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Review

New insights on the neuroprotective role of sterols and sex steroids: The seladin-1/DHCR24 paradigm

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ABSTRACT

In 2000 a new gene, i.e. *seladin-1* (for selective Alzheimer's disease indicator-1) was identified and found to be down regulated in vulnerable brain regions in Alzheimer's disease. Seladin-1 was considered a novel neuroprotective factor, because of its anti-apoptotic properties. Subsequently, it has been demonstrated that *seladin-1* corresponds to the gene that encodes 3-beta-hydroxysterol delta-24-reductase (*DHCR24*), that catalyzes the synthesis of cholesterol from desmosterol. There is evidence that cholesterol plays a fundamental role in maintaining brain homeostasis. Because of its enzymatic activity, seladin-1/DHCR24 has been considered the human homolog of the plant protein DIMINUTO/DWARF1, that is involved in the synthesis of sterol plant hormones. We have recently demonstrated that seladin-1/DHCR24 is a fundamental mediator of the protective effects of estrogens in the brain. This review describes how this protein interacts with cholesterol and estrogens, thus generating a neuroprotective network, that might open new possibilities in the prevention/treatment of neurodegenerative diseases.

1. Introduction

The identification at the beginning of the new millennium of the seladin-1 gene, considered the human homolog of the plant *DIMINUTO/DWARF1* gene primarily described in *Arabidopsis thaliana* [89,42], promised to significantly contribute to the advancement of our knowledge about neurodegenerative processes [33]. In that study reduced expression levels of seladin-1 in the brain were associated to the most common neurodegenerative disease in the elderly, i.e. Alzheimer's disease (AD) [84]. Soon after its first description, it was noticed that seladin-1 is identical to the gene encoding the enzyme 3-beta-hydroxysterol delta-24-reductase (*DHCR24*), involved in the cholesterol biosynthetic pathway. There is evidence that the intracellular amount of cholesterol, that is also a fundamental component of the membrane microdomains named lipid rafts, plays an important role in brain physiology. In addition, the apparent close link between the neuroprotective properties of seladin-1 and those ascribed to sex steroids, and in particular to estrogens, led us to hypothesize that the newly described protein might be a mediator of the neuroprotective effects of these hormones. This review will summarize the initial observations regarding the identification and characterization of seladin-1, together with its plant counterpart DIMINUTO/DWARF1. Then, the review will address the two main issues relating seladin-1 to neuroprotection, i.e. its relationship with cholesterol on one hand and with estrogens on the other hand. Undoubtedly, these are major issues in the field of neurodegenerative diseases and in particular of AD, considering for instance that there is still no reliable way of preventing or curing this disease.

2. Seladin-1/DHCR24

2.1. The identification and the expression pattern of seladin-1

The story of seladin-1 began in 2000, when Greeve and colleagues identified a new gene in the attempt to identify, by using a differential mRNA display approach, genes that were differentially expressed in selective vulnerable brain regions in AD [33], such as the hippocampus, the amygdala, the inferior temporal cortex and the enthorhinal cortex [84]. Among the over 30 genes differentially expressed in AD vulnerable brain regions vs. unaffected areas, the authors identified a novel cDNA with a markedly reduced expression in the inferior temporal cortex of AD patients compared to the frontal cortex, obtained shortly post-mortem. The new gene was named *seladin-1* from selective Alzheimer's disease INdicator-1. Conversely, *seladin-1* was evenly expressed in the brain of unaffected individuals. A subsequent study demonstrated that the down-regulation of seladin-1 expression in vulnerable AD brain areas is paralleled by an increase in the amount of

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hyperphosphorylated tau, a protein component of neurofibrillary tangles [39]. This gene (GenBank accession number AF261758) spans 46.4 kb, maps to chromosome 1p31.1–p33, and comprises nine exons and eight introns; it encodes an open reading frame of 516 amino acid residues. Seladin-1 is located in the endoplasmic reticulum and, although to a lesser extent, in the Golgi apparatus [33].

Seladin-1 expression has been also detected in many different organs apart the brain, where it is also detectable for instance in the thalamus, substantia nigra, caudate nucleus and corpus callosum [33], including endocrine glands, such as adrenal [33,81,51,6], pituitary [33,53], thyroid gland [33], ovary [33,31], testis [33], or endocrine-related organs, such as the prostate [10,36,24,12].

2.2. The anti-apoptotic role of seladin-1

Seladin-1 was originally found to confer resistance against β-amyloid and oxidative stress-induced apoptosis and to effectively inhibit the activation of caspase-3, a key mediator of the apoptotic process. Interestingly, in PC12 cells (rat adrenal pheocromocytoma) that were selected for resistance against β-amyloid toxicity, the level of expression of seladin-1 was remarkably high [33]. The anti-apoptotic property of seladin-1 has been confirmed in other cell models, including human pituitary adenoma cells. In this case, seladin-1, by inhibiting the apoptoptic cascade, was associated both to a more aggressive behavior of tumor cells and to resistance to pharmacological treatment [53]. Similarly, seladin-1 was associated to resistance against oxidative stress-induced apoptosis in melanoma cells [23].

Very recently it has been demonstrated that the ability of seladin-1 to protect against apoptosis elicited by oxidative stress is due, at least in part, to the scavenger activity of this protein [50]. The authors of the study showed that intracellular generation of reactive oxygen species (ROS) in response to H_2O_2 was diminished in embryonic mouse fibroblasts expressing seladin-1, compared to cells in which the expression had been abolished, thus suggesting a ROS-scavenging activity of this protein. This hypothesis was validated by the observation that intact seladin-1 determined high H_2O_2-scavenging activity, whereas an N-terminal deletion caused loss of this activity. The scavenger activity of seladin-1 was only moderately lower than that of catalase, a well known H_2O_2-scavenging enzyme. Another study addressed this protein as a key mediator of Ras-induced senescence [104]. In this study it was shown that, following oncogenic and oxidative stress, seladin-1 binds p53 in fibroblasts and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. Ablation of seladin-1 caused the bypass of Ras-induced senescence, and allowed Ras to transform cells. Wild-type seladin-1 cells, but not mutants that disrupt its association with either p53 or Mdm2, were able to suppress the transformed phenotype. These results showed an unanticipated role for seladin-1 in integrating cellular response to oncogenic and oxidative stress. Noteworthy, a very recent publication proposed an unifying interpretation of these data. In this study neuroblastoma SH-SY5Y cells were subjected to acute or chronic oxidative stress. Following acute stress, seladin-1 expression increased and the over expression conferred resistance to H_2O_2-induced toxicity. Conversely, chronic exposure to oxidative stress diminished the expression of seladin-1, but the protective effect was maintained. In fact, reduced seladin-1 levels prevented apoptosis in a p53-dependent manner, via increased p53 ubiquitination and degradation [44].

