures, as we will discuss below.

We note that some members of other subfamilies of NRs specifically respond to steroid hormones, for instance, the vitamin D3 receptor (VDR/NR1I1), the bile

Key Words

- androgen receptor
- glucocorticoid receptor
- protein structure
- ligand-binding domain
- multimerization
- prostate cancer
- androgen insensitivity syndromes (AIS)
- hormone resistance

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acid receptor (FXR/NR1H4), oxysterol receptors α and β (LXRα/NR1H3 and LXRβ/NR1H2, respectively) and the retinoid-related orphan receptors α, β and γ (ROα/1F1, ROβ/1F2 and ROγ/1F3, respectively). However, for space reasons, we will focus in the current review almost exclusively on the members of the sequence and structurally related ‘classical’ steroid family of NRs, most notably on the AR.

Similar to other NRs, the members of the steroid family display a marked modular structure: a variable N-terminal region called the N-terminal domain (NTD) is followed by a highly conserved DNA-binding domain (DBD) comprising two zinc finger motifs and a C-terminal ligand-binding domain (LBD) that contains the internal ligand-binding pocket (LBP) (Jenster et al. 1995, McEwan 2012a, Gallastegui et al. 2015, Tien & Sadar 2018, Weikum et al. 2018, Fuentes-Prior et al. 2019, Veras Ribeiro Filho et al. 2019). DBD and LBD modules are separated by a poorly conserved interdomain linker called ‘hinge’, which harbors a nuclear localization signal (NLS) (Fig. 1 for a schematic representation of the domain organization in steroid NRs). All the NR-comprising domains are proposed drug targets, but the LBD remains still the main module for therapeutic intervention and, in spite of intense efforts, no molecule targeting other receptor domains has advanced into late clinical trials (Sadar 2011, Dalal et al. 2014, Nadal et al. 2017, Veras Ribeiro Filho et al. 2019).

Over the years, several physiologically relevant protein partners of NRs have been reported to selectively fine-tune their actions in various tissues. These factors, collectively termed coregulators, can either enhance or inhibit transcription of target genes and are therefore known as coactivators or corepressors, respectively (Hermanson et al. 2002, Perissi & Rosenfeld 2005, Dasgupta et al. 2014). These NR coregulators underlie the tissue-selective actions of NR ligands and are also emerging therapeutic targets per se, although they have proved hard to target so far (Yi et al. 2015, Wang et al. 2016, Ruggero et al. 2018). All NR domains have been involved in coregulator recruitment and macromolecular complex formation, although the atomic details still remain elusive (Dasgupta et al. 2014, Foley & Mitsiades 2016, Giudici et al. 2016, Lempiäinen et al. 2017).

Although the NTDs are variable in length and intrinsically disordered (i.e., isolated NTDs lack stable secondary (2D) and/or tertiary (3D) structures under physiological conditions), the results of different biochemical and biophysical studies suggest that they undergo at least partial folding (‘helicity induction’) to modulate the formation of competent transcription...
activation complexes around the NRs (Kumar et al. 2004, McEwan et al. 2007, De Mol et al. 2016, 2018). This is particularly relevant for a polypeptide stretch termed activation function-1 (AF-1; residues 142-485 in the human AR), which is a hormone-independent transactivation function (Fig. 1). An important result of early investigations on the role of the NTD was the realization of at least transient contacts with the C-terminal LBD moiety. These intra- or intermolecular amino/carboxy terminal (N-C) interactions play key roles in regulating the functions of some steroid receptors, both by stabilizing the overall protein structure and by modulating interactions with DNA and coregulators, thus ultimately controlling gene expression (Langley et al. 1998, He & Wilson 2002, Bai et al. 2005). On the LBD, a solvent-exposed pocket responsible for recognizing both AF-1 and coregulators has been extensively studied, which is called activation function 2 (AF-2) or ligand-dependent coactivator-binding site (Figs 1 and 2A). At least in the case of the AR, this is in addition to a nearby pocket that contributes to allosterically regulate receptor functions and which has been termed binding function-3 (BF-3) (A note of caution must be added here, as most of the structural and functional evidence comes from studies performed using short peptides derived from known coregulators, which might not correctly reproduce interactions found in multiprotein complexes in vivo.) (Estébanez-Perpiñá et al. 2005, 2007b, Buzón et al. 2012, Ravindranathan et al. 2013, Badders et al. 2018) (Fig. 2B).

In the following section, we will briefly discuss recently presented results on the structure-and-function relationship of the AR, but will refer to and compare with other steroid NRs when appropriate.

