**Extended and Structurally Supported Insights into Extracellular Hormone Binding, Signal Transduction and Organization of the Thyrotropin Receptor**

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**Abstract**

The hormone thyrotropin (TSH) and its receptor (TSHR) are crucial for the growth and function of the thyroid gland. The TSHR is evolutionary linked with the receptors of follitropin (FSHR) and lutropin/choriogonadotropin (LHR) and their sequences and structures are similar. The extracellular region of TSHR contains more than 350 amino acids and binds hormone and antibodies. Several important questions related to functions and mechanisms of TSHR are still not comprehensively understood. One major reason for these open questions is the lack of any structural information about the extracellular segment of TSHR that connects the N-terminal leucine-rich repeat domain (LRRD) with the transmembrane helix (TMH) 1, the hinge region. It has been shown experimentally that this segment is important for fine tuning of signaling and ligand interactions. A new crystal structure containing most of the extracellular hFHSR region in complex with hFSH has recently been published. Now, we have applied these new structural insights to the homologous TSHR and have generated a structural model of the TSHR LRRD/hinge-region/TSH complex. This structural model is combined and evaluated with experimental data including hormone binding (bTSH, hTSH, thyrostimulin), super-agonistic effects, antibody interactions and signaling regulation. These studies and consideration of significant and non-significant amino acids have led to a new description of mechanisms at the TSHR, including ligand-induced displacements of specific hinge region fragments. This event triggers conformational changes at a convergent center of the LRRD and the hinge region, activating an “intramolecular agonistic unit” close to the transmembrane domain.

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**Introduction**

Previous knowledge of the extracellular TSHR and GPHR regions

The general topology of GPHR structures. The general structural topology of the homologous glycoprotein-hormone receptors (GPHRs) is identical with that of other G-protein-coupled receptors (GPCRs) and is characterized by an extracellular N-terminal region, seven transmembrane helices (TMHs) connected by three intracellular loops (ICLs) and three extracellular loops (ECLs) and with an intracellular tail. The TMHs and loops constitute the serpentine domain (SD), which spans the membrane from the extra- to the intracellular site.

A common special structural feature of all GPHRs, in contrast to other family A GPCRs (rhodopsin-like), is that they contain a large N-terminal extracellular region, comprising more than 320 amino acids (reviewed in [1]). This receptor region interacts with the hormone(s) and is the initial mediator of signal transformation (reviewed in [2]). This N-terminal extracellular region can be subdivided into: i. the N-terminal tail with the signal peptide, ii. cysteine-box 1 (C-b1) containing four interacting cysteines, which are part of iii. the leucine-rich repeat domain (LRRD) that is constrained C-terminally by disulfide bonds between two cysteines of cysteine-box 2 and two cysteines of cysteine-box 3 that is part of iv. the hinge region, which connects the LRRD and the transmembrane spanning domain (reviewed in [2]).

The endogenous hormone-ligand binds to the leucine-rich repeat domain. The major binding region for the hormones TSH, LH, CG and FSH has been identified by experimental studies on the GPHR LRRDs [3–5]. The receptor/hormone interactions and molecular differences between hormone/receptor subtypes have already been intensively studied [6–8]. Each full repeat of the LRRD comprises 20–30 amino acids. In the case of the GPHRs, each repeat contains a conserved eleven residue segment with the consensus sequence LxxLxLxx;C/xL (x = I, V, I, or F), that constitutes a β-strand at the hormone binding site. These β-strands are arranged as a β-sheet [9]. The hNogo-66 receptor ectodomain (PDB entry code: 1OZN [10]) has been used previously as an advanced structural GPHR LRRD template that predicted the GPHR LRRDs as shaped like a “scythe-blade” [11], rather than like a “horse-shoe”, as had been assumed [12]. This GPHR LRRD model based on the Nogo-66 receptor also indicated the N-terminal cysteine-box 1 of GPHRs as an integral part of the LRRD structure, representing an LRRD flanking ‘N-cap’ motif [13,14]. Furthermore, this TSHR LRRD model predicted that the folds are not stabilized by helices at the concave
site - as is known for other types of LRRDs [15] - but instead by interaction between aromatic side chains in the hydrophobic interior core designated as ‘Phe-spine’. Interestingly, the sequence (amino acid positions 261–281) following the previously assumed 9 full repeats [8,16] is composed of a typical sequence pattern of leucine-rich repeats, indicated a full tenth repeat and an eleventh β-strand on the convex surface [11]. Site-directed mutagenesis of specific residues in this region and modeling approaches supported the hypothesis of an extended LRRD through Cb-2 that interacts with Cb-3 through disulfide bridges [11,17]. In summary, these extracellular components were predicted to be arranged in close spatial proximity, in accordance with functional findings [18–20].

The first crystal structure of the FSHR LRRD (published in 2005, PDB entry code: 1XWD) confirmed the predicted “scythe-blade” shape and the absence of helical secondary structural elements [21]. The N-terminal site of the LRRD was characterized by an anti-parallel β-strand and participation of Cb-1 amino acids. The C-terminus of the solved LRRD structure terminates with the tenth β-strand of an incomplete repeat-loop around amino acid position 255 (TSHR numbering with signal peptide) (reviewed in [2]). Moreover, the structural features of 9 LRRD repeats were subsequently confirmed by crystal structure complexes between the TSHR LRRD and an activating [22] (PDB entry code: 3G04) or inactivating [23] autoantibody (PDB entry code: 2XWT), respectively.

