HHS Public Access

Author manuscript

Biochim Biophys Acta Mol Basis Dis. Author manuscript; available in PMC 2024 February 01.

Published in final edited form as:

Biochim Biophys Acta Mol Basis Dis. 2023 February ; 1869(2): 166596. doi:10.1016/j.bbadis.2022.166596.

Regulation of retinoid mediated StAR transcription and steroidogenesis in hippocampal neuronal cells: Implications for StAR in protecting Alzheimer's disease

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Abstract

Retinoids (vitamin A and its derivatives) play pivotal roles in diverse processes, ranging from homeostasis to neurodegeneration, which are also influenced by steroid hormones. The rate-limiting step in steroid biosynthesis is mediated by the steroidogenic acute regulatory (StAR) protein. In the present study, we demonstrate that retinoids enhanced StAR expression and pregnenolone biosynthesis, and these parameters were markedly augmented by activation of the PKA pathway in mouse hippocampal neuronal HT22 cells. Deletion and mutational analyses of the 5'-flanking regions of the StAR gene revealed the importance of a retinoic acid receptor

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Credit authorship contribution statement

Conceptualization and supervision, PRM and PHR; performed experiments and generated data, PRM, APR, JAP, and SK; writing and original draft preparation, PRM; review, editing, and finalization of manuscript, PRM, APR, and PHR; funding acquisition, APR and PHR. All authors have read and agreed to the final version of manuscript for publication.

Declaration of competing interest

The authors declare that there are no known competing interests that could be perceived as prejudicing the impartiality of this work.

(RAR)/retinoid X receptor (RXR)-liver X receptor (LXR) heterodimeric motif at −200/−185bp region in retinoid responsiveness. The RAR/RXR-LXR sequence motif can bind RARα and RXRα, and retinoid regulated transcription of the StAR gene was found to be influenced by the LXR pathway, representing signaling cross-talk in hippocampal neurosteroid biosynthesis. Steroidogenesis decreases during senescence due to declines in the central nervous system and the endocrine system, and results in hormone deficiencies, inferring the need for hormonal balance for healthy aging. Loss of neuronal cells, involving accumulation of amyloid beta (Aβ) and/or phosphorylated Tau within the brain, is the pathological hallmark of Alzheimer's disease (AD). HT22 cells overexpressing either mutant APP (mAPP) or mutant Tau (mTau), conditions mimetic to AD, enhanced toxicities, and resulted in attenuation of both basal and retinoid-responsive StAR and pregnenolone levels. Co-expression of StAR with either mAPP or mTau diminished cytotoxicity, and concomitantly elevated neurosteroid biosynthesis, pointing to a protective role of StAR in AD. These findings provide insights into the molecular events by which retinoid signaling upregulates StAR and steroid levels in hippocampal neuronal cells, and StAR, by rescuing mAPP and/or mTau-induced toxicities, modulates neurosteroidogenesis and restores hormonal balance, which may have important implications in protecting AD and age-related complications and diseases.

Keywords

Retinoids; RAR/RXR; StAR; hippocampal neuronal cells; mAPP; mTau; neurosteroid biosynthesis; Alzheimer's disease

1. Introduction

Vitamin A (retinol) and its derivatives, especially all-trans retinoic acid (atRA) and 9-cis RA (referred to as retinoids), possessing antioxidant properties, play pivotal roles in a spectrum of developmental, metabolic, and physiological processes [1–4]. The biological action of retinoids is principally mediated through two families of ligand-activated nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptor (RXRs), both of which have 3 subtypes (α, β, and γ), with additional isoforms due to alternative splicing [5, 6]. While both atRA and 9-cis RA trigger RARs, RXRs are uniquely activated by 9-cis RA. Both RARs and RXRs form either homo- or heterodimers and bind to a retinoid response element, called the RARE/RXRE, present in the regulatory region of target genes [5, 7]. These receptors are expressed in numerous tissues, including adrenal, gonads, brain, and skin, suggesting they respond to diverse ligands, perform distinct cellular functions, and regulate independently [8]. Accumulating evidence indicates that RAR-RXR heterodimers interact with many signaling pathways and result in a large array of combinatorial actions that transduce the pleiotropic effects of retinoids. Among different isoforms, RARα and RXRα are the functional subtypes *in vivo*, and mice lacking these isoforms result in various reproductive anomalies, including embryonic lethality and/or sterility [8, 9]. Studies have reported that systemic administration of retinoids reverses reproductive and developmental blocks and restores steroidogenesis in vitamin A deficient (VAD) mice and rats [1, 8].

The StAR protein (also known as STARD1), by facilitating the transport of intra-mitochondrial cholesterol, the substrate for all steroid hormones, regulates steroid biosynthesis, which involves endocrine, autocrine, and paracrine signaling in various tissues [4, 10–12]. At the inner mitochondrial membrane, the cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone (the first steroid), which is then metabolized to various steroid hormones by a series of tissue-specific enzymes. It is unequivocal that agents/factors that influence StAR expression also influence steroid biosynthesis, underlining the crucial involvement of StAR in the regulation of steroidogenesis. Regulation of the StAR protein is mediated by numerous signaling events, including the protein kinase A (PKA) and PKC pathways, and involves transcriptional and translational activation [11, 12]. In accordance with this, inhibition of transcription and/or translation of StAR has been reported to decrease steroid biosynthesis by ~90%, whereas remaining steroid production occurs via StAR independent signaling [10, 12–14]. Transcription of the StAR gene has been shown to be mediated by combinatorial actions of multiple cis-acting elements, including cAMP response element-binding protein/cAMP-responsive element modulator/activator protein-1 (AP1), steroidogenic factor 1, sterol regulatory element-binding protein (SREBP), and RXR-LXR, all of which can recognize directly or indirectly to DNA binding motifs located within the ~250bp upstream of the transcription start site [10, 12, 15, 16]. Noteworthy, however, no canonical RARE/RXRE sequence is identified in the 5'-flanking region of the StAR promoter; thus, retinoid-driven StAR gene transcription involves a discrete mechanism(s). We reported that retinoid signaling influences a number of genes involved in cholesterol trafficking and metabolism, including hormone-sensitive lipase, ATP-binding cassette transporter A1 (ABCA1), apolipoprotein E, and LXR, and that retinoids can reverse the declines in StAR and steroid biosynthesis in aged mouse Leydig and human epidermal keratinocytes [2, 12, 17, 18], suggesting the importance of retinoids in the restoration of steroidogenesis during aging.

Alzheimer's disease (AD) is an irreversible, age-related neurological disease and is the most common cause of dementia [19, 20]. AD is characterized by a progressive memory loss and multiple cognitive impairments that affect behavioral and social skills of a person's ability to live and/or act independently. Risk factors and etiology of AD remain elusive, however, multiple changes associated with the disease process include accumulation of amyloid beta (Aβ), Tau, and neurofibrillary tangles. AD is associated with mitochondrial dysfunction, synaptic damages and cognitive declines in learning and memory regions of the brain, including hippocampal and cortex [21–25]. Aging is predominantly influenced by hormone deficiencies, including steroids, and it is the key risk factor in the progression of AD. Therefore, preservation of hormonal homeostasis, involving hippocampal neurosteroidogenesis, is a prerequisite not only for healthy aging, but also for protection of AD. While hormonal imbalance, involving sex steroid hormones, especially androgens and estrogens, in men and women respectively, is connected with a host of pathologies that are most prevalent in geriatric populations, the relationships between sex steroids and AD pathogenesis remain elusive. It is worth noting that AD and age-related complications are linked with decreases in retinoid metabolism and signaling, and a correlation between perturbed retinoid signaling and AD pathology has been demonstrated

[26, 27]. Both RAR and RXR isoforms are expressed in diverse regions of the aged brain, especially RARα and RXRα in the hippocampus, suggesting their relevance in cognitive functions [28]. Furthermore, studies have identified downregulation of RARα signaling in AD patients, an event that is reflected by an accumulation of Aβ in VAD rats [27, 29]. A central question is whether retinoids motivate hippocampal neurosteroidogenesis, involving StAR, for alleviating AD and age-related complications and diseases. Utilizing a hippocampal neuronal HT22 cell line (that closely resembles its normal counterpart and has been frequently used in studying various brain functions) as an *in vitro* model, our data provide evidence that retinoid signaling was capable of upregulating StAR and pregnenolone biosynthesis and that StAR, by protecting mAPP- and/or mTau-induced cytotoxicity, reverses the decline in hippocampal neurosteroid biosynthesis.

2. Materials and methods

2.1. Reagents

All-trans retinoic acid (atRA), 9-cis RA, TTNPB (4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), SR11233 (4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzoic acid), T0901317 (LXR agonist), and dibutyryl-cyclic adenosine 3', 5'-monophosphate ($(Bu)_2$cAMP), were purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine 3000, OPTI-MEM, and Purelink RNA mini kit, were obtained from Invitrogen Life Technologies (Carlsbad, CA). DNAs, random hexamers, and Taqman Mastermix were obtained from Applied Biosystems (Foster City, CA). Reverse transcriptase and RNasin were from Promega Corp. (Madison, WI). Antibodies were obtained from the following sources: StAR (AbCam, Cambridge, MA), CYP11A1 (Chemicon International Inc., Temecula, CA), and β-actin (Ambion, Austin, TX).

2.2. Cells, plasmids, transfections, and luciferase assay

Mouse hippocampal neuronal HT22 cells (ATCC, Manassas, VA) were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS and antibiotics [30, 31].

The 5-flanking −254/−1bp (relation to the translation initiation codon as −1bp) region of the mouse StAR promoter was synthesized using a PCR based cloning strategy and inserted into the *Xho*I and *Hind*III sites of the pGL3 basic vector (Promega) that contains firefly luciferase as a reporter gene [2, 32]. Various StAR reporter deletion constructs, i.e. −966/−1bp, −426/−1bp, −254/−1bp, −151/−1bp, and −68/−1bp were synthesized using a PCR based cloning strategy [2, 4, 33]. The −254/−1bp StAR luciferase segment was used for generating mutations in the RAR/RXR-LXR (−200/−185bp), SREBP (−188/−179), AP1 (−187/−181bp), and specificity protein 1 (Sp1; −157/−151bp) motifs (sequences corresponding to mouse StAR promoter) were generated using the Quick-change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), as described previously [32–34]. Specifically, the oligonucleotide sequence used in mutating RAR/RXR-LXR site was 5'-CCGTGA**att**CTGCTT**gat**CTATATG-3' (sense strand in which mutated bases in bold), which was verified by restriction digestion using *Eco*RI and *Sau*3A1 [2, 4]. Both pGL3-basic vector (pGL3) and the pRL-SV40 plasmid containing the Renilla luciferase gene

driven by SV40 promoter were obtained from Promega. Expression plasmids for pCMV5-StAR (StAR), mutant pRP-mAPP (mAPP), mutant Tau pRP-mTau (mTau), pLXREx3-Luc, pCMX-RARα (RARα,), and pCMX-RXRα (RXRα) have been previously described [2, 18, 30, 31]. All plasmids were confirmed by either restriction endonuclease digestion or sequencing on a PE Biosystems 310 Genetic Analyzer (Perkin-Elmer, Boston, MA).

