



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

Gonadotropin-releasing hormone modulates cholesterol synthesis and steroidogenesis in SH-SY5Y cells.

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Gonadotropin-releasing hormone modulates cholesterol synthesis and steroidogenesis in SH-SY5Y cells / F. Rosati;N. Sturli;M. C. Cungi;M. Morello;F. Villanelli;G. Bartolucci;C. Finocchi;A. Peri;M. Serio;G. Danza. - In: JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY. - ISSN 0960-0760. - STAMPA. - 124:(2011), pp. 77-83. [10.1016/j.jsbmb.2011.01.012]

Availability:

The webpage <https://hdl.handle.net/2158/433461> of the repository was last updated on

Published version:

DOI: 10.1016/j.jsbmb.2011.01.012

Terms of use:

Open Access

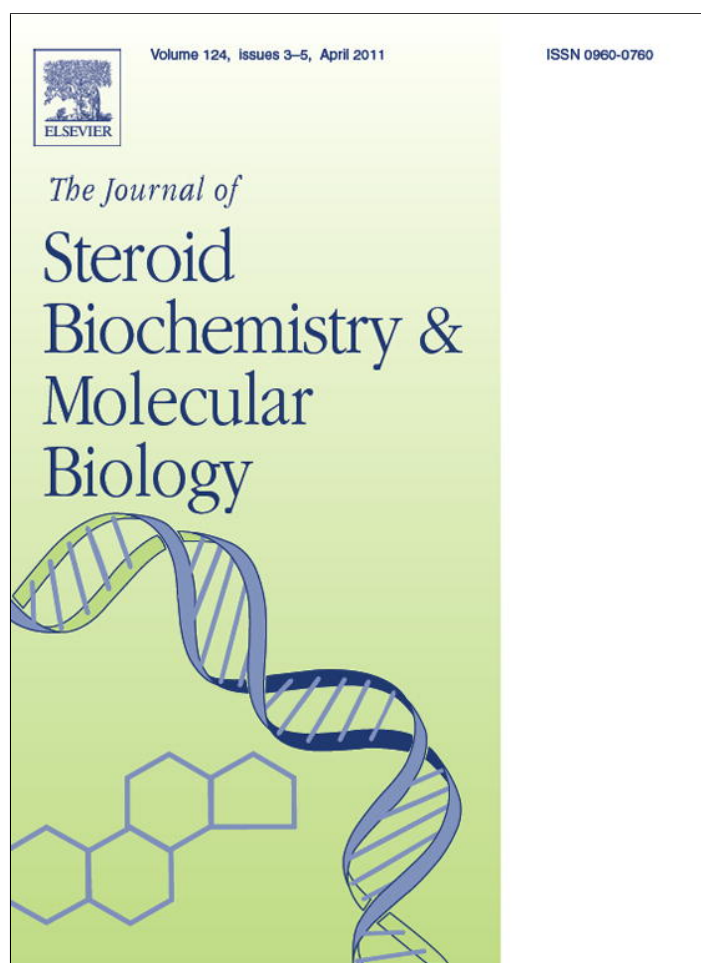
La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Gonadotropin-releasing hormone modulates cholesterol synthesis and steroidogenesis in SH-SY5Y cells

Fabiana Rosati^a, Niccolò Sturli^a, Maria Chiara Cungi^a, Matteo Morello^a, Fabio Villanelli^a, Gianluca Bartolucci^b, Claudia Finocchi^a, Alessandro Peri^a, Mario Serio^a, Giovanna Danza^{a,*}^a Endocrine Unit, Department of Clinical Physiopathology, University of Florence, Viale G. Pieraccini 6, 50139 Firenze, Italy^b Department of Pharmaceutical Science, University of Florence, Via U. Schiff 6, 50019 Firenze, Italy

ARTICLE INFO

Article history:

Received 2 September 2010

Received in revised form 26 January 2011

Accepted 27 January 2011

Keywords:

Gonadotropin-releasing hormone

Neurosteroids

Cholesterol

Steroidogenesis

Seladin-1/DHCR24

ABSTRACT

Neurosteroids are involved in Central Nervous System development, brain functionality and neuroprotection but little is known about regulators of their biosynthesis. Recently gonadotropins, Gonadotropin-releasing Hormone (GnRH) and their receptors have been localized in different brain regions, such as hippocampus and cortex.

Using human neuronal-like cells we found that GnRH up-regulates the expression of key genes of cholesterol and steroid synthesis when used in a narrow range around 1.0 nM. The expression of Hydroxysteroid D24-reductase (seladin-1/DHCR24), that catalyzes the last step of cholesterol biosynthesis, is increased by 50% after 90 min of incubation with GnRH. StAR protein and P450 side chain cleavage (P450_{scc}) are up-regulated by 3.3 times after 90 min and by 3.5 times after 3 h, respectively. GnRH action is mediated by LH and 1.0 nM GnRH enhances the expression of LHβ as well.

A two fold increase of cell cholesterol is induced after 90 min of GnRH incubation and 17β-estradiol (E2) production is increased after 24, 48 and 72 h. These data indicate for the first time that GnRH regulates both cholesterol and steroid biosynthesis in human neuronal-like cells and suggest a new physiological role for GnRH in the brain.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Steroid hormones have been traditionally considered as exclusively originating from adrenals and gonads with their synthesis regulated by the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axes, respectively. Steroids are small cholesterol-derived, lipophilic molecules that can cross the plasma membrane and the blood-brain barrier; thus the spatial specificity of their action has been attributed to steroid receptors expression by target cells. However, from the past twenty years new evidences are contributing to a redefinition of these concepts: local *de novo* steroidogenesis has been demonstrated in many peripheral tissues [1–5]. Moreover, for cholesterol, the starting molecule of steroids biosynthesis, the *de novo* synthesis has now been completely elucidated [6] and recent data demonstrate that gonadotropins (in particular LH) are able to regulate this biosynthetic pathway, suggesting a strong association between cholesterol and steroid biosynthesis [7]. One of the most important local site where peripheral steroidogenesis has been demonstrated

is the Central Nervous System (CNS). The concept of endogenous steroid synthesis in the brain, or neurosteroidogenesis, arises from the observations, made in the 1980s by Baulieu and colleagues [4,8,9], of the presence of steroids such as dehydroepiandrosterone, pregnenolone and their sulphates in rat brain. Their concentration was greater than those found in the circulation and remained high after adrenalectomy and gonadectomy. Starting from these data, neuroactive steroids have received great attention as autocrine and paracrine regulators of brain function. Neurosteroids are a class of neuroactive compounds involved in CNS development, in adult brain functionality and in neuroprotection [10–13]. The absence or reduced concentrations of neurosteroids during development and in adults may be associated with neurodevelopmental, psychiatric, or behavioural disorders and neurodegeneration [13]. Despite the continuous identification of new and unexpected functions of these compounds, little is known about the regulators of their biosynthetic pathway in the brain and existing data are mainly focused on neurotransmitters and neuropeptides [14].