The general spatial orientation of the hormone bound at the LRRD was also described by the complexed FSHR LRRD/FSH X-ray structure. FSH interfaces with particular β-strands from 2–9 at the concave inner surface of the LRRD [24,25]. Thyrotropin, a ligand for TSHR, probably binds in a similar mode to that of FSH to the concave site of the LRRD. A complementary pattern of amino acid side-chain properties is responsible for specific hormone recognition [7].

The hinge region as a second hormone binding site also regulates signaling properties. The hinge region is located between the LRRD and the transmembrane SD. Most of the differences between the amino acids in the three homologous GPHR subtypes TSHR, FSHR, and LHR can be found in this region [1]. The cysteines of Cb-2 (TSHR: Cys283, Cys284, Cys301) at the border between LRRD and the hinge region are linked by disulfide bonds to the cysteines of Cb-3 (TSHR: Cys390, Cys398, Cys408) close to TMH1. Indirect evidence has permitted the global assignment of disulfide bridges between these six cysteines (reviewed in [2]).

On the basis of site-directed mutagenesis at particular amino acids and insights from deletions leading to receptor activation, it has been concluded that the extracellular hinge regions of GPHRs are necessary for stabilization of a signaling-competent basal receptor conformation [19,20,26–34]. Constitutively active GPHR variants at the extracellular site were found at pathogenic TSHR positions 288–410, such as the polar and negatively charged Gln297, Gln303, Asp308 or Asp306 [17,40–53]. Note-worthy, is a sulfated tyrosine 385, which was found to have a prominent role in hormone binding to TSHR [49,50], but also in other GPHR subtypes [39,54].

Generally, it has been widely accepted that the hinge region is a receptor determinant that implements fine tuning of signaling with respect to basal signaling activity, constitutive activation and hormone induced signaling. There have been several reports of cooperative intramolecular interactions between receptor components [19,30,33,47,55–57]. Nevertheless, there have only been partial or indirect answers to two important questions: Which amino acids constitute the intramolecular interfaces between the LRRD/hinge region - serpentine domain and how are these structural parts arranged relative to each other? How does the hormone interact with the hinge region?

Implications of the new crystal structure of the extracellular FSHR region in complex with FSH

Some answers to these questions have now been provided by the recently crystalized LRRD/fragmental hinge region/FSH complex [34] (figure 1). The milestone character of this new structure is due to the addition of hinge region fragments, which were not included in the previous structure of FSHR LRR/FSH complex [21].

It is noteworthy that several aspects of hormone binding and orientation observable in the new complex were predicted 17 years earlier on the basis of a combination of functional and homologous structural information [58]. Furthermore, this FSHR crystal structure confirmed the hypothesis that the GPHR hinge region is not a self-folding domain [17]. The specific structural properties are determined by intra- and intermolecular interactions.

The new FSHR LRRD/hinge region/FSH crystal structure [54] contains trimeric receptor/ligand complexes (figure 1). The relevance of such a trimer for the natural GPHR formation should be examined in future studies, because it is well known that GPHRs form higher order complexes [47] and the extracellular region participates in oligomerization [59]. However, the previous FSHR/FSH crystal structure - with a shorter LRRD containing only 9 full repeats and without the new fragments of the hinge region [21] - has a dimeric complex which does not overlap with the recently published structure with interactions between the monomers. Experimental studies to confirm the previously observed crystal structure dimer-contacts failed to support the significance of these amino acid contacts [60]. However, this
current study will mainly focus on structural information on the monomeric TSHR/ligand complex.

Beside the predicted [11] and detected [23] structural participation of TSHR Cb-1 in the N-terminal fold of the LRRD (figure 1), this new structure revealed that the LRRD continues through specific amino acids of Cb-2, with a C-terminal end at amino acid position Asn280 (figure 1). Thus, the eleven LRRs predicted by us [17] and others [12] are indeed present in this new LRRD structure. It is to be noted that previously crystallized ligand/receptor complexes contained only nine completed repeats [21,23], implying that the complex was already stable for protein expression and crystallization. The termination of the LRRD with a cysteine-box is consistent with a general type of LRRD architecture in which a cysteine-box represents a fold-stabilizing ‘C-cap’ motif [15]. In the particular case of the GPHRs, these cysteines are linked to cysteines of Cb-3 at the C-terminal of the hinge region. In conclusion, the previous prediction of tight spatial

proximity between Cb-2 and Cb-3 [11] is also confirmed as well as the suggested cysteine-bridges [27,28,39]. However, the helical structure for positions 272–280 contains the two consecutive cysteines (Cys275, Cys276) that are bridged to the last two extracellular cysteines of Cb-3. They are located on one side (Cys346) at a short β-strand element parallel to the eleventh strand of the concave LRRD β-sheet. The C-terminus of the hinge region at Cys346 forms a β-strand parallel to the last repeat strand of the LRRD and ends with residues that are conserved among the GPHRs and that are known from studies on TSHR to be relevant to signaling [11,44].