HT22 cells were cultured in either 6- or 12-well plates to ~70% confluence and transfection studies were carried out using Lipofectamine 3000 reagent (Invitrogen), under optimized conditions [2, 4, 30, 31]. The amount of DNA used in different transfection experiments was equalized with an empty expression vector.

For reporter gene analyses, cells were prepared to ~70% confluence and transfected using Lipofectamine 3000 reagent, within the context of −254/−1bp StAR-Luc segment, without or with StAR, RARα, RXRα, mAPP, and mTau, either alone or in combination, as specified, and where appropriate in the presence or absence of 10–20 ng pRL-SV40 vector (for determining transfection efficiency). Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay system (Promega) [14, 33, 34]. Briefly, following treatments, cells were washed with 0.01M PBS and 300μl of the reporter lysis buffer was added to cells. Cellular debris was pelleted by centrifugation at 12,000 × g for 10 min at 4°C, and the supernatant was measured for relative light units (luciferase/renilla), as described previously [2, 14, 18, 34].

2.3. Determination of pregnenolone by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine pregnenolone levels in cell culture media using a Kit from Novus Biologicals (Centennial, CO), under optimized conditions [35]. Briefly, treatments were carried out in the presence of SU-10603 and cyanoketone for blocking pregnenolone metabolism, and media from different groups were collected, diluted appropriately, and pregnenolone levels were measured and expressed (ng/mg protein). The sensitivity of E2 assay was 47 pg/ml, and coefficient of variation was below 10%. Pregnenolone assays were performed in duplicates and absorbance was read at 450nm in a Microplate Reader, as described previously [35].

2.4. Immunoblotting

Immunoblotting studies were carried out using total cellular protein [2, 4, 17, 18]. Briefly, cells were homogenized in RIPA lysis buffer (Thermo Scientific, Rockford, IL), and the supernatant was assayed for whole cell protein. Equal amounts of total protein (50–60μg) were solubilized in sample buffer and loaded onto 10–12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were electrophoretically transferred onto Immuno-Blot PVDF membranes, which were probed with primary antibodies that recognize StAR, CYP11A1, and β-actin for ~16h. Following overnight incubation, the membranes were washed with Tris buffer saline with Tween® 20 and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature (RT). The immunodetection of proteins was determined using a Super Signal Chemiluminescence Imaging Kit (Thermo Scientific), and the intensity of bands was visualized and quantified [2, 4, 18, 35].

2.5. Determination of cell survival/apoptotic assay

Cell survival was determined with Annexin V-FITC/PI apoptosis assay using Cellometer Vision CBA Image Cytometry System (Nexcelom Bioscience, Lawrence, MA), as described previously [30, 31]. In brief, HT22 cells from different transfected groups were trypsinized, collected, resuspended in 1x PBS, and counted. Cells (1.0–1.5 × 10^5 cells) were then resuspended in 40μl of Annexin V binding buffer and processed according to the manufacturer's instructions. Finally, cell pellet was resuspended in Annexin V binding buffer and counted using Cellometer [30, 31].

2.6. RNA extraction and qRT-PCR

Total RNA was extracted from different groups using Purelink RNA Mini kit, following instructions of the manufacturer (Invitrogen). Real-time PCR was performed following procedures described previously [2, 17]. PCR primers used were the following: StAR (forward), 5'-CCGGGTGGATGGGTCAA-3', StAR (reverse), 5'-CACCTCTCCCTGCTGGATGTA-3' and GAPDH (forward), 5'-GCAGTGGCAAAGTGGAGATTG-3', GAPDH (reverse), 5'-GTGAGTGGAGTCATACTGGAACATG-3'. GAPDH was used as a house-keeping gene. Quantitative PCR data were normalized against GAPDH and evaluated using the comparative Ct method [2, 17].

2.7. Determination of the PKA activity

The PKA activity was determined using the SignaTECT PKA assay system (Promega), as described previously [17, 36, 37]. HT22 cells were pretreated without or with H89 and then treated in the absence or presence of different retinoids. Cells were then washed with 0.01M cold PBS and collected in an extraction buffer with protease inhibitor cocktails. Cells were then sonicated, centrifuged at 14,000 × g for 5 min at 4°C, and the supernatant (5μl from each group) was added to 20μl of kinase reaction mixture and incubated for 5 min at RT. The reaction was stopped by adding 12μl of termination buffer and samples were processed for determining the PKA activity [17, 36].

2.8. Statistical analysis

All experiments were repeated at least three times as specified in different figure legends. Statistical analysis was performed by analysis of variance using Statview (Abacus Concepts Inc., Berkeley, CA) followed by Fisher's protected least significant differences test. T-tests were used for analyzing significance between two groups using GraphPad Prism (GraphPad Software, La Jolla, CA). Data presented are the mean ± SE, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Assessment of retinoids on StAR expression and steroidogenesis in neuronal cells

Mouse hippocampal neuronal HT22 cells treated with atRA (10μM, a ligand for RARs; [2]), in the presence of SU-10603 (20μM) and cyanoketone (5μM) for blocking pregnenolone metabolism [4], resulted in 3.4 ± 0.4, 2.8 ± 0.3, and 5.1 ± 0.9-fold increases in StAR mRNA

(**A**), StAR protein (**B & C**), and pregnenolone (**D**) levels, over untreated values, respectively (Fig. 1). Whereas a suboptimal concentration of a cAMP analog, $(Bu)_2$cAMP (0.2mM), displayed moderate effects on StAR and steroid levels, it markedly elevated ($p<0.001$) atRA induced steroidogenic responses (**A-D**). Under similar experimental conditions, basal expression of CYP11A1 protein was ~1.7-fold over the level seen with StAR (**B & C**). Further, while both atRA and $(Bu)_2$cAMP, individually, had no apparent effects, their combination showed ~2-fold increases in CYP11A1 expression when compared their responses with basal values, suggesting the chronic effect of steroidogenesis involves increased transcription/translation of the genes encoding steroidogenic enzymes. Both SU-10603 (20μM) and cyanoketone (5μM) were used for determining pregnenolone levels in all subsequent experiments.

To ascertain the effect of atRA on the steroidogenic response, cells were treated without or with atRA, 9-cis RA (10μM, [2]; ligands for both RARs and RXRs), and selective analogs with affinities to both RAR (TTNPB, 5μM; and RXR (SR11233, 5μM; [2, 18]), in the presence of SU-10603 and cyanoketone, for 24h (Fig. 2). These incubations were carried out without or with two different doses of $(Bu)_2$cAMP (0.2mM and 1.0mM). The results show that treatments with atRA, 9-cis RA, TTNPB, and SR11233 resulted in ~5, 5.6 ± 0.9, 6.2 ± 1.3, and 6.0 ± 1.1-fold increases in pregnenolone levels, over untreated controls, respectively. Addition of 0.2mM $(Bu)_2$cAMP to different retinoid incubations further elevated (2–3-fold) pregnenolone biosynthesis compared with their responses individually. Pregnenolone production was robust in response to a maximal stimulating dose of $(Bu)_2$cAMP (1.0mM), an effect in agreement with our previous findings [14, 37]. These results indicate that retinoid signaling can enhance StAR expression and hippocampal pregnenolone biosynthesis, and these responses were further increased in the presence of $(Bu)_2$cAMP. The doses of retinoids, RAR, and RXR analogs used were maintained in different experiments below unless specified.

The PKA activity was determined to understand the molecular events in retinoid and $(Bu)_2$cAMP induced steroidogenesis. It can be seen from Fig. 3 that HT22 cells pretreated with a pathway-specific inhibitor of PKA, H89 (20μM; [14, 38]), diminished basal PKA activity by 54 ± 7%, suggesting endogenous pregnenolone production involves PKA signaling. While treatments with either 9-cis RA or TTNPB, by themselves, displayed no significant effects, addition of $(Bu)_2$cAMP (0.2mM) to those incubations demonstrated 4.1 ± 0.4 and 4.4 ± 0.5-fold increases in PKA activity. $(Bu)_2$cAMP treated PKA activity was inhibited ($p<0.05$) by H89 (data not shown). Moreover, H89 inhibited ($p<0.01$) both 9-cis RA and TTNPB plus $(Bu)_2$cAMP mediated PKA activity. $(Bu)_2$cAMP (1.0mM) resulted in 7.3 ± 0.6-fold increase in PKA activity, an effect was markedly affected by H89 (Fig. 3). These results indicate that the induction of retinoid mediated StAR expression, thus, steroid synthesis, is impacted by the PKA pathway, reinforcing the importance of cAMP/PKA-dependent mechanisms in regulating steroidogenesis [14, 17].

3.2. Involvement of retinoid signaling in StAR promoter activity in HT22 cells

The 5'-flanking ~1-kb StAR promoter region, containing multiple tarns-regulating factors, plays a crucial role in StAR gene expression [2, 10, 15–17, 39]. To assess the effects of

retinoids in StAR gene transcription, HT22 cells were transfected with the −966/−1bp StAR promoter luciferase (StAR-Luc) segment for 24h, and then treated without or with atRA, 9-cis RA, TTNPB, and SR11233, in the absence or presence of $(Bu)_2$cAMP (0.2mM), for additional 24h. The results presented in Fig. 4 show that cells transfected with the −966/−1bp StAR-Luc resulted in 2.9 ± 0.4, 3.0 ± 0.3, 3.1 ± 0.5, and 3.0 ± 0.5-fold increases in StAR promoter activity in response to atRA, 9-cis RA, TTNPB, and SR11233, over untreated cells, respectively. While $(Bu)_2$cAMP, by itself, had a moderate effect ($p<0.05$), it synergistically elevated atRA, 9-cis RA, TTNPB, and SR11233 induced StAR promoter activity, over their responses seen individually. These findings indicate the importance of the −966/−1bp region in retinoid mediated StAR gene transcription in hippocampal neuronal HT22 cells.