Recent studies indicate that FSH, LH, ACTH and their receptors are locally expressed in tissues that are target of peripheral hormones (i.e. CNS and the immune system), suggesting the presence of miniature HPG and HPA axis homologs that are able to autonomously regulate local steroidogenesis [15]. GnRH receptors

Abbreviation: E2, 17β-estradiol.

* Corresponding author. Tel.: +39 0554271372; fax: +39 0554271371.

E-mail address: g.danza@dfc.unifi.it (G. Danza).

(GnRHs) have been localized in many regions of the brain such as the hippocampus and cortex and detected in human neuronal cell models like the neuroblastoma SH-SY5Y and M17 cells [16,17]. In M17 cells GnRH induces an up-regulation of the LH β subunit [17] and LH mediates neuronal pregnenolone production increasing Steroidogenic Acute Regulatory protein (StAR) expression [18]. Moreover, GnRH regulates estrogen synthesis and spine synapse density in rat primary hippocampal cultures [19].

On the other hand, cholesterol that serves as a precursor for neurosteroids, derives totally from *de novo* synthesis in this compartment [20,21]. The enzyme that catalyzes the last step of cholesterol biosynthesis is 3 β -hydroxysterol Δ 24-reductase (DHCR24) that is also named seladin-1 (for SElective Alzheimer Disease INDicator-1) because its expression is markedly reduced in brain areas affected by Alzheimer's disease [22]. We have recently demonstrated that the expression of seladin-1/DHCR24 is up-regulated by estrogen in a human long term primary culture of GnRH secreting foetal neuroblasts (FNC) [23,24]. Nothing is known about the influence of GnRH on cholesterol biosynthesis although the possibility of shared regulators for cholesterologenesis and steroidogenesis has been hypothesised [7].

A better understanding of how the biosynthetic pathways leading to cholesterol and steroids are controlled in CNS and the possible identification of common regulators could improve our knowledge of the physiology of the human brain and open new perspectives in the pharmacological treatment of its disturbances.

For this purpose we examined whether GnRH is able to stimulate cholesterol synthesis and steroidogenesis in two human neuronal cell models: SH-SY5Y, a neuroblastoma cell line used as a human neuronal model and possessing the GnRH receptor (GnRH-R) [25,26], and FNC, a model of human neuroblast [27]. FNCs are a long term primary culture of neuroblast deriving from foetal olfactory epithelium and retains both olfactory and neuroendocrine characteristics, producing GnRH and expressing its receptor.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade or the highest purity available. Cholesterol, GnRH (type I), LH, Bradford reagent, BSTFA and other chemicals were from Sigma (Milan, Italy), unless otherwise stated. Stigmasterol was from Steraloids Inc. (Newport, RI, USA).

2.2. Cell cultures and hormone treatments

Human SH-SY5Y neuroblastoma cells were obtained from A.T.C.C. (Manassas, VA, USA) and were cultured in DMEM supplemented with 10% FBS, 1.0% glutamine and 1.0% antibiotics from Sigma. FNC were isolated from human foetal olfactory neuroepithelium, cloned, and long-term cell cultures were established and propagated in Coon's modified Ham's F12 from Life Technologies (Grand Island, NY, USA), supplemented with 10% FBS and 1.0% antibiotics, as described previously [27]. The cells were maintained in a 5.0% CO₂ humidified atmosphere at 37 °C in tissue plasticware from PBI International (Milan, Italy).

Stock solution of GnRH was prepared at 10 mg/ml concentration in ultrapure water and stored at –20 °C in working aliquots. Stock solution of LH was prepared at 50 IU/ml in water and stored at 4 °C. Cells were plated 48 h prior to treatment at a density of approximately 2.0 \times 10⁵ cells/well in six-wells plates and of 1.0 \times 10⁶ cells/plate in 10-cm dishes. The following day, cultures were placed in serum-free medium for 24 h prior to treatment with 0.01–1000 nM GnRH or 100 mIU/ml LH at 37 °C for the incubation times indicated in Section 3. Medium was changed with fresh

medium containing the appropriate hormone concentration every 24 h.

LH-receptor was blocked using the LHR(H-50) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), raised against an extracellular domain of human LH-receptor. SH-SY5Y cells were incubated for 1 h at 37 °C with the LHR(H-50) antibody diluted 1:200 in serum-free medium before the hormone treatment.

2.3. Isolation of RNA, synthesis of cDNA and RT-PCR assay

Cells were plated at a density of 1.0 \times 10⁶ cells in 10-cm dishes, maintained in serum free medium for 24 h and then treated with various concentrations of GnRH for the indicated periods.

Total RNA was isolated from SH-SY5Y and FNC cells using the RNEasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase I (Qiagen) during RNA purification following the manufacturer's protocol. The concentration of total RNA was determined spectrophotometrically with Nanodrop[®] ND-1000 (National Instruments Corporation, Texas, USA).

Total RNA of SH-SY5Y and FNC (100 ng) was amplified with the primer set for LH α subunit [28] using the SuperScript[™] One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

1–2 μ g of total RNA was reverse transcribed into cDNA by using TaqMan reverse transcription reagents with random examers (Applied Biosystems Inc., Foster City, CA, USA). The profile of the reverse transcription reaction was 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. Each reverse transcription was carried out in triplicate.