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Figure 1. The FSHR LRRD and hinge region with bound FSH. The crystal structure of the FSHR LRRD/hinge region fragment/FSH complex is trimeric [54] (insert, boxed window). A monomeric LRRD/FSH/hinge region complex was extracted from the trimeric arrangement to visualize specific important structural and functional features. The LRRD was previously assumed to end at LRR9 with an additional tenth β-strand [21,23]. The new structure shows an additional eleventh repeat and extends the LRRD through amino acid Asn280 (green backbone). This is followed by a short helical secondary structure, comprising the first two cysteines (Cys275, Cys276, yellow lines) of cysteine box 2 (Cb-2, N-terminal hinge region). These form disulfide bridges with cysteines (Cys346, Cys356, yellow) of cysteine-box 3 (Cb-3, C-terminal hinge region, chocolate brown), in which a short β-strand is additionally assembled parallel to the eleventh strand of the concave LRRD β-sheet. The N-terminal section of the hinge region after the LRRD is crystallized till residue Ser295. The inner cysteine bridge (Cys292-Cys338) between the two remaining extracellular cysteines connects this section with the C-terminal hinge region. It was already known from mutagenesis studies that the sulfated Tyr335 (at the C-terminal hinge region that is crystallized from position Thr331-Ile339) is mandatory for hormone binding and signaling and interacts tightly with amino acids of the hormone subunits, between the β-subunit loop 2 and the α-loop 1. The side chains of the hormone constitute a binding pocket for the sulfated tyrosine; light green sticks represent aromatic residues, green sticks hydrophobic residues and orange sticks hydrophilic residues. The adjacent negatively charged Asp334 interacts directly with the positively charged Arg53 of the hormone β-subunit. The C-terminus of the hinge region at Cys346 forms a β-strand parallel to the last repeat strand of the LRRD and ends with residues that are conserved among the GPHRs and that are known from studies on TSHR to be relevant to signaling [11,44].

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Extended Insights into the Thyrotropin Receptor

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binding [39,49]. Interestingly, the homologous GPHRs in complex with their corresponding hormones (figure 2A) were suggested to have structural differences at this hormone binding-sensitive hinge region since a sulfated and binding-relevant tyrosine is common, but this is not localized at the exact position of TSHR/LHR corresponding to FSHR, as seen in figure 2B. Details of the interaction between a sulfated tyrosine and hormone are now visible in the ECD/FSH crystal structure [54] and confirm that the hinge region contains a second hormone binding site and is obligatory for conducting a signal from the extracellular site to other receptor parts [17]. The negatively charged Asp334 adjacent to Tyr335 contributes to the charge-charge hormone/receptor interactions and supports the exact adjustment of this receptor part to the hormone.

In summary, the new FSHR crystal structure of an extracellular complex between the FSHR in interaction with hFSH [54] is a milestone in GPHR research. This FSH/FSHR complex visualizes an activated state of the extracellular region. This new information supports a general subdivision of two separated binding sites at the extracellular region as follows: I. at the LRRD, II. at the hinge region around Tyr335. Binding of the hormone modifies the hinge region at specific sites, commonly at a sulfated tyrosine (TSHR Tyr385) as also shown in studies with chimeric GPHRs [39,55]. This hormone - hinge region interplay is conveyed by a currently unknown information-pathway to the serpentine domain as it is not quite clear how the still unknown inactive conformation of the extracellular region is modified by the hormone to switch into the active conformation. On the basis of the FSHR/FSH crystal structure it has been suggested that the loop harbouring the sulfated tyrosine may be lifted [54].

What can we learn from this new FSHR crystal structure for the homologous TSHR? Due to the high sequence similarity between GPHR and their respective hormones, this new FSHR structure offers a solid basis for models of the TSHR LRRD/hinge region/ligand complex. This includes the general mode of endogenous ligand binding and detailed interactions despite known differences in GPHR-subtype specific interactions [6,7,48].

Results and Discussion

On the basis of the recently published structure of the FSHR extracellular region [54] we have designed and extended our previous TSHR LRRD model with the hinge region fragments in complex with TSH, thyrostimulin, and antibodies. This model answers important questions about structural and functional relations at the TSHR: How do the endogenous TSHR ligands interact with the receptor? How does the receptor become activated?

The structural assembly of the TSHR LRRD and hinge region

The TSHR Cb-1 constitutes the N-terminus of the LRRD as an “N-cap” motif (figure 3A). The LRRD fold is stabilized by hydrophobic and aromatic interactions localized in the interior

![Figure 2. Amino acid sequences of hormone subtypes and specific receptor fragments. A) Alignment of hormone subtype amino acid sequences with signal peptides (numbering with signal peptides throughout the manuscript). The α-subunits of hTSH, bTSH, hTSH analogue (TR1401, Trophogen Inc., Rockville, MD) and thyrostimulin are shown, together with the β-subunit sequences of hFSH, hLH and hCG. Residues are colored according to their biophysical properties: blue - positively charged, red - negatively charged, green - hydrophobic, orange - hydrophilic, magenta - histidine, brown – cysteine, black – proline, light green - aromatic residues). B) Comparison of the amino acid sequences of different GPHR subtypes and LGRs. Residues are presented from the cysteine-box 2 through the transmembrane helix 1. Specificities are highlighted, including the positions of CAMs and amino acids relevant to binding. doi:10.1371/journal.pone.0052920.g002]
space between the repeats. This “Phe spine” pattern also holds true for the new extension of the LRRD structure. According to the new TSHR LRRD model, the aromatic Phe269 at LRR10 interacts with Phe206 localized at the short helix as an integral part of repeat 11 (figure 3B). In support of this, mutations to non-aromatic residues at position 206 impaired the cell-surface expression of LHR [61] and TSHR signaling capacity [53] indicating that Phe206 is of structural significance.