3.3. Functional analyses of the 5'-flanking −966/−1bp region for determining retinoid responsiveness and their relevance to StAR transcription

Deletion and mutational analyses were performed to identify elements responsive to retinoids within the −966/−1bp region of the StAR promoter (Fig. 5). HT22 cells were transfected with various StAR-Luc deletion segments i.e. −966/−1bp, −426/−1bp, −254/−1bp, −151/−1bp, and −68/−1bp for 24h, and subsequently treated without or with 9-cis RA for an additional 24h. As illustrated in Fig. 5A, cells transfected with the −966/−1bp StAR-Luc resulted in a ~2.8-fold increase in reporter activity in response to 9-cis RA. Deletion of the −966/−1bp to −426/−1bp demonstrated similar basal and 9-cis RA induced responses as those of the −966/−1bp. Transfection of the −254/−1bp segment also displayed qualitatively similar responses to the −426/−1bp StAR-Luc, suggesting element(s) responsive to 9-cis RA remained within this region. Both basal and 9-cis RA induced reporter activities decreased by 62% when cells were transfected with the −151/−1bp StAR-Luc segment. Cells transfected with the −68/−1bp StAR-Luc further diminished ($p<0.05$) both basal and 9-cis RA mediated StAR promoter responsiveness, signifying the presence of retinoid response element(s) between −254/−1bp and −151/−1bp 5'-flanking region of the mouse StAR promoter.

The −254/−151bp region contains DNA recognition motifs for a RAR/RXR-LXR (−200/−185bp), a SREBP (−188/−179bp), an AP1 (−187/−181bp), and a Sp1 (−157/−151bp) transcription factors [2, 10]; thus, their relevance to retinoid mediated StAR gene transcription was evaluated by generating mutation in each of these sites. HT22 cells transfected with the −254/−1bp StAR-Luc (wild-type) showed ~2.9-fold increase in StAR reporter activity in response to 9-cis RA (Fig. 5B). Mutation in the RAR/RXR-LXR (Mut-RAR/RXR-LXR) motif decreased basal and 9-cis RA mediated StAR promoter activity by 66%. Conversely, disruption of SREPB, AP1, and Sp1 sites (Mut-SREBP, Mut-AP1, and Mut-Sp1, respectively) resulted in basal StAR propter responses between 19% and 27%, but did not affect 9-cis RA induced reporter activity. These results suggest retinoid mediated StAR gene expression is influenced by multiple trans-acting elements, in which the RAR/RXR-LXR motif plays the most predominant role.

The functional relevance of the RAR/RXR-LXR region in trans-regulation of the StAR gene was further verified in conjunction with RARα and RXRα. HT22 cells were transfected

with either pCMX (EV) or pCMX-RARα, and pCMX-RXRα, within the context of the −254/−1bp StAR-Luc with either wild-type (Wt-RAR/RXR-LXR) or mutant (Mut-RAR/RXR-LXR) segments, and then treated without or with atRA and 9-cis RA, individually. RARα and RXRα isoforms chosen were based on previous findings [2, 4, 8]. As depicted in Fig. 6, cells treated with atRA and 9-cis RA, resulted in a 2.7 and 2.9-fold increase in StAR promoter activity, over mock (EV) controls, respectively. Overexpression of either RARα or RXRα showed ~2-fold increase in basal activity, but coordinately increased ($p<0.001$) atRA/9-cis RA mediated StAR reporter responsiveness. Conversely, the −254/−1bp StAR-Luc containing Mut-RAR/RXR-LXR noticeably diminished both basal and atRA/9-cis RA induced StAR promoter activity, in which RARα and RXRα displayed no apparent effects. These results suggest that the RAR/RXR-LXR heterodimeric motif plays a central role in retinoid regulated transcription of the StAR gene in hippocampal neuronal cells.

3.4. Influence of retinoid signaling in LXR activity, and cooperation/interaction between RAR/RXR and LXR in StAR gene expression and pregnenolone biosynthesis.

Since the −200/−185bp region can recognize both RAR/RXR and LXR [2, 4, 16], it was of interest to examine the contribution of the LXR pathway in retinoid mediated StAR transcription. HT22 cells transfected with an LXRE luciferase reporter plasmid (pLXREx3-Luc) resulted in 2.9 ± 0.3, 3.1 ± 0.4, 3.3 ± 0.4, 3.2 ± 0.5-fold increases in LXR activity in response to atRA, 9-cis RA, TTNPB, and SR11233, over untreated cells, respectively (Fig. 7A). Addition of $(Bu)_2$cAMP (0.2mM), to different retinoid incubations, further enhanced ($p<0.001$) retinoid induced LXR activity, when compared with their responses individually, suggesting retinoid mediated hippocampal neurosteroidogenesis involves the LXR signaling cascade.

To understand the cooperation between RAR/RXR and LXR in retinoid induced StAR gene expression, HT22 cells were transfected with the −254/−1bp StAR-Luc, and then treated without or with suboptimal doses of TTNPB (2μM), SR11233 (2μM), T0901317 (T1317, 1μM; an LXR agonist; [2, 18]) either individually, or a combination of them, as indicated. As shown in Fig. 7B, TTNPB, SR11233, and T1317 resulted in 2.4 ± 0.4, 2.3 ± 0.3, and 2.1 ± 0.2-fold increases in StAR promoter activity, over untreated cells, respectively. Cells treated with either TTNPB or SR11233 in the presence of T1317 demonstrated an additional 2–2.5-fold increases in StAR promoter activity. Combined treatments with TTNPB, SR11233, and T1317 strongly elevated ($p<0.0001$) StAR reporter responsiveness, suggesting an involvement of RAR/RXR and LXR crosstalk signaling in retinoid mediated trans-regulation of the StAR gene.

The functional cooperation/interaction between RAR/RXR and LXR in pregnenolone biosynthesis was examined to gain insights into the mechanisms. HT22 cells were treated without or with TTNPB, SR11233, and T1317, or their combination, at doses utilized in Fig. 7B, in the presence of SU-10603 and cyanoketone. The results presented in Fig. 8 demonstrate that treatments with suboptimal doses of TTNPB, SR11233, and T1317, individually, resulted in 3.5 ± 0.2, 3.6 ± 0.4 and 3.9 ± 0.5-fold increases in pregnenolone levels, over untreated cells, respectively. Combined effects of either TTNPB plus T1317 or SR11233 plus T1317 demonstrated 8 ± 0.7 and 7.7 ± 0.5-fold increases in pregnenolone

production. In addition, incubation of TTNPB, SR11233 and T1317 together further elevated ($p<0.0001$) pregnenolone biosynthesis. These findings corroborate with the data presented in Fig. 7B, and demonstrate the cooperation/interaction of RAR/RXR and LXR in StAR gene trans-activation and results in retinoid mediated upregulation of hippocampal neurosteroid biosynthesis.

3.5. Assessment of the roles of APP and Tau in StAR gene transcription

Since accumulation of APP and/or Tau is a primary event in AD, effects of APP and Tau on StAR gene expression were evaluated. HT22 cells were transfected with either EV or mutant pRP-APP (mAPP) and mutant pRP-Tau (mTau) expression plasmids, individually, within the context of the −254/−1bp StAR-Luc for 24h. Both mAPP and mTau chosen were based on our recent findings [30, 31]. It can be seen from Fig. 9A that concentration-dependent increases in StAR promoter responsiveness were progressively affected with increasing doses (0.5 to 2.5μg) of mAPP and/or mTau cDNAs (conditions mimetic to AD). The decrease in StAR promoter activity was evident at 1μg of either mAPP or mTau ($p<0.05$), and affected further with increasing DNA concentrations, suggesting mAPP and/or mTau induced, AD-mimetic, attenuation of StAR promoter responsiveness in hippocampal neuronal cells could be due to cytotoxicity.

To determine if both RAR/RXR and LXR events are functional in mAPP and mTau overexpressing HT22 cells, StAR promoter activity, in response to both RAR/RXR and LXR agonists, was evaluated. The results presented in Fig. 9B reveal that treatments with either 9-cis RA or T1317 resulted in ~2.8-fold increase in StAR promoter activity, an effect was further enhanced ($p<0.001$) when cells were incubated with these agonists together. Conversely, cells overexpressing either mAPP or mTau decreased basal StAR promoter activity between 55% and 63%, and coordinately diminished 9-cis RA, T1317, and 9-cis RA plus T1317 induced reporter responsiveness. These findings indicate that both RAR/RXR and LXR signaling events are capable of activating StAR gene transcription in both mAPP and mTau overexpressing AD mimetic conditions.

3.6. Overexpression of StAR with either mAPP or mTau, and their correlation to cell survival

The hypothesis that an increase in StAR level reduces mAPP and mTau induced cytotoxicity, thereby restoring cell survival/function, was examined. HT22 cells were transfected in six different groups: i) EV, ii) StAR, iii) mAPP, iv) StAR plus mAPP, v) mTau, and vi) StAR plus mTau, for 24h, as indicated (Fig. 10A). Overexpression of StAR, mAPP, and mTau demonstrated ~2 to 2.5-fold increases in their endogenous protein levels (data not illustrated). Cellular images captured by the EVOS digital imaging system showed that cells overexpressing StAR revealed an increase in cell numbers by ~2-fold compared with EV control. Conversely, overexpression of either mAPP or mTau decreased cell survival between 48 and 57%. Inclusion of StAR, with either mAPP or mTau overexpression, demonstrated a noticeable reversal ($p<0.01$) in cell survival, suggesting a protective role of StAR in mAPP and mTau mediated cell viability.

To better understand the protective influence of StAR in mAPP and/or mTau induced cytotoxicity, cells from different transfected groups, as those of Fig. 10A, were processed for Annexin V-FITC/PI assay for determining cell survival. As illustrated in Fig. 10B, overexpression of StAR resulted in a 1.9 ± 0.3-fold increase in cell numbers/survival compared to EV control. Cells overexpressing either mAPP or mTau decreased cell survival by ~50%, reinforcing their cytotoxic effects [30, 31, 40]. Overexpression of StAR together with either mAPP or mTau significantly increased ($p<0.01$) cell numbers in both cases. These results suggest that StAR is capable of protecting/rescuing mAPP/mTau induced cytotoxicity in AD memetic hippocampal neuronal cells.