2.3. The enzymatic activity of seladin-1 and desmosterolosis

In addition to its clearly described role in modulating apoptosis, an additional corner stone in unraveling the biological properties of seladin-1 was represented by the demonstration that this protein has also a specific enzymatic activity, which was found to be markedly reduced in desmosterolosis, a rare autosomal recessive disorder characterized by multiple congenital anomalies [28]. Patients affected by this disease have elevated plasma levels of the cholesterol precursor desmosterol and this abnormality suggested a deficiency of the *DHCR24* enzyme, that catalyzes the reduction of the Δ^{24} double bond in desmosterol to produce cholesterol (Fig. 1). Waterham and colleagues identified the human *DHCR24* cDNA, that was identical to the *seladin-1* cDNA [102]. Seladin-1 will be therefore more properly indicated as seladin-1/DHRC24 for the remaining part of this review. *DHCR24* activity was confirmed *in vitro* by enzymatic assay following heterologous expression of the *DHCR24* cDNA in *Saccharomyces cerevisiae*. In yeast homogenates *DHCR24* activity was strictly NADPH dependent, although no common consensus sequence for an NADPH-binding site can be found in the *DHCR24* amino acid sequence. In this enzyme-activity assay the production of cholesterol from desmosterol increased twofold in the presence of FAD, indicating the functionality of the conserved domain characteristic of FAD-dependent oxidoreductases found in the *DHCR24* amino acid sequence [102]. Conversely, in constructs containing mutant *DHCR24* alleles from patients with desmosterolosis the conversion from desmosterol into cholesterol was absent or markedly reduced.

Desmosterolosis belongs to a group of several inherited disorders, linked to enzyme defects in the cholesterol biosynthetic pathway at the post-squalene level, which have been described in recent years [37]. These diseases include Smith–Lemli Opitz syndrome, that was the first to be described in 1964 and in which 3β-hydroxisterol Δ^7-reductase is defective, lathosterolosis (defective 3β-hydroxisterol Δ^5-desaturase), CHILD syndrome (defective 3β-hydroxysteroid dehydrogenase), Greenberg displasia (defective 3β-hydroxisterol Δ^{14}-reductase and Conradi-Hunermann syndrome (defective 3β-hydroxisterol Δ^8–Δ^7 isomerase. These genetic diseases are biochemically characterized by reduced plasma cholesterol levels and their clinical presentation shares some common features, including major developmental malformations and in most cases severe neuropsychological alterations, thus suggesting an important role for cholesterol in brain homeostasis.

2.4. Seladin-1/DHCR24 is the human homolog of the plant DIMINUTO/DWARF1 gene

DIMINUTO/*DWARF1* is a gene encoding an enzyme involved in the biosynthetic pathway of the most active brassinosteroid,

Fig. 1. The enzymatic step catalyzed by seladin-1/DHCR24.

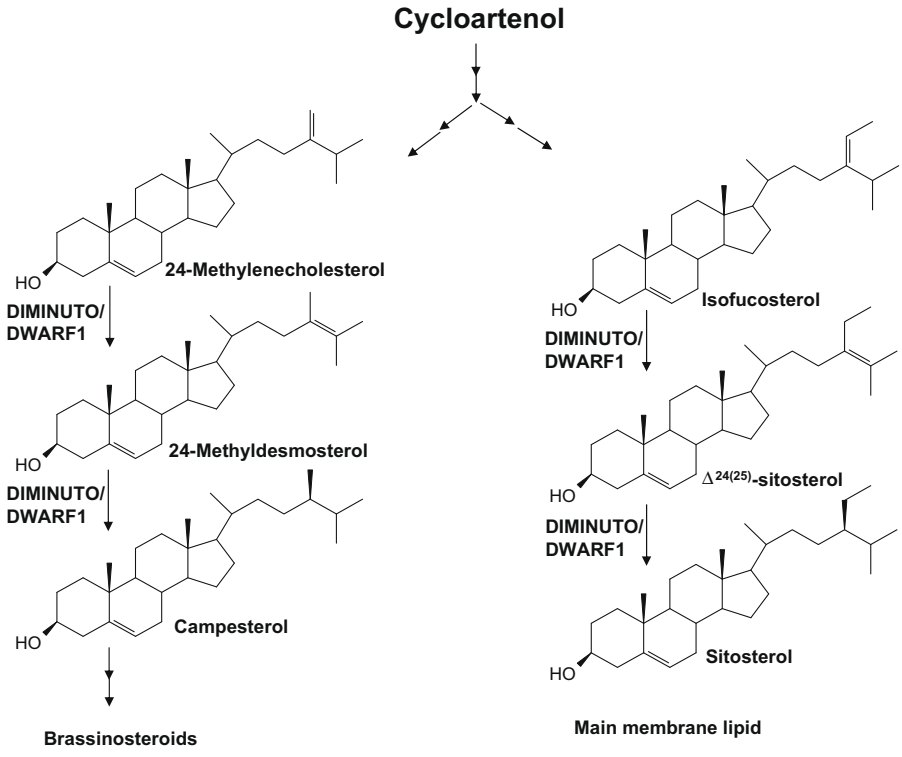

Fig. 2. The enzymatic steps catalyzed by DIMINUTO/DWARF1.

brassinolide [82] (Fig. 2). Brassinosteroids (BRs) are a class of sterols plant hormones that can be considered as the counterpart of animal steroid hormones. They have been shown to regulate gene expression, stimulate cell division and differentiation, and modulate reproductive plant biology [4]. BRs also mediate growth response unique to plants, including the promotion of cell elongation in the presence of a complex cell wall and the multiple developmental responses to darkness and light.