The ‘life cycle’ of the AR: from chaperone-bound monomer to multimers on chromatin

Newly synthesized AR is present in the cytoplasm associated with major molecular chaperones (Prescott & Coetzee 2006, Centenera et al. 2013a, Guy et al. 2015, Foley & Mitsiades 2016, Kita et al. 2017). AR first binds to the heat shock protein 70 (Hsp70)–Hsp40 complex, which is followed by binding of Hsp90 and co-chaperones and subsequent dissociation of Hsp70–Hsp40. Hsp90 binding ensures that the AR retains a high-affinity conformation for the androgenic hormones, testosterone and its more potent derivative, dihydrotestosterone (DHT) (Hernández et al. 2002, Azad et al. 2015). Another chaperone, Hsp27, is regulated by cell stress and prevents aggregation and degradation of the AR, besides promoting nuclear trafficking of the receptor and its binding to DNA (Zoubeidi et al. 2007).

Binding of androgens in the cytoplasm induces AR dissociation from the chaperone complex, likely...
accompanied by significant conformational changes, nuclear translocation of the receptor and its dimerization, which is a critical step within this signaling pathway (Centenera et al. 2008, van Royen et al. 2012, Nadal et al. 2017). Pioneer studies suggested that AR oligomerization precedes binding to chromatin and demonstrated that DNA binding was redox dependent (Kokontis & Liao 1999). More recently, AR dimerization has been shown to enhance chromatin interaction and remodeling to modulate gene expression (Jin et al. 2013). However, it must be stressed that the exact cycle of AR monomer-to-oligomer transition in the cell has not been precisely elucidated (Schaufele et al. 2005, Centenera et al. 2008, van Royen et al. 2012, Nadal et al. 2017). It is usually assumed that this transition follows exactly the same steps as in related NRs, although data supporting this assumption are incomplete or simply lacking.

Recent studies of steroid NR trafficking in living cells have dramatically advanced our understanding of this process, forcing a paradigm shift in the field. In particular, careful quantitation of GR, AR and PR dynamics at the single-molecule level have unexpectedly revealed that the dimeric forms of these receptors are intermediate states toward their biologically active, tetrameric arrangements (‘dimers of dimers’) (Presman et al. 2016, Paakinaho et al. 2017, Presman & Hager 2017, Presman et al. 2017). Further, it has been established that the regulatory complexes assemble around steroid NRs and undergo a rapid exchange in the time scale of seconds (the ‘hit-and-run’ model; Voss et al. 2011, Sung et al. 2014, Swinstead et al. 2016, Presman & Hager 2017). However, detailed information regarding the activities of these signaling complexes in normal and tumor cells is still missing. The intricate links between NR oligomeric state, affinity for ligands (hormones), binding cooperativity and recruitment to specific chromatin sites remain to be worked out (Fuentes-Prior et al. 2019).

Quaternary structure of the AR–LBD: functional implications

Several AR features have complicated elucidation of the physiologically relevant tertiary and quaternary structures of full-length AR (FL-AR) and other steroid receptors, and it is still a matter of debate for example whether the biological units are dimers or ‘dimers of dimers’ (see above and Presman et al. 2016, Nadal et al. 2017, Presman & Hager 2017, Presman et al. 2017). In addition to the conformational flexibility that is a prerequisite for the functional versatility of the individual AR modules, they are known to interact with each other to precisely regulate the NR functions in a context-dependent manner.

Further, it is worth noting that post-translational modifications (PTMs) profoundly modulate AR actions, both under physiological and pathological conditions (Faus & Haendler 2006, Gioeli & Paschal 2012, Treviño & Weigel 2013, van der Steen et al. 2013, Koryakina et al. 2014).

The intrinsically disordered nature of the long NTD and its unique interactions with the LBD (the ‘N/C contacts’) raised over the years the issue of whether FL-AR dimerizes in a parallel, ‘head-to-head’ manner or alternatively in an anti-parallel, ‘head-to-tail’ configuration (Langley et al. 1995, 1998, Schaufele et al. 2005, Centenera et al. 2008, Minges et al. 2013). On the other hand, the crystal structure of rat AR–DBD revealed a head-to-head homodimer bound to its cognate DNA (Shaffer et al. 2004), in an arrangement quite similar to that adopted by DBD dimers of GR (Luisi et al. 1991) and ER (Schwabe et al. 1993). In this conformation, the C-terminal residues of both monomers, corresponding to L627 in human AR, are located far away (about 50 Å). However, the length of the G628–E669 linker does not allow for an accurate prediction of the likely arrangement of LBD monomers in a hypothetical dimer. In fact, limited and contradictory information in support of AR–LBD dimerization had been presented over the years, with some authors even suggesting that the LBD–LBD interactions may not significantly contribute to receptor oligomerization (Schaufele et al. 2005, Centenera et al. 2008, van Royen et al. 2012, Nadal et al. 2017).