3.7. Protective role of StAR in mAPP and/or mTau mediated pregnenolone synthesis

Since StAR reduces the decline in cell survival generated by both mAPP and mTau, it was of interest to examinepregnenolone biosynthesis. HT22 cells were transfected with EV, mAPP, StAR, StAR plus mAPP, mTau, and StAR plus mTau, and then treated without or with TTNPB and SR11233. The results summarized in Fig. 11 demonstrate that both TTNPB and SR1233 elevated pregnenolone levels by ~6-fold in EV control. Cells overexpressing StAR resulted in a 1.8 ± 0.3-fold increase in basal, and concomitantly enhanced TTNPB and SR1233 induced pregnenolone levels. Overexpression of either mAPP or mTau diminished endogenous pregnenolone production by 50–60% and coordinately repressed TTNPB/SR1233 mediated pregnenolone levels. Inhibitory effect of mTau, on both basal and TTNPB/SR1233 induced pregnenolone synthesis, was relatively higher than those observed with mAPP, suggesting mTau appeared more cytotoxic. Co-expression of StAR with either mAPP or mTau considerably reversed basal response and concurrently enhanced/restored both TTNPB and SR11233 responsive pregnenolone levels (Fig. 11). These findings support the results presented in Fig. 10 and demonstrate that StAR resulted in reduction of mAPP and mTau induced cytotoxicity and, as a consequence, enhanced pregnenolone production; events were further influenced by retinoid signaling, pointing to the neuroprotective function of StAR in hippocampal steroidogenesis in AD mimetic conditions.

4. Discussion

Regulation of the hypothalamic-pituitary-thyroid-adrenal-gonadal (HPTAG) axis, involving a well-balanced circadian rhythmicity, is crucial to bodily homeostasis, thus, the proper functioning of a variety of physiological functions. These processes, however, are affected as life progresses from adulthood into senescence [12, 41–44]. Aging is an inevitable heterogeneous phenomenon, in which the functional efficacies of a multitude of organs, involving malfunction of the HPTAG auto-regulatory system, result in hormonal imbalance that is linked to numerous health complications along with a host of pathologies. Hormone deficiencies in aging, including steroids, modulate the immune system, develop into an array of physiological anomalies, including impaired memory and cognitive function, and contribute to increased morbidity and mortality [43–48]. AD is the most prevalent neurological disorder in aging and it is influenced by diverse events, including deposition Aβ plaques and Tau in the hippocampal and cortex regions within the brain [23–25]. Noteworthy, retinoids play unique modulatory and integrative roles across many physiological and pathophysiological processes [2–4, 9, 49, 50]. Accordingly, retinoid

and its receptors, transporters, and metabolizers have been frequently used as therapeutic targets in numerous complications and diseases [27, 51–53]. Both retinoid metabolism and signaling are also affected in multiple disorders [3, 52, 54], and supplementation of retinoids have been shown to rescue/reverse a number of defects in a variety of animal models [8, 55–57]. In line with these findings, we previously demonstrated that retinoid signaling influences the steroidogenic machinery in a number of tissues and reverses the declines in StAR expression and steroid biosynthesis in aged mouse Leydig cells and human epidermal keratinocytes [2, 4, 17, 18]. The present findings extend our understanding by elucidating the molecular events by which retinoid signaling upregulates StAR and pregnenolone biosynthesis, and StAR, by protecting mAPP- and/or mTau-induced cytotoxicity (conditions mimetic to AD), modulates hippocampal neurosteroidogenesis for preserving bodily homeostasis, events may be beneficial in protecting/delaying AD or age-related diseases.

The results of the present findings demonstrate that retinoids, especially atRA and/or 9-cis RA, moderately, but consistently, enhanced StAR expression and pregnenolone biosynthesis in HT22 neuronal cells. However, activation of the cAMP/PKA pathway strikingly elevated the steroidogenic response mediated by retinoid signaling, strengthening the notion that a low level of PKA is instrumental in modulating steroidogenesis. Consistent with this, we reported that cAMP/PKA dependent mechanisms, by activating StAR phosphorylation at Ser194/195 (mouse/human), play an indispensable role in optimal production of steroid biosynthesis [2, 4, 14]. In contrast, without impacting StAR phosphorylation, cAMP independent signaling displays moderate effects on steroid production. The inhibition of PKA activity attenuated, but not abolished, the steroidogenic response, suggesting involvement of additional pathway(s) in retinoid signaling. As such, retinoid mediated steroidogenesis involves both cAMP-independent and cAMP-dependent events and likely represents crosstalk between the signaling. Even so, while the effect of retinoid signaling on hippocampal neurosteroid synthesis is moderate, it could play an important role in regulating diverse cellular functions, especially with AD and age-related complications [27].

An overwhelming amount of evidence indicates that transcription of the StAR gene is coordinated by both enhancer (switching on) and silencer (switching off) elements, and a balance between inducer and repressor functions of these factors seemingly allows for a fine-tuning of the steroidogenic machinery [2, 10, 15, 58]. Employing a variety of experimental approaches, our present data document that retinoid mediated upregulation of StAR gene transcription is predominantly influenced by an overlapping RAR/RXR-LXR heterodimeric motif located at position −200/−185bp region in the mouse StAR promoter, although a number of trans-acting factors play permissible roles. Both RAR/RXR and LXR family members can recognize the RAR/RXR-LXR motif in the StAR promoter and regulates its transcription. In support of this, we previously demonstrated the functional relevance of RAR/RXR and LXR interacting events to maintain the steroidogenic response in adrenal and gonadal cells [2, 4, 17]. Our current data provide evidence that the −254/−1bp region of the StAR promoter, possessing the RAR/RXR-LXR heterodimeric motif, in addition to sequence specific DNA binding sites of a number of transcription factors, appears to function as the ‘retinoid response unit’ in trans-regulation of the StAR gene in hippocampal neuronal cells.

A new gateway to hippocampal neurosteroidogenesis is the involvement of the LXR pathway in retinoid mediated regulation of StAR transcription. Oxysterols are ligands for LXRs, and RAR/RXR-LXR heterodimers have been implicated in controlling cholesterol trafficking, metabolism, and balance [16, 59]. Our data demonstrate that retinoid signaling enhanced the functional cooperation/interaction between RAR/RXR and LXR in influencing the steroidogenic response; an event was further triggered by cAMP/PKA signaling. We reported that LXR activation, involving SREBP and ABCA1, facilitates cholesterol esterification and contributes to the effects of retinoids in increasing StAR in diverse cell models [2, 4, 17]. Hence, it is conceivable that an increase in StAR level/expression (either by retinoid and/or LXR signaling), involving efficient cholesterol transport, may be a potential therapeutic tool against a number of cholesterol/steroid-led pathophysiological events that frequently evolve during aging. Noteworthy, there are 15 distinct StAR-related lipid transfer domain proteins (STARD1-STARD15), thus, the involvement of additional factor(s) in neurosteroid biosynthesis cannot be excluded.

An intriguing aspect of the present findings is the protective role of StAR in mAPP and/or mTau induced toxicities, resulting in reversal of hippocampal neurosteroidogenesis for sustaining hormonal equilibrium, in AD mimetic conditions, for ameliorating age-related complications and diseases. Both mAPP and mTau were observed to noticeably diminish StAR gene transcription and pregnenolone biosynthesis in hippocampal neuronal cells. Immunohistochemical analyses, involving co-expression studies, revealed that StAR, by decreasing endogenous mAPP and mTau expression, acts as a neuroprotector, with discrete mechanisms connecting mAPP and mTau (Manna PR et. al, a manuscript in preparation), implying that hippocampal neurosteroidogenesis is frequently attenuated and/or dysregulated in AD and/or aged brain. These results are reminiscent of previous findings that demonstrate that StAR mRNA expression is significantly decreased in human AD brains compared to age-matched cases [60]. Our data also show that both RAR/RXR and LXR interacting events effectively increased both StAR transcription and pregnenolone biosynthesis in mAPP and/or mTau induced AD-mimetic hippocampal neuronal cells. It is plausible that retinoid linked therapeutic drugs, for AD and age-related complications and diseases [27, 61, 62], may influence hormonal stability, by controlling brain Aβ and Tau clearance, as those of in the present study. Regardless of the mechanism involved, mitochondrial StAR protein can reverse the declines in cell survival and neurosteroid biosynthesis that are particularly affected in mAPP and/or mTau-driven AD mimetic conditions. In addition to hippocampal aggregation of APP and Tau, AD pathogenesis is impacted by multiple events, including defective autophagy and mitophagy, microRNA dysregulation, and dysfunction in mitochondrial networks affecting synaptic damage and cognitive impairment [22, 25, 63–68]. Recent findings from our laboratory demonstrated that mitophagy enhancers and a small molecule inhibitor DDQ decreases in mAPP and/or mTau induced synaptic toxicities in both hippocampal neuronal cells and mouse model of AD [30, 31, 69]. Retinoid mediated upregulation of StAR transcription, concomitant with neurosteroid biosynthesis, and its protective and/or restorative influence in mAPP- and mTau-induced/AD mimetic cytotoxicity indicates that retinoid therapy, by mitigating hormonal homeostasis, can alleviate AD and age-associated complications and diseases, thus improving the health and quality of life in aging populations.

5. Conclusions

In summary, our current study findings provide new insights into the molecular events by which retinoid signaling acts to drive both StAR transcription and pregnenolone biosynthesis in hippocampal neuronal HT22 cells, and these events are primarily mediated by an RAR/RXR-LXR heterodimeric motif located at −200/−185bp region in the mouse StAR promoter. Additionally, retinoid responsive steroidogenesis is influenced by the cAMP/PKA signaling cascade. Upregulation of hippocampal StAR expression, concomitant with steroid biosynthesis, in response to retinoids, involves modulation of the LXR pathway, including the RAR/RXR-LXR regulatory events, resulting in hormonal stability that is fundamental for healthy aging. HT22 cells overexpressing StAR, in the presence of either mAPP or mTau (a condition mimetic to AD), reduces cytotoxicity and reverses the declines in cell survival and steroidogenic responsiveness. Therefore, StAR, by restoring neurosteroidogenic machinery, is capable of protecting and/or delaying AD and other physiological anomalies that especially occur during the process of aging.

Acknowledgements

The authors would like to thank Drs. D.J. Mangesldorf (UT Southwestern Medical Center, Dallas, TX), H-Y Kao (Case western Reserve University, Cleveland, OH), and M.B. Elam (University of Tennessee Health Science Center, Memphis, TN) for the generous gifts of RXRα, RARα, and pLXREx3-Luc plasmids, respectively.

