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Genome-wide analysis of DHEA- and DHT-induced gene expression in mouse hypothalamus and hippocampus

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ABSTRACT

Dehydroepiandrosterone (DHEA) is the most abundant steroid in humans and a multi-functional neuroactive steroid that has been implicated in a variety of biological effects in both the periphery and central nervous system. Mechanistic studies of DHEA in the periphery have emphasized its role as a prohormone and those in the brain have focused on effects exerted at cell surface receptors. Recent results demonstrated that DHEA is intrinsically androgenic. It competes with DHT for binding to androgen receptor (AR), induces AR-regulated reporter gene expression *in vitro*, and exogenous DHEA administration regulates gene expression in peripheral androgen-dependent tissues and LnCAP prostate cancer cells, indicating genomic effects and adding a level of complexity to functional models. The absence of information about the effect of DHEA on gene expression in the CNS is a significant gap in light of continuing clinical interest in the compound as a hormone replacement therapy in older individuals, patients with adrenal insufficiency, and as a treatment that improves sense of well-being, increases libido, relieves depressive symptoms, and serves as a neuroprotective agent. In the present study, ovariectomized CF-1 female mice, an established model for assessing CNS effects of androgens, were treated with DHEA (1 mg/day), dihydrotestosterone (DHT, a potent androgen used as a positive control; 0.1 mg/day) or vehicle (negative control) for 7 days. The effects of DHEA on gene expression were assessed in two regions of the CNS that are enriched in AR, hypothalamus and hippocampus, using DNA microarray, real-time RT-PCR, and immunohistochemistry. RIA of serum samples assessed treatment effects on circulating levels of major steroids. In hypothalamus, DHEA and DHT significantly up-regulated the gene expression of hypocretin (Hcrt; also called orexin), pro-melanin-concentrating hormone (Pmch), and protein kinase C delta (Prkcd), and down-regulated the expression of deleted in bladder cancer chromosome region candidate 1 (Dbccr1) and chitinase 3-like 3 (Chi3l3). Two-step real-time RT-PCR confirmed changes in the expression of three genes (Pmch, Hcrt and Prkcd) using the same RNA sample employed in the microarray experiment. Immunohistochemistry showed augmentation of prepro-hypocretin (pHcrt) neuropeptide protein expression by DHEA and DHT in hypothalamus, consistent with the localization of orexin neurons. In hippocampus, DHT down-regulated the expression of Prkcd, while DHEA did not have significant effects. RIA results supported the view that DHEA-induced effects were mediated through AR. The current study identified neurogenomic effects of DHEA treatment on a subset of genes directly implicated in the regulation of appetite, energy utilization, alertness, apoptosis, and cell survival. These changes in gene expression in the CNS represent a constellation of effects that may help explain the diverse benefits attributed to replacement therapy with DHEA. The data also provide a new level of detail regarding the genomic mechanism of action of DHEA in the CNS and strongly support a central role for the androgen receptor in the production of these effects. More broadly, the results may be clinically significant because they provide new insights into processes that appear to mediate the diverse CNS effects attributed to DHEA.

Abbreviations: Adione, androstenedione; AR, androgen receptor; B2m, beta-2 microglobulin gene; Chi3l3, chitinase 3-like 3 gene; CNS, central nervous system; C_T, threshold cycle; Dbccr1, deleted in bladder cancer chromosomal region candidate 1; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; FDR, false discovery rate; Hcrt, hypocretin, orexin; IHC, immunohistochemistry; IPD, integrated particle density; MCH, melanin-concentrating hormone; MCHR1, melanin-concentrating hormone receptor 1; OVX, ovarietomized; pHcrt, prepro-hypocretin neuropeptide; Pmch, melanin-concentrating hormone gene; Prkcd, protein kinase C delta gene; REV, relative expression value; T, testosterone.