The long-standing questions of whether the AR–LBD domain dimerized and the relative arrangement of monomers have been answered with the resolution of the crystal structure of human AR–LBD bound to DHT and a peptide derived from the coregulator, ubiquitin-activating enzyme 3 (UBA3) (Nadal et al. 2017). All the AR–LBD structures previously solved by us and others had captured essentially the same conformation of monomeric AR–LBD (Matías et al. 2000, Sack et al. 2001, He et al. 2004, Estébanez-Pépiñá et al. 2005, Bohl et al. 2007, Estébanez-Perpiña et al. 2007). In striking contrast, our recent crystal structure features four independent, helically arranged LBD molecules (Nadal et al. 2017) (Fig. 2B). Two of these LBD monomers are arranged in a symmetrical ‘core dimer’, while the other two peripheral partners are associated in a less compact manner to the BF-3 sites of each of these monomers (Fig. 2B). The core AR–LBD dimer exhibits a head-to-head arrangement and displays the corresponding coactivator-binding sites (AF-2 pockets) facing opposite directions, thus able to independently interact with coregulators (Fig. 2A).
Helices 5 (H5) from both AR–LBD moieties occupy the center of the dimerization interface (Fig. 3A), which is thus topologically distinct from the ‘canonical’, H10–11-centered arrangements identified, among others, in ER homodimers and in RXR-mediated heterodimers (Fig. 3C) (Wurtz et al. 1996, Brzozowski et al. 1997, Bourguet et al. 2000, Chandra et al. 2008, 2017, Khorasanizadeh & Rastinejad 2016, Nadal et al. 2017, Fuentes-Prior et al. 2019). The H5-centered dimerization mode of human AR–LBD has been confirmed in solution and in cells (Nadal et al. 2017). This non-canonical dimeric conformation might be shared by other NRs, not only of the oxosteroid class (Fuentes-Prior et al. 2019). In fact, a topologically equivalent dimer of GR–LBD had been previously reported (Bledsoe et al. 2002) (Fig. 3B), although its biological relevance has been questioned upon careful comparison of currently available GR structures, and alternative GR dimeric conformations have been suggested (Bianchetti et al. 2018). Clearly, further experimental work is needed to fully clarify the physiologically relevant conformation(s) of the GR and other members of the oxosteroid subfamily to integrate structural, biochemical and cellular studies (Fig. 3) (Bledsoe et al. 2002, Kauppi et al. 2003, Robertson et al. 2013a,b, Presman et al. 2016, 2017, Paakinaho et al. 2017, Presman & Hager 2017, Weikum et al. 2017, Wilkinson et al. 2018).

Although specific functions have been commonly ascribed to the different NR domains (e.g., DNA and hormone binding), seminal X-ray crystallography studies of non-steroidal NRs indicate that DBD, hinge and LBD...
are more intricately interconnected than initially thought, both structurally and functionally (Chandra et al. 2008, 2013, 2017, Lou et al. 2014, reviewed in Rastinejad et al. 2015, Fuentes-Prior et al. 2019). Regarding the AR and other steroid receptors, current knowledge of their interdomain interactions derives mostly from biochemical and mutagenesis studies. A recent analysis of multidomain ER by small-angle X-ray scattering (SAXS), however, suggests an important cross-talk between DBD and LBD moieties (Huang et al. 2018). These findings, at the light of the results of Rastinejad and coworkers with non-steroidal NRs, point to important DBD-linker-LBD interactions in vivo (Chandra et al. 2008, 2013, 2017, reviewed in Fuentes-Prior et al. 2019). This is in addition to the well-known intra- or intermolecular N–C interactions (see above), which restrict the possible DBD/LBD relative orientations (Fig. 4B).

**Allosteric modulation of AR activity**

NRs are allosteric proteins *par excellence* (Kuriyan 2004, del Sol et al. 2006, McEwan 2012b, Mackinnon et al. 2014), and the AR is no exception in this regard (Estebanez-Perpiñá et al. 2007, Buzón et al. 2012, Grosdidier et al. 2012). Indeed, FL–AR functions as an allosteric switch alternating between inactive, chaperone-bound/ligand-free states and active, hormone- and coactivator-bound conformations. Ligand binding and the exchange of chaperones by coactivators are allosterically coupled, but the sequence of molecular events and detailed conformational changes associated are only partially understood (Hur et al. 2004, Estebanez-Perpiñá et al. 2005). In particular, hormone binding to the LBP has been shown to trigger allosteric remodeling of the AF-2 and BF-3 interaction surfaces. In this manner, occupancy of the LBP by ligands regulates the dynamics and stability of surfaces that recognize coregulators and is thus allosterically coupled to the recruitment of these proteins to the AR (Estebanez-Perpiñá et al. 2005, Estebanez-Perpiña et al. 2007). Albeit a recent proteomics study has identified the essentially overlapping, agonist-specific interacotmes of both AR and GR (Lempiainen et al. 2017), the detailed molecular determinants of NR binding to a large number of coregulators are far from being well understood.