Expression of mRNA of Seladin-1/DHCR24, StAR, P450scc and LH β was determined by quantitative RT-PCR. The relative amounts of the mRNAs of the target genes were determined by calculating the ratio between these mRNAs and the mRNA of the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH). In our experimental systems, GnRH treatment did not change the expression of GAPDH. We observed comparable results using another housekeeping gene, HPRT1, to normalize the target gene expression (data not shown).

The quantification of Seladin-1/DHCR24 mRNA was performed by real-time RT-PCR. TaqMan primers, probe and thermal cycling conditions were as previously described [29]. PCR mixture (25 μ l final volume) included 1 \times final concentration of Universal PCR Master Mix (Applied Biosystems), and 50 ng of cDNA.

For the quantification of P450scc gene expression, 50 ng of cDNA were amplified in a reaction mixture (25 μ l final volume) containing Power SYBR Green Master Mix (Applied Biosystems) at 1 \times final concentration and the primer pair [30] at 0.5 μ M concentration. The cDNA was then subjected to PCR using the following conditions: 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min in the ABI Prism 7900 Sequence Detector (Applied Biosystems).

Primer for StAR (Hs.STAR.1.SG QuantiTect Primer Assay n. QT00091959), LH β (Hs.LHB.1.SG QuantiTect Primer Assay n. QT00207858) and GAPDH (Hs.GAPDH.2.SG QuantiTect Primer Assay n. QT01192646) were purchased from Qiagen. PCR mixture (25 μ l final volume) included 1 \times final concentration of QuantiTect Primer assay mix, 1 \times final concentration of Power SYBR Green Master Mix (Applied Biosystems) and 50 ng cDNA. The thermal cycling condition was 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min in the ABI Prism 7900 Sequence Detector (Applied Biosystems).

According to the comparative threshold cycle (Ct) method, the amount of target mRNA normalized to GAPDH and relative to an internal control was calculated by 2^{– $\Delta\Delta$ Ct}. The results (mean \pm SE) were expressed as fold mRNA variations compared with control. Each sample of the RT triplicate, of any experimen-

tal point, was measured in duplicate and 3–5 experiments were performed.

2.4. Western blotting analysis for StAR

For Western blot analysis of SH-SY5Y, cell pellets from one 10-cm culture plate were lysed in 200 μ l RIPA buffer (150 mM NaCl, 50 mM TRIS pH 7.5, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS) with Protease inhibitor cocktail (Sigma), vortexed and centrifuged at 13,000 \times g for 10 min at 4 °C. Supernatants from pellet extractions were analyzed for total protein content using the BCA protein determination kit (Pierce, Rockford, IL, USA). Twenty micrograms of total protein was loaded for each sample, resolved by 10% SDS polyacrylamide gel electrophoresis at 100 V and transferred to polyvinylidene difluoride (PVDF) membrane from Millipore (Billerica, MA, USA). The membrane was blocked in blocking buffer (TBS-Tween20 0.1% (v/v), 5% (w/v) skim milk) for 1 h and incubated for 2 h at room temperature with anti-StAR antibody (Abcam, Cambridge, UK) diluted 1:3000 in blocking buffer. After washing the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and revealed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore). The intensities of the immunoreactive bands were quantified by Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories, Hercules, CA, USA). The StAR protein signals were corrected by F-actin signal.

2.5. Cholesterol measurement

Cells were plated at a density of 2×10^5 cells in six-wells plates, maintained in serum free medium 24 h and then treated with various concentrations of GnRH for the indicated periods.

The amount of cholesterol was determined by gas chromatography/mass spectrometry (GC/MS) as previously described with minor modifications [31]. Briefly, cells from six wells plate were harvested and lysed in NaOH 1 N (200–300 μ l/well) using a cell scraper and frozen at -20°C . The protein concentration was determined by using the Bradford method [32]. 5–10 μ g of proteins was used for cholesterol determination. After the addition of stigmaterol (1000 ng) as internal standard, sterols were extracted with n-hexane. After evaporation, sterols were derivatized in bis-N,O-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 10% trimethyl-chlorosilane at 70 °C for 30 min. BSTFA solutions (2 μ l) were automatically injected for analysis in a Hewlett-Packard GC-MS system composed of a 6890 series II gas-chromatograph equipped with a 5973 Mass Spectrometry Detector, a 6890 automatic injector and a GC column was a J&W DB1 (15 m \times 0.25 mm \times 0.25 μ m). A six point calibration curve in the 25–1000 ng cholesterol range with 1000 ng of stigmaterol was used for cholesterol quantification. The peak area ratios (PAR) were calculated using the signals at 458 m/z and 484 m/z for cholesterol and stigmaterol, respectively.

The results (mean \pm SE) were expressed as fold cholesterol variations compared with control. All measurements were carried out in triplicate and three experiments were performed.

Inter-assay CV% was less than 10% in all the concentration range of the calibration curve.

2.6. E2 measurement

After incubation of SH-SY5Y with 1 nM GnRH for 24, 48 and 72 h the medium was harvested and its E2 content was determined with an immunoassay using the E2 kit for IMMULITE 2000 (Siemens Healthcare diagnostics, Deerfield IL, USA) after an extraction step. Briefly, for each point 750 μ l of medium was extracted with 1 ml of tertbutylmethyl ether (TBME) for two times. The TBME extract was