Funding

This investigation was supported by NIH grants AG063162 and AG071560 to (APR), and R01AG042178, R01AG47812, R01NS105473, AG060767, AG069333, AG066347 and AG079264 to PHR.

Data Availability

Data generated and analyzed during this study are included in this article.

Abbreviations:

StAR	steroidogenic acute regulatory protein
AD	Alzheimer’s disease
RAR	retinoic acid receptor
RXR	retinoid X receptor
LXR	liver X receptor
PKA	protein kinase A
$(Bu)_2$cAMP)	dibutyryl-cyclic adenosine 3’, 5’-monophosphate
APP	amyloid precursor protein
RA	retinoic acid
atRA	all-trans retinoic acid

TTNPB	(4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid)
SR11233	(4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzoic acid)
Wt	wild-type
Mut	mutant
ELISA	enzyme-linked immunosorbent assay

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Highlights:

- Retinoids upregulate StAR transcription and steroidogenesis in hippocampal neuronal cells.
- Retinoid regulated hippocampal neurosteroidogenesis involves RAR/RXR and LXR signaling.
- Overexpression of either mAPP or mTau in neuronal cells results in an AD mimetic condition.
- StAR reduces mAPP/mTau-induced cytotoxicity and reverses neurosteroidogenic machinery.
- StAR plays a vital role in protecting and/or regressing AD and age-related complications.

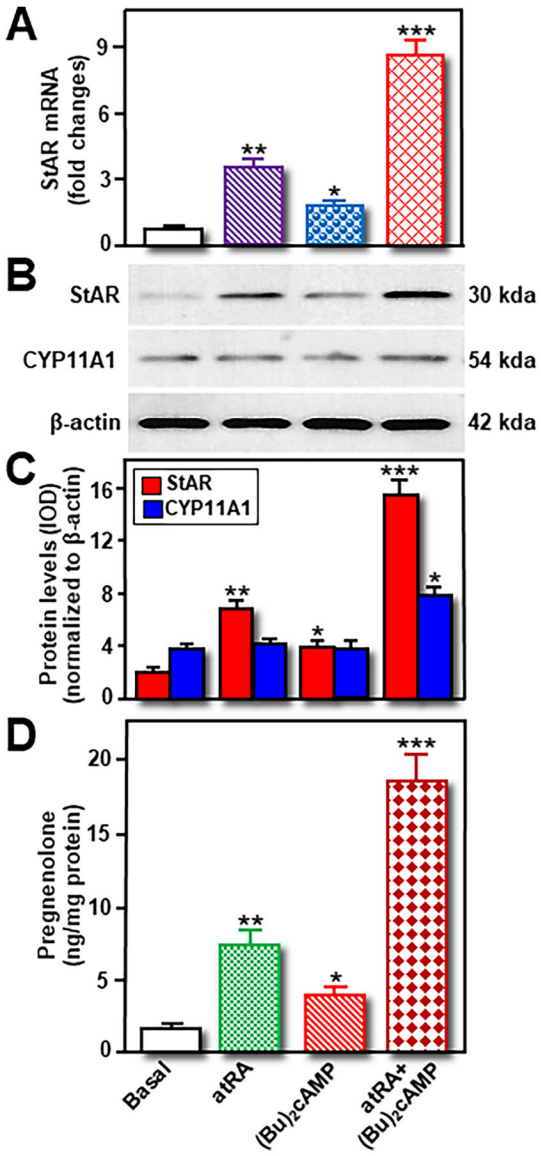

Fig. 1.

Influence of atRA on StAR mRNA, StAR protein, CYP11A1, and pregnenolone levels in HT22 cells. These cells were treated without (Basal) or with atRA (10μM), $(Bu)_2$cAMP (0.2mM), and their combination for 24h, in the presence of SU-10603 (20μM) and cyanoketone (5μM) for blocking pregnenolone metabolism. Following treatments, cells were processed for either total RNA extraction or cellular protein preparation. StAR mRNA levels were determined by qRT-PCR and represented as fold change (**A**). Representative immunoblots illustrate expression of StAR and CYP11A1 proteins in different groups with 50μg of total protein (**B**). Integrated optical density (IOD) values, for both StAR and CYP11A1 in each band, were quantified and normalized with the corresponding β-actin bands, and compiled data (N=3) are presented (**C**). Accumulation of pregnenolone in media was determined from different groups and expressed as ng/mg protein (**D**). Data represent

the mean ± SE of 3–4 independent experiments. *, $p<0.05$. **, $p<0.01$, ***, $p<0.001$ vs. Basal.

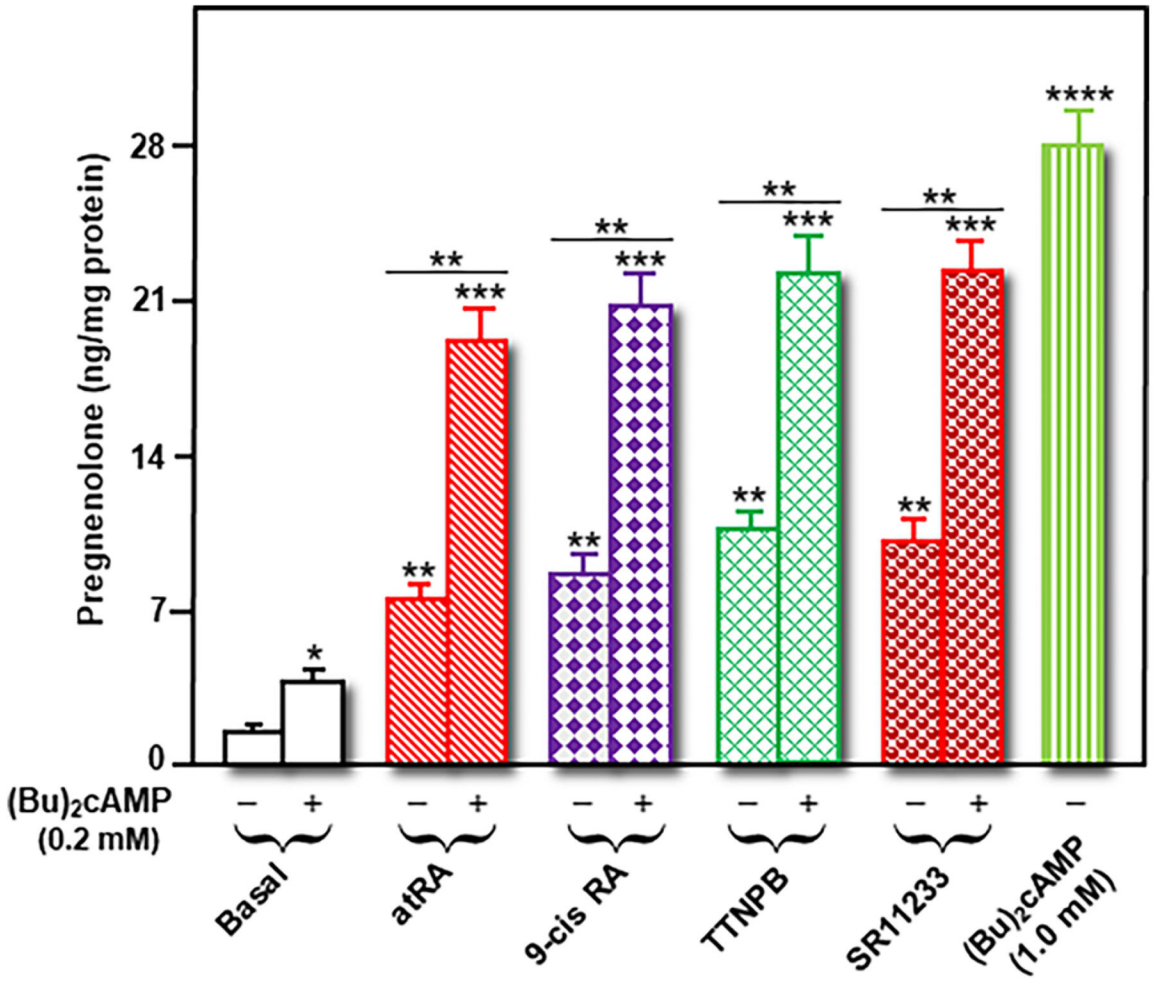

Fig. 2.

Effects of atRA, 9-cis RA, TTNPB, and SR1233 in $(Bu)_2$cAMP mediated pregnenolone biosynthesis. HT22 cells were treated without or with atRA (10μM), 9-cis RA (10μM), TTNPB (5μM), and SR11233 (5μM), in the absence or presence of $(Bu)_2$cAMP (either 0.2mM or 1.0mM) for 24h, as indicated. Treatments were made in the presence of SU-10603 (20μM) and cyanoketone (5μM) for blocking pregnenolone metabolism. Following treatments, media from different treatment groups were collected, and pregnenolone levels were determined and expressed as ng/mg protein. Data represent the mean ± SE of 3–4 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$ vs. Basal, and +/− $(Bu)_2$cAMP.