The new TSHR model reflects the fact that the LRRD continues through specific amino acids of Cb-2 (TSHR amino acids between Cys283-Cys301) (figure 3A,B). It is important that only the eleventh repeat contains a helical secondary structure element. Initially, it had been assumed that this was the case for each repeat at the convex side of GPHR LRRDs on the basis of crystal structures of other homologous LRRDs such as the ribonuclease A inhibitor (PDB entry code 1BNH, as reviewed in [2]). This helix contains the first two cysteines of Cb-2 (Cys283 and Cys284) that are connected by disulfide bridges to Cb-3 (C-terminal hinge region) (figure 3B). Thus, bearing in mind the covalent linkage between the LRRD-Cb-2 and Cb-3 and the close proximity of the latter to the serpentine domain, this helix might be a central element and pivot of any hinge-like movement upon hormone binding or during activation. This is in line with the fact that residues Pro280 and probably also Ser281 are involved in N-terminal stabilization of this helix and are in direct spatial proximity to amino acids Pro400–Pro407 of Cb-3 (figure 3B).

Several CAMs have been found, particularly at five positions in this region [11,44] as well as mutations at Pro280 and Ser281 [18]. These wild type amino acids are highly conserved in GPHRs and have already been suggested as determinants of an intramolecular agonistic unit [11,17]; it has now been confirmed that they are in close vicinity. Because Cb-3 is directly attached to TMH1, this agonistic unit is then localized close to the extracellular loops [11]. Interactions between these extracellular regions and the SD appear to interact cooperatively indicating tight structural interplay [19,30,46,47,53,57]. An aromatic environment has been proposed for Ser281 and Tyr279 based on the fact that mutations of Ser281 to aromatic side-chains cause only slight receptor activation [10].

The middle of the TSHR hinge region between positions 304–381 that also includes a cleavable peptide (C-peptide, positions 317–369) [63–66] is not provided in the crystal structure of the FSHR and therefore, it is missing in the TSHR model.

Figure 3. The TSHR LRRD and hinge region in complex with TSH. A-B Homology model of the extracellular TSHR region complexed with bTSH. The new LRRD (pale beige backbone ribbon) repeat LRR 11 (green) contains a helical secondary element with two consecutive cysteines (yellow) of cysteine-box 2. The hormone is visualized as a surface (α-subunit in orange, β-subunit in greenblue). The sulfated tyrosine 385 (TSHR number, red stick) [49] location is comparable to that in the FSHR/FSH complex interacting with the hormone in a pocket constituted by hydrophobic (orange sticks), aromatic (light green sticks) and hydrophobic residues (green sticks). In contrast to FSH and human TSH, bovine TSH contains additional positively charged residues at the α-subunit (blue surface regions in A) and blue sticks at B) which are known to increase hormone binding properties compared to hormone variants without those positive charges at the corresponding positions [70]. Our TSHR model suggests that these additional positively charged residues at the α-subunit interact with negatively charged asparagines and glutamates (red sticks) in the TSHR hinge region (Asp382, Glu303) that have been shown experimentally to interplay with positively charged hormone side chains of bTSH [51,52]. These interactions are responsible for the super-agonistic effects of bTSH compared to hTSH and are perfectly matched in this TSHR/TSH model based on the FSHR/FSH complex. Met300 and Leu299 (hydrophobic, green surface and sticks) and the negatively charged Glu297 (red stick in B) are already known to be sensitive to TSHR activation [18]. Moreover, Cb-2 and C-b3 interact tightly. Amino acids at the N- and C-terminal sites (magenta) close to TMH1 (the serpentine domain) have been shown by mutation studies to activate TSHR constitutively. In Cb-2 (A), Pro280 and Ser281 at the end of helical repeat 11 region are in direct spatial proximity to amino acids Pro400–Pro407 of Cb-3. One difference between the β-subunits of bTSH and hTSH is not directly explained by the current TSHR/TSH model. The positively charged βArg89 of bTSH is absent in hTSH and substitution of this residue into hTSH leads to greatly enhanced binding of hTSH to TSHR [70,71]. The TSHR monomer model reveals that this residue is located at the β-L3 loop and is not involved in any intra- or intermolecular interaction to the targeted receptor.
Ligand binding at the extracellular TSHR region

The hormone interacts at two main receptor sites: I. at the LRRD, II. at the hinge region (figures 1 and 3A). Hormone binding at the LRRD as site I has been characterized in detail by several experimental studies [6,7,59]. The sulfated tyrosine 385 at hormone binding site II in the C-terminal hinge region is a key player [49,50] as well as the negatively charged Asp306. The latter probably imitates the interaction of Asp334 in FSHR since Asp306 and not His384 (corresponding position to FSHR Asp334) has been found to be sensitive to hormone binding [53]. Therefore, in our TSH model the backbone of the sulfated tyrosine vicinity is slightly different to the FSHR structure which is in accordance to experimental findings [62]. Just as in the FSHR/FSH complex, the sulfated Tyr385 interacts with TSH in a pocket between the α- and β-subunits, which has a nearly identical composition of hydrophilic, aromatic and hydrophobic residues contributed from TSH loops α-L1 and β-L2 (figures 1 and 3).