The function of DIMINUTO/DWARF1 was identified analyzing the *dim* mutant of *A. thaliana*, which shows a severe dwarf phenotype with reduced fertility [42]. The mutant phenotype could be rescued by the addition of exogenous brassinolide or brassinolide precursors, indicating a role in BRs biosynthesis. In particular, DIMINUTO/DWARF1 is involved in the synthesis of camposterol, the key precursor of BRs, and of the main plant membrane sterol lipid sitosterol. Animals mainly synthesize cholesterol, which serves as a precursor of steroid hormones after the cleavage of the alkyl side chain, and as a membrane sterol lipid. Plants use campesterol as a hormone precursor and sitosterol as membrane lipid together with stigmasterol. Thus, the plant protein DIMINUTO/DWARF1 and seladin-1/DHCR24 share a similar function as sterols biosynthetic enzymes. The defect of this function does not influence plant embryogenesis, that seems to be influenced by upstream precursors of 24-methylencholesterol (24-methylenlophenol) [18]. Moreover, the disruption of the *DIMINUTO*/*DWARF1* gene seems to expand the life span of the *dim* mutant and this phenomenon is probably associated with the reduced fertility of the plant [17]. Interestingly, *Arabidopsis* with a mutation in one of the genes homologs of 3-hydroxy-3-methylglutaryl-CoA reductase [88], the first step in the isoprenoid biosynthesis, shows an early senescence indicating that the roles of plant sterols are probably more diversified and structure-dependent than in animals, in which cholesterol seems to perform multiple functions.

Similarity between BRs and animal sterols/steroids synthesis is not limited to DIMINUTO/DWARF1. As an example DET2, the enzyme that catalyzes the 5α reduction of campestenone in 5α-campestanone, shares a consistent grade of similarity with mammalian 5α-reductase, involved in the production of dihydrotestosterone from testosterone. Similarly to mammals, we have demonstrated and characterized two separate 5α-reductase activities also in plant tissues (*Solanum malacoxylon*) and we have shown that plant enzymes can recognize human substrates and *vice versa* [77,78].

The ability of human enzymes to use plant substrates is a very interesting issue and, noteworthy, plant-derived sterols have been found in mammal brain [54], opening a new window on their possible therapeutic use in human diseases.

3. Seladin-1/DHCR24 and AD: a multifaceted association

The initial identification of seladin-1/DHCR24 in vulnerable brain regions of AD patients established a close relationship between this protein and the disease. The main aim of the experimental work performed in the last few years was to unravel different aspects underlying this association, such as: the reason for reduced expression in vulnerable brain areas in AD, the relationship between seladin-1/DHCR24, cholesterol and neuroprotection, and the relationship between seladin-1/DHCR24, estrogens and neuroprotection. The remaining part of this review will cover these issues.

3.1. Seladin-1/DHCR24 down-regulation in AD brain: possible hypotheses

The fact that some of the brain regions affected in AD, namely the hippocampus and the subventricular zone, correspond to the areas which host stem cells with neurogenic potential and migratory activity in the adult brain, led us to hypothesize that seladin-1 might be a predominant product of multipotent cells. Therefore, we compared the amount of expression of the *seladin-1*/*DHCR24*

gene in stem cells and in neuronal-like cells derived from them. We used human mesenchymal stem cells (hMSC) as a multipotent cell model, because they are much more easily obtainable than neuronal stem cells and can be readily differentiated into neurons. We found that seladin-1/DHCR24 is abundantly expressed in hMSC, whereas the level of expression markedly decreased when these cells were induced to differentiate into mature neurons. In addition, we detected high levels of expression of seladin-1/DHCR24 in the human adult hippocampus and spinal cord, which have been shown to contain neural stem cells with neurogenic activity [9]. These findings led us to hypothesize that defective seladin-1/DHCR24 expression detected in AD vulnerable brain regions might be linked to an impaired neuronal stem cell compartment, that could be a potential risk factor to develop this disease. However, in the absence of a direct demonstration, it cannot be excluded at present that reduced seladin-1/DHCR24 expression in AD may be due to a modification of the neurogenic properties of the adult brain as a consequence of neuronal damage and therefore of AD itself.

Additional explanations for the reduced seladin-1/DHCR24 expression in brain areas affected in AD may be hypothesized, including for instance an altered methylation pattern. With regard to this point, we have recently demonstrated that the reduced expression of this gene detected in adrenocortical carcinomas compared to adenomas is inversely related to the methylation status of the promoter region (L. Simi, F. Malentacchi, P. Luciani, S. Gelmini, C. Deledda, M. Mannelli, A. Peri and C. Orlando, submitted for publication). These evaluations should be obviously extended to neuronal cells, in order to ascertain whether a different methylation status may determine the altered expression pattern of seladin-1/DHCR24 in the brain of AD patients.

Finally, the possible presence of heterozygous mutations (homozygous mutations yield desmosterolosis) of the *seladin-1/DHCR24* gene in AD patients was investigated. To this purpose, 100 Italian patients with a strong family history of AD, and negative for mutations in *amyloid precursor protein*, *presenilin 1 and presenilin 2* genes were investigated. No seladin-1/DHCR24 mutation was detected in genomic DNA extracted from peripheral blood samples [91]. In another study performed in the Finnish population, four single nucleotide polymorphism sites were genotyped in more than 400 AD cases and control subjects and the allelic and genotypic distribution was calculated. Both risks and protective haplotypes were identified and the authors concluded that the *seladin-1/DHCR24* gene may be associated with AD risk, although they admitted that such an association should be more thoroughly studied using more markers [45].