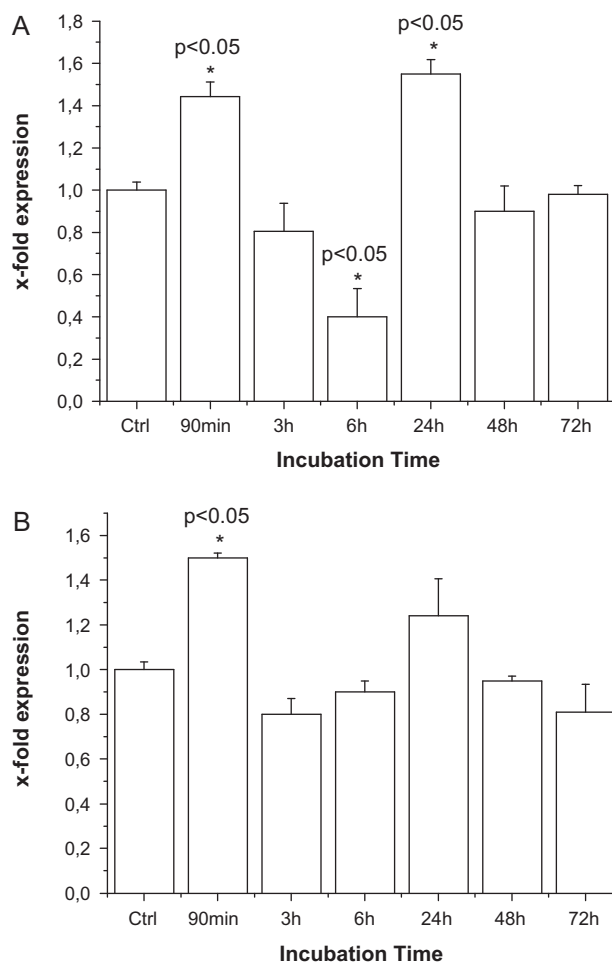


Fig. 1. Seladin-1/DHCR24 expression after GnRH stimulus. Seladin-1/DHCR24 mRNA in SH-SY5Y cells (A) and in FNC cells (B) stimulated with 1.0 nM GnRH. Values are normalized using GAPDH as housekeeping gene and reported as x-fold variation vs control. Each sample represents the mean \pm SE of the RT triplicate measured in duplicate (6 measures in total). Five experiments were performed.

dried under a nitrogen flux, samples were reconstituted with 250 μ l of the specific diluent and used for E2 quantification according to the manufacturer's instructions.

The sensitivity of the method was 15 pg/ml and, assessed by inter assay CV%, ranged from 7% at 1800 pg/ml to 16% at 89 pg/ml.

2.7. Statistical analysis

All data are expressed as mean \pm standard error (SE). Comparisons between the different groups were performed by ANOVA followed by Bonferroni's *t*-test. A *P*-value less than 0.05 was accepted as statistically significant.

3. Results

3.1. GnRH modulates the expression of seladin-1/DHCR24 in SH-SY5Y and FNC cells

To assess the role of GnRH in the modulation of cholesterol biosynthesis SH-SY5Y cells were stimulated with GnRH at doses from 0.01 to 1000 nM and the seladin-1/DHCR24 mRNA was quantitatively determined by real time RT-PCR after the following incubation times: 90 min and 3, 6, 24, 48 and 72 h. GnRH was able to modulate seladin-1/DHCR24 expression only in a narrow range between 0.2 and 2.0 nM and the 1.0 nM dose was selected for

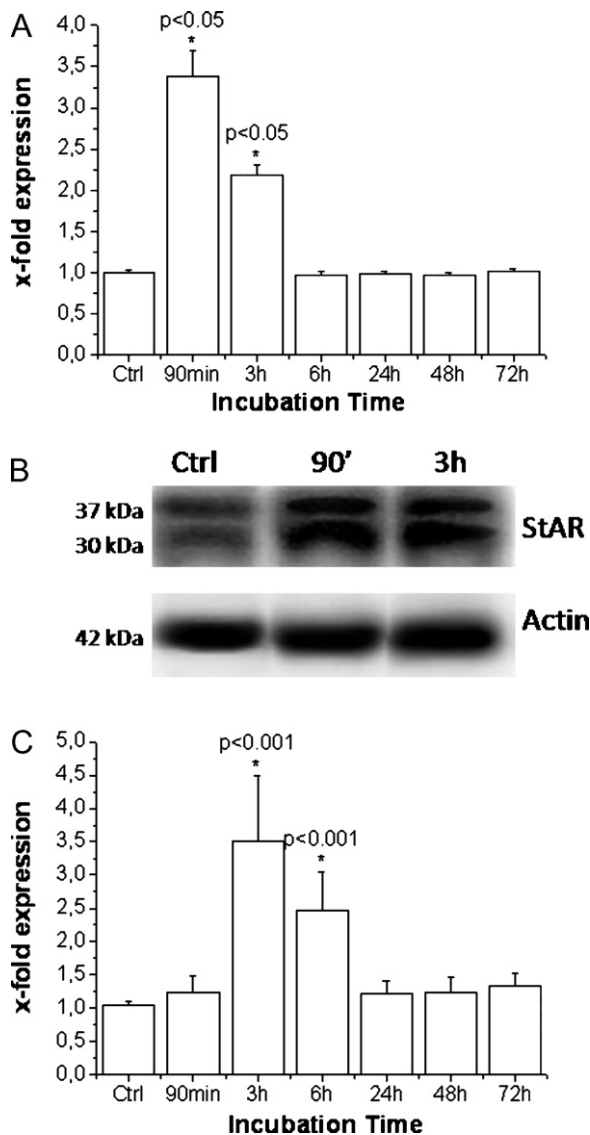


Fig. 2. StAR and P450scc expression after GnRH stimulus. StAR mRNA levels (A) and western blotting analysis for StAR protein (B) in SH-SY5Y cells stimulated with 1.0 nM GnRH. P450scc mRNA levels (C) in SH-SY5Y cells stimulated with 1.0 nM GnRH. For both target genes, values are normalized using GAPDH as housekeeping gene and reported as x-fold variation vs control. Values for each sample represent the mean \pm SE of the RT triplicate measured in duplicate (6 measures in total). Three experiments were performed.

most of the studies. Seladin-1/DHCR24 expression was significantly up-regulated after 90 min of incubation, down-regulated after 6 h, then it was again up-regulated after 24 h and returned to its basal value at 48 and 72 h (Fig. 1A). Similar results were obtained with FNC, in particular also in these cells only 1.0 nM GnRH was able to modulate seladin-1/DHCR24 expression, that was up-regulated after 90 min but resulted unchanged at the other incubation times (Fig. 1B).