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1. Introduction

Dehydroepiandrosterone (DHEA), together with its sulfate ester (DHEAS), is the principal secretory product of the adrenal glands and the most abundant steroid in humans [1]. These compounds also are synthesized *de novo* in the brain, leading to their additional designation as neuroactive neurosteroids [2,3]. In recent years, DHEA has attracted significant attention from clinicians based on multiple observations, including a steady decline in production from early adulthood to age 70, a growing literature showing changes in DHEA associated with various pathologies, and observations that replacement therapy with DHEA may mitigate or alleviate age-associated declines in a range of functions. For example, in elderly humans, patients with adrenal insufficiency, and animal models of affective illness, DHEA replacement generally produced antidepressant effects, improved psychological well-being, mood, and libido, and decreased fatigue [1,4–9]. In rodents, DHEA administration also reportedly enhanced memory and cognition, and protected neurons from damage induced by excitatory amino acids [10–14]. These and related observations have led to the proposed use of DHEA as a therapeutic agent for patients with adrenal insufficiency and as a general replacement therapy in elderly individuals [1,15,16]. There also is a public health concern related to DHEA because it is sold as an unregulated dietary supplement and may act as an anabolic steroid [17]. These considerations engendered a call for additional research on both the potential clinical utility and mechanism of action of DHEA (e.g. [17–19]).

Although multiple beneficial effects of DHEA on CNS function have been reported in human populations and rodent models, the mechanism of action of this compound in the brain has not yet been fully characterized. Earlier studies focused on the modulatory effects of DHEA on membrane receptors, particularly $GABA_A$, NMDA, and σ_1 receptors [20–23]. However, modulation of cell surface receptors alone may not provide a satisfactory explanation for the range of effects attributed to DHEA. More specifically, several findings showed that DHEA is intrinsically androgenic. The observations include the ability to displace DHT from AR in a cell-free assay system, the induction of AR-mediated transcriptional activity in the presence of trilostane, which inhibits metabolism by blocking 3-beta hydroxysteroid dehydrogenase, promoting nuclear trafficking of AR in transfected CHO cells, and up-regulation of neural AR in female mice, an effect characteristic of androgens [24–26]. Furthermore, AR is particularly abundant in the hypothalamus and hippocampus of rodents and this nuclear receptor was up-regulated by DHEA, T, and DHT in mice and rats [24,27–29]. These observations provide a strong rationale for further characterization of potential neurogenomic effects of DHEA mediated through AR.

In the present study, DNA microarrays were used to identify genes regulated by DHEA and DHT in CF-1 mouse hypothalamus and hippocampus. The differential expression of a subset of regulated genes was further confirmed using real-time RT-PCR and immunocytochemistry. The expression of the neuropepetide genes hypocretin (Hcrt) and pro-melanin-concentrating hormone (Pmch), which are implicated in energy utilization, appetite, and alertness, and three others (Prkcd, Dbccr1, and Chi313) involved in apoptosis/cell survival, were significantly changed by both DHEA and DHT. The differential expression of these genes advances our understanding of the mechanism of action of DHEA in the central nervous system and may help explain the multiple effects of DHEA reported in humans.

2. Materials and methods

2.1. Animals, treatments and brain tissue collection

CF-1 female mice (Charles River Lab.), an established model for the assessment of androgenic effects in the CNS [22,23,25], were housed in groups of 3–4 per cage at $23 \pm 2\,^{\circ}C$ under a 12-h light/dark cycle with free access to food and water. All maintenance procedures were in full accord with federal guidelines for animal care. Mice were about 2 months of age and were allowed a 5-day adaptation period in the colony before use in the experiments. They were ovariectomized under nembutal anesthesia 10 days before treatment. DHEA and DHT were dissolved in 90% corn oil and 10% dimethyl sulfoxide. Ten days after surgery, mice were randomly divided among three groups ($n = 8$) and then given seven consecutive daily injections of DHEA (1 mg/day), DHT (0.1 mg/day), or vehicle. The dose of DHEA was based on previous investigations and its lower relative affinity for AR compared to DHT (DHEA:Ki = 3.48 μM; DHT:Kd = 1.73 nM) [22–24]. Six hours after the last treatment, they were sacrificed, the brains were immediately frozen in dry ice plus alcohol solution and then stored in $-72\,^{\circ}C$ before isolating the hypothalamic and hippocampal regions, and trunk blood was collected for RIA. The hypothalami and hippocampi were rapidly isolated on an ice-cold glass stage and placed into a 1.5-ml microcentrifuge tube with TRI_{ZOL} reagent. Four hypothalami or hippocampi were pooled for RNA isolation, which produced two RNA replicates for each condition (treatment × brain region).

