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ent-Steroids: Novel Tools for Studies of Signaling Pathways

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Abstract

Membrane receptors are often modulated by steroids and it is necessary to distinguish the effects of steroids at these receptors from effects occurring at nuclear receptors. Additionally, it may also be mechanistically important to distinguish between direct effects caused by binding of steroids to membrane receptors and indirect effects on membrane receptor function caused by steroid perturbation of the membrane containing the receptor. In this regard, *ent*-steroids, the mirror images of naturally occurring steroids, are novel tools for distinguishing between these various actions of steroids. The review provides a background for understanding the different actions that can be expected of steroids and *ent*-steroids in biological systems, references for the preparation of *ent*-steroids, a short discussion about relevant forms of stereoisomerism and the requirements that need to be fulfilled for the interaction between two molecules to be enantioselective. The review then summarizes results of biophysical, biochemical and pharmacological studies published since 1992 in which *ent*-steroids have been used to investigate the actions of steroids in membranes and/or receptor-mediated signaling pathways.

Keywords

ent-steroid; enantioselectivity; enantiomer; diastereomer; lipid perturbation

1. Introduction

The plasma cell membrane is an extraordinarily complex platform for transmembrane cell signaling pathways. This platform is typically composed of many different types of membrane bound receptors, such as ligand-gated and voltage-gated ion channels, G-coupled protein receptors, membrane bound steroid receptors and receptor kinases, whose signaling mechanisms are the focus of study for many of the investigators at this workshop. Additionally, this platform also contains a large array of different classes of lipids and steroids (chiefly, cholesterol in mammalian cells) which determine membrane physical properties and/or become involved as second messengers in signaling pathways. Consequently, when evaluating the modulation of membrane bound receptors by steroids, it is necessary to consider both the direct actions of the steroid caused by its binding to the receptor of interest and the indirect actions of the steroid on receptor function caused by steroid alteration of the membrane environment. *A priori* there is no reason to disregard the possibility that both types of steroid-mediated effects may be occurring simultaneously.

Distinguishing between the direct and indirect effects of steroids on membrane receptor function can be difficult. One potential way to make the distinction relies on a stereochemical approach based on the fact that enantiomers (non-superimposible mirror images of optically

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active molecules –steroids and *ent*-steroids in this case) have mirror image shapes but identical physicochemical properties. Because enantiomers have mirror image shapes and receptors have well-defined and structurally maintained binding pockets, receptors generally can discriminate between ligands of different shapes. Hence, it is more probable than not, that binding of a ligand to its receptor will be enantioselective (i.e., one enantiomer will bind more effectively than the other enantiomer). By contrast, membrane lipids present a dynamic environment that does not maintain structurally well-defined binding sites for steroids. Hence, in the membrane, the physiochemical properties of the steroid, not it's absolute configuration (one enantiomer or the other), will be the dominant factor that determines the degree to which the steroid affects membrane properties. Since both enantiomers have identical physicochemical properties, their effects on membrane properties will be essentially equivalent (non-enantioselective). Therefore, the direct receptor binding and indirect membrane perturbation effects of the steroid on receptor function could potentially be distinguished by differences in the magnitude of enantioselectivity observed for each mechanism of receptor modulation.

This review provides a background for understanding the difference between steroids and *ent*-steroids, references for the preparation of *ent*-steroids, a short discussion about relevant forms of stereoisomerism and the requirements that need to be fulfilled for the interaction between two molecules to be enantioselective. The review then summarizes results of biophysical, biochemical and pharmacological studies published since 1992 in which *ent*-steroids have been used to investigate the membrane and/or receptor-mediated actions of steroids. Additional information on the chemistry and biology of steroid enantiomers can be found in a previous review [1].

2. Occurrence of steroids and *ent*-steroids

The structures of cholesterol and *ent*-cholesterol are shown in Figure 1. Cholesterol has eight chiral centers (C_3,C_8,C_9,C_{10},C_{13},C_{14},C_{17},C_{20}) in its structure and the stereochemistry at each of these chiral centers is reversed in *ent*-cholesterol. Since these two molecules are non-superimposable mirror images, they are enantiomers of each other. Only cholesterol is a naturally occurring steroid. The reason for this is likely related to the biosynthetic pathway for steroid formation. Steroids are formed by the enzymatic epoxidation and cyclization of squalene to form lanosterol, which is a precursor to cholesterol and other steroids [2]. Since the cyclization occurs under strict stereoelectronic control in the chiral environment of an enzyme active site, the squalene is folded in a manner that leads to lanosterol and never to its mirror image, *ent*-lanosterol. By analogy, this is similar to using the left hand as a mold for making left hand gloves. The left hand is never going to be a mold for making right hand gloves.

Since *ent*-steroids do not occur naturally, they must be chemically synthesized. At this time, they are not commercially available. The process we currently use for making *ent*-steroids [3] is an adaptation of methods developed in the pharmaceutical industry for the production of steroid hormones. Other methods for preparing *ent*-steroids are discussed in the earlier cited review by Biellmann. There is also a recent comprehensive review on enantioselective methods for steroid synthesis [4]. Most of these methods have not been adapted for the synthesis of *ent*-steroids, but there is no reason that they could not be applied for this purpose. For example, an enantioselective approach for making the secosteroid (−)-astrogorgiadiol [5] was recently adapted for the synthesis of *ent*-cholesterol [6].

