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Dehydroepiandrosterone formation is independent of cytochrome P450 17α-hydroxylase/17, 20 lyase activity in the mouse brain

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ABSTRACT

Cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17) is a microsomal enzyme reported to have two distinct catalytic activities, 17α-hydroxylase and 17, 20 lyase, that are essential for the biosynthesis of peripheral androgens such as dehydroepiandrosterone (DHEA). Paradoxically, DHEA is present and plays a role in learning and memory in the adult rodent brain, while CYP17 activity and protein are undetectable. To determine if CYP17 is required for DHEA formation and function in the adult rodent brain, we generated CYP17 chimeric mice that had reduced circulating testosterone levels. There were no detectable differences in cognitive spatial learning between CYP17 chimeric and wild-type mice. In addition, while *CYP17* mRNA levels were reduced in CYP17 chimeric compared to wild-type mouse brain, the levels of brain DHEA levels were comparable. To determine if adult brain DHEA is formed by an alternative Fe^{2+}-dependent pathway, brain microsomes were isolated from wild-type and CYP17 chimeric mice and treated with $FeSO_4$. Fe^{2+} caused comparable levels of DHEA production by both wild-type and CYP17 chimeric mouse brain microsomes; DHEA production was not reduced by a CYP17 inhibitor. Taken together these *in vivo* studies suggest that in the adult mouse brain DHEA is formed via a Fe^{2+}-sensitive CYP17-independent pathway.

1. Introduction

Changes in 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone; DHEA) levels have been implicated in behavioral disorders such as depression and anxiety [1,2]. DHEA is produced by the adrenal cortex and serves as the precursor of androgenic and estrogen sex hormones. In rodents, DHEA has neuroactive steroid activity that enhances memory [3], enhances long-term potentiation in the hippocampus [4], and affects cortical organization in development [5].

DHEA biosynthesis by the adrenal gland and gonads in the periphery is well characterized. The initial step in steroid biosynthesis is the metabolism of cholesterol to pregnenolone. This process is catalyzed by the cytochrome P450 side chain cleavage enzyme (CYP11A1) [6] in the inner mitochondrial membrane. Pregnenolone is then converted to DHEA by cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17) in the microsomal compartment of steroidogenic cells [6].

While DHEA is present in high levels in the blood under normal conditions, it is found in the brain at even higher levels than in the periphery [7–9]. Moreover, DHEA concentrations in rat brain are not affected by adrenalectomy and castration suggesting that DHEA is made *de novo* within the brain [8,9]. The default conclusion is that DHEA is produced in the brain by the same CYP17 pathway that exists in the periphery. Steroidogenic enzymes exist in brain [10] and some steroids in brain are formed via the same enzymatic pathways as those described in adrenals and gonads [9,10]. However, studies have repeatedly demonstrated that CYP17 expression and activity are undetectable in the adult brain [11–14]. Although *Cyp17* mRNA has been found by *in situ* hybridization to be present during rat embryonic development [15], the presence of *Cyp17* mRNA in adult tissue has been controversial [16–19]. CYP17 activity was also undetectable in rodent brain slices, brain homogenates, brain microsomes, primary cultures of mixed glial cells, glial tumors, astrocytes, and neurons of rat and mouse embryos [11–13,20]. In one report, CYP17 immunoreactivity and activity were found in principal neurons; however, the transformation rate of pregnenolone to DHEA for the amount of immunoreactive protein present was 1:500,000 in this study, which is close to background activity seen with all enzymatic reactions

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[21]. Moreover, a detailed gas chromatography–mass spectrometry analysis of all steroids present in the adult rat brain did not detect 17α-hydroxyprogesterone, thus confirming the lack of CYP17 enzyme activity in brain [22]. Taken together, these studies strongly suggest that the CYP17 pathway is not the primary pathway for DHEA biosynthesis in the adult brain.