Interestingly, a potential further endogenous ligand for TSHR is thyrostimulin [63-66], the ancestral hormone for glycoproteins, which is 4-fold more potent in stimulating cAMP than rhTSH [67] (sequence in figure 2A). On the assumption that the general orientation of the heterodimeric thyrostimulin at the LRRD is as suggested for hTSH we generated a model with bound thyrostimulin (figure 4A), which should also interact with tyrosine 385 in the C-terminal hinge region. However, in contrast to the sulfated tyrosine binding site of TSH the α-subunit GPA2 of thyrostimulin contains a positively charged residue (Arg44) instead of two uncharged hydrophilic residues from the hormone α-subunit (Gln and Asn from α-L1 loop). Moreover, a second positively charged residue, Lys69 from the β-L2 loop (shorter than in TSH), participates in this binding mechanism (figures 3B and 4A) and might influence binding properties.

Furthermore, for the TSHR crystal structures of the LRRD are available with either an activating [23] or an inactivating [22] autoantibody (AB). Both the activating antibody M22 and the inactivating antibody K1–70 are bound at the concave site of the LRRD like the hormone. The binding site of the activating AB M22 is displaced to the C-terminal site of the LRRD compared to the inactivating AB K1–70 (figure 4B). If we incorporate these ABs in our current TSHR LRRD/hinge region model, it is observed that the bulky body of M22 is in very tight spatial proximity to residues in Cb-3 which is sensitive for activation and also M22 might directly interact with more residues at the extended LRRD than previously assumed (figure 4B). The inactivating antibody K1–70 does not establish any contact to amino acids in the hinge region in our model.

It has to be noted here that the orientation of the hinge region fragments that participate in hormone binding was identified on the basis of an active/bound FSHR LRRD/hormone complex and might be in different locations in TSHR/antibody-complexes. However, neither the sulfated Tyr385 nor any negatively charged receptor amino acid interacts with the antibody as suggested for the hormone (figure 4B). Tyr385 should probably contact the surface of M22, but is not embedded tightly in a specific pocket. The lack of significance of Tyr385 for binding an activating antibody is in accordance with experimental findings [49]. In conclusion, these activating and inactivating antibodies contact the LRRD in an area which largely overlaps with hormone binding, but the second binding site at the hinge region seems to be specific to hormone binding. Therefore, the molecular activation mechanisms might be different in detail for M22 and TSH. The bulkier antibody should achieve activating effects by steric displacement of the hinge region fragment and directly by contact and modification of signaling sensitive hinge region fragments.

Moreover, further TSHR binding sensitive residues have been previously identified by experiments on the N-terminal hinge region including Leu299 and Met300 or the negatively charged Glu297 [52,53,68]. In the current TSHR/TSH model they interact with complementary (hydrophilic or positively charged) amino acids in the hormone (details in figure 3B).

Furthermore, additional signaling or binding relevant amino acids at the C-terminus of the hinge region have been suggested based on mutagenesis studies [52,53,68] such as Ghu394 and Asp393. We speculate here in accordance to a recent experimental report [69] that those residues (also Gly294 at Cb-2 [53]) participate in signal transduction by intramolecular contacts rather than by direct intermolecular interactions. Mutations at these positions might induce structural changes and thus modify the arrangement of binding relevant amino acids in close proximity.

Bovine TSH contains positively charged residues at the α-subunit (figure 2A), which are known to increase hormone binding properties compared to hormone variants without these positive charges at corresponding positions such as hFSH and hTSH [70]. There is experimental evidence that these residues interact with the positively charged hormone side chains of bTSH [51,52] and they are responsible for super-agonistic effects mediated by bTSH compared to hTSH (figure 2A). These complementary charge-charge interactions were already suggested in 2009 [review [2]] by combining functional insights with previous structural implications and have now been substantiated. Indeed, our current (FSHR crystal structure based) TSHR model suggests complementary interactions between these four side chains and the negatively charged side-chains of Asp382 and Ghu303 at the N- and C-terminal hinge region.

However, one difference between the β-subunits of bTSH and hTSH cannot be directly explained by the current monomeric TSHR/TSH model. The positively charged Arg89 of the bTSH β-subunit is absent in hTSH and substitution of this residue into hTSH greatly enhances the binding properties of hTSH [70,71]. The structural model (figure 3) reveals that this residue at the β-L3 loop is located opposite to the second binding region and is not involved in any intra- or intermolecular interaction at this monomeric model. Therefore, the functional effect of this substitution is not explained by a one receptor - one hormone molecule constellation and might point to a functional impact in an oligomeric receptor scenario. This would be in accordance with recent findings at the TSHR, where the receptor-hormone stoichiometry was found not to be symmetric in an oligomeric constellation [47,72]. Furthermore, it might be speculated that this positively charged residue is a determinant for extracellular trans-activation [59]. This means that not the targeted receptor but a second TSH receptor molecule in close proximity becomes activated by this hormone contact [39].