3. Diastereomers, enantiomers and their physiochemical properties

Optically active molecules that have only one chiral center can exist only as a pair of enantiomers. However, optically active molecules with two or more chiral centers have stereoisomers that are either diastereomers or enantiomers. The maximum number of

stereoisomers that can exist for an optically active molecule is 2^n stereoisomers, where *n* equals the number of chiral centers in the molecule. Figure 2 demonstrates the difference between diastereomers and enantiomers using 17β-estradiol as an example. 17α-Estradiol is the C_{17} diastereomer of 17β-estradiol. 17α-Estradiol is also referred to as the C_{17} epimer of 17β-estradiol. The stereochemical term epimer is commonly used when only one of several chiral centers in a molecule is inverted. Because 17β-estradiol has five chiral centers, and no plane or center of symmetry, it has an enantiomer and an additional thirty diastereomers. These additional diastereomers are obtained by inverting a different chiral center or any combination of up to four of the five chiral centers. Only when all five chiral centers are inverted is the enantiomer, *ent*-17β-estradiol, obtained. In nearly all cases in the biological literature the stereoselective actions of a steroid described are diastereoselective actions because the stereochemistry at one chiral center bearing an important substituent, as in the case of 17β- and 17α-estradiol, has been inverted. Steroid actions that are enantioselective have rarely been studied because *ent*-steroids have not been readily available.

The reason for stressing the difference between the two forms of stereoisomers is that diastereomers have different physicochemical properties and enantiomers do not (see Table 1). Thus, whereas enantiomers affect membranes in the liquid state in an identical manner (discussed in Section 4), diastereomers may not. For example, cholesterol and its C_3 diastereomer, epicholesterol, orient differently in membranes to alter membrane properties in different ways [7,8]. Likewise, anesthetic steroids and their C_3 diastereomers have different mobilities in membranes [9]. Hence, in the absence of information showing that a steroid and one of its diastereomers behave in an acceptably similar manner in membranes, these stereoisomers are poorly suited for use as tools to distinguish between the direct binding and indirect membrane perturbing actions of a steroid on membrane receptor function.

4. Requirements for enantioselective interactions between molecules

Enantiomers of a molecule can be distinguished from each other by the direction in which they rotate linearly polarized light. Additionally, they can usually be distinguished by their interactions with a different optically active molecule provided that the enantiomers cannot interact with this optically active molecule in an identical manner. For example, as shown in Figure 3 for the enantiomers of ligand X with a generic receptor ABCD, one enantiomer has four favorable interactions with the receptor, whereas the other enantiomer has only two favorable interactions with the receptor. Hence, the enantiomers are distinguishable from each other by their differential interactions with the receptor. In molecular terms, these interactions generally consist of hydrogen bonds, dipole-dipole interactions, π-bond interactions, ion-pair interactions, hydrophobic interactions and steric factors. Geometrical arguments establish that a minimum of 4 interaction constraints are needed for enantiomer discrimination by another optically active molecule [10,11].

Enantioselectivity expectations for steroid-protein and steroid-lipid interactions are summarized in Table 2. Because macromolecules like enzymes, receptors, specific transporters and antibodies that interact with steroids have architecturally well-defined binding sites that are sterically and electronically complimentary to those of the steroids they bind, it is generally expected that their interactions with steroids will be enantioselective. By contrast, lipid membrane bilayers in the liquid state, although they are composed of optically active lipids, do not maintain a defined architecture because of rapid movement of the lipids. Hence, enantioselective sterol-lipid interactions for a membrane bilayer in the liquid state would not be expected.

In Table 2, membrane proteins are placed in a column where an expectation for enantioselectivity is not given since either (or both) outcome(s), depending on whether steroid

modulation is caused by direct binding to the protein and/or by membrane perturbation, can be expected. A finding of steroid enantioselectivity strongly suggests that a steroid binding site exists on the membrane protein. The failure to observe steroid enantioselectivity either means that steroid effects are mediated by membrane perturbation or that the receptor cannot discriminate between steroid enantiomers. In such a case, additional information from other types of studies (e.g., site-directed mutagenesis, protein structural data) would be needed to distinguish between the two types of steroid modulation.

5. Biophysical studies of steroid enantioselectivity in lipid monolayers, bilayers and micelles

The issue of enantioselective interactions between cholesterol and phospholipids was first addressed using phospholipid enantiomers (reviewed in [12]). No enantioselective effects were observed. Because our rationale for using steroid enantiomers to discriminate between the direct and indirect effects of steroids on membrane receptor function is critically dependent on the hypothesis that steroid enantiomers modulate membrane properties in a non-enantioselective manner, the issue has been addressed again using cholesterol enantiomers in a variety of biophysical experiments. No enantioselectivity was observed for cholesterol effects on egg sphingomyelin bilayer properties by differential scanning calorimetry, x-ray diffraction and neutral density measurments [13]. Furthermore, the phase-transition properties of a mixture of 1-stearoyl-2-oleoylphosphatidylcholine and phosphatidyl inositol 4,5 diphosphate are not altered in an enantioselective manner by cholesterol [14].

Cholesterol enantioselectivity effects on the packing of different lipid monolayers on the surface of water have also been examined. No reproducible enantioselective effects were observed. Our group initially reported enantioselectivity for the interactions of cholesterol and *ent*-cholesterol with egg sphingomyelin [15,16]. However, later results from biophysical studies with our collaborators [13] raised questions about the validity of our intial report. Subsequently, when these monolayer studies were repeated in another laboratory as part of a study of cholesterol effects on epidermal growth factor signaling, our initial results were not reproduced as no enantioselectivity was found for cholesterol-sphingomyelin interactions [17]. Most recently, the interactions of cholesterol enantiomers with dipalmitolylphosphatidylcholine enantiomers in monolayers on the surface of water were examined. Again, no enantioselectivity was observed [18].