Noteworthy, in addition to allosteric rearrangements elicited by hormone binding, the target DNA sequences to which NRs bind also induce important remodeling of the receptor structure (Fuentes-Prior et al. 2019). Most impressively, cognate DNA-binding sequences of
the GR have been shown to function as true allosteric modulators, which are capable of affecting conformation and regulate receptor activity (Meijsing et al. 2009, Love et al. 2017, Weikum et al. 2017, Frank et al. 2018). Thus, a change in a single base pair in the GR-binding site resulted in up to 10-fold higher affinity in the activation of a glucocorticoid response element (GRE) reporter gene in response to ligand. More recently, it has been reported that nucleotides directly flanking the core-binding site not only modulate the 3D structure of this site, but also that of the GR–DBD and even the quaternary conformation of the dimeric receptor (Schöne et al. 2016). This implies that GR activity can be modulated by both sequence composition and DNA shape to achieve fine-tuning of the GR structure and activity downstream of binding. These features are likely to be shared by other members of the oxosteroid class of NRs, at the light of the almost identical sequences of DBDs and relative conservation of interdomain hinges. This flanking effect could explain how NRs predicted to recognize similar binding motifs show distinct DNA-binding preferences in vivo. It is feasible that while two related NRs can bind to the same DNA motif, the competent conformation required for an optimal transcriptional response is only achieved through specific flanking sequences. In particular, we assume that AR–DBD binding to its cognate DNA sequences will trigger intra- and interdomain conformational changes that would affect the overall quaternary structure of the receptor, including its hormone-binding domain.

The precise molecular mechanisms underlying allosteric transitions in FL–AR, including the equilibrium between different conformational states and the impact of ligand binding to allosteric modulatory sites (e.g., AF-2, BF-3) on receptor oligomerization, remain to be worked out (Fig. 2A) (Nadal et al. 2017). In multidomain AR, both intra- and interdomain allosterity may occur simultaneously (Fernandez et al. 2017, Fernandez 2018). The first may take place within the NTD, DBD or LBD moieties, while interdomain signal transduction appears to be essential in coordinating AR functions (e.g., through the well-studied N-C contacts, but also upon as of yet uncharacterized DBD–LBD and LBD–DNA interactions). Similar considerations might apply to other steroid NRs.

**Dysregulation of steroid receptors and human disease**

Given the essential roles of steroid hormones and their cognate NRs in development, homeostasis and metabolism, it is not surprising that dysregulation of their activities is directly responsible for a number of important human conditions (Huang et al. 2010, Evans & Mangelsdorf 2014, Luo et al. 2018). For instance, the ER is a key driver of 70% of breast cancer subtypes that require estrogen hormones for progression, and different point mutations in the ER genes are linked to acquired resistance to commonly used estrogen-blocking drugs such as tamoxifen (Kojetin et al. 2008, Katzenellenbogen et al. 2018, Nasrazadani et al. 2018, Reinert et al. 2018). Regarding oxosteroid receptors, single-residue mutations in GR and MR genes have mainly been associated to loss-of-function phenotypes (Zennaro & Fernandes-Rosa 2017). However, the most conspicuous association between a nuclear receptor and human disease links the AR to several biomedical conditions, as we briefly discuss below.

**Genetic bases of drug resistance in prostate cancer**

AR is encoded by a ubiquitously expressed gene located in the X chromosome at Xq11-12 and is particularly important in prostate development and homeostasis (Lubahn et al. 1988). When dysregulated, however, AR activity is central to the onset, development and progression to metastasis of prostate cancer (PCa), the most common cancer diagnosed in males worldwide (Matias et al. 2000, Gottlieb et al. 2004, Knudsen & Penning 2010, Arora & Barbieri 2018, Centenera et al. 2018, Cioni et al. 2018, Li et al. 2018, Luo et al. 2018, Nevedomskaya et al. 2018, Paschalis et al. 2018). In addition, AR mutations are linked to disorders of male sexual differentiation and development termed androgen insensitivity syndromes (AIS) (Hughes et al. 2012, Mongan et al. 2015, Gibson et al. 2018) and to the rare adult-onset hereditary neurodegenerative disorder known as spinal and bulbar muscular atrophy (SBMA or Kennedy’s disease; OMIM #313200) (Spada et al. 1991, Batters et al. 2018, Cortes & La Spada 2018, Lieberman 2018, Pennuto & Rinaldi 2018). Finally, AR malfunction is also associated to androgenic alopecia or loss of scalp hair and skin malignancies (Ellis et al. 2001, Clocchiatti et al. 2018).