A CYP17-independent, ferrous ion (Fe^{2+})- dependent alternative DHEA biosynthetic pathway may exist in the adult brain [23]. In agreement with these findings, our laboratory has shown that DHEA formation increases in the presence of Fe^{2+} ions and pregnenolone further potentiates this effect, in rat C6-2B glioma cells, immature oligodendrocyte precursors, mature oligodendrocytes, bovine microsomes, and human brain specimens [12,13,20,24,25]. DHEA formation under these conditions was not inhibited by the CYP17 inhibitors SU10603 or ketoconazole. However, all of the above mentioned studies were performed *in vitro*, raising the possibility that the alternative pathway is an artifact of the *in vitro* conditions. To determine if the Fe^{2+}-dependent alternative DHEA biosynthetic pathway exists *in vivo*, we created a chimeric mouse model carrying a *Cyp17* gene deletion [26]. CYP17 chimeric mouse Leydig cells had dramatically reduced levels of *Cyp17* mRNA, and intratesticular and circulating testosterone levels. Despite the absence of CYP17 expression and activity, Fe^{2+}-dependent DHEA biosynthesis was intact in chimeric mouse brain tissue and isolated microsomes, demonstrating the presence of a CYP17-independent, ferrous ion Fe^{2+}-dependent alternative DHEA biosynthetic pathway in the brain.

2. Materials and methods

2.1. Materials

^{3}H-DHEA and ^{3}H-testosterone were obtained from NEN/DuPont (Boston, MA). Anti-DHEA and anti-testosterone polyclonal antibodies were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Primers were synthesized by Bio-Synthesis Inc. (Lewisville, TX). PCR supplies were from Clonetech (San Diego, CA). Plasmid purification and DNA gel extraction kits were from Qiagen (Valencia, CA). Organic solvents were of HPLC grade purchased from Fluka (New York, NY) and Fisher Scientific (Pittsburg, PA).

2.2. Animals

Wild-type C57BL/6 mice and *Cyp17* gene targeted chimeric mice were previously described [26]. Animal protocols were approved by the Georgetown University Institutional Animal Care and Use Committee.

2.3. Water maze

Water maze tests were performed as described [27,28]. Tests were performed using a white circular pool (1.2 m diameter with walls 60 cm high) and a stationary platform (11 cm diameter) submerged (1 cm) below the water surface. The water was made opaque with non-toxic white paint and maintained at 24 °C. The movement of the mice was recorded on VHS tape. Wild-type C57BL/6 mice and CYP17 chimeric mice were given four trials per day for five days, a two day break, and then another four days of trials. A probe trial (the platform was removed and the mice were allowed to search for it for 60 s) was given at the end of the last training day (day 12). Each trial consisted of releasing the mouse into the water facing the outer edge of the pool at one of the quadrants, and timing how long it took them to escape to the platform. A trial terminated when the animal reached the platform and remained there for 15 or 90 s elapsed, whichever occurred first. Mice that failed to find the platform within the 90 s time limit were placed onto the platform by the experimenter and had to stay there for 15 s before being returned to their cages.

2.4. Steroid measurements

Blood was drawn from wild-type and CYP17 chimeric male mice into Serum Separation Blood Collection Tubes (BD Bioscience, San Jose, CA). Testosterone levels in collected sera were measured by radioimmunoassay (RIA) [26]. Wild-type and CYP17 chimeric adult mice were sacrificed by decapitation. Brain tissues were homogenized on ice and organic extracts were prepared with diethyl-ether/ethyl acetate (1:1, v/v) extraction [12,13,20]. Extracts were applied to Sep-Pak Silica cartridges (Waters Corp., Milford, MA). DHEA synthesis was measured by RIA [12,20]. Protein levels were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.5. Semi-quantitative RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated from wild-type mouse brain and CYP17 chimeric mouse brain using the TRIZOL Reagent (Invitrogen, Carlsbad, CA). For semi-quantitative RT-PCR 1 μg of total RNA as template and 20 μM of *Cyp17* specific primers (sense CCCATCTATTCTCTTCGCCTGGGTA and antisense GCCCCAAAGATGTCTCCCACCGTG) were used. Reactions were carried out using an RNA PCR kit from Perkin Elmer (Branchburg, NJ), according to the manufacturer's protocol. Preliminary experiments were run to determine the optimal number of cycles required to perform the PCR reactions in exponential phase; GAPDH was detected after running 17 cycles and Cyp17 at 27 cycles. PCR products were resolved on 1.5% agarose electrophoresis gels containing 1 μg/ml ethidium bromide. The amplified fragments were recovered and purified using the Quiaquick gel extraction kit (Quiagen, Chatsworth, CA). The identity of the generated PCR products was confirmed by DNA sequencing performed at the Lombardi Cancer Center Sequencing Core Facility (Georgetown University Medical Center). Quantitative RT-PCR was performed as we previously described [26].