3.2. Cholesterol and the brain: the role of seladin-1/DHCR24

Following the identification of the ε4 allelic variant of the apolipoprotein E as a major genetic risk factor for AD, a role for cholesterol in the pathogenesis of this disease was suggested. However, this is still an open and controversial issue at present and the published studies are divided between those who support the idea that cholesterol may favor the onset of the disease and those who, on the contrary, believe that cholesterol may play a protective role against AD. In particular, on one hand some reports showed that elevated cholesterol levels increase β-amyloid formation in *in vitro* systems and in animal models of AD [106,37]. Accordingly, epidemiological studies suggest that statin therapy may provide protection against AD, although the clinical benefit of statins might be also due to their cholesterol-independent effects on cerebral circulation and inflammation [74]. Furthermore, most of the commercially available statins do not cross the blood–brain barrier. It has to be considered that the central nervous system contains as much as 25% of the total amount of unesterified cholesterol in the entire body, that is mostly produced via local *de novo* synthesis [11]. Thus, it is not surprising that several studies pointed out the fact that the cellular content of cholesterol, particularly the amount contained in the cell membrane, should be addressed much more than the plasma levels [106]. If cell cholesterol is considered, an appropriate amount in the cell membrane would create a barrier against toxic insults, whereas a cholesterol-depleted membrane would facilitate the interaction with toxic factors such as β-amyloid. One of the mechanisms of β-amyloid toxicity is the generation of membrane pores permeable to toxic calcium ions. This mechanism is enhanced in cholesterol-depleted membranes [3]. Accordingly, reduced membrane lipids in the cortex of AD transgenic mice have been detected [107]. We have very recently provided evidence that over expression of seladin-1/DHCR24, as well as PEG-cholesterol treatment, increases resistance to β-amyloid toxicity and prevents calcium influx in neuroblastoma cells, whereas the exposure to a selective inhibitor of *DHCR24* enhances the toxic effect of β-amyloid, similarly to cholesterol depletion with methyl-β-cyclodextrin [16]. The amount of cell cholesterol may also affect amyloidogenesis. In particular, the link between cholesterol and β-amyloid production is related to the membrane localization of the enzymes involved in the processing of the Amyloid Precursor Protein (APP). The complete proteolytic machinery required for β-amyloid generation is located within lipid rafts, that are discrete liquid-ordered microdomains floating in the less-ordered liquid domains of the surrounding cell membrane [41,98]. Lipid rafts, or detergent-resistant membranes, consist of a dynamic assembly of cholesterol, sphingomyelin and glycosphingolipids. One of the most important properties of lipid rafts is that they can selectively include or exclude specific proteins to a variable extent. Because of this property, they play a fundamental role in the regulation of a wide range of important biological processes, including numerous signal transduction pathways, cell adhesion and migration, synaptic transmission, organization of the cytoskeleton, and protein sorting during both exocytosis and endocytosis [14,87,40,15]. Membrane cholesterol content is crucial for lipid rafts organization and it seems to regulate, in a dose dependent manner, the membrane localization and the activity of secretases involved in the metabolism of APP [90] Numerous molecular and cellular studies support the hypothesis that the amyloidogenic processing of APP occurs more efficiently in cholesterol-rich lipid rafts, whereas the non-amyloidogenic processing occurs mainly in other regions of the membrane [100,99,76]. Another hypothesis is that a moderate decrease in cholesterol levels results in increased β-amyloid production, whereas a more severe reduction in cholesterol decreases β-amyloid generation [19,1,41]. These authors suggested that cleavage of APP by β-secretase occurs outside of rafts and that a moderate reduction in cholesterol levels promotes the movement of β-secretase from rafts to non-raft regions of the membrane where it can reach the major pool of non-raft APP. The observation that in the brain from AD patients there are both reduced cholesterol levels and disrupted rafts is consistent with this theory. Moreover, plasmin, a lipid raft resident proteins, is involved in β-amyloid degradation and the impairment of its activity by lipid rafts disruption leads to β-amyloid accumulation [47].

Besides their involvement in β-amyloid production and degradation, lipid rafts seems to have an important physiological role in brain functionality. For this reason their deregulation may be detrimental for the central nervous system and promotes neurodegeneration by many other mechanisms, although this possibility has not been extensively studied, so far. A relationship between seladin-1/DHCR24 expression and lipid rafts has been proposed [19,49]. This issue might be investigated in principle in *DHCR24* null mice (*DHCR24*$^{-/-}$). These animals were generated for the first time in 2003 and, as expected, their plasma and tissues did not contain cholesterol, whereas desmosterol accumulation was

observed [103]. These animals were around 25% smaller in size than *DHCR24*$^{+/+}$ and *DHCR24*$^{+/-}$ littermates at birth and were not fertile. Therefore *DHCR24*$^{-/-}$ mice must be generated from heterozygous *DHCR24*$^{+/-}$ pairs. Surprisingly, in contrast to initial reports, animals that were subsequently generated showed a different viability. Some *DHCR24*$^{-/-}$ mice die within a few hours after birth and show a lethal dermopathy, associated with retention of epidermal water, in agreement with similar observations in patients with desmosterolosis [64,63]. Other *DHCR24*$^{-/-}$ mice have a longer lifespan. Brain cholesterol deficiency in 3-weeks-old *DHCR24*$^{-/-}$ animals was associated with altered membrane composition, including disrupted lipid rafts. In mice surviving up to 16 weeks an age-dependent accumulation of desmosterol in brain membranes led to the formation of desmosterol-containing lipid rafts rescuing the membrane-related functional deficits [43]. Besides the variable results obtained with knockout mice, overall these data appear to confirm the existence of a relationship between seladin-1/DHCR24 and lipid rafts composition. Human neuronal cell models, if available, might represent an additional choice to properly address this issue. To this purpose we have used human fetal neuroepithelial cells (FNC, whose isolation and characterization will be described later on in Section 3.3.2). These cells were transiently transfected with the seladin-1/DHCR24 open reading frame-enhanced green fluorescence protein fusion construct [33]. Thereafter, live cells were labeled with the red-fluorescent Alexa Fluor 594 conjugate of cholera toxin subunit B (CT-B). This CT-B conjugate binds the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into lipid rafts. Lipid rafts were visualized by confocal microscopy. As shown in Fig. 3 seladin-1/DHCR24 transfected cells present an enhanced signal compared to control cells indicating an increased content of lipid rafts at the cell membrane. This preliminary result confirms the role of seladin-1/DHCR24 in modulating lipid rafts content and suggests that its neuroprotective effects may depend, at least in part, on the assembly of these membrane microdomains. Nevertheless, a thorough experimental investigation is needed in order to verify whether this hypothesis is correct.