3.2. GnRH modulates the expression of StAR and P450scc

The same GnRH doses and incubation times were used to verify if GnRH was able to promote steroidogenesis by modulating the expression of the genes codifying for StAR and P450scc. StAR protein is responsible for cholesterol transport into the inner mitochondrial membrane and this represents the rate limiting step of steroidogenesis. Cholesterol is then converted into the first steroid,

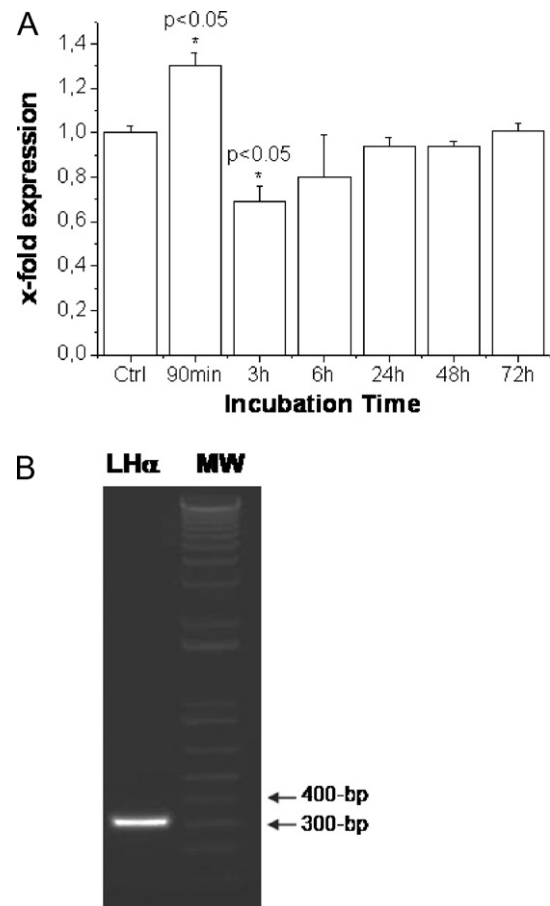


Fig. 3. LH expression after GnRH stimulus. A: LHβ mRNA in SH-SY5Y cells stimulated with 1.0 nM GnRH. Values are normalized using GAPDH as housekeeping gene and reported as x-fold variation vs control. Values for each sample represent the mean \pm SE of the RT triplicate measured in duplicate (6 measures in total). Three experiments were performed. B: PCR amplification of LHα subunit in untreated SH-SY5Y cells. The size of the PCR product is 320-bp.

pregnenolone, by P450scc [33]. Similarly to what reported for seladin-1/DHCR24, GnRH was active in the same narrow range around 1.0 nM in SH-SY5Y. A significant up-regulation of StAR mRNA expression was observed after 90 min and 3 h of incubation, values returned to the basal levels after 6 h and then remained unchanged (Fig. 2A). Moreover, protein expression of StAR was confirmed by western blot analysis. Results showed a strong increase of 37 kDa and 30 kDa forms of StAR protein after 90 min and 3 h of incubation with 1.0 nM GnRH (Fig. 2B).

In addition, in these cells we registered a significant, up-regulation of the expression of P450scc after 3 and 6 h of treatment with 1.0 nM GnRH that subsequently come back to the basal levels (Fig. 2C). In FNC GnRH was unable to modulate StAR protein and P450scc expression at any concentration tested from 0.01 to 1000 nM (not shown).

3.3. GnRH activity is mediated by LH

GnRH induces the expression of LHβ subunit in human neuroblastoma cells M17 [17]. We examined whether the same phenomenon occurs in SH-SY5Y cells. We used these cells because, differently from FNC, they were able to respond to GnRH through the modulation of the expression of steroidogenesis related genes. GnRH 1 nM was able to up-regulate the expression of the LHβ subunit after 90 min of incubation in SH-SY5Y (Fig. 3A). LH is composed

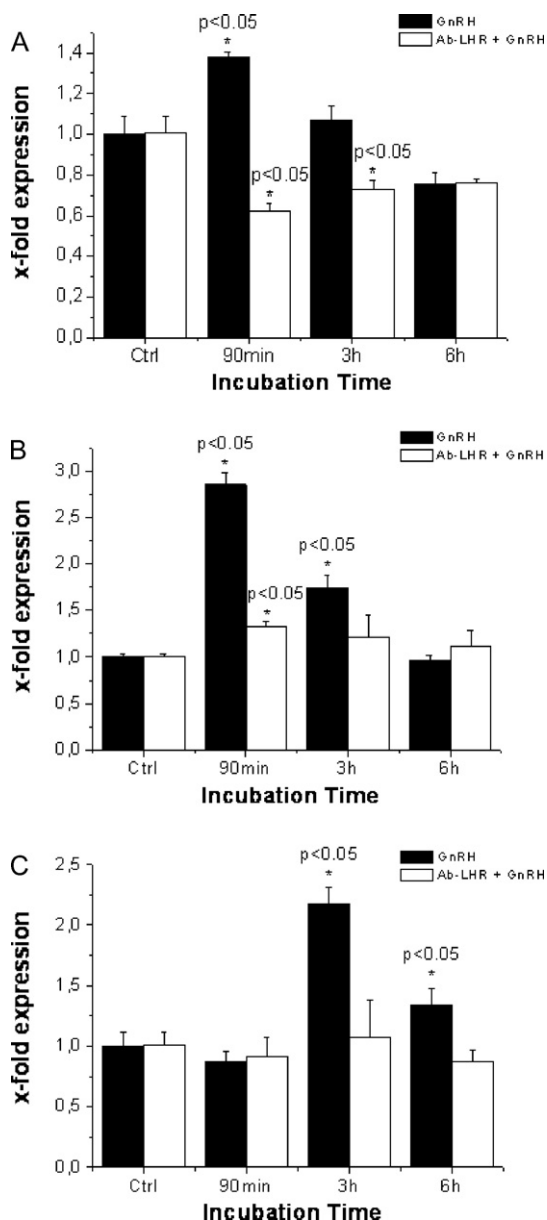


Fig. 4. Seladin-1/DHCR24, StAR and P450scc expression after GnRH stimulus and LH-Receptor blockage. Seladin-1/DHCR24 mRNA (A), StAR mRNA (B) and P450scc mRNA (C) in SH-SY5Y cells stimulated with 1.0 nM GnRH after the blockage of LH-Receptor with Ab-LHR (1:200). Values are normalized using GAPDH as housekeeping gene and reported as x-fold variation vs control. Values for each sample represent the mean \pm SE of the RT triplicate measured in duplicate (6 measures in total). Three experiments were performed.

by two subunits. We verified by PCR if these cells constitutively express the LH α subunit (Fig. 3B).