The interactions of anesthetic steroid enantiomers with phospholipid monolayers have also been explored. No enantioselectivity was observed for the actions of the enantiomers of either pregnanolone (3α5βP) or allopregnanolone (3α5αP) on the biophysical properties of structurally diverse lipids [19]. We also measured the critical micelle concentration (cmc) of three different pairs of enantiomers of bile steroids (lithocholic acid, chenodeoxycholic acid and deoxycholic acid) and no differences in the cmc for any of the enantiomer pairs were observed [20,21]. Most recently, we found that enantiomers of 25-hydroxycholesterol, an oxysterol that influences cellular cholesterol trafficking, affected the packing of phospholipid monolayers in an identical manner [22]. Lastly, the enantioselective effect of pregnenolone sulfate (PS) on membrane capacitance has been examined and found to be non-enantioselective [23]. Overall, these results extend the enantioselectivity studies beyond studies of cholesterol-lipid interactions and support the general conclusion that steroid enantiomers perturb membranes in an essentially equivalent manner.

6. Biological studies of cholesterol, desmosterol and U18666A enantioselectivity

Table 3 lists the outcomes of enantioselectivity studies obtained with enantiomers of cholesterol, desmosterol and U18666A. Cholesterol enantioselectivity results that were published prior to 2004 are discussed in greater detail in an earlier review [12]. As is apparent from Table 3, some membrane proteins and small molecules that localize to membranes interact with cholesterol in an enantioselective manner while others do not. Clearly, these results suggest that both indirect membrane effects and direct binding effects of cholesterol are important interactions that modulate the activity of membrane proteins and other optically active biomolecules. It can be argued that those membrane bound proteins that do not discriminate between cholesterol enantiomers have non-enantioselective binding sites for cholesterol. However, the strength of this argument seems weakened when considering the observed frequency of non-enantioselective cholesterol action.

Cholesterol oxidase and ACAT1 (acyl-CoA:cholesterol acyltransferase1), enzymes that use cholesterol as a substrate are highly enantioselective [17,24,25]. Likewise the interactions of desmosterol with the liver X receptor (LXR) is highly enantioselective [26] as are the interactions of cholesterol with the cholesterol transporters ABCG5 and ABCG8 [27]. It would be surprising if enantioselectivity were not observed in these studies, since these proteins are clearly involved in direct interactions with their steroid ligands.

The nematode *Caenorhabditis elegans* cannot maintain proper growth or produce subsequent generations of viable progeny when cholesterol is replaced in their diet by *ent*-cholesterol [28]. Since cholesterol plays only a minor structural role in this nematode [29], the result may indicate that steroid hormone(s) derived from cholesterol are involved in maintaining the growth and reproduction of this nematode and that no substitutes for them can be produced from *ent*-cholesterol. In a cultured mammalian cell line where bulk membrane effects of cholesterol can be satisfied with phytosteroids, those processes which require small amount of cholesterol are not completely enantioselective [30].

The enantioselectivity of oral cholesterol absorption in hamsters was explored utilizing tracer amounts of deuterated forms of cholesterol enantiomers. Uptake of the cholesterol and *ent*-cholesterol tracers into the intestinal mucosa at 30 min was similar, but the cholesterol tracer was retained there while the *ent*-cholesterol tracer rapidly entered the systemic circulation and was returned to the gut lumen. All of the *ent*-cholesterol tracer was excreted in the stool in 3 days, whereas ~50% of the cholesterol tracer was retained in the hamsters after this time. The mechanism(s) that explain these results remains to be delineated [31].

The amphipathic steroid U18666A inhibits multiple enzymes involved in sterol biosynthesis and causes numerous pathologies. Because of the complex actions of this compound, it was proposed that apoptosis of cultured bovine lens epithelial cells was due to membrane effects of U18666A [32]. To gain further support for this hypothesis, the enantioselectivity of U18666A action was investigated. The apoptotic actions of U18666A and *ent*-U1866A were found to be equal [33]. The enantioselectivity of other actions of U18666A remain to be investigated.

The ability of peptide enantiomers that induce cholesterol-rich domain formation has also been found to be influenced in a differential manner by cholesterol enantiomers. In one case, a peptide formed cholesterol-rich domains with cholesterol, but not with *ent*-cholesterol [14].

7. Biological studies of bile steroid enantiomers

Bile steroids are endogenous steroid detergents and they have both nuclear and membrane receptors that mediate their actions. As mentioned above in Section 5, the cmc values for the enantiomers of lithocholic acid (LCA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) were found to be identical to those of their natural counterparts. These results suggest that the detergent effects of the enantiomer pairs on lipid membranes will be non-enantioselective. By contrast, when the actions of the LCA and CDCA enantiomers were examined at the human farnesoid X receptor (hFXR), the heterodimeric receptor formed by this receptor and the human retinoid X receptor (hFXR/hRXR), human vitamin D receptor (hVDR), human pregnane X receptor (hPXR) and the G-protein coupled receptor TGR5, their actions were, nearly exclusively, enantioselective [20].

As summarized in Table 4, enantioselectivity for receptor activation was highest for the preferred ligand at its cognate receptor (CDCA at hFXR, hFXR/hRXR; LDA at hVDR) with *ent*-steroids not causing significant receptor activation. Additional studies show binding of *ent*-CDCA and *ent*-LCA to hFXR does occur as both *ent*-steroids are able to displace a high affinity ligand at this receptor. Enantioselectivity for activation of hPXR, a receptor known to be activated by structurally diverse ligands [34], was not significant for LCA and in the case of CDCA activation, *ent*-CDCA was the more potent activator. Significant enantioselectivity for the activation of TGR5 was observed for both LCA and CDCA, with both the *ent*-steroids being the weaker activators. This last result may be the first report of enantioselective actions of a steroid at a G-protein coupled receptor.