2.6. Immunoblot analysis

Protein lysates from homogenized brain and testis were used for immunoblot analysis performed as previously described [26]. Rabbit anti-CYP17 antiserum was a gift from Drs. D.B. Hales (University of Illinois College of Medicine, Chicago, IL) and A. Payne (Stanford University) and anti-GAPDH (Trevigen, Gaithersburg, MD) antisera were used as loading control.

2.7. DHEA synthesis by microsomes

Mouse brain microsomes were prepared by homogenization and differential centrifugation. Brains were washed several times with washing buffer (150 mM KCl, 10 mM Tris–HCl pH 7.4), homogenized in buffer [250 mM sucrose, 50 mM Tris–HCl pH 7.4, 1 mM dithiothreitol, 1 mM Ethylenediaminetetraacetic acid (EDTA), 20% glycerol] and centrifuged for 10 min at 1000 × *g*. Supernatants were collected and centrifuged for 30 min at 10,000 × *g* and then again for 1 h at 300,000 × *g*. Microsomal pellets were washed and suspended in 50 mM Tris–maleate buffer (pH 7.4) to a final protein concentration of 10 mg/ml (17, 18). A 1 ml reaction volume was created with the following components: 100 μg of the microsomal protein fraction in 50 mM Tris–maleate buffer [pH 7.4] containing NADPH (600 μM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (1.5 units/ml), the CYP17 inhibitor SU10603 (5 μM) (26), the 3β-hydroxysteroid dehydrogenase inhibitor trilostane (5 μM), in the absence or presence of $FeSO_4$ (10 mM). All the incubations were carried out at 37 °C in a 5% CO_2 atmosphere for 1 h

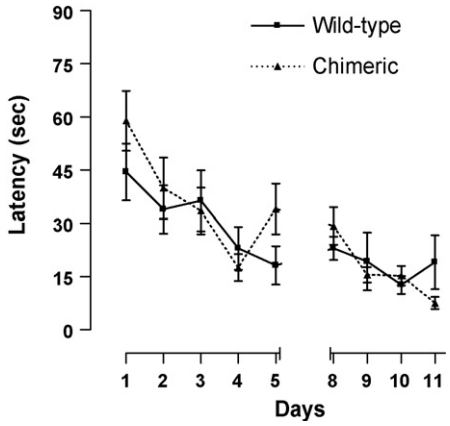

Fig. 1. Spatial learning in the water maze. Wild-type ($n = 4$) and *Cyp17* gene targeted chimeric ($n = 4$) mice were trained for five days, given a two day break, then given additional training for four days. Animals received four trials each day from four separate start positions designed as North, South, West and East ($n = 4$). The average latency of four trails each day to reach the hidden platform is plotted. If the mouse could not find the platform in 90 s, as the trial was terminated at 90 s. Probe trials were given on the last day of training. Results are shown as the means ± SEM ($n = 4$).

and stopped by the addition of cold ethanol (250 μl). Steroids were extracted with 4 ml of diethyl–ether/ethyl acetate (1:1) and evaporated to dryness. Extracts were applied to Sep-Pak Silica cartridges (Waters Inc). DHEA synthesis was measured by RIA. In separate experiments, the presence of DHEA was confirmed by GC-MS [29] on a capillary gas chromatograph GC-17A Ver. 3 coupled to a mass spectrometer (Shimadzu, Kyoto, Japan). Identities of the compounds were established by the Rt of their trimethylsilyl derivative on GC and by the ratio of the *m/e* values of diagnostically important ions 360, 345, 304, and 270.

2.8. Statistics

Statistical analysis of the data was performed by either ANOVA or unpaired *t*-test using the Prism (version 4.0) package from GraphPad (San Diego, CA).