An extended and refined scenario of TSHR activation

We conclude that the new structural information is in good accordance with the experimental data for the TSHR - at least for the monomeric complex. The newly derived TSHR model is also applicable to further aspects of signaling such as antibody binding and action. We summarize our insights for the TSHR as follows:

The bound hormone is fixed at the TSHR LRRD (extended through Asn288) and employs a lever-like mechanism to displace the hinge region by interacting specifically with amino acids at the N- and C-terminal hinge region in spatial proximity to the sulfated Tyr385 (figures 3 and 5). In the case of bTSH, but not hTSH, additional charge-charge interactions are of importance for binding at the second receptor binding site.
Upon hormone binding, this spatial displacement triggers conformational changes at the LRRD and the N- and C-terminus of the hinge region including the pivotal helix of the eleventh repeat and twelfth b-strand at Cb-3. A second signal processing path via rigid body movement of the LRRD cannot be excluded. In either case, the extracellular modifications converge at the pivotal Cb-2 helix. Conformational changes at this helix and structural regions in direct vicinity activate the intramolecular agonistic unit close to TMH1. In this context, it is striking that with the exception of the disulfide-bridge Cys229-Cys338 (figure 1) no side chain or backbone interaction can be observed in the FSHR crystal structure that stabilizes the hairpin-like fold of the hinge region between the remaining residues of Cb-2 and Cb-3. One can speculate that this indicates conformational movability of this region in support of the suggested scenario. In accordance with this, it is exactly this region between positively charged residues K290-K293 at the N-terminal hinge region Cb-2 in TSHR that is accessible for tryptic clipping [40] (figure 3). Tryptic clipping also partially activates the TSHR [73]. The hypothesis that displacement of the hinge region is relevant to signaling is further supported for the TSHR by constitutive activation caused by deletions of extracellular regions (e.g. the fragment between positions 371–384 (figure 5) [30]; the corresponding region is not resolved in the FSHR structure. Such constitutive activation has also been reported for the FSHR [26]. This hinge region fragment either indirectly masks or might even be located in direct proximity to the agonistic unit (figure 3 and 5) and thus participates in restraining the basal conformation of the receptor.

Moreover, so far it is not understood how the TSHR specific insertion of around 50 amino acids (C-peptide) in the middle of the hinge region [63–68] influences the function of the described hinge region segments that are relevant for signaling. One could speculate that its flexibility and functionality might be impeded in a non-cleaved stage by the intact C-peptide and is increased in the cleaved stage. In this regard, it was postulated in a recent study that the occurrence of TSHR cleavage might be dependent on cell–cell contacts since an almost complete cleavage was observed in confluent cells (like in thyroid tissue) while in sparse cells (cultured thyrocytes and non-thyroid cells) most of the TSHR was in an uncleaved form [74]. Furthermore, activation of Gq was found to be reduced in non-cleaved TSHR (cultured cells) [74]. This finding would reveal that presence or absence of cleavage is of potential relevance for simultaneous induction of both Gs and Gq-mediated signaling pathways. Interestingly, the capacity for dual signaling of TSHR was also found to be dependent on the receptor-hormone stoichiometry in an oligomeric constellation [72]. Two TSH molecules bound at a TSHR homodimer are necessary to activate both effectors Gs (cAMP) and Gq (IP3) while activation of a TSHR dimer by just one TSH molecule only induces Gs signaling. However, concrete dependencies between the phenomena of cleavage, oligomerization, hormone binding and dual signaling capacity associated with activation mechanisms...
at the hinge region are not yet provided by direct experimental data, but shall be an aspect of future studies.

Anyhow, the activation-sensitive key participants in the agonistic unit (as so far investigated and recognized) are Pro280 and Ser281 at Cb-2, and Pro400, Asp403, Glu404, Asn406, Pro407 at Cb-3 and are located in close spatial proximity to each other. They are linked by disulfide bridges (figure 3 and 5). This unit is adjacent to TMH1 and is probably located between the ECLs as an interface between the extracellular and transmembrane regions. The transition between the extracellular region and TMH1 is made up of negatively charged amino acids, in which side chain substitutions lead to impaired signaling capacity. These are key to interactions with the serpentine domain spanning the membrane. Several residues identified as sensitive to hormone binding are enclosed in a red box (Glu297, Glu303, Asp382 and Asp386) and represent a spatial cluster that is linked by a disulfide bridge between Cys301 and Cys390. Asp382 (and Glu303) are important for super-agonistic effects of bTSH but are not important for hTSH. Sulfation at Tyr385 is obligatory for hormone binding and is accompanied by negatively charged amino acids close to Tyr385 (Asp386, Asp382). Two cleavage sites define the so-called cleavable peptide (C-peptide, 50 amino acids). Deletions at the transition between the C-peptide and the Cb-3 are reported to activate the TSHR (and also the FSHR) constitutively (magenta trapezoid). In contrast, single point mutations lead to partial inactivation of TSHR signaling at this region. The dashed arrows indicate the potential signal processing path for activation upon ligand binding from the hinge region and/or potentially via the LR. In either case, the extracellular modifications converge at the pivotal helix that links the intramolecular agonistic unit together (dashed box).