upstream enhancer of the AR are found in up to 87% of metastatic castration-resistant PCa (mCRPC) cases, compared to <2% of primary prostate cancers (Takeda et al. 2018, Viswanathan et al. 2018, Wu et al. 2018). In addition, it has been recently reported that deletion of the gene encoding the chromatin remodeler, chromatin helicase DNA-binding protein (CHD1), redistributes the AR cistrome in a manner that favors the expression of PCa-specific oncogenic pathways (Augello et al. 2019). Not surprisingly, CHD1 loss had been previously reported as one of the most common and deleterious genetic alterations in PCa (Grasso et al. 2012, Huang et al. 2012, Rodrigues et al. 2015, Zhao et al. 2017) and an early event in cancer development (Wedge et al. 2018).

Further, AR gene duplications and AR-mediated chromosomal rearrangements are common events. Among them, about half of the patients with PCa feature fusions of the AR-regulated gene, TMPRSS2, with different fragments of the ETS-related gene (ERG), a member of the ETS family of transcription factors. The presence of these TMPRSS2:ERG fusions defines the predominant molecular subtype of PCa (Yu et al. 2010, Park et al. 2014, Reig et al. 2016, Stelloo et al. 2018, Berghlund et al. 2019). The encoded ERG truncated variants are resistant to degradation and transform the AR from a factor promoting lineage-specific differentiation of the prostate to one that potentiates de-differentiation into a stem cell-like state (Yu et al. 2010). However, TMPRSS2:ERG fusions alone do not appear to be transforming (Casey et al. 2012), and the precise mechanism(s) by which they contribute to PCa initiation and/or progression are still a matter of debate. For instance, it has been reported that these fusions synergize with deletion of the tumor suppressor, PTEN, to promote prostatic intraepithelial neoplasia (PIN) (Carver et al. 2009, King et al. 2009, Casey et al. 2012). In this regard, and also connecting to the work by Augello and coworkers cited above, it has been proposed that ETS factors alter the AR cistrome to prime the prostate epithelium to respond to aberrant signals such as PTEN loss, thus ultimately resulting in prostate-specific transformation (Chen et al. 2013). Carver and coworkers also observed that two genes strongly associated with cell migration, ADAMTS1 and CXCR4, were upregulated upon ERG overexpression (Carver et al. 2009). Besides, overexpression of MMP9 and plexin B correlates with the presence of the fusion in samples of PCa patients and has been linked to migration and invasion of prostate cancer cells (Liu et al. 2017).

On the other hand, several studies have described the synthesis in vivo of alternatively spliced transcripts encoding truncated AR isoforms. The identification of these constitutively active AR variants (AR-Vs), which lack portions or the entire hormone-sensitive LBD, has added an unanticipated level of complexity to the AR signaling pathways (Dehm & Tindall 2011, Centenera et al. 2013b, Ho & Dehm 2017). Many of these truncated AR-Vs seem to support androgen-independent expression of AR-target genes and therefore androgen-independent growth of PCa cells (Dehm & Tindall 2011). However, the androgen independency of AR-Vs does not imply that expressing cells are totally independent of the presence of androgenic hormones. The (patho)physiological implications of the coexistence of full-length AR with one or several AR-Vs in the same cell are still a matter of intense debate. The likely formation of AR-Vs (hetero)dimers with FL-AR, either through DBD-mediated or N–C interactions may still result in hormone-dependent cell growth leading to PCa progression (Liu et al. 2014, Uo et al. 2017). Further, potential differences in the interactomes of the different AR-Vs in comparison with that of the full-length receptor have not been explored so far.

The AR–LBD dimerization interface is a hot spot of disease-linked mutations

Inspection of the 3D structure of the AR–LBD homodimer immediately revealed that a large number of hitherto unexplained mutations of solvent-exposed residues identified in PCa (Fig. 5A) and AIS patients cluster at the dimerization interface (Nadal et al. 2017; see also Fig. 5B). We stress that these mutations might have additional consequences for AR protein structure and function, such as impaired interaction with chaperones in the monomeric state (see above). Accordingly, the situation in vivo does not fit a simplistic dichotomy of gain-of-function mutations causing PCa, while loss-of-function variants of the AR gene are linked to AIS (Hay & McEwan 2012).