3. Results

We first examined reference learning and memory abilities of wild-type and CYP17 chimeric mice using the Morris water maze. Measurement of the mean memory latency on each day of testing revealed no significant spatial learning or memory differences between the wild-type and CYP17 chimeric mice (Fig. 1). Memory latency was reduced by 70% over the 11 days trial in both wild-type and CYP17 chimeric mice. There were no significant differences in the rate of memory latency decline between the two groups.

To determine the level of *Cyp17* mRNA levels in the brains of CYP17 chimeric mice and control wild-type mice, we extracted total RNA from the entire brain. Fig. 2A shows the level of *Cyp17* mRNA by RT-PCR. The bands obtained were confirmed to be mouse *Cyp17* by DNA sequencing. Further quantitative RT-PCR analysis revealed that the level *Cyp17* mRNA was reduced by 50% in chimeric mouse brain tissue compared to wild-type mouse brain tissue (Fig. 2B). Fig. 3 shows that the 54 kDa immunoreactive CYP17 protein is present in mouse testis. However, we did not detect a CYP17 immunoreactive protein in brain extracts from either wild-type (Fig. 3A) or CYP17 chimeric mice (data not shown).

No significant differences were found between DHEA levels in extracts from homogenized wild-type or CYP17 chimeric mouse brains (Fig. 4A). In contrast, serum testosterone levels were reduced by 65% in the chimeric mice (Fig. 4B). As expected, microsomes isolated from wild-type and CYP17 chimeric mouse brains that were

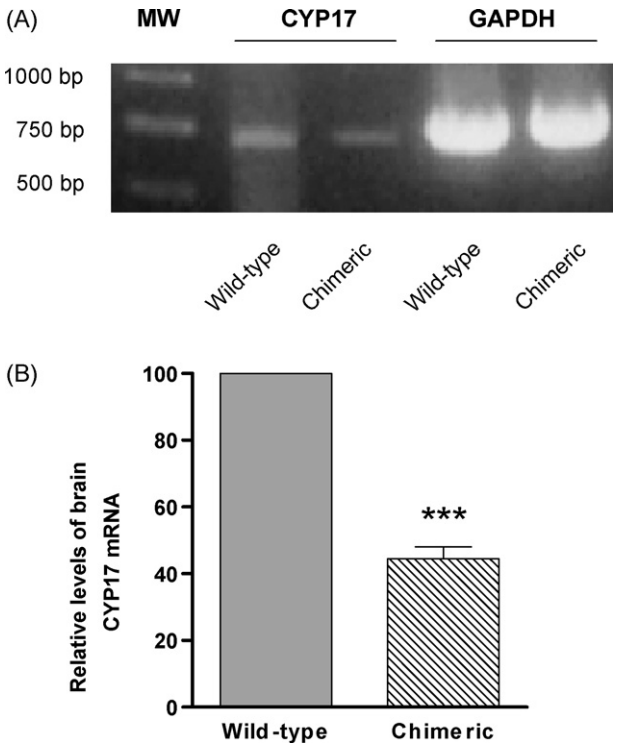

Fig. 2. *Cyp17* mRNA expression in wild-type and CYP17 chimeric mouse brain tissue. (A) Semi-quantitative RT-PCR. Total RNA was isolated from mouse brain tissue and amplified by RT-PCR with specific *Cyp17* primers. GAPDH was used as endogenous control. (B) Quantitative real-time PCR for *Cyp17* mRNA. The level of wild-type *Cyp17* mRNA was set as 100%. Results are shown as means ± SEM ($n = 4$); $^{***}p < 0.001$ compared to wild-type.

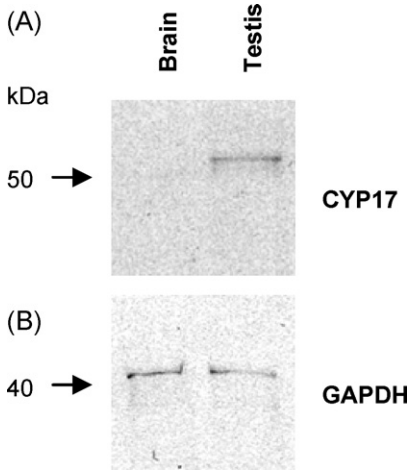

Fig. 3. Immunoblot analysis of CYP17 expression. Protein extracts from wild-type mouse brain and testis were separated by SDS-PAGE on a 10% gel, electrotransferred to nitrocellulose membranes and submitted to immunobloting using an anti-CYP17 antiserum (A). Anti-GAPDH was used as loading control (B).

incubated in the presence of SU10603 and trilostane, did not form DHEA (Fig. 5). However, addition of $FeSO_4$ (10 mM) to the reaction media induced DHEA formation by both wild-type and CYP17 chimeric mouse brain microsomes (Fig. 5).