The overall results from these studies conform to the expectation that receptors will generally discriminate between steroid enantiomers. The enantioselectivity appears to be greatest for the highest affinity ligands of the receptor. This is in agreement with Pfeiffer's rule which states that the degree of ligand enantioselectivity observed is directly correlated with receptor binding affinity of the natural enantiomer [35]. The finding that hPXR activation was greater for *ent*-CDCA than for CDCA is somewhat surprising, but as discussed in Section 8, it has also been observed in enantioselectivity studies of steroid binding to γ-aminobutyric acid type A receptors ($GABA_A$ receptors). This outcome appears to be more likely when either the receptor binds structurally diverse ligands or the ligand/receptor interactions are less than optimal.

The first use of an *ent*-bile steroid to answer a biological question has also been reported [21]. Bile steroids are known to decrease proliferation and induce apoptosis in colon cancer cell lines. DCA and *ent*-DCA were used to study the enantioselectivity of these actions. In two human colon cancer cell lines, the effects of the enantiomers on cell proliferation were very similar. By contrast, *ent*-DCA showed a markedly decreased ability to induce apoptosis in comparison to DCA. Studies are currently in progress to extend these enantioselectivity studies to other bile steroids and to further elaborate the mechanism of bile steroid-induced apoptosis. Because of the enantioselectivity observed thus far, it seems unlikely that detergent effects are the exclusive explanation for the apoptotic effects of bile steroids.

8. Enantioselectivity of steroid modulation of ligand-gated and voltage-gated ion channels

The reason that we first undertook the preparation of *ent*-steroids was to provide evidence for the existence of anesthetic steroid binding sites on $GABA_A$ receptors. In 1996, we reported that the actions of 3α5αP were enantioselective [36]. Whereas 3α5αP is a potent positive allosteroic modulator of $GABA_A$ receptors, *ent*-3α5αP is not (see Table 5). Until 2006, when site-directed mutagenesis studies provided direct support for the existence of these sites [37],

the enantioselective actions of 3α5αP and other steroid analogues [38–41] were the strongest evidence that these sites existed.

More recently, the enantioselectivity of androgen action at $GABA_A$ receptors has been examined. The androgens, androsterone and etiocholanolone, are weak positive allosteric modulators of $GABA_A$ receptors. Interestingly, their enantiomers are more potent and in the case of etiocholanolone, cleary more efficacious [42]. Further modifications of the unnatural enantiomers of these two *ent*-steroids led to compounds which have modulatory actions comparable to those of the most active steroid modulators [42]. Since the structure of the group at steroid postion C_{17} is the only structural feature that distinguishes these enantiomer pairs from those showing the expected enantioselectivity (steroid > *ent*-steroid), the result indicates that the C_{17} functional group is the major factor responsible for enantioselectivity of anesthetic steroid modulation of $GABA_A$ receptors. Surprisingly, site-directed mutagenesis studies suggest that etiocholanolone enantiomers bind to different sites than their steroid counterparts [43]. The reason why these receptors would have different binding sites for *ent*-steroids is not obvious and further studies are needed to identify these putative sites and determine if there are endogenous ligands for them.

The enantioselective actions of dehydroepiandrosterone sulfate (DHEAS), pregnenolone sulfate (PS) and (3α,5β)-3-hydroxypregnan-20-one sulfate, compounds that are negative allosteric modulators of $GABA_A$ receptors, have also been investigated using rat $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Only the actions of DHEAS were found to be enantioselective [44]. By contrast, a form of the *Caenorhabditis elegans* $GABA_A$ receptor displayed enantioselectivity for PS, but not DHEAS [45]. These opposite enantioselectivity results in the two different systems suggest that the selectivity of $GABA_A$ receptors for steroid enantiomers is likely a function of the specific way that a particular steroid contacts its binding site on the receptor.

The human ρ1 GABA-C receptor is another type of GABA receptor that is modulated by 3α5αP, 3α5βP and other steroids. Mutations of a single key residue in a transmembrane domain (Ile307 in domain TM2) of the receptor can have major effects on steroid modulation [46]. For example, depending on the mutation, 3α5βP inhibits channel function, potentiates channel function, or has both actions depending on 3α5βP concentration. These different actions of the steroid were not attributed to different modes of steroid binding to the receptor, but were attributed instead to steroid effects on membrane lateral pressure [46].

Steroid enantioselectivity studies are not in agreement with the conclusion that effects on membrane lateral pressure account for ρ1 GABA-C receptor modulation by steroids [47,48]. As noted in Section 5, there is no enantioselectivity for the actions of either 3α5αP or 3α5βP on membrane properties [19] so the effects of each enantiomer pair on membrane lateral pressure should be equivalent. Moreover, when the actions of the enantiomer pairs were examined at ρ1 GABA-C receptors, enantioselective steroid actions were observed. For example, enantiomers of 3α5βP had opposite actions on receptor function [47]. These enantioselectivity results suggest that steroid action in the membrane to change a membrane property, such as lateral pressure, is less likely than a direct interaction of the steroids with binding sites on ρ1 GABA-C receptors.

Other ligand-gated ion channels for which enantioselectivity of steroid allosteric modulation has been examined are the $r\alpha_4\beta_2$nAchR (rat $\alpha_4\beta_2$ neuronal nicotinic acetylcholine receptor) (enantioselectivity observed for one of two steroid modulators) [49], the $h\alpha_4\beta_2$nAchR (human $\alpha_4\beta_2$ neuronal nicotinic acetylcholine receptor) (enantioselectivity observed) [50] and the rat NMDA-type *N*-methyl-D-aspartate-type) glutamate receptor (enantioselectivity not observed) [51]. Because no enantioselectivity for PS potentiation of NMDA receptors was observed, and because it is known that PS potentiation of this receptor improves learning and memory [52],

ent-PS was tested *in vivo* for its ability to facilitate learning and memory. In two different experimental paradigms this was found to be the case [51,53].