Nevertheless, there are several correlations between for example the nature of the mutant residue and disease severity, which strongly point to impaired homodimer formation as a more likely cause of the observed phenotype. This is in particular the case of repeatedly identified mutations that affect residues such as F755, N757, V758, N759, R761 and P767 (Figs 4A and 5A, C). Most notably, the mutant Y764C has been found both in PCa and AIS. Furthermore, residue F755 has been found conservatively replaced by either leucine, in some patients with partial AIS, or by another aliphatic residue, valine, in cases of complete androgen insensitivity.
The side chain of F755 makes important Van der Waals interactions with P802 from the second monomer (Fig. 4A), and modeling experiments indicate that a leucine residue at position 755 might still interact with this proline, supporting homodimer formation. However, a less bulky valine would not be able to contact P802, with concomitantly impaired dimerization. A second example of strong genotype-phenotype correlation is given by a mutant p.V758I identified in PCa patients. Again, a fully conservative replacement of an aliphatic residue leading to PCa most likely reflects the higher stability of the homodimer formed by mutant AR–LBD molecules, as the bulkier I758 side chain fills better the intermonomer space. These findings, among others, lend extraordinary support to the (patho)physiological relevance of the AR homodimer interface and suggest that this area might be targeted for pharmacological intervention (see below).

**NR3C1/GR mutations linked to either glucocorticoid resistance or hypersensitivity syndromes**

Glucocorticoids (GCs) are major regulators of many physiological functions, and thus contribute substantially to tissue homeostasis. Dysfunction of GC-mediated actions underlies two human pathologic conditions, Cushing syndrome and Addison’s disease, which are due to GC excess or deficiency, respectively. On the other hand, alterations in GR function (either due to mutations or polymorphisms of the encoding gene, NR3C1, or to other molecular changes along the GR signaling pathway) result in impaired tissue-specific sensitivity to GCs, which may manifest as GC resistance or hypersensitivity, both of which are associated with significant morbidity (Nicolaides & Charmandari 2015, Nicolaides et al. 2015a,b, Wilkinson et al. 2018).

For instance, in primary generalized GC resistance (PGGR or Chrousos syndrome, MIM #615962), NR3C1 heterozygous variants (point mutations, insertions or deletions) result in impaired GR function and thus decreased sensitivity to GCs in all organs, although with a high degree of variability among different tissues. Alterations in the GR function include dysregulation of the GC-mediated negative feedback mechanisms, which results in compensatory activation of the hypothalamic–pituitary–adrenal (HPA) axis. In turn, HPA overactivation leads to hypersecretion of ACTH and cortisol (highly variable, ranging from severe symptoms to subclinical hypercortisolism) and may result in increased production of other adrenal steroids such as androgens and mineralocorticoids. Patients affected by this syndrome are thus commonly diagnosed by clinical signs of mineralocorticoid and/or androgen excess (hypertension due to hyperaldosteronism and/or hyperandrogenic signs), rather than those associated with GC deficiency (Kino & Chrousos 2001, Kino et al. 2002, Nicolaides et al. 2010, 2015a,b, Charmandari et al. 2013, Nicolaides & Charmandari 2015, Wilkinson et al. 2018).

So far, 26 mutations have been described in the human NR3C1 gene, most of which are missense mutations and affect the GR–LBD (Vitelli et al. 2016, 2018, 2019)...
Nicolaides & Charmandari 2017) (Fig. 5C). Functional studies indicate that most NR3C1 mutations impair several steps of GC signaling including hormone recognition, GR nuclear translocation, DNA binding and interactions with the coactivator, GRIP1, which ultimately leads to reduced or absent GR-dependent transactivation. Structural studies have contributed to a better understanding of how conformational changes in the receptor cause GC resistance, although a systematic analysis of all reported mutants is lacking. Computer simulations suggest that the primary cause of GC resistance in Chrousos syndrome is an overall destabilization of the LBD, as the result of single amino acid replacements that affect LBP, AF-2 or both (Hurt et al. 2016). The possible role of impaired receptor multimerization on this syndrome, however, has not been explored so far.

In addition, it has been recently reported that NR3C1 mutations also cause another pathological condition called incidentaloma (incidentally discovered bilateral adrenal hyperplasia), with clinical features similar to those of Chrousos syndrome such as asymptomatic hypercortisolism and/or hypertension. Five novel heterozygous NR3C1 mutations that cause impaired GR signaling were identified, which represents a relatively high prevalence of 5% in the analyzed cohort. These findings suggest that the overall prevalence of NR3C1 mutations may have been previously underestimated and advise to perform GR mutation screening in patients with adrenal incidentalomas (Vitellius et al. 2016, 2018). Although most of the identified NR3C1 mutations mapped at the GR–LBD, other (p.R477S, p.R477C, p.R477H and p.Y478C) were located at the C-terminal end of the second zinc finger, implicated in the dimerization of the receptor.