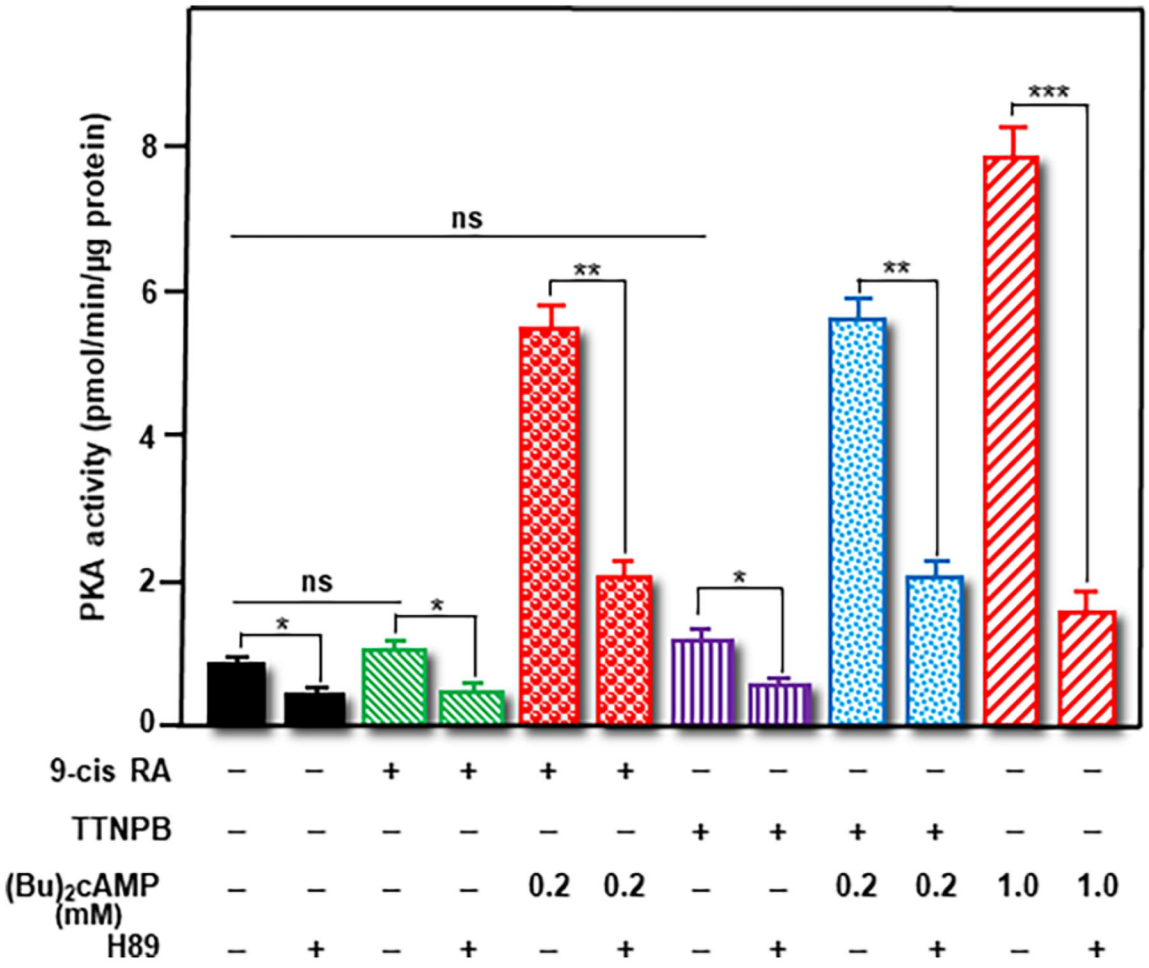

Fig. 3.

The contribution of PKA activity in retinoid responsiveness. HT22 cells were pretreated with H89 (20µM) for 45 min, then treated without or with 9-cis RA (10µM) and TTNPB (5µM), in the absence or presence of $(Bu)_2$cAMP (either 0.2mM or 1.0mM), as indicated, for 24h. Cells were then processed to determine PKA activity. Data represent the mean ± SE of 3 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs. H89; ns = not significant, untreated cells vs. 9-cis RA and TTNPB.

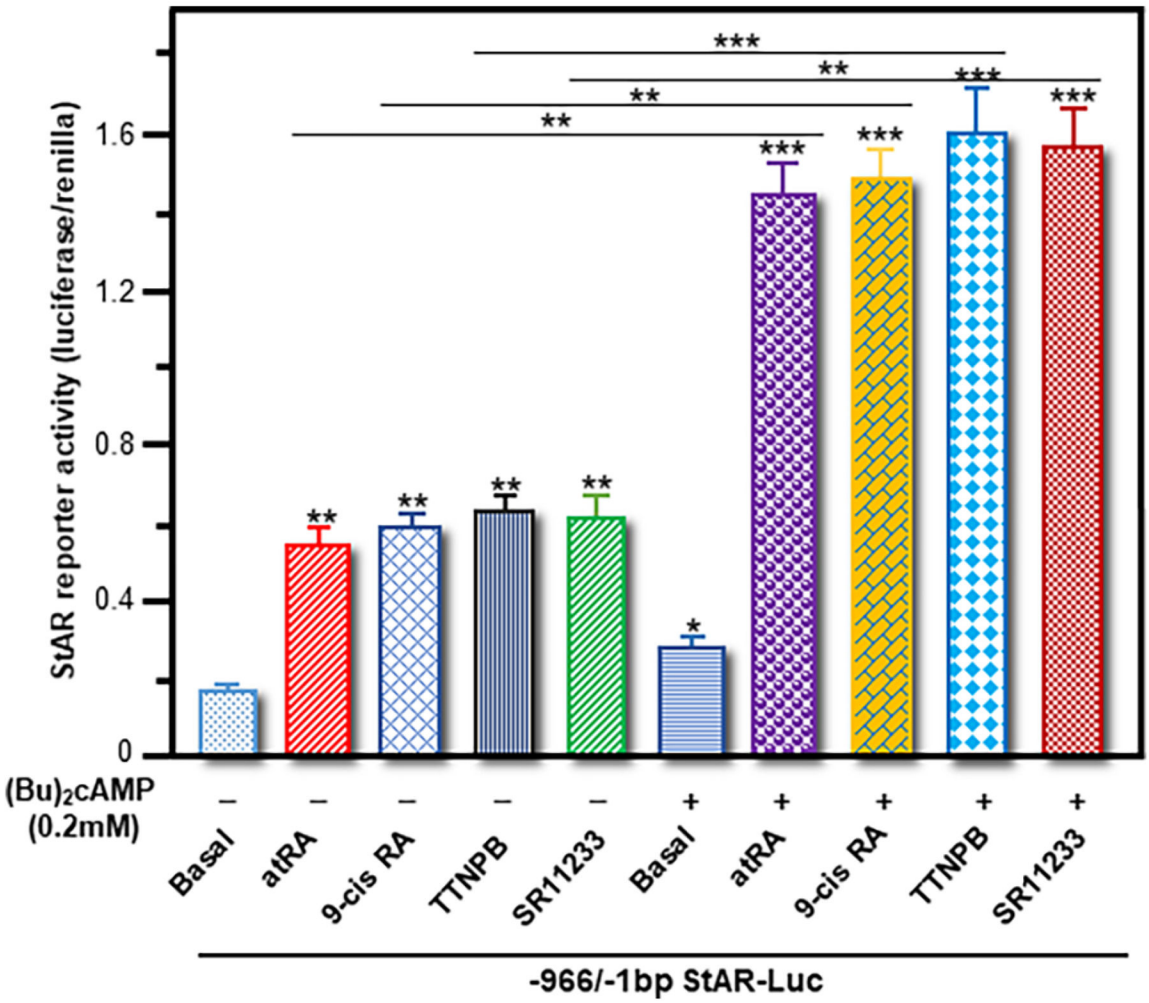

Fig. 4.

Effects of retinoids and RAR/RXR analogs in $(Bu)_2$cAMP mediated StAR promoter activity. HT22 cells were transiently transfected with the −966/−1bp StAR-Luc segment in the presence of pRL-SV40, using lipofectamine 3000 reagent. Following 24h of transfection, cells were treated without or with atRA (10μM), 9-cis RA (10μM), TTNPB (5μM), and SR11233 (5μM), in the absence or presence of $(Bu)_2$cAMP (0.2mM) for an additional 24h, as indicated. Luciferase activity in the cell lysates was determined and expressed as StAR promoter activity (luciferase/renilla). Data represent the mean ± SE of 4 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs. Basal and +/− $(Bu)_2$cAMP.

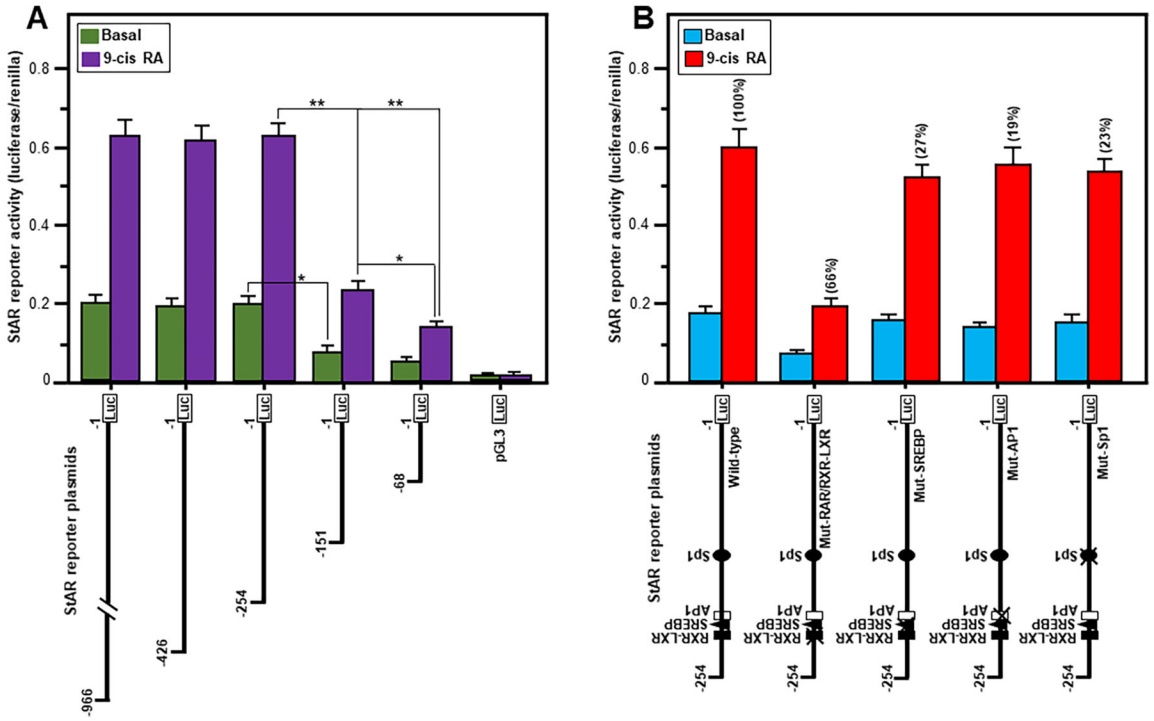

Fig. 5.

Deletion and mutation analyses of the 5'-flanking region of the StAR promoter for identification of retinoid response element(s), and their relevance to 9-cis RA mediated StAR promoter activity. HT22 cells were transiently transfected with different StAR promoter/reporter deletion plasmids i.e. −966/−1bp, −426/−1bp, −254/−1bp, −151/−1bp, and −68/−1bp, in the presence of pRL-SV40, using lipofectamine 3000 reagent, for 24h (**A**). Cells were also transfected with either the −254/−1bp StAR reporter plasmid (wild-type) or the −254/−1bp segment containing mutations in the putative binding sites (RAR/RXR-LXR, SREBP, AP1, and Sp1), in the presence of pRL-SV40, as indicated (**B**). Schematic representations illustrate different StAR reporter plasmids (**A** and **B**; bottom panels). Following 24h of transfection, cells were treated without or with 9-cis RA (10μM) for an additional 24h. Luciferase activity in the cell lysates was determined in different groups and expressed as StAR promoter activity (luciferase/renilla), which represent the mean ± SE of 3–4 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs. Basal or (−254/−1bp vs. −151/−1bp).