Enantioselectivity for steroid inhibition of LVA (low voltage-gated) and HVA (high voltage-gated) calcium channels (I_{Ca}^{++}) has been observed with the steroids being more potent blockers of calcium currents than the *ent*-steroids [54–56]. It should also be noted that ligand-gated glycine and 5-hydroxytryptamine$_3$ (5-HT$_3$) channels are also modulated by steroids [57,58], but the enantioselectivity of steroid modulation has not been examined in these cases.

Finally, although not a direct effect on an ion-channel, enantioselectivity for a steroid effect on transmitter release has been examined. The σ1-like receptor has a presynaptic effect on glutamate release and whereas PS acting through this receptor enhanced glutamate release to augment NMDA receptor mediated currents, *ent*-PS did not [59].

The results summarized for steroid enantioselectivity at ion-channels cover all possible outcomes for steroid modulation: steroid > *ent*-steroid, steroid = *ent*-steroid, steroid < *ent*-steroid and opposite actions of steroid and *ent*-steroid. While lipid perturbation could explain some results in which enantioselectivity was not apparent, it is difficult to understand how the complexity of other results can be explained solely by lipid perturbation of ion channel function.

9. Steroid neuroprotection and enantioselectivity

Both 17β-estradiol and progesterone have neuroprotective actions and studies to establish the molecular basis for neuroprotection are currently of widespread interest [60–62]. Interestingly, for both steroids, neuroprotection correlates with the abilities of these steroids to reduce oxidative damage to neurons. Exactly how this occurs remains to be determined. This is an especially interesting issue for progesterone, which unlike the free radical scavenger 17β-estradiol, is not a molecule that can directly scavenge free radicals [63].

17β-Estradiol, because it is a phenolic compound, is a chemical antioxidant and this physicochemical property can contribute to its neuroprotective effects. Since 17β-estradiol and *ent*-17β-estradiol have identical physiochemical properties, the *ent*-steroid was also evaluated as a neuroprotective agent. In cell culture models of oxidative damage and in an *in vivo* stroke model, *ent*-17β-estradiol and 17β-estradiol were equally effective neuroprotectants [64,65]. Enantiomers of other compounds structurally-related to *ent*-17β-estradiol also have neuroprotective actions [66–68].

The exact mechanism(s) whereby *ent*-17β-estradiol exerts it neuroprotective actions is still being determined. However, since this *ent*-steroid antagonizes uterine growth stimulated by 17β-estradiol, and has only poor affinity for nuclear estrogen receptors, it seems unlikely that its actions at estrogen receptors adequately account for its neuroprotective actions [64]. Recent studies suggest that 17β-estradiol modulation of the ERK signaling pathway is involved in the neuroprotective actions of this steroid. Both enantiomers of 17β-estradiol were found to preserve phosphatase activity in cultured neuronal cells subjected to oxidative insults thereby attenuating the persistent phosphorylation of ERK associated with neuronal death [69]. Exactly how phosphatase activity is maintained and why these actions of 17β-estradiol are non-enantioselective needs to be addressed by future studies.

Progesterone and its metabolite 3α5αP both have neuroprotective actions in a rodent model of traumatic brain injury (TBI) [70]. Mechanistically, both progesterone signaling through nuclear progesterone receptors and 3α5αP signaling through potentiation of GABA$_A$ receptor function have been evaluated by a progesterone enantioselectivity study [71]. Like progesterone, *ent*-progesterone was found to be neuroprotective against TBI. Unlike

progesterone, the *ent*-steroid binds only weakly to the human nuclear progesterone receptor and it does not activate the receptor. Moreover, as mentioned in Section 8, *ent*-3α5αP is a very weak modulator of $GABA_A$ receptors, so if the conversion of *ent*-progesterone to *ent*-3α5αP were occurring *in vivo*, it would still not be expected that *ent*-progesterone would be as effective as progesterone in this TBI model if modulation of $GABA_A$ receptors was the key signaling pathway. Moreover, the conversion of *ent*-progesterone into *ent*-androgens which are somewhat effective positive allosteric modulators of $GABA_A$ receptors (see Section 8) seems unlikely since *ent*-progesterone has been shown to be an inhibitor of the human form of the lyase enzyme responsible for this transformation [72]. Overall, the results suggest that other non-enantioselective signaling pathways remain to be elaborated.

3α5αP was also recently found to prolong the lifespan of mice in a mouse model of human neurodegenerative Niemann Pick Type C disease. In an initial study, these actions of 3α5αP appeared to correlate with 3α5αP potention of $GABA_A$ receptor function [73]. However, when *ent*-3α5αP was subsequently evaluated in this mouse model, this *ent*-steroid prolonged lifespan as effectively as 3α5αP [74]. Since *ent*-3α5αP is not an effective potentiator of $GABA_A$ receptors, this result indicates that pathways other than $GABA_A$ receptor signaling pathways are operative. Using fibroblasts from patients with NPC disease it was recently shown that these cells were oxidatively stressed and that 3α5αP and *ent*-3α5αP equally attenuated oxidative stress in these cells [63]. It is hoped that future studies will reveal the mechanism that explains this interesting result as such studies should provide new insights into the mechanism(s) of neuroprotection by steroids.

10. Summary on steroid enantioselectivity

Our group initially became interested in steroid enantioselectivity as a way to provide evidence for the existence of anesthetic steroid binding sites on $GABA_A$ receptors. Subsequently, our collaborators have provided us with opportunities to expand our steroid enantioselectivity studies far beyond this initial question. To state the obvious, these studies of steroid enantioselectivity have proven to be very fruitful. Thus far, the expectation that steroid effects on membrane physical properties will be non-enantioselective has been fulfilled. Hence, enantioselective modulation of membrane protein function by steroids provides strong evidence for the existence of steroid binding sites on membrane proteins that respond to steroids in this manner. Surprisingly, not all *ent*-steroids are less effective modulators of protein function than their natural counterparts. In cases where *ent*-steroids have actions equal to or greater than their natural counterparts, there may be opportunities to develop *ent*-steroids as drugs. Possible advantages of *ent*-steroid drugs would include a potential lack of agonist activity at nuclear receptors and reduced interference with the modification of endogenous steroids by steroid transforming enzymes. However, even if *ent*-steroid drugs are not forthcoming, the utility of *ent*-steroids as tools to address the direct and indirect effects of steroids on membrane protein function now seems established.