By contrast, in the rare syndrome known as primary generalized glucocorticoid hypersensitivity (PGGH), patients exhibit clinical signs of metabolic syndrome without hypercortisolism, due to compensatory hypoaactivation of the HPA axis (Nicolaides & Charmandari 2017). This disease mostly correlates with activating NR3C1 polymorphisms while to date, a single patient harboring a point mutation in the NTD (p.D401H) exhibited clinical symptoms of GC hypersensitivity including obesity, type 2 diabetes and hypertension (Charmandari et al. 2008).

Further, loss-of-function mutations in the NR3C2/MR gene are responsible for renal pseudohypoaldosteronism type 1 (MIM #177735), a rare disease of mineralocorticoid resistance characterized by sodium and potassium imbalances that manifests at birth with weight loss and dehydration despite elevated plasma levels of aldosterone. Conversely, an activating MR mutation that reshapes its LBP (p.S810L) has been associated with a severe form of inherited hypertension (Kino & Chrousos 2001, Zennaro & Fernandes-Rosa 2017). Finally, PR mutations are less frequent but they have been associated to an increased risk of breast, endometrial and colon cancer (Agoulnik et al. 2004).

Impact of mutations affecting the non-canonical dimerization interface in other non-steroidal NRs

We finish this section on the linkage between dysregulation of NR activities and pathologic conditions by presenting the case of a non-steroidal NR that has also been shown to dimerize in an AR-like, non-canonical conformation, the thyroid receptor (TR; Estévez-Perpiñá et al. 2007a, Jouravel et al. 2009 and Fig. 5D). Vertebrates possess actually two TR isoforms, termed TRα (NR1A1) and TRβ (NR1A2), and which are encoded by two independent genes, THRA and THRβ, respectively. In spite of their close structural similarity (70% sequence identity), these two isoforms differ strongly both in expression profile. TRα is ubiquitously expressed, while TRβ is mainly expressed in liver, pituitary, inner ear and some brain areas (Chatonnet et al. 2013), but in particular because they regulate different sets of genes. TRs respond to iodinated biomolecules known as thyroid hormones (THs), mostly to the active form 3,5,3′-triiodo-l-thyronine (T3), which is generated in turn from the prohormone, l-thyroxine (T4) (Holzer et al. 2017).

Since THs have fundamental functions in development, growth and metabolic homeostasis, alterations in TR structure affect these functions at many different levels. This is in particular the case of generalized thyroid hormone resistance (GTHR), a syndrome characterized by elevated serum TH but impaired action of these hormones within the hypotalamic–pituitary–thyroid axis and variable tissue hyposensitivity to them (Huber et al. 2003a,b, Onigata & Szinnai 2014). GTHR is linked to mutations located in the LBD of TRβ (Weiss et al. 1993, Adams et al. 1994). Similar to the results discussed above for GR, most of these mutations result in a lower affinity for T3 or a defective interaction with TR coregulators. Whether are least some of these variants would impair dimer formation has not been experimentally confirmed. However, inspection of the 3D structure of the TRβ homodimer (Estévez-Perpiñá et al. 2007a, Jouravel et al. 2009) immediately reveals that some of the reported missense mutations actually map to the homodimer interface and are therefore likely to disrupt receptor homodimerization (Fig. 5D). Particularly interesting mutations in this regard affect

In summary, residues responsible for non-canonical homodimer formation in NRs play a critical functional role in disease.

Table 1  Mutations within the non-canonical dimerization interface are linked to various human conditions.

<table>
<thead>
<tr>
<th>Androgen receptor</th>
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<th>Estrogen receptor</th>
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Residues from the non-canonical dimerization interface of the AR–LBD were aligned to those of the corresponding domains in GR, ER and TR. Missense mutations that affect these topologically equivalent residues are given, along with the associated disease. Note in particular that several mutations affect topologically equivalent residues in AR–LBD and TR–LBD. Note also that replacement of the highly conserved residue R753 of AR–LBD, which corresponds to R611 of GR–LBD, R394 of ER–LBD and R320 of TR–LBD, causes hormone resistance in all receptors.

GCR, glucocorticoid resistance; THR, thyroid hormone resistance.

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role, leading to various pathological conditions when dimerization is disrupted. A list of mutations that are likely to interfere with non-canonical homodimer formation of some NRs and their associated conditions is given in Table 1.