3.3. Estrogens and the brain: the role of seladin-1/DHCR24

3.3.1. Estrogens and neuroprotection

This topic has been extensively reviewed by many authors and it is covered also in this issue of *Frontiers in Neuroendocrinology*, and therefore it will be briefly summarized in this review. It is well known, based on *in vitro* evidence, that estrogens exert neurotrophic and neuroprotective effects by stimulating the expression of neurotrophins and cell-survival factors, enhancing synaptic plasticity, and acting as an antioxidant factor [7,57,94]. In addition to the hypothalamus, which is the traditional site of estrogens action in the brain, both the estrogen receptor α (ERα) and β (ERβ) have been found in different brain areas such as the neocortex and the hippocampus, two areas highly involved in AD [7]. Experimental evidence supports a favorable effect of estrogens in neurons, in agreement with the knowledge that AD is more common in women and that decreased estrogen levels after menopause are a risk factor for the disease [69]. Furthermore, *in vitro* studies indicate that estrogens are able to effectively reduce the production of β-amyloid, the histopathological hallmark of AD, from its precursor APP [94]. Thus, estrogens therapy has been considered a rationale option for the treatment of this disease. To date, despite the lack of general consensus, several studies indicated that estrogens treatment may decrease the risk or delay the onset of AD in post-menopausal women [27]. Conversely, the data from the Women's Health Initiative Memory Study (WHIMS) trial showed that hormone replacement therapy (HRT) has no benefit [86,73]. However, it has to be remembered that different factors may determine the efficacy of estrogens or HRT, such as age, the menopausal status, the route of administration and the dose, the starting cognitive function, and the presence of pre-existing risk factors (i.e. smoking, apolipoprotein E genotype) [56,94]. In particular, there seems to be a critical time for estrogens treatment. In fact, early and prolonged therapy has been found to produce the maximum benefit in terms of reduced risk for AD [108,35]. In addition, estrogens therapy is not the same as HRT and the type of progestogen used may determine the outcome of the therapeutic intervention [83]. In the WHIMS study a significant impact on dementia risk appeared for instance to be related to treatment with estrogens plus medroxyprogesterone acetate and not to estrogens alone. These data suggest that the progestin used in that study may have been responsible for the effect on dementia risk.

With regard to the ER involved in neuroprotection, the observations from ERα (ERKO) and ERβ (βERKO) knockout mice suggest a critical role for ERα. In fact, whereas 17β-estradiol exerted a protective effect in the brain of ovariectomized βERKO mice, it did not in ERKO mice [26]. This finding appears in agreement with the reported decreased expression of ERα in hippocampal neurons of AD patients [38]. However, a possible role of ERβ in neuroprotection has been postulated, based on the evidence that βERKO mice undergo increased neuronal loss throughout life compared to wild-type controls [101]. It has to be added that, in addition to classical nuclear ERs, more recent findings suggest that the brain contains a plethora of ERs, such as ERγ and a variety of nuclear

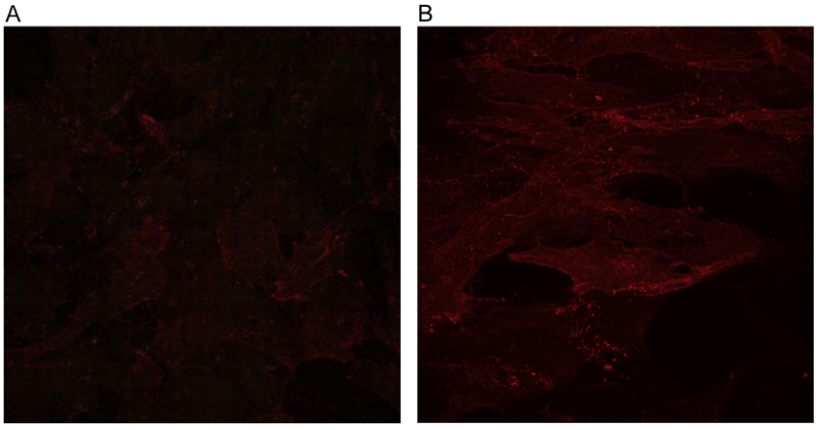

Fig. 3. (A) Control FNC cells (mean red fluorescence intensity = 15.5, arbitrary units) and (B) FNC cells transiently transfected with seladin-1/DHCR24 (mean red fluorescence intensity = 41.1).

as well as cytoplasmic and plasma membrane receptors [34,93,96,2].

The neuroprotective role of the Selective Estrogen Receptor Modulators (SERMs) has been less extensively investigated. Nonetheless, a neuroprotective effect of tamoxifen and raloxifene has been observed [22] and a beneficial role of tamoxifen and raloxifene against β-amyloid toxicity has been demonstrated in nervous cells [67,68,25]. There is increasing evidence that SERMs may also be neurotrophic, by increasing for instance synaptic density and stimulating neurite outgrowth [22]. Data regarding the clinical use of SERMs in AD are very limited, so far. However, the Multiple Outcomes of Raloxifene Evaluation trial evaluated the cognitive function in more than 5000 women with osteoporosis assigned to receive raloxifene (60 mg or 120 mg) or placebo daily for 3 years. Compared to those taking placebo, women receiving 120 mg/day of raloxifene had a 33% lower risk of mild cognitive impairment and somewhat lower risks of AD and any cognitive impairment [105].

In summary, the basic science strongly supports a neuroprotective role of estrogens/SERMs. Although there is no clear cut evidence yet that these molecules can decrease the risk or ameliorate the clinical course of AD, it is conceivable that there might be a proper space for a hormonal-based intervention in this disease. Undoubtedly, a more profound knowledge of the molecular mechanisms by which ERs activation determines neuroprotective effects may further support this conclusion.

3.3.2. Estrogens and seladin-1/DHCR24

With that idea in mind, we questioned whether seladin-1/DHCR24 might be targeted as a downstream effector of the activation of ERs in the brain. Admittedly, a parallelism between some of the biological properties of this protein and the neuroprotective effects of estrogens and SERMs exists. In order to answer that question, we took advantage of the above mentioned FNC cells. These cells were established, cloned and propagated previously by Vannelli and colleagues at the Department of Anatomy, Histology, and Forensic Medicine of the University of Florence, Italy [95]. FNC are GnRH-secreting neuroblast long-term cell cultures derived from human fetal (8–12 weeks of gestational age) olfactory epithelium. They show unique features, because they express both neuronal and olfactory markers that are typical of maturing olfactory receptor neurons [95]. FNC cells are electrically excitable and following exposure to a number of different aromatic chemicals show a specific increase in intracellular cAMP, indicating some degree of functional maturity. Thus, FNC cells appear to originate from the stem cell compartment that generates mature olfactory receptor neurons. In addition, they express both ERα and β [5]. For these reasons they represented a suitable human *in vitro* model, that could be of help in: (i) providing further information on the role of estrogens in neurons, and (ii) answering the question whether seladin-1/DHCR24 might be an effector of ERs activation. Furthermore, such an *in vitro* model appeared to be in principle much more informative than other cell models, that had been used in most of the previously published studies, i.e. cells of animal origin or human cells, yet transformed or of neoplastic origin.