4. Discussion

This is the first *in vivo* report demonstrating that there exists a DHEA biosynthetic pathway in brain tissue that is distinct from the CYP17-dependent pathway present in peripheral steroidogenic

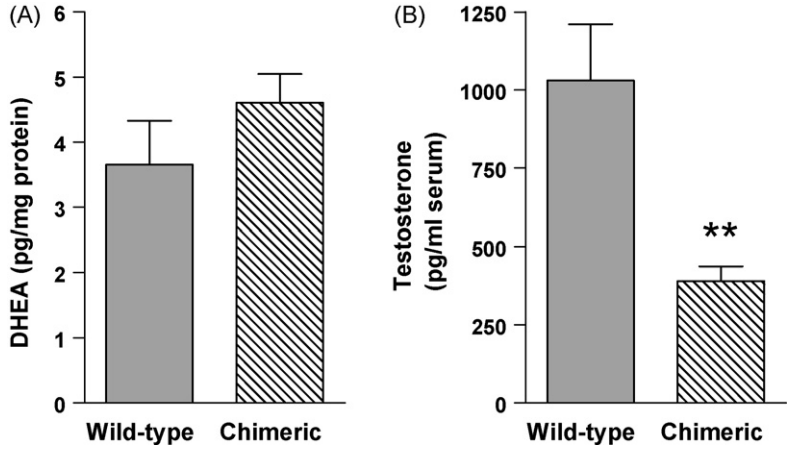

Fig. 4. DHEA levels in brain tissue and circulating testosterone levels. (A) DHEA levels in brain tissue of wild-type and CYP17 chimeric mice. (B) Serum testosterone level of wild-type and CYP17 chimeric mice. Results are shown as the means ± SEM ($n = 4$); $^{**}p < 0.01$ compared to wild-type.

tissues. Although in CYP17 chimeric mice the Leydig cell *Cyp17* mRNA, and intratesticular and circulating testosterone levels were dramatically reduced, the lowered levels of testosterone remained sufficient to support spermatogenesis [26]. However, male chimeras consistently failed to generate heterozygous Cyp17 mouse progeny and further studies demonstrated that CYP17 is essential for sperm function. Deletion of one *Cyp17* allele prevented genetic transmission of mutant and wild-type alleles and resulted in infertility. Circulating testosterone levels were reduced by 65% in the chimeric mice, which is in agreement with our previous data [26] and consistent with the role of CYP17 in peripheral steroidogenesis. In the present study, we observed that although the CYP17 protein is undetectable in adult mouse brain extracts, *Cyp17* mRNA is present and it is reduced by 50% in CYP17 chimeric mouse brain compared to the wild-type brain tissue. Despite this difference in *Cyp17* mRNA levels and lack of CYP17 protein, CYP17 chimeric mice contained the same endogenous levels of DHEA as wild-type mice.

These data raised the question of whether CYP17 activity is required for the synthesis of DHEA by adult brain tissue. This question was first raised by Lieberman et al. [30] and in 1994, Prasad et al. [23] reported that oxidizing and reducing agents could induce DHEA production by organic extracts of rat brain tissue. This report proposed the existence of alternative precursors for neurosteroid