To assess whether the effect of GnRH is mediated by LH, we incubated SH-SY5Y cells with 1.0 nM GnRH in the presence of an antibody directed towards an extracellular domain of the LH-receptor. mRNA levels of seladin-1/DHCR24, StAR and P450scc were quantified by quantitative RT-PCR after 90 min, 3 and 6 h of incubation. The LH-receptor blockage operated by the antibody completely reverted the up-regulation pattern observed with 1.0 nM GnRH for all the three genes considered so demonstrating the involvement of the LH signal in this phenomenon (Fig. 4A–C). To definitely demonstrate this point we successively incubated SH-SY5Y cells with LH 100 mIU/ml finding a modulation pattern of

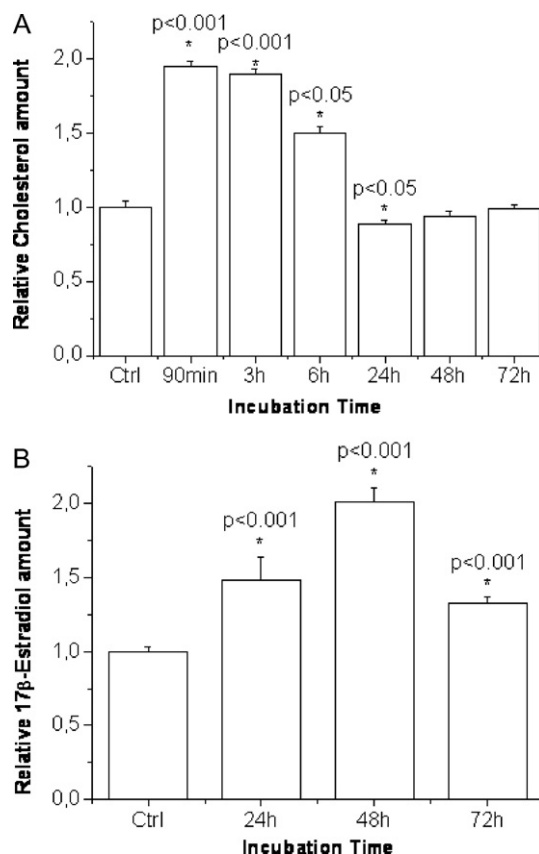


Fig. 5. Cell Cholesterol and E2 production after GnRH stimulus. A: Total cell cholesterol in SH-SY5Y cells stimulated with 1.0 nM GnRH. Values are reported as fold variation vs control. The absolute amount of cholesterol measured for the control is 19.51 ± 0.62 ng cholesterol/ μ g protein. Each point is performed in triplicate and the results represent the mean \pm SE of five experiments. B: E2 amount released in the medium of SH-SY5Y cells stimulated with 1.0 nM GnRH. The absolute amount of E2 measured for the control is 1.75 ± 0.19 pg/ml. Values are reported as fold variation vs control. Each point is performed in quadruplicate and the results represent the mean \pm SE of two experiments.

the three genes similar to that induced by 1.0 nM GnRH (data not shown).

3.4. GnRH modulates cell cholesterol and E2 synthesis in SH-SY5Y cells

To verify whether the modulation of the expression of seladin-1/DHCR24 operated by 1.0 nM GnRH induces a variation of cell cholesterol we quantified this sterol by GC/MS in total cell lysates after incubating SH-SY5Y for 90 min, 3, 6, 24, 48 and 72 h. The results demonstrate a strong increment of cell cholesterol after 90 min, values remain up-regulated at 3 and 6 h, significantly decrease after 24 h to definitively return to the basal level after 48 h (Fig. 5A). These variations can be linked to the modulation of the expression of seladin-1/DHCR24 supposing that the up-regulation of this enzyme after 90 min induces a rapid increment of cholesterol synthesis. The decrement of cell cholesterol after 24 h, that could be due to its consumption during the steroidogenic process, provokes a new up-regulation of seladin-1/DHCR24 expression that restores basal cholesterol levels. Cholesterol levels were not significantly increased after 1.0 nM GnRH treatment in FNC cells (data not shown).

Recently it was described that GnRH stimulates E2 synthesis in rat primary hippocampal cultures. To assess whether GnRH exposure causes E2 production in our human cell model we quantified the amount of E2 released in the culture medium after incubation

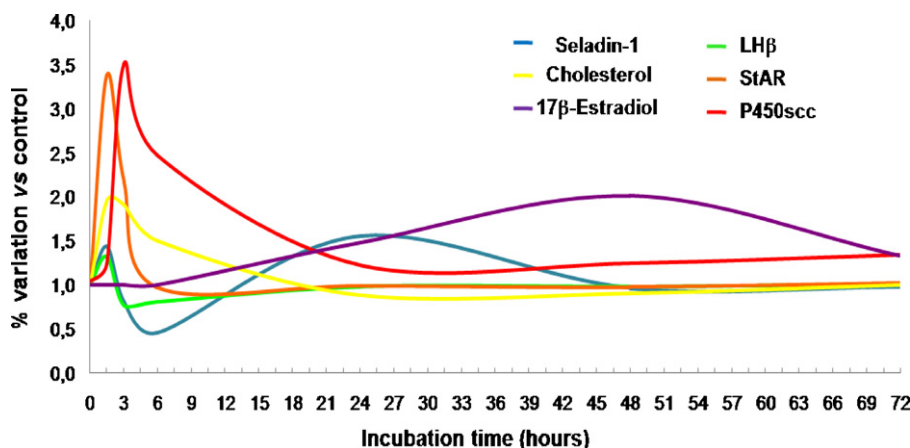


Fig. 6. Effect of GnRH on steroidogenesis in SH-SY5Y cells. Summary of the modulation of seladin-1/DHCR24, StAR, P450scc and LH β expression, cell cholesterol amount and E2 production after treatment of SH-SY5Y cells with 1.0 nM GnRH. For each target the variation is represented by a smoothed curve connecting the experimental points.

with 1.0 nM GnRH. E2 concentration results significantly increased after 24, 48 and 72 h of incubation indicating that 1.0 nM GnRH induces a steroidogenic impulse directed to the synthesis of E2 (Fig. 5B).