This review reflects my perspective as a medical chemist/pharmacologist on the use of *ent*-steroids to study steroid modulation of particular membrane protein targets. However, Section 9 of the review indicates that *ent*-steroids can be useful tools for identifying steroid effects that are likely not mediated by the classical steroid receptors. There is already a well-established literature on the rapid non-genomic actions of steroids and the Mannheim Classification has been proposed as a way to classify mechanisms for these types of steroid effects [75–77]. It is hoped that future studies with *ent*-steroids will allow their mechanisms of action to be determined and categorized according to the Mannheim Classification.

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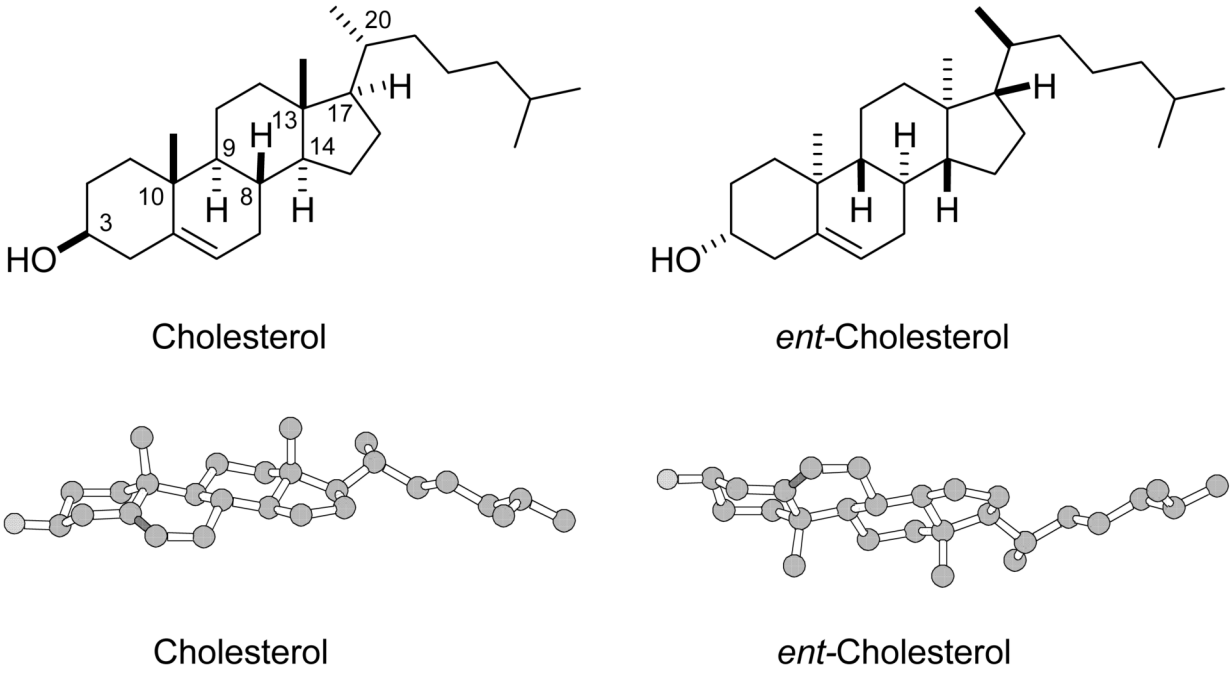

Figure 1.

The structures of cholesterol and *ent*-cholesterol in two dimensional (top line) and three dimensional (bottom line) drawings. Hydrogen atoms are not shown in the three dimensional drawings. The stereochemistry at all chiral centers (C_3,C_8,C_9,C_{10},C_{13},C_{14},C_{17} and C_{20}) is opposite in the enantiomer pair.