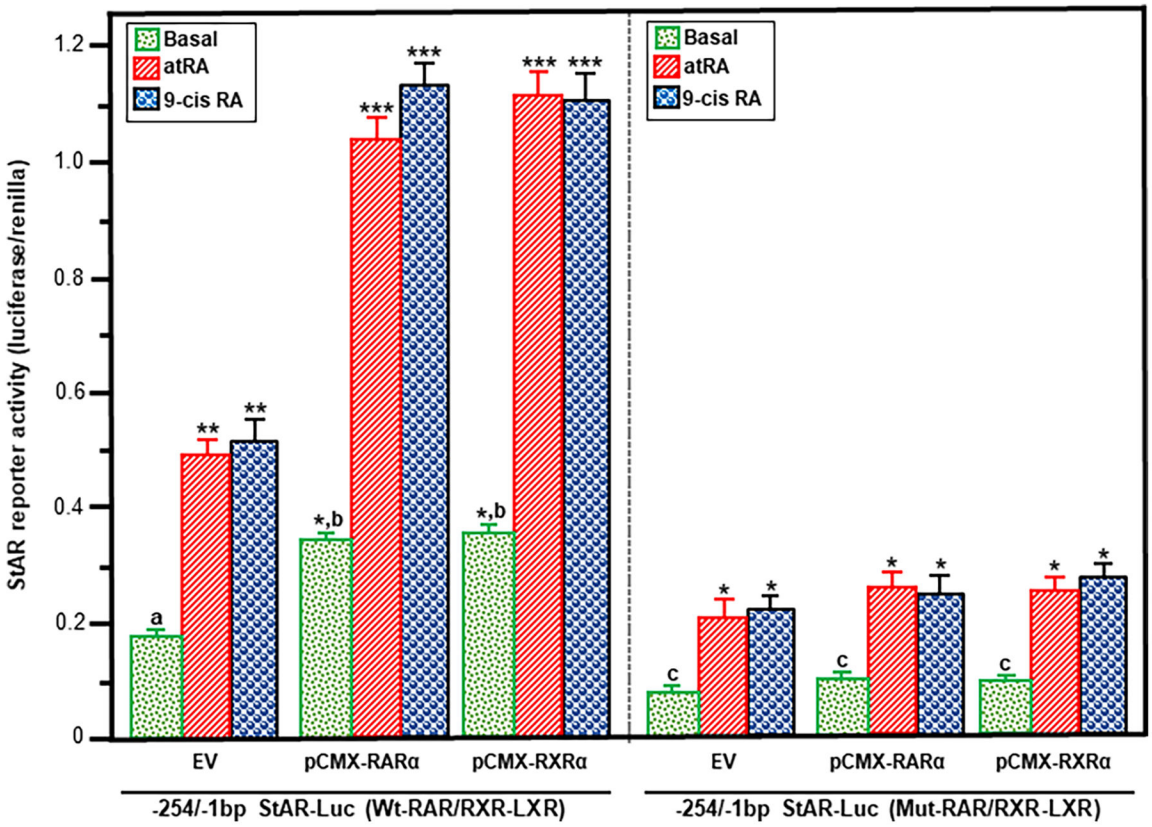

Fig. 6.

Functional relevance of the RAR/RXR-LXR motif in RARα and RXRα driven StAR promoter activity. HT22 cells were transiently transfected with either EV or pCMX-RARα (RARα) and pCMX-RXRα (RXRα) expression plasmids, within the context of the −254/−1bp StAR-Luc segment, containing either wild type RAR/RXR-LXR (Wt-RAR/RXR-LXR) or mutant RAR/RXR-LXR (Mut-RAR/RXR-LXR) motifs, in the presence of pRL-SV40, for 24h. Following 24h of transfection, cells were treated without or with atRA (10μM) and 9-cis RA (10μM) for an additional 24h, and luciferase activity in the cell lysates was determined in different groups and expressed as StAR promoter activity (luciferase/renilla). Results represent the mean ± SE of 3 independent experiments. Letters above the bars indicate that these groups differ significantly from each other at least at $p<0.05$. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs. respective Basal.

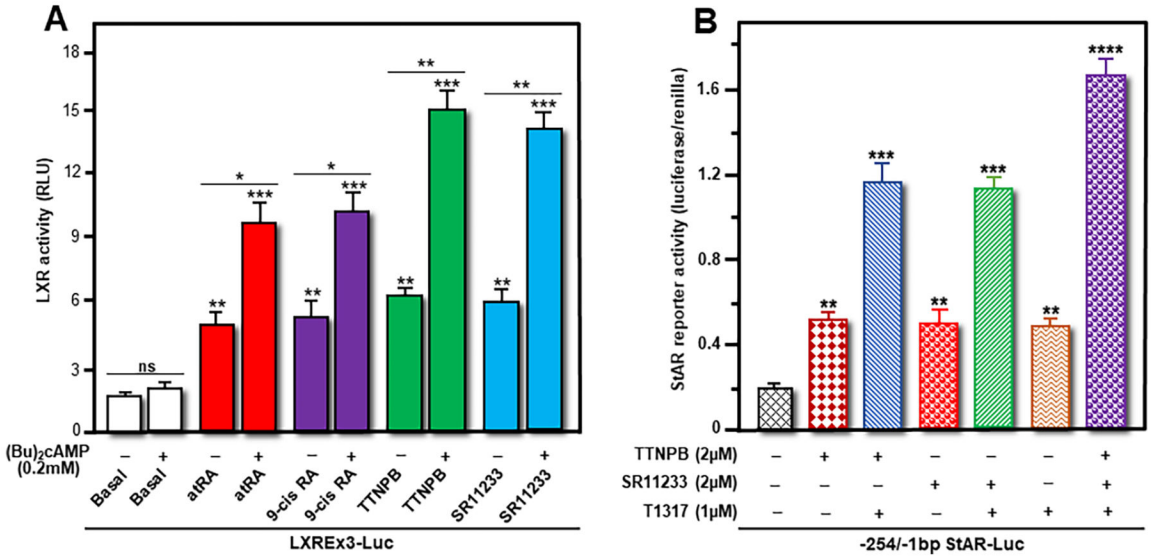

Fig. 7.

Role of retinoid signaling in LXR activity, and cooperation between RAR/RXR and LXR in StAR promoter activity. HT22 cells were transiently transfected with the pLXREx3-Luc, using lipofectamine 3000 reagent. Following 24h of transfection, cells were treated without or with atRA (10μM), 9-cis RA (10μM), TTNPB (5μM), and SR11233 (5μM), in the absence or presence of $(Bu)_2$cAMP (0.2mM) for an additional 24h (**A**). HT22 cells were also transfected with the −254/−1bp StAR-Luc segment in the presence of pRL-SV40 cells, for 24h, and then treated with suboptimal doses of either TTNPB (2μM), SR11233 (2μM), and T1317 (1μM), individually, or a combination of them, for an additional 24h (**B**). Luciferase activity in the cell lysates was determined and expressed as LXR activity (RLU, relative light unit; **A**) or StAR promoter activity (luciferase/renilla; **B**). Results represent the mean ± SE of 3–4 independent experiments. *, $p<0.01$; **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$ vs. Basal/none, and +/− $(Bu)_2$cAMP, ns = not significant.

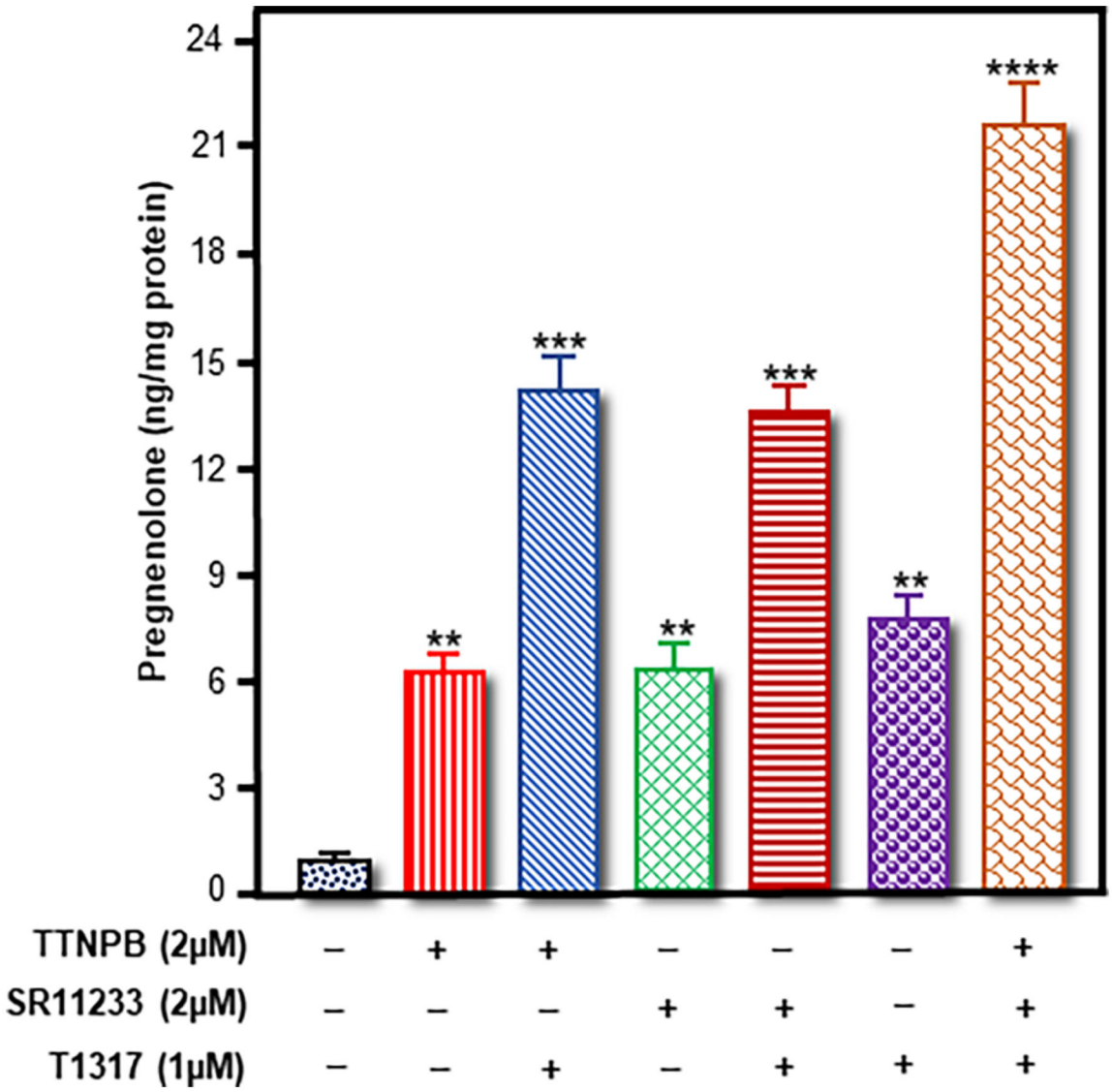

Fig. 8.