Fig. 6 summarizes the modulation of all the targets analyzed in SH-SY5Y cells after treatment with 1 nM GnRH each represented by a smoothed curve connecting the experimental points. This figure well represents the global neurosteroidogenic process triggered by GnRH. This process starts with a rapid rise of LH β subunit, Seladin-1/DHCR24 and cell cholesterol, followed by an up-regulation of P450scc and a contemporary consumption of cholesterol to form successively E2 as the end product.

4. Discussion

These results demonstrate, for the first time in a human neuronal cell model, that GnRH stimulates steroidogenesis enhancing both cholesterol and estrogen biosynthesis. GnRH is active only at a narrow range around 1 nM while lower or higher concentrations are unable to stimulate steroidogenesis. The first GnRH effect is registered after 90 min with the up-regulation of LH β mRNA. At this incubation time we observed the increase of seladin-1/DHCR24 expression, cell cholesterol amount and StAR protein. This is followed by the up-regulation of P450scc expression after 3 h. The antibody used to block the LH-receptor completely reverts the GnRH effect strongly suggesting that, similar to the HPG axis, GnRH action is mediated by LH. The data obtained stimulating cells directly with LH further support this hypothesis. The final event of GnRH action is the enhancement of E2 synthesis that is registered after 24, 48, and 72 h. Our results are in agreement with data obtained by Prange-Kiel et al. [19] on rat hippocampal slices and primary cells treated with GnRH. In fact also in these rat samples GnRH acts with a bell shaped dose response curve and the final steroid synthesized is estradiol. Our data add new interesting information demonstrating that GnRH action, in a human neuronal-like cell model, is mediated by LH and modulates also the last step of cholesterol biosynthesis, enhancing cell cholesterol.

Finally the studies presented here, although based on a neuroblastoma cell line, suggest a new physiological mechanism for estrogen local synthesis in the brain through the action of GnRH on GnRH responsive cells. This hormone could act as a common modulator of cholesterol and estrogen biosynthesis by an autocrine-paracrine mechanism, strongly supporting the presence of a local miniature endocrine axis. It remains to be seen whether neurons responsive to GnRH in the CNS may respond similarly

in situ. Estrogens are fundamental neuro-steroids with a trophic and protective role in the CNS [34,35] and the elucidation of this regulatory mechanism for their local synthesis will add new information on the CNS. In FNC cells, differently from SH-SY5Y cells, 1.0 nM GnRH does not represent a steroidogenic input. This point can be explained considering that FNC cells are a long term primary culture of foetal neuroblast described as already committed to become GnRH secreting neurons even if not completely differentiated. The physiological role of GnRH secreting cells is to respond to circulating levels of sex steroids and regulate GnRH secretion for a correct functionality of the HPG axis. For this reason an autocrine-paracrine production of E2 triggered by secreted GnRH could represent an interference for their physiological role and results inhibited. In agreement with this hypothesis it has been reported that the hypothalamus, that is under the control of circulating steroids for the regulation of its neuroendocrine function, shows a relatively low expression of StAR compared with other brain areas. This reflects the low local steroid production in an area whose function is dependent on systemic hormonal steroids [36].

Acknowledgements

This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale: PRIN2006 n.2006069900, to G.D.) and from Ente Cassa di Risparmio di Firenze.

References

- [1] M.A. Zmijewski, R.K. Sharma, A.T. Slominski, Expression of molecular equivalent of hypothalamic-pituitary-adrenal axis in adult retinal pigment epithelium, *J. Endocrinol.* 193 (2007) 157–169.
- [2] A. Slominski, J. Wortsman, R.C. Tuckey, R. Paus, Differential expression of HPA axis homolog in the skin, *Mol. Cell. Endocrinol.* 265–266 (2007) 143–149.
- [3] K.M. Kayes-Wandover, P.C. White, Steroidogenic enzyme gene expression in the human heart, *J. Clin. Endocrinol. Metab.* 85 (2000) 2519–2525.
- [4] C. Corpéchet, P. Robel, M. Axelson, J. Sjövall, E.E. Baulieu, Characterization and measurement of dehydroepiandrosterone sulfate in rat brain, *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 4704–4707.
- [5] J. Matsuzaki, T. Tsuji, I. Imazeki, H. Ikeda, T. Nishimura, Immunosteroid as a regulator for Th1/Th2 balance: its possible role in autoimmune diseases, *Autoimmunity* 38 (2005) 369–375.
- [6] H.R. Waterham, Defects of cholesterol biosynthesis, *FEBS Lett.* 580 (2006) 5442–5449.
- [7] S.M. Eacker, N. Agrawal, K. Qian, H.L. Dichek, E.Y. Gong, K. Lee, R.E. Braun, Hormonal regulation of testicular steroid and cholesterol homeostasis, *Mol. Endocrinol.* 22 (2008) 623–635.
- [8] C. Corpéchet, M. Synguelakis, S. Talha, M. Axelson, J. Sjövall, R. Vihko, E.E. Baulieu, P. Robel, Pregnenolone and its sulfate ester in the rat brain, *Brain Res.* 270 (1983) 119–125.