Figure 5. Scheme of initial TSHR activation. New functional-structural TSHR features at the extracellular site are described for the hinge region and the LDRD in a schematic overview. Repeat 11 with the pivotal helix (green) is part of the LDRD that ends at Asn288 and therefore includes Cys283 and Cys284 of cysteine-box 2 (Cb-2). These cysteines are linked by disulfide bridges to Cys398 and Cys408 of cysteine-box 3, respectively. Constitutively activating mutations at Pro280 and Ser281 (magenta) influence the conformation of this helix and underline the importance of this region for receptor function. Further positions of CAMs are located at the C-terminal part of Cb-3, in close spatial proximity to Cb-2. Mutations at both fragments probably cause conformational changes at this region which induce receptor activation. Thus, these wild type amino acids constitute an activation-sensitive intramolecular agonistic unit (magenta, boxed). This unit is adjacent to TMH1 and is probably located between the ECLs as an interface between the extracellular and transmembrane regions. The transition between the extracellular region and TMH1 is made up of negatively charged amino acids, in which side chain substitutions lead to impaired signaling capacity. These are key to interactions with the serpentine domain spanning the membrane. Several residues identified as sensitive to hormone binding are enclosed in a red box (Glu297, Glu303, Asp382 and Asp386) and represent a spatial cluster that is linked by a disulfide bridge between Cys301 and Cys390. Asp382 (and Glu303) are important for super-agonistic effects of bTSH but are not important for hTSH. Sulfation at Tyr385 is obligatory for hormone binding and is accompanied by negatively charged amino acids close to Tyr385 (Asp386, Asp382). Two cleavage sites define the so-called cleavable peptide (C-peptide, 50 amino acids). Deletions at the transition between the C-peptide and the Cb-3 are reported to activate the TSHR (and also the FSHR) constitutively (magenta trapezoid). In contrast, single point mutations lead to partial inactivation of TSHR signaling at this region. The dashed arrows indicate the potential signal processing path for activation upon ligand binding from the hinge region and/or potentially via the LR. In either case, the extracellular modifications converge at the pivotal helix that links the intramolecular agonistic unit together (dashed box).

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Materials and Methods

Molecular homology modeling and bioinformatics

For the design of a hTSHR C-terminal hinge region fragment model (hTSHR positions Ser383-Ile111) the corresponding region in the recently published hFSHR crystal structure (PDB entry code: 4AY0 [54]) was used as a structural template (FSHR positions Thr331-Ile359). In addition, a template is not provided for Asp382, which is located adjacent to Ser303 in the TSHR.
Consecutively to Cb-2, the FSHR LRRD crystal structure included the N-terminal fragment of the hinge region (FSHR positions Trp291-Ser295, TSHR positions Gln289-Ser304). This portion was also added to the TSHR LRRD model and side-chains of TSHR were substituted. Disulfide bridges between cysteines of Cb-2 and Cb-3 were built as suggested by the FSHR crystal structure (figures 1 and 2).

The hormones bTSH, hTSH and thyrostimulin were modelled based on FSH (sequence alignment provided in figure 2A), whereby the mode of orientation between hormones and LRRD were maintained in the models of the complex as suggested by the FSHR/FSH complex. In consequence, the activated LRRD-hinge region-hormone complexes postulated by the FSHR LRRD/FSH structure were designed and used for comparison.

Structural modifications and homology modeling procedures were performed with Sybyl X2.0 (Tripos Inc., St. Louis, Missouri, 63144, USA). Side-chains of the hormone models were subjected to conjugate gradient minimizations (until converging at a termination gradient of 0.05 kcal/(molÅ)). The AMBER F99 force field was used. Finally, the models were minimized without constraints.

Structure images were produced using PyMOL software (DeLano WL, version 1.03, San Carlos, CA, USA).

**Author Contributions**
Conceived and designed the experiments: G. Krause AK G. Kleinau. Performed the experiments: G. Krause AK G. Kleinau. Analyzed the data: G. Krause AK G. Kleinau. Contributed reagents/materials/analysis tools: G. Krause AK G. Kleinau. Wrote the paper: G. Krause AK G. Kleinau.