Implications for the development of novel therapeutic strategies

All steroid NRs are major therapeutic targets to treat several endocrine-related diseases (Moore et al. 2006, Evans & Mangelsdorf 2014, Carroll 2016, Nasrazadani et al. 2018). Most notably, the dependence of PCa tumors on the AR protein and its cognate endogenous hormones is the basis for its pharmacological exploitation as a drug target. Indeed, inhibition of AR functions by means of ligand depletions and/or the use of AR antagonists (antiandrogens) is the first line of therapeutic intervention against PCa (Chen et al. 2008, Mohler et al. 2012, Lorente et al. 2015, Aggarwal et al. 2017, Ponnumasy et al. 2017, Narayanan et al. 2018, Nevedomskaya et al. 2018). However, recurrent resistant and incurable tumors arise as a result of inappropriately restored AR function associated with various genetic and epigenetic accidents. In particular, emergence of point mutations as a response to antiandrogen therapy is a quite common event, which may result in antiandrogens acting as agonists rather than antagonists with severe clinical consequences (Knudsen & Penning 2010, Balbas et al. 2013, Joseph et al. 2013, Schrecengost & Knudsen 2013, Lorente et al. 2015, Watson et al. 2015, Jernberg et al. 2017, Giacinti et al. 2018). The identification of constitutively active, truncated splice AR variants (AR-Vs) lacking parts or the entire LBD also poses important pharmacological challenges (Dehm & Tindall 2011, Centenera et al. 2013b, Ho & Dehm 2017, Paschalis et al. 2018). This is in addition to the cross-reactivity of therapeutic steroidal androgens with other, highly related steroid NRs (i.e., GR) in PCa patients, which derives in unwanted side effects that pose additional limits to their clinical use (Arora et al. 2013, Karamouzis et al. 2016, Narayanan et al. 2016). Altogether, there is an unmet need for the development of novel therapeutic strategies including tissue-selective AR modulators (SARMs; Estébanez-Perpiñá et al. 2007b, Dalton 2017, Narayanan et al. 2018) that may overcome the problems encountered by currently used drugs.

Most therapeutic strategies to date have focused on the development of small-molecule compounds that compete with receptor binding to natural hormones (so-called ‘LBP-focused strategies’). However, the recent advances in the structure and function of NRs discussed above have largely expanded the space for pharmacological intervention. First, we mention that AR chaperones had been proposed as potential therapeutic targets against PCa and, after initial failures, heat shock proteins, and in particular Hsp90, are currently considered viable targets to treat PCa (Eskew et al. 2011, Centenera et al. 2012, 2013a, He et al. 2013). Indeed, inhibitors of Hsp90 C-terminal domain have been recently shown to inhibit growth of various PCa cell lines without inducing the expression of other chaperones (Armstrong et al. 2016).

Novel strategies that directly target the NR include blocking receptor interactions with specific coactivators (Azad et al. 2015, Guy et al. 2015, Foley & Mitsiades 2016), directly targeting NR binding to cognate DNA sequences or interfering with DBD dimerization (Dalal et al. 2017), but also modulating ligand-independent functions (reviewed in Caboni & Lloyd 2013). Also along these lines, the AR–NTD has been explored as drug target for AR function control, in particular for management of its ligand-independent truncated variants (De Mol et al. 2016, Imamura & Sadar 2016, Kumar et al. 2016, Monaghan & McEwan 2016).

It is believed that conformational changes elicited upon ligand binding are ultimately responsible for the different activity profiles exhibited by therapeutic compounds in different cell types, similar to what has been shown for other NRs (Srinivasan et al. 2013, Nwachukwu et al. 2016). Given that these signals might propagate through the whole multidomain protein, controlling NR functions by targeting novel unexplored sites is an attractive but challenging alternative. Most importantly, apart from the classical modulators targeting the LBP site, synthetic allosteric modulators are currently under investigation, thus substantially broadening the chemical space for novel antiandrogens (Buzón et al. 2012). In this manner, the AR LBP shall no longer be seen as the only pocket suitable for pharmacological intervention, but all NR domains and their protein–protein interaction sites are envisioned as potential drug targets (Estébanez-Perpiñá et al. 2007b, Buzón et al. 2012, Nadal et al. 2017, Badders et al. 2018). In this regard, we recall that both AF-2 and BF-3 sites are druggable and are ideal candidates for off-LBP strategies (Estébanez-Perpiñá et al. 2007b, Buzón et al. 2012, Ravindranathan et al. 2013, Badders et al. 2018). Most notably, AF2-targeting compounds have been recently validated as potent androgen-sparing strategies for SBMA therapy (Badders et al. 2018). Last but not least, interfering with the non-canonical dimerization intera...
of the AR and other oxosteroid receptors (Nadal et al. 2017, Fuentes-Prior et al. 2019) offers hitherto unforeseen possibilities for the regulation of NR activities, and thus, for the development of novel pharmaceutical drugs.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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b α


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