- [9] M. Schumacher, Y. Akwa, R. Guennoun, F. Robert, F. Labombarda, F. Desarnaud, P. Robel, A.F. De Nicola, E.E. Baulieu, Steroid synthesis and metabolism in the nervous system: trophic and protective effects, *J. Neurocytol.* 29 (2000) 307–326.
- [10] I. Charalampopoulos, E. Remboutsika, A.N. Margioris, A. Gravanis, Neurosteroids as modulators of neurogenesis and neuronal survival, *Trends Endocrinol. Metab.* 19 (2008) 300–307.
- [11] S.R. King, Emerging roles for neurosteroids in sexual behavior and function, *J. Androl.* 29 (2008) 524–533.
- [12] R.C. Melcangi, L.M. Garcia-Segura, A.G. Mensah-Nyagan, Neuroactive steroids: state of the art and new perspectives, *Cell. Mol. Life Sci.* 65 (2008) 777–797.
- [13] S.H. Mellon, Neurosteroid regulation of central nervous system development, *Pharmacol. Ther.* 116 (2007) 107–124.
- [14] J.L. Do Rego, J.Y. Seong, D. Burel, J. Leprince, V. Luu-The, K. Tsutsui, M.C. Tonon, G. Pelletier, H. Vaudry, Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides, *Front. Neuroendocrinol.* 30 (2009) 259–301.
- [15] K.L. Schmidt, D.S. Pradhan, A.H. Shah, T.D. Charlier, E.H. Chin, K.K. Soma, Neurosteroids, immunosteroids, and the Balkanization of endocrinology, *Gen. Comp. Endocrinol.* 157 (2008) 266–274.
- [16] A.J. Albertson, A. Navratil, M. Mignot, L. Dufourny, B. Cherrington, D.C. Skinner, Immunoreactive GnRH type I receptors in the mouse and sheep brain, *J. Chem. Neuroanat.* 35 (2008) 326–333.
- [17] A.C. Wilson, M.S. Salamat, R.J. Haasl, K.M. Roche, A. Karande, S.V. Meethal, E. Terasawa, R.L. Bowen, C.S. Atwood, Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression, *J. Endocrinol.* 191 (2006) 651–663.
- [18] T. Liu, J. Wimalasena, R.L. Bowen, C.S. Atwood, Luteinizing hormone receptor mediates neuronal pregnenolone production via up-regulation of steroidogenic acute regulatory protein expression, *J. Neurochem.* 100 (2007) 1329–1339.
- [19] J. Prange-Kiel, H. Jarry, M. Schoen, P. Kohlmann, C. Lohse, L. Zhou, G.M. Rune, Gonadotropin-releasing hormone regulates spine density via its regulatory role in hippocampal estrogen synthesis, *J. Cell Biol.* 180 (2008) 417–426.
- [20] M. Mulder, Sterols in the central nervous system, *Curr. Opin. Clin. Nutr. Metab. Care* 12 (2009) 152–158.
- [21] J.M. Dietschy, Central nervous system: cholesterol turnover, brain development and neurodegeneration, *Biol. Chem.* 390 (2009) 287–293.
- [22] I. Greeve, I. Hermans-Borgmeyer, C. Brellinger, D. Kasper, T. Gomez-Isla, C. Behl, B. Levkau, R.M. Nitsch, The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress, *J. Neurosci.* 20 (2000) 7345–7352.
- [23] S. Benvenuti, P. Luciani, G.B. Vannelli, S. Gelmini, E. Franceschi, M. Serio, A. Peri, Estrogen and selective estrogen receptor modulators exert neuroprotective effects and stimulate the expression of selective Alzheimer's disease indicator-1, a recently discovered antiapoptotic gene, in human neuroblast long-term cell cultures, *J. Clin. Endocrinol. Metab.* 90 (2005) 1775–1782.
- [24] P. Luciani, C. Deledda, F. Rosati, S. Benvenuti, I. Cellai, F. Dichiarara, M. Morello, G.B. Vannelli, G. Danza, M. Serio, A. Peri, Seladin-1 is a fundamental mediator of the neuroprotective effects of estrogen in human neuroblast long-term cell cultures, *Endocrinology* 149 (2008) 4256–4266.
- [25] M.J. Cooper, G.M. Hutchins, P.S. Cohen, L.J. Helman, R.J. Mennie, M.A. Israel, Human neuroblastoma tumor cell lines correspond to the arrested differentiation of chromaffin adrenal medullary neuroblasts, *Cell Growth Differ.* 1 (1990) 149–159.
- [26] S. Pahlman, S. Mamaeva, G. Meyerson, M.E. Mattsson, C. Bjelfman, E. Ortoft, U. Hammerling, Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function, *Acta Physiol. Scand. Suppl.* 592 (1990) 25–37.
- [27] G.B. Vannelli, F. Ensoli, R. Zonefrati, Y. Kubota, A. Arcangeli, A. Becchetti, G. Camici, T. Barni, C.J. Thiele, G.C. Balboni, Neuroblast long-term cell cultures from human fetal olfactory epithelium respond to odors, *J. Neurosci.* 15 (1995) 4382–4394.
- [28] I. Casella, H. Lindner, C. Zenzmaier, D. Riitano, P. Berger, T. Costa, Non-gonadotropin-releasing hormone-mediated transcription and secretion of large human glycoprotein hormone alpha-subunit in human embryonic kidney-293 cells, *Endocrinology* 149 (2008) 1144–1154.
- [29] P. Luciani, P. Ferruzzi, G. Arnaldi, C. Crescioli, S. Benvenuti, A. Valeri, I. Greve, M. Serio, M. Mannelli, A. Peri, Expression of the novel adrenocorticotropin-responsive gene selective Alzheimer's disease indicator-1 in the normal adrenal cortex and in adrenocortical adenomas and carcinomas, *J. Clin. Endocrinol. Metab.* 89 (2004) 1332–1339.
- [30] X. Wu, T. Iguchi, N. Itoh, K. Okamoto, T. Takagi, K. Tanaka, T. Nakanishi, Ascorbic acid transported by sodium-dependent vitamin C transporter 2 stimulates steroidogenesis in human choriocarcinoma cells, *Endocrinology* 149 (2008) 73–83.
- [31] C. Cecchi, F. Rosati, A. Pensalfini, L. Formigli, D. Nosi, G. Liguri, F. Dichiarara, M. Morello, G. Danza, G. Pieraccini, A. Peri, M. Serio, M. Stefani, Seladin-1/DHCR24 protects neuroblastoma cells against amyloid toxicity by increasing membrane cholesterol content, *J. Cell. Mol. Med.* 12 (2008) 1990–2002.
- [32] M.M. Bradford, A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [33] I. Hanukoglu, Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis, *J. Steroid Biochem. Mol. Biol.* 43 (1992) 779–804.
- [34] C.M. Brown, S. Suzuki, K.A. Jelks, P.M. Wise, Estradiol is a potent protective, restorative, and trophic factor after brain injury, *Semin. Reprod. Med.* 27 (2009) 240–249.
- [35] R.D. Brinton, Estrogen-induced plasticity from cells to circuits: predictions for cognitive function, *Trends Pharmacol. Sci.* 30 (2009) 212–222.
- [36] E. Lavaque, A. Sierra, I. Azcoitia, L.M. Garcia-Segura, Steroidogenic acute regulatory protein in the brain, *Neuroscience* 138 (2006) 741–747.