Cooperation and/or interaction between RAR/RXR and LXR in pregnenolone biosynthesis. HT22 cells were treated with suboptimal doses of either TTNPB (2μM), SR11233 (2μM), and T1317 (1μM), individually, or a combination of them, in the presence of SU-10603 and cyanoketone, for 24h. Following treatments, media from different treatment groups were collected, and pregnenolone levels in media were determined and expressed as ng/mg protein, which represent the mean ± SE of 4 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$ vs. none.

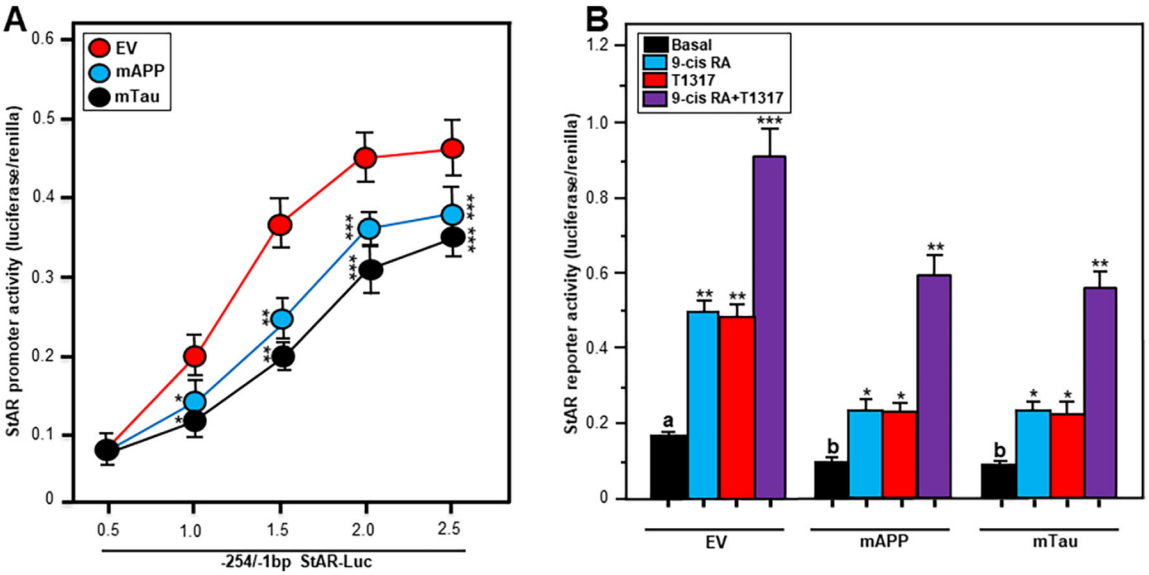

Fig. 9.

Roles of mAPP and mTau in retinoid mediated StAR promoter activity. HT22 cells were transfected with either EV or mAPP, and mTau expression plasmid, individually, with either increasing (0.5 to 5μg; **A**) or a fixed dose (2μg; **B**), within the context of the −254/−1 bp StAR-Luc, in the presence of pRL-SV40, using lipofectamine 3000 reagent. After 24h of transfection, cells were collected and processed to determine luciferase activity (**A**). Cells were treated without or with suboptimal doses or either 9-cis RA (2μM) and T1317 (1μM), individually, or a combination of them, for an additional 24h (**B**). Luciferase activity in the cell lysates was determined in different groups and expressed as StAR promoter activity (luciferase/renilla), which represent the mean ± SE of 4 independent experiments. Letters above the bars indicate that these groups differ significantly from each other at least at $p<0.05$. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs. EV (**A**) or Basal in each category (**B**).

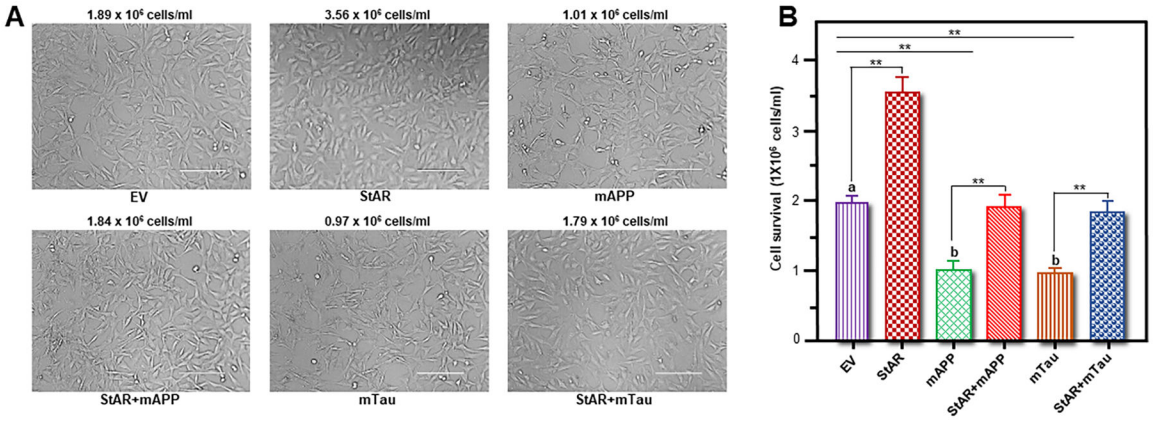

Fig. 10.

Overexpression of StAR, mAPP, and mTau, on cell survival. HT22 cells were transfected with either EV, StAR, mAPP, and mTau expression plasmid, either individually or a combination of them, using lipofectamine 3000 reagent. Following 24h of transfection, cells from different groups were visualized by EVOS digital imaging system (**A**). Cellular images from a representative experiment (N=5) are shown along with cell numbers on top. These cells were then processed for Annexin V-FITC/PI assay for determining cell survival (**B**). Results represent the mean ± SE of 4 independent experiments. Letters above the bars indicate that they differ significantly from each other at least at $p<0.05$. **, $p<0.01$ vs. different groups specified. Scale bar 200μm.

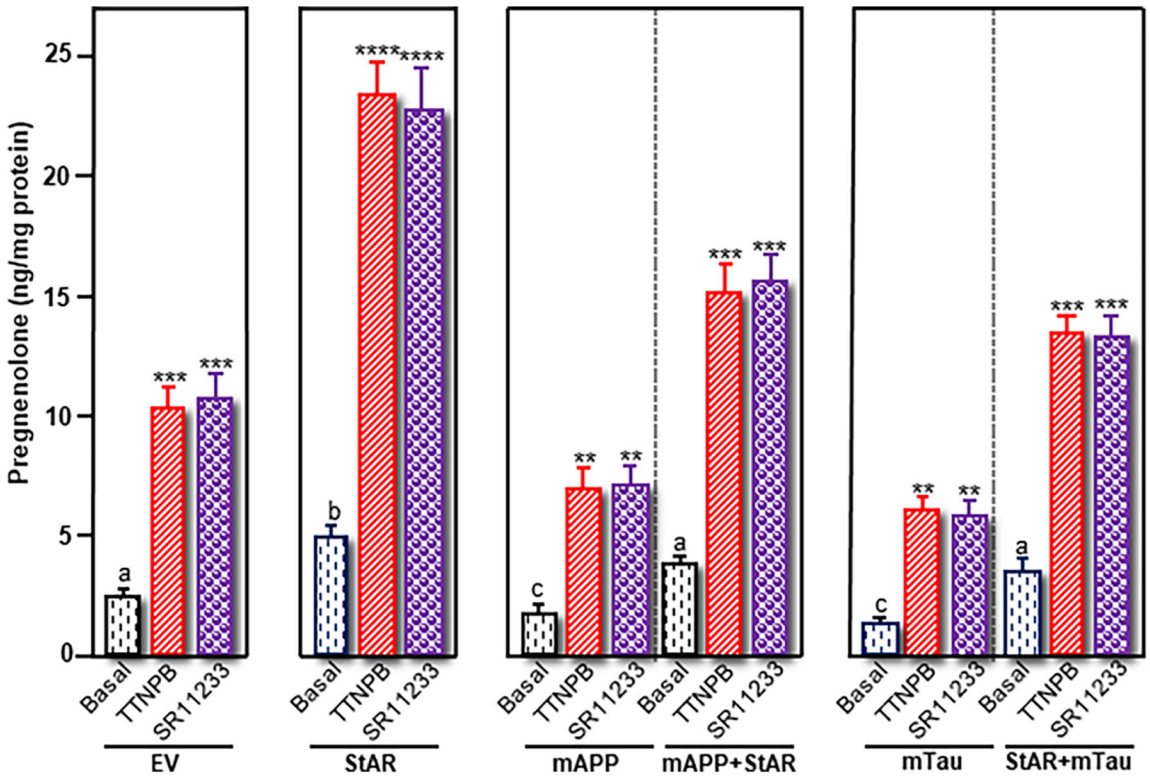

Fig. 11.

Functional relevance of StAR in the reversal of mAPP and mTau induced cytotoxicity and pregnenolone biosynthesis. HT22 cells were transfected with EV, StAR, mAPP, and mTau expression plasmid, either individually or a combination of them, using lipofectamine 3000 reagent. Following 24h of transfection, cells were treated without or with TTNPB (5μM) and SR11233 (5μM) for additional 24h. Accumulation of pregnenolone in media was determined in different groups and expressed as ng/mg protein. Data represent the mean ± SE of 3 independent experiments. Letters above the bars indicate that these groups differ significantly from each other at least at $p<0.05$. **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$ vs. Basal.