Our findings confirmed the protective role of estrogens/SERMs in the brain. In fact, we observed that, whereas in the absence of pre-incubation with estrogens β-amyloid and H_2O_2 significantly reduced cell viability, the pre-treatment with 17β-estradiol (100 pM–100 nM) effectively counteracted β-amyloid- or oxidative stress-induced toxicity [8]. In agreement with 17β-estradiol, also the SERM tamoxifen (100 pM–100 nM) effectively protected FNC cells from the toxic effects of β-amyloid, whereas partially different results were observed using raloxifene. In fact, cell viability after exposure to β-amyloid was preserved at low concentrations of raloxifene (100 pM and 1 nM). Conversely, 10 and 100 nM did not exert protective effects. In addition, we found that 17β-estradiol effectively counteracted β-amyloid-induced apoptosis in FNC, as demonstrated by the strong inhibition of the activation of caspase-3.

Finally, in order to answer the question whether estrogens and/or SERMs have an effect on seladin-1/DHCR24 expression, we evaluated the expression of seladin-1 mRNA in FNC cells, treated or not with 17β-estradiol, tamoxifen or raloxifene. We found that FNC cells constitutively express seladin-1/DHCR24 and that 17β-estradiol and tamoxifen significantly increased the amount of mRNA. Raloxifene determined a similar increase of seladin-1/DHCR24 mRNA, compared to an equal concentration of 17β-estradiol (1 nM). However, higher concentrations of raloxifene (10–100 nM) determined a marked reduction of the expression of this gene, in keeping with the observed lack of a neuroprotective effect at these concentrations. A selective ERα agonist (propylpyrazoletriol) determined a significant increase of seladin-1/DHCR24 expression, whereas a selective ERβ agonist (diarylpropionitrile) produced a weaker effect. These additional findings suggested a predominant role of ERα in mediating the stimulatory effect of estrogens on seladin-1/DHCR24 expression. In conclusion, this study led us to hypothesized that this factor might be a mediator of the neuroprotective effects of estrogens/SERMs. In particular, the parallelism between the concentrations of raloxifene that conferred neuroprotection on one hand, and stimulated seladin-1/DHCR24 expression on the other hand, was highly predictive that this was the truth.

This hypothesis appeared supported by additional recent findings. In fact, we demonstrated that, upon silencing seladin-1/DHCR24 expression by small interfering RNA methodology, the protective effect against β-amyloid and oxidative stress toxicity exerted by 17β-estradiol was lost (Fig. 4). To further elucidate the role of estrogens in stimulating the neuroprotective effect of seladin-1/DHCR24, we performed an *in silico* analysis on a 6 kb region upstream the gene promoter, in order to identify Estrogen Resposive Elements (EREs), which confer responsiveness to estrogens acting as transcriptional *enhancers*. Although we could not detect any perfect palindromic ERE, our analysis revealed the presence of a region rich in half-ERE elements spanning from −4384 to −2887 bps. To verify their functionality, we performed luciferase transcriptional transactivation assays in response to the administration of ERs-activating molecules in CHO cells co-transfected with vectors containing the putative *enhancer* ERE sequences of seladin-1/DHCR24 and *ERα*. Luciferase activity was significantly increased after treatment with 17β-estradiol, raloxifene and tamoxifen, thus demonstrating the responsivity of the promoter of seladin-1/DHCR24 to estrogens [52] (Fig. 5). Subsequently, we tested the responsivity of the promoter to phytoestrogens. These compounds are naturally occurring non-steroidal chemicals derived from plants, that can bind ERs and induce either estrogen or anti-estrogen effects in many cell types [70]. For this reason, phytoestrogens have been proposed as natural SERMs [85]. We found that genistein and zearalenone, but not quercetin, were able to induce luciferase activity [52]. These molecules are therefore active on seladin-1 EREs, although the complex interaction with co-activator or co-repressor molecules is still to be evaluated. Admittedly, these data provide a direct demonstration that this protein is a fundamental mediator of the neuroprotective effects of estrogens, at least in the experimental model that we used.

In addition to estrogens responsiveness, in a study by Nelson PS and colleagues the androgen-responsiveness of seladin-1/DHCR24 was demonstrated and was hypothetically related to an androgen responsive element (ARE) identified in the gene promoter region, even though functional evidence was not reported [65]. Interestingly, this sequence is located just downstream the ERE sequences described in our study [52]. In another recent study performed by

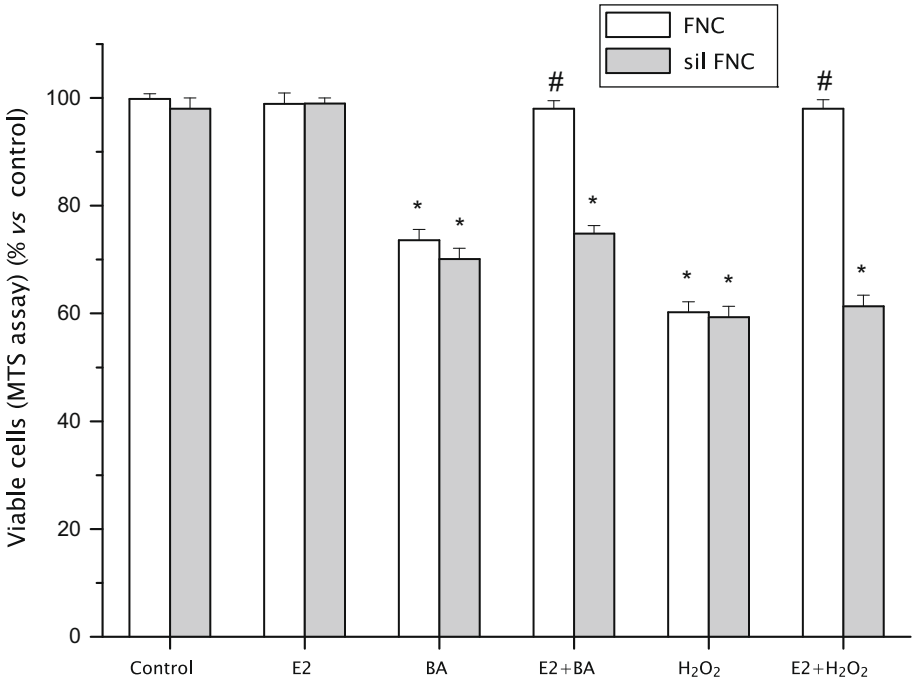

Fig. 4. Effect of 17β-estradiol (10 nM for 48 h) (E2) against ß-amyloid (BA) (100 nM for 18 h) or H_2O_2 (200 μM for 20 h) toxicity in control FNC or in cells subjected to seladin-1/DHCR24 silencing (silFNC). The results were expressed as mean percentage ± SE of viable cells/well in three different experiments. $^{*}P < 0.05$ vs. the corresponding untreated control cells; $^{\#}P < 0.05$ vs. the corresponding cells exposed to ß-amyloid or to H_2O_2. Modified from Ref. [52].