**References**

1. Vassart G, Pardo L, Costagliola S (2004) A molecular dissection of the glycoprotein hormone receptors. Trends Biochem Sci 29: 119–126.
2. Kleinau G, Krause G (2009) Thyrotropin and homologous glycoprotein hormone receptors: structural and functional aspects of extracellular signaling mechanisms. Endocr Rev 30: 133–151.
3. Kern T, Schofer FI, Sprengel R (1991) Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. EMBO J 10: 1883–1890.
4. Nagayama Y, Rapoport B (1992) Role of the carboxyl-terminal half of the human thyrotropin receptor in signal transduction. Endocrinology 131: 548–552.
5. Nagayama Y, Wadhwa HL, Chazenbalk GD, Ruso D, Seto P, et al. (1991) Thyrotropin-horminizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for thyrotropin receptor function. Proc Nat Acad Sci U S A 88: 902–905.
6. Angelova K, de Jonge H, Granneman JC, Puett D, Bogerd J (2010) Functional constraints.
7. Caltabiano G, Campillo M, De Leener A, Smits G, Vassart G, et al. (2008) The Thyrotropin-luteinizing hormone/chorionic gonadotropin receptor extracellular domain of the glycoprotein hormone receptors. J Biol Chem 283: 34813–34827.
8. Jaeschke H, Neumann S, Kleinau G, Mueller H, Cajaia, et al. (2006) An aromatic environment in the vicinity of serine 281 is a structural requirement for thyrotropin receptor function. Endocrinology 147: 1753–1760.
9. Vlaeminck-Guillen V, Ho SC, Roden P, Vassart G, Costagliola S (2002) Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. Mol Endocrinol 16: 736–746.
10. Zhang M, Tong KP, Fremont V, Chen J, Narayan P, et al. (2009) The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. Endocrinology 141: 3514–3517.
11. Fan QR, Hendrickson WA (2005) Structure of human follicle-stimulating hormone in complex with its receptor. Nature 435: 269–277.
12. Sanders P, Young S, Sanders J, Kabelis K, Baker S, et al. (2011) Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. J Mol Biol 416: 81–99.
13. Sanders J, Chiaradley DY, Sanders P, Baker S, Sullivan A, et al. (2007) Crystal structure of the TSH receptor in complex with a thyroid-stimulating hormone autoantibody. Thyroid 17: 395–410.
14. Fan QR, Hendrickson WA (2007) Assembly and structural characterization of an authentic complex between human follicle stimulating hormone and a hormone-binding ectodomain of its receptor. Mol Cell Endocrinol 260–262: 73–82.
15. Kajava AV (1998) Structural diversity of leucine-rich repeat proteins. J Mol Biol 287: 519–527.
16. Bhowmick N, Huang J, Puet D, Isaacs NW, Lapthorn AJ (1996) Determination of residues important in hormone binding to the extracellular domain of the luteinizing hormone/chorionic gonadotropin receptor by site-directed mutagenesis and modeling. Mol Endocrinol 10: 1147–1159.
17. Kleinau G, Mueller S, Jaeschke H, Grzesik P, Neumann S, et al. (2011) Defining structural and functional dimensions of the extracellular thyrotropin receptor region. J Biol Chem 286: 29292–29293.
18. DeLano WL, version 1.03, San Carlos, CA, USA.
19. Swindells ML, et al. (1994) Structural principles of leucine-rich repeat (LRR) proteins. Nature 374: 183–186.
43. Kopp P, Muirhead S, Jourdain N, Gu WX, Jameson JL, et al. (1997) Congenital

42. Karges B, Gidenne S, Aumas C, Haddad F, Kelly PA, et al. (2005) Zero-length

41. Karlsson E, Rau M, Stoffel W, Rice EE, Brown CD, et al. (2002) Whole-transcript

40. Jiang X, Dreano M, Buckler DR, Cheng S, Ythier A, et al. (1995) Structural

39. Bruysters M, Verhoef-Post M, Themmen AP (2008) Asp330 and Tyr331 in the

38. Jiang X, Dreano M, Buckler DR, Cheng S, Ythier A, et al. (1995) Structural

37. Kreuchwig A, Kleinau G, Kreuchwig F, Worth CL, Krause G (2011) Research

36. Kleinau G, Brehm M, Wiedemann U, Labudde D, Leser U, et al. (2007)

35. Kleinau G, Brehm M, Wiedemann U, Labudde D, Leser U, et al. (2007)

34. Zhang ML, Sugawa H, Kosugi S, Mori T (1995) Constitutive activation of the

33. Nishihara E, Chen CR, Mizutori-Sasai Y, Ito M, Kubota S, et al. (2012)

32. Nurwakagari P, Breit A, Hess C, Salman-Livny H, Ben-Menahem D, et al.

31. Kollipara R, Fadok VA, Longaker MT, Ignatoff JA, Schroit AJ (2011) Structural

30. Mizutori Y, Chen CR, McLachlan SM, Rapoport B (2008) The thyrotropin

29. Kollipara R, Fadok VA, Longaker MT, Ignatoff JA, Schroit AJ (2011) Structural

28. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

27. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

26. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

25. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

24. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

23. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

22. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

21. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

20. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

19. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

18. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

17. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

16. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

15. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

14. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

13. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

12. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

11. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

10. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

9. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

8. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

7. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

6. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

5. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

4. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

3. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

2. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

1. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S (2001) Effects of

Extended insights into the Thyrotropin Receptor
from thyrotropin to retro-orbital tissue plasma membranes. J Biol Chem 250: 6516–6521.

77. Chazenbalk GD, Kakinuma A, Jaume JC, McLachlan SM, Rapoport B (1996) Evidence for negative cooperativity among human thyrotropin receptors overexpressed in mammalian cells. Endocrinology 137: 4586–4591.

78. Tate RL, Schwartz HI, Holmes JM, Kohn LD (1975) Thyrotropin receptors in thyroid plasma membranes. Characteristics of thyrotropin binding and solubilization of thyrotropin receptor activity by trypic digestion. J Biol Chem 250: 6509–6515.

79. Jeoung M, Lee C, Ji I, Ji TH (2007) Trans-activation, cis-activation and signal selection of gonadotropin receptors. MolCell Endocrinol 260–262: 137–143.

80. Alvarez E, Cahoreau C, Combarnous Y (2009) Comparative structure analyses of cystine knot-containing molecules with eight aminocyl ring including glycoprotein hormones (GPH) alpha and beta subunits and GPH-related A2 (GPA2) and B5 (GPB5) molecules. Reprod Biol Endocrinol 7: 90.

81. Okajima Y, Nagasaki H, Suzuki C, Suga H, Ozaki N, et al. (2008) Biochemical roles of the oligosaccharide chains in thyrostimulin, a heterodimeric hormone of glycoprotein hormone subunits alpha 2 (GPA2) and beta 5 (GPB5). Regul Pept 148: 62–67.