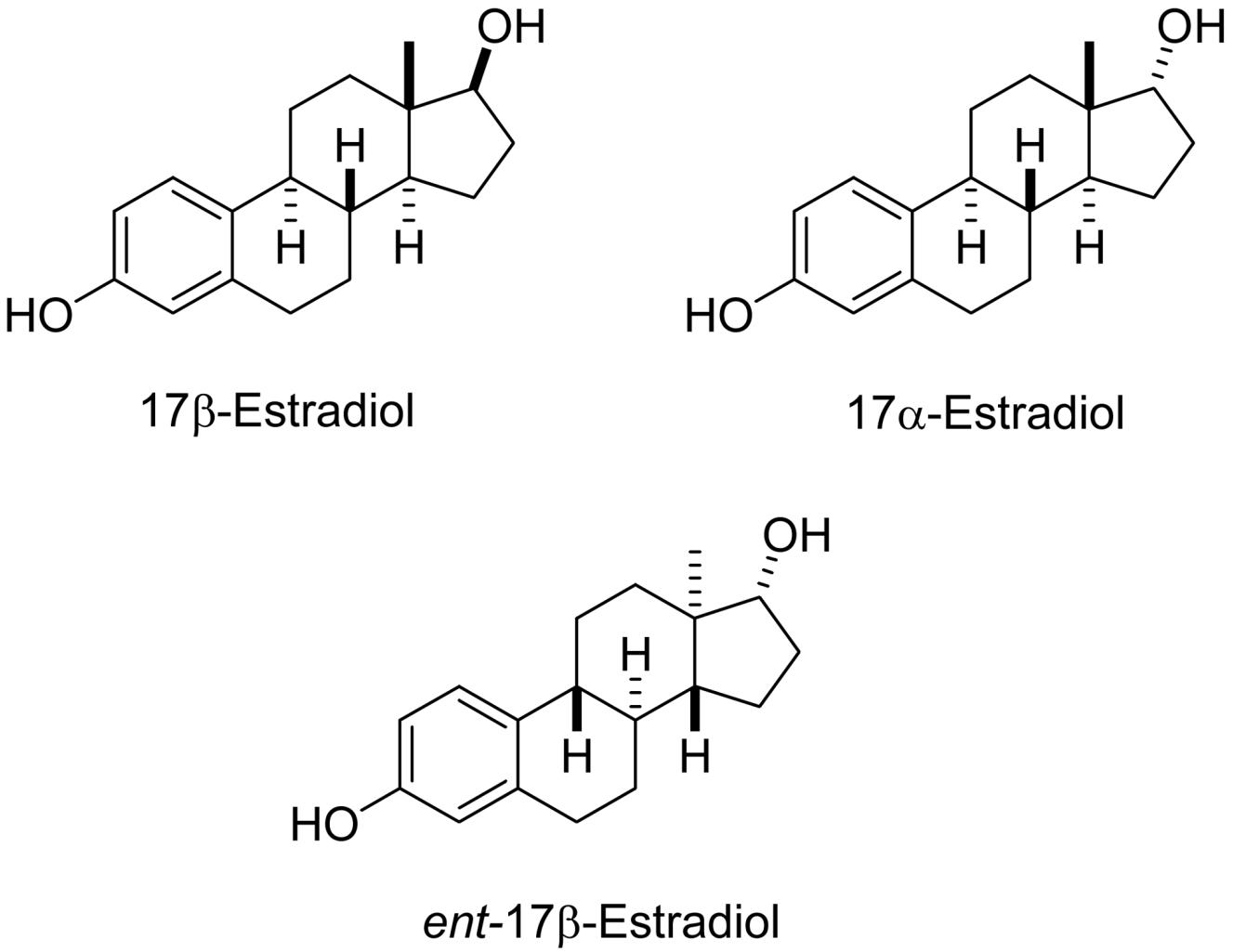

Figure 2.
An example of the difference between diastereomers and enantiomers. 17β-Estradiol and 17α-estradiol are a diastereomer pair which differ only in stereochemistry at C_{17}. 17β-Estradiol and *ent*-17β-estradiol are the enantiomer pair. All five chiral centers in the enantiomer pair have opposite stereochemistry.

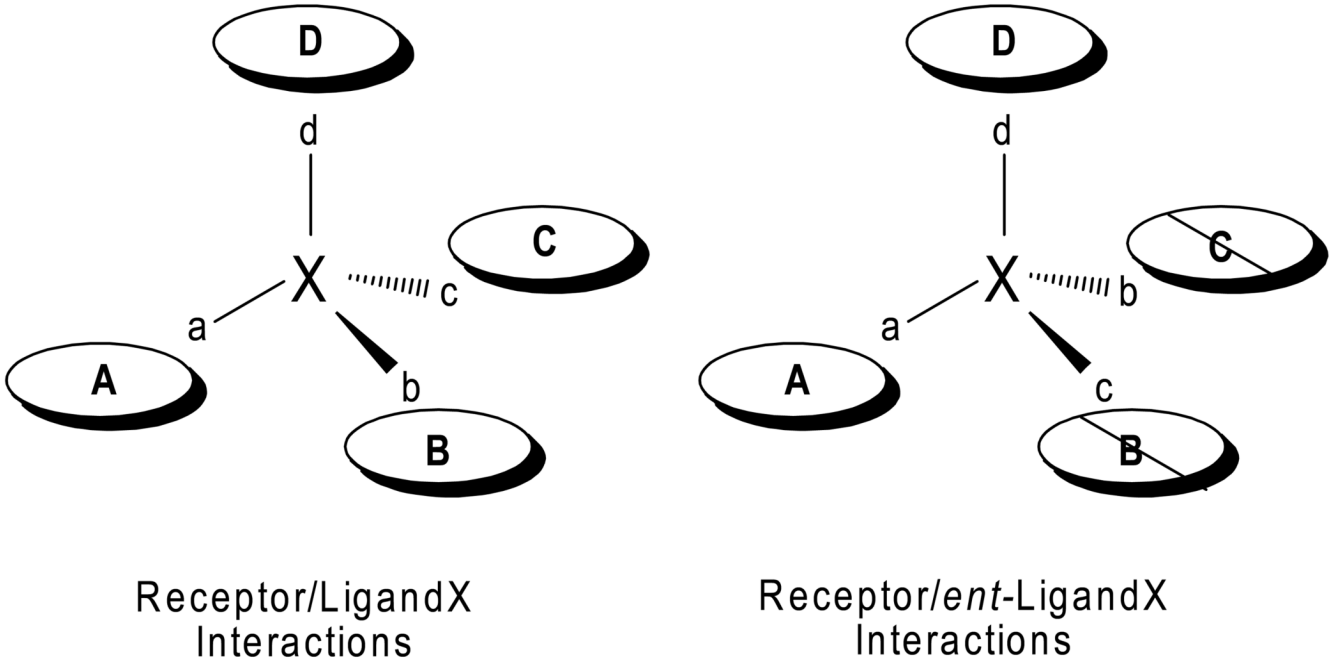

Figure 3.