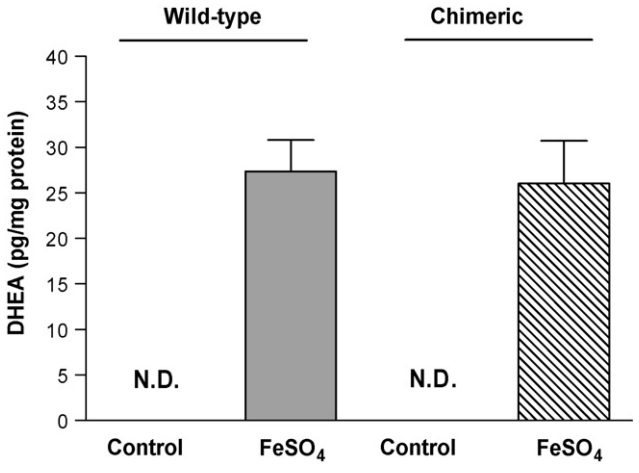

Fig. 5. DHEA synthesis by mouse brain microsomes. DHEA formation in mouse brain microsomes from wild-type and CYP17 chimeric mice incubated *in vitro* with the CYP17 inhibitor SU10603 (5 μM), the 3β-hydroxysteroid dehydrogenase inhibitor trilostane (5 μM), with and without $FeSO_4$ (10 mM). Steroids were extracted, measured, and shown as the means ± SEM ($n = 4$); N.D., not detected.

biosynthesis in the brain that could be metabolized in appropriate oxidative conditions. This was the first indication that there could be a CYP17-independent mechanism for steroid biosynthesis in the brain. Studies in our laboratory using Fe^{2+} ions as a redox tool demonstrated an alternative pathway for DHEA synthesis is present in rat glioma cells, which lack *Cyp17* mRNA and protein expression [13,20]. These results were subsequently extended to show that other redox reagents, such as ferrous sulfate and β-amyloid (Aβ) peptide, also induced DHEA synthesis by human glioma cells, bovine microsomes, and human brain extracts [13,20,24,25]. Moreover, CYP17 has been shown to exert catalytic activities distinct of those involved in steroid biosynthesis [31].

$FeSO_4$-induced comparable levels of DHEA synthesis by wild-type and CYP17 chimeric mouse brain microsomes, suggesting that Fe^{2+} acts either directly or indirectly on the transformation of an unknown precursor. We previously proposed that this putative precursor is a hydroxyperoxy derivative of either cholesterol or pregnenolone [20]. Treatment of brain tissue microsomes with Fe^{2+}-induced DHEA synthesis by both wild-type and CYP17 chimeric mouse brain microsomes. These data suggest that the redox environment affects the ability of the endogenous biosynthetic pathway to transform the unknown precursor to DHEA. In this context, Fe^{2+} would be expected to increase either the amounts of the precursor available for synthesis or the rate of its transformation to DHEA. In support of these findings, Maayan et al. [32] reported that the increased DHEA synthesis seen in the brain of castrated male mice was completely blocked by the antioxidant N-acetylcysteine amide. The CYP17 and 3β-hydroxysteroid dehydrogenase inhibitors SU10693, and trilostane did not block the $FeSO_4$-induced DHEA formation providing additional evidence that CYP17 does not mediate the Fe^{2+}-induced DHEA synthesis

DHEA has been shown to play a critical role in learning, memory, and brain development [3–5]. Thus, a reduction of DHEA levels throughout development would be expected to affect the cognitive process of the CYP17 chimeric mice. However, no spatial memory deficiency was seen in the CYP17 chimeric mice in comparison to wild-type mice, suggesting that *Cyp17* deletion and the reduction of *Cyp17* mRNA levels in the brain did not affect learning or memory. This finding is supported by clinical findings from patients with CYP17 defects [33–35]. Deficiency of CYP17 in humans is not lethal; however, because CYP17 is important for testosterone and estrogen production, CYP17-deficient patients exhibit male pseudohermaphroditism or an absence of pubertal development [33–35]. In contrast, there are no reports of cognitive deficiencies in CYP17-deficient patients. It is important to note that although the findings reported herein demonstrate the lack of relationship

between learning and memory and CYP17 expression in brain they do not question or address the role of DHEA in these brain functions. Considering the proposed roles of DHEA in neuronal function, DHEA production defects in humans would be expected to have a dramatic impact on brain development and function. The only explanation for the lack of brain pathology in CYP17-deficient patients and our mouse model is that DHEA is synthesized by a CYP17-independent Fe^{2+}-sensistive pathway that maintains or compensates for reduced DHEA synthesis.

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