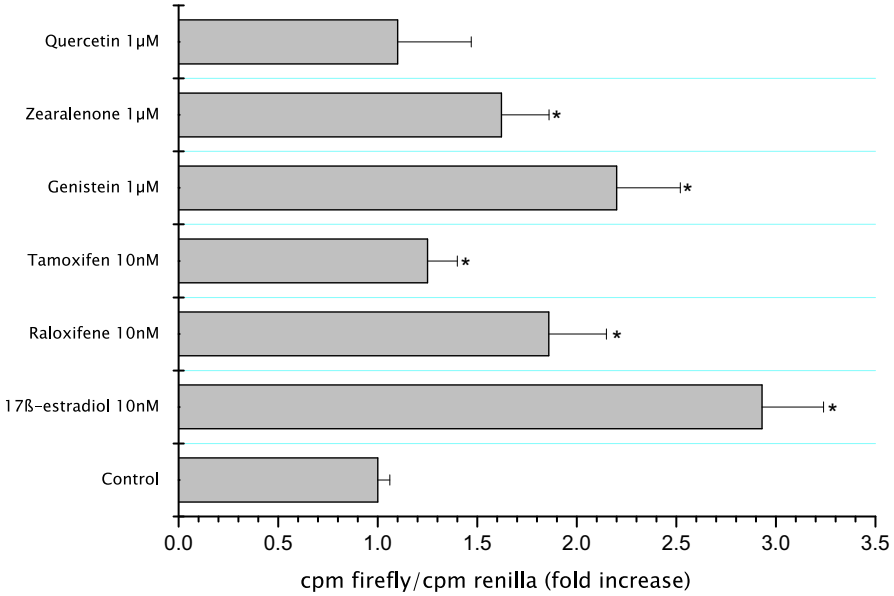

Fig. 5. Normalized luciferase activity elicited by 17β-estradiol, raloxifene, tamoxifen, genistein, zearalenone or quercetin in CHO cells co-transfected with a plasmid containing the putative *enhancer* ERE sequences in the promoter region of seladin-1/DHCR24 and an ERα-overexpressing plasmid. $^{*}P < 0.05$ vs. untreated cells. Modified from Ref. [52].

our group, we investigated the functional activity of these ARE sequences using again a luciferase reporter gene assay. The transcriptional activation in response to the androgen receptor agonist R1881 suggests a classical AR-mediated mechanism of transcriptional regulation of seladin-1/DHCR24 expression. This finding was confirmed by the increase of the amount of transcript elicited by R1881 in AR positive prostate cancer cells as well as in benign prostate hyperplasia cells [12]. The identification of close hormone responsive elements suggests a multi-hormone responsivity of this region, as reported for other genes [48].

3.3.3. Relationship between estrogens, seladin-1/DHCR24 and IGF-I in neuroprotection

There is strong evidence that the IGF system plays an important role in the nervous system by favoring for instance neuronal development, metabolism, survival and regeneration [58,79,80,61,110]. In addition, a tight link between ERs and the IGF-I receptor (IGF-IR) occurs in the brain, as beautifully reviewed by Mendez et al [60]. Many neurons and astrocytes express these receptors and 17β-estradiol is able to activate IGF-IR and its signaling pathways. In particular, in the model proposed by these authors, 17β-estradiol

regulates the activity of protein kinases, thus resulting in increased levels of phosphorylated IGF-IR and Akt. The activation of the latter regulates synaptic plasticity [109] and favors neuronal survival by suppressing for instance Bad-induced cell death [20,21]. 17β-estradiol also decreases the activity of glycogen synthase kinase, which in turn decreases the phosphorylation of Tau protein, that is hyperphosphorylated in AD. On the other hand, IGF-I may regulate the transcriptional activity of ERs. In fact, IGF-I may activate ERs in the absence of estrogens [55,71] and induces ER-mediated gene expression in different cell types including neuronal cells [29,30,62]. In view of those findings, we hypothesized that a relationship between seladin-1/DHCR24 and the IGF-I system might occur, similarly to the previously demonstrated association between this neuroprotective factor and estrogens. Thus, recently we have addressed these issues using again FNC as the *in vitro* cell model and high glucose concentrations as the neurotoxic factor. It is known that high glucose concentrations may be detrimental to nerves and it is generally accepted that one of the mechanisms leading for instance to diabetic neuropathy may be related to a direct or an indirect effect of glucose levels. Glucose, and particularly intermittent high glucose concentrations as it may occur in poorly controlled diabetes, may cause a number of alterations in nervous cells, including for instance altered transcription and translation, ion channel dysfunction, altered axonal transport, demyelination, AGE formation and impaired neurotrophic support [92,72]. We preliminarily demonstrated that these cells express IGF-IR and synthesize and release in the culture medium different members of the IGF system, such as IGF-I, IGF Binding Protein (IGFBP)-2 and -4, but not -1, -3, -5 and -6 [32]. The exposure to IGF-I stimulated cell growth, reduced apoptosis and increased the release of IGFBP-2, whereas it decreased the amount of IGFBP-4 in FNC. It is known that IGFBP-2 may facilitate the binding of IGF-I to its receptor [13], whereas IGFBP-4 is generally considered as a potent inhibitor of the biological effects of IGF-I [59]. Conversely, intermittent (20 mM or 10 mM, alternatively), but not constant (20 mM), high glucose concentrations significantly reduced FNC cell growth, increased apoptosis and disrupted the IGF system, as demonstrated by the marked reduction of IGF-I and IGFBP-2 release. The addition of IGF-1 to the culture medium counteracted the effects of intermittent high glucose on cell proliferation and apoptosis. Interestingly, we found that IGF-I significantly increased *seladin-1/DHCR24* expression, whereas high glucose markedly reduced it. Finally, 17β-estradiol treatment determined a significant increase of the release of IGF-I in the culture medium, indicating that a cross-talk between estrogens and IGF-I occurs in FNC cells. Overall, these results suggest that seladin-1/DHCR24 might be a mediator of the pro-survival effects of IGF-I in the nervous system, although the exact mechanism of action needs to be addressed in future studies, designed for instance to evaluate the effects of IGF-1 and high glucose after silencing the expression of the gene. In addition, these findings indicate that the disruption of the IGF system may be one of the mechanisms through which glucose toxicity causes diabetic neuropathy. An interplay between seladin-1/DHCR24, estrogens and IGF-I may be also envisaged. In particular, both IGF-I and 17β-estradiol directly stimulate the expression of this neuroprotective factor; furthermore, the latter hormone appears to have also an indirect stimulatory effect by increasing the release of IGF-I, which in turn can bind to IGF-IR via an autocrine loop (Fig. 6). It remains to be clarified whether the stimulatory effect of IGF-I on the expression of seladin-1/DHCR24 is a direct consequence of IGF-I/IGF-IR binding or is mediated via an interaction between the complex IGF-I/IGF-IR and ERs.