Receptor discrimination between enantiomers. Molecules X and *ent*-X are an enantiomer pair containing structural elements a,b,c and d which can interact with areas A,B,C and D on receptor ABCD. The enantiomer on the left has four favorable interactions (a,A; b,B; c,C; d,D) with the receptor whereas the enantiomer on the right has two favorable interactions (a,A; d,D) and two unfavorable interactions (b,C; c,B; indicated by diagonal line through the interaction sites) thus allowing for enantiomer discrimination by the receptor.

Table 1

Identical physicochemical properties of enantiomers in a non-chiral environment

Solubility in water and other non-optically active solvents
Oil/water, octanol/water and similar partition coefficients
Melting points (provided each enantiomer is in the identical crystalline form)
Chromatographic mobility on non-chiral columns and adsorbents
Thermodynamic properties
Spectroscopic properties
Chemical reactivity with non-optically active reagents

Table 2

Expected outcomes for steroid enantioselectivity studies

None to Low	Moderate to High	Unknown
Lipid packing in monolayer and bilayer membranes	Binding to nuclear receptors	Membrane Proteins
Membrane perturbation effects on receptor function	Binding to specific transporters	
	Binding to enzymes	

Table 3

Biological studies involving cholesterol, desmosterol and U18666A enantiomers.

Biological focus	Enantioselectivity	Reference
Cholesterol		
Amphotericin B Channel Behavior	*ent* gives different response	[78] [79]
Crystalline Cholesterol Antibody	Not enantioselective	[80]
Cholesterol Oxidase Activity[a]	*ent* is very poor substrate	[17,81]
SERCA2b Activity	Not enantioselective	[82]
ACAT1 Activity	*ent* is a very poor substrate and is not an allosteric activator	[25]
Cholesterol transporters ABCG5 and ABCG8	*ent* is not efficiently transported	[83]
Lipid domain inducing peptides	D- and L-peptides affect domain formation differently with cholesterol enantiomers	[14]
Vibrio Cholera Cytolysin Pore Formation	*ent* essentially inactive	[84]
Streptolysin O Pore Formation	*ent* less effective	[84]
BAX Pore Activation	Not enantioselective	[85]
Multidrug Resistance P-glycoprotein	Enantioselective for some transported drugs	[81] [24][b]
Nicotinic Acetycholine Receptor	Not enantioselective	[86]
Epidermal Growth Factor Receptor	Not enantioselective	[17]
Caenorhabditis elegans Health & Reproduction	*ent* lethal for progeny development	[28]
Desmosterol		
LXR activation	*ent* is very poor activator	[26]
U18666A		
Bovine lens epitheal cells	Drug induced apoptosis and inhibition of sterol biosynthesis are not enantioselective	[33]

[a] This enzyme from *Rhodococcus erythropolis* has been shown to oxidize the hydroxyl groups in androsta-5,9(11)-diene-3β,17β-diol and its enantiomer with similar kinetics [87].

[b] This reference addresses enantioselective actions of DHEAS on cholesterol trafficking and esterification mediated by cholesterol transport through MDR1.

Table 4

Enantioselectivity for bile steroid-receptor activation[a]

Receptor	CDCA	*ent*-CDCA	LCA	*ent*-LDA
hFXR	+++[b]	−	+	+
hFXR/hRXR	++++	+	++	++
hVDR[c]	−	−	+++	−
hPXR	+	++	+	+
TGR5	+	−	++	−

[a] Summary of results from [20].

[b] Increasing number of plus signs indicates increasing degree of receptor activation and a minus sign indicates no receptor activation.

[c] It was previously reported that the enantiomer of 1α,25-dihydroxyvitamin D_3 had no significant affinity for VDR [88].

Table 5

Steroid enantioselectivity for modulation of ligand- and voltage-gated channels.

Channel	Compound	Enantioselectivity of Modulation	Reference
Rat $\alpha_1\beta_2\gamma_2$ $GABA_A$	3 α5αP 9 (↑)[a]	steroid ≫ *ent*-steroid	[36,38–40][b]
	3 α5βP (↑)	steroid > *ent*-steroid	[41]
	Androsterone (↑)	*ent*-steroid > steroid	[42]
	Etiocholanolone (↑)	*ent*-steroid ≫ steroid	[42]
	PS (↓)	Not enantioselective	[44]
	DHEAS (↓)	steroid > *ent*-steroid	[44]
Human ρ1 GABA-C	3 α5αP	steroid (↑) *ent*-steroid (no effect)	[47]
	3α5βP	steroid (↓) *ent*-steroid (↑)	[47]
C. elegans $GABA_A$ (UNC-49B-PS7)	PS (↓)	steroid > *ent*-steroid	[45]
	DHEAS (↓)	Not enantioselective	[45]
$h\alpha_4\beta_2$ nAchR	17β-Estradiol	No modulation by *ent*-steroid	[50]
$r\alpha_4\beta_2$ nAchR	ACN[c] (↓)	steroid > *ent*-steroid	[49]
	ECN[d] (↓)	Not enantioselective	[49]
Rat NMDA	PS (↑)	Not enantiselective	[51]
Rat HVA I_{Ca}^{++}	ACN (↓)	steroid > *ent*-steroid	[56]
Rat LVA I_{Ca}^{++}	3α5αP (↓)	steroid > *ent*-steroid	[55]
	Alphaxalone[e] (↓)	steroid > *ent*-steroid	[55]
	ACN (↓)	steroid > *ent*-steroid	[54,55]
	ECN (↓)	steroid > *ent*-steroid	[54,55]

[a] Upward pointing arrow indicates the compound potentiates the channel and downward pointing arrow indicates the compound inhibits the channel.

[b] References [38–40] also contain information on the enantioselectivity of tricyclic benz[*e*]indene analogues.

[c] ACN, (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile.

[d] ECN, (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile.

[e] Alphaxalone, (3α,5α)-3-hydroxypregnane-11,20-dione.