3.3.4. A unified model for cholesterol, estrogens and seladin-1/DHCR24

We have addressed separately the relationship between seladin-1/DHCR24 and cholesterol or estrogens, respectively, so far. However, if we remember that cholesterol represents the precursor of all steroids, it appears obvious to think that a relationship involving cholesterol, estrogens and seladin-1/DHCR24 together might exist. To this purpose, we propose a "circular" model, in which this neuroprotective factor may be considered both the α and the ω. Estrogens stimulate the expression of seladin-1/DHCR24, which in turn increases the synthesis of cholesterol (Fig. 7). There is evidence that both low density (LDL) or high density (HDL) lipoproteins, enriched in cholesterol, up-regulate the expression of the steroidogenic acute regulatory protein (StAR) [75,66]. StAR is required for the movement of cholesterol from the outer to the inner mitochondrial membrane, the site of cholesterol side chain cleavage, and therefore is fundamental for the initiation of steroid synthesis. Thus, seladin-1/DHCR24 may be viewed as the crucial point of a virtuous cycle. If we imagine this cycle in nervous cells, a multifaceted mechanism of neuroprotection, that unifies the specific properties of estrogens and cholesterol, appears to be kept together by seladin-1/DHCR24. This model is in keeping with other similar proposals from previous studies, that addressed StAR and its related protein named mito-

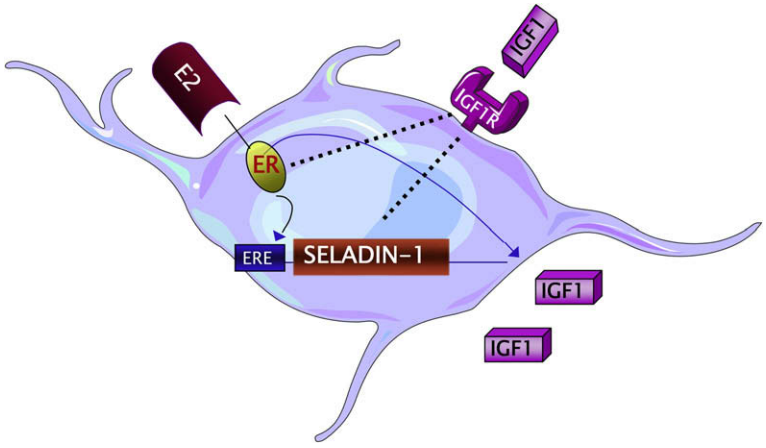

Fig. 6. The cartoon represents the working hypothesis on the relationship between seladin-1/DHCR24, estrogens and IGF-I in nervous cells. The cartoon depicts a neuroblast, because this is the cell model in which the experimental studies covering this issue have been performed by our group. This does not exclude that the same relationship may apply to other cells of the nervous system. 17β-Estradiol (E2) stimulates the expression of the seladin-1/DHCR24 gene via functionally active half-palindromic EREs contained in its promoter region. In addition, 17β-estradiol increases the release of IGF-I. IGF-I, in turn, binds to IGF-I receptors (IGF-IR) and stimulates the expression of seladin-1/DHCR24, too. The dotted lines suggest that the latter might be a direct effect or it might be mediated via an interaction between the complex IGF-I/IGF-IR and ERs.

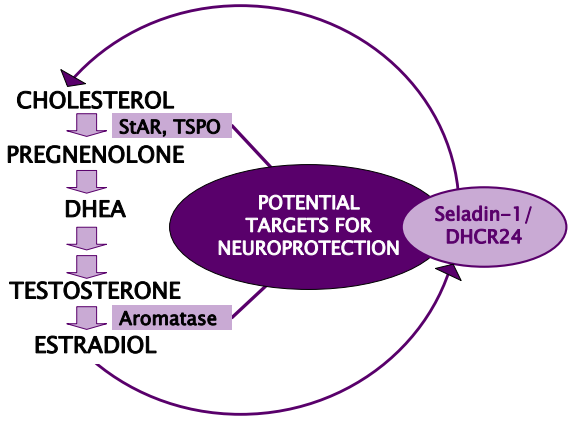

Fig. 7. Potential molecular targets to increase the production of neuroprotective steroids. Seladin-1/DHCR24 induces the synthesis of cholesterol and hence of estradiol, that in turn stimulates the expression of this neuroprotective factor. Modified from Ref. [97].

chondrial translocator protein of 18 kDa (TSPO), formerly known as the Peripheral type Benzodiazepine Receptor (PBR), or aromatase as potential targets for neuroprotection [97,46].

4. Conclusions

We feel that the data summarized in this review indicate that seladin-1/DHCR24 has maintained its initial promises and that this protein may certainly contribute to open a new possible scenario on several very interesting issues, including for instance: (i) the role of cell cholesterol and, in a broader view, of cholesterol-rich lipid rafts in neuroprotection and (ii) the role of hormone-mediated neuroprotection in preventing or in treating neurodegenerative diseases. Obviously, these issues need further thorough investigation in order to fully elucidate both the role of seladin-1/DHCR24 in maintaining nervous cells homeostasis and the alterations leading to pathological conditions affecting the nervous system. The ultimate goal of these upcoming studies is to possibly identify new molecular targets, in order to design novel, and hopefully effective, molecules for pharmacological intervention against neurodegenerative diseases.

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