2.2. RNA isolation, fluorescent cRNA synthesis and microarray hybridization

Total RNA was extracted by using TRI_{ZOL} reagent according to the manufacturer's instruction (Invitrogen). The integrity and concentration of total RNA were determined using an Agilent 2100 Bioanalyzer and a NanoDrop Spectrophotometer (Agilent), respectively. High quality undegraded RNA (A260/A280 > 1.86) was used for further analysis. Agilent Low RNA Input Fluorescent Amplification Kit and Qiagen's RNeasy Mini Kit were used to synthesize and purify fluorescent cRNA target. Two biological replicates of cRNAs for each condition were labeled with Cy3 or Cy5, respectively, using a dye-swap strategy. Equal amounts of cRNAs from conditions being compared were prepared with different labels mixed, and then hybridized to the Mouse 60-mer Oligo Microarray (Agilent). For example, to compare the relative gene expression for DHEA vs. vehicle in the hypothalamus, Cy5-labeled cRNA from DHEA-treated animals and Cy3-labeled cRNA from vehicle animals were hybridized to a microarray. The second pair of cRNA replicates from these conditions was prepared with reversed labels and hybridized to another microarray.

2.3. Microarray data analysis

The red (Cy5) and green (Cy3) signal intensities were obtained by scanning the microarrays using an Agilent microarray scanner, with Agilent G2567AA Feature Extraction Software (v. 7.5). The relative expression value (REV) of a gene for two different samples was represented by base 2 log-ratios of the two signal intensities (e.g. $\log_2(R/G)$ or $\log_2(G/R)$, depending on the labels for the cRNAs; R: red signal intensity; G: green signal intensity). The original data contained information for about 22,000 spots. After data cleaning, 14,744 spots with high quality data were left for further analysis. The data were normalized using global Loess algorithm implemented in the function *normalizeWithinArrays* in R *limma* package [30]. The data were analyzed based on an ANOVA model:

$$y_{gki} = \mu_{gk} + \varepsilon_{gki}, \quad \varepsilon_{gki} \sim F,$$

where the y_{gki} is the REV for gene g, condition k and array i; $g = 1, 2, 3, \ldots, G$; $k = 1, 2, 3, 4$; $i = 1, 2$; the μ_{gk} is fixed unknown parameters of REV; the ε_{gki} is independent and identically distributed as some distribution function F. The least-squares estimator of μ_{gk} is

$\hat{\mu}_{gk} = 1/2\sum_i y_{gki}$, and the residuals are $e_{gki} = y_{gki} - \hat{\mu}_{gk}$. For gene '*g*', the null and alternative hypotheses are: H_{g0}: $\mu_{gk} = 0$ and H_{g1}: *at least one of* $\mu_{gk} \neq 0$. Using the extra-sum-of-squares method that fit the data to the full and reduced model, the hypothesis for gene '*g*' can be tested by the statistic $F_g = \frac{2G\sum_k \hat{\mu}_{gk}^2}{\sum_{lki}(y_{lki}-\hat{\mu}_{lk})^2}$, where $l = 1, 2, \ldots, G$; $k = 1$–4; $i = 1, 2$. A bootstrap-based method was used to compute the raw *p*-value ([31]; algorithm 3.1, p. 80).

(1) Compute $\hat{\mu}_{gk}$, F_g, and e_{gki}, for $g = 1, 2, \ldots, G$, $k = 1$–4, $i = 1, 2$.
(2) Draw a sample $\{e^*_{gki}\}$ with size 8G with replacement from $\{e_{gki}\}$. Then the bootstrap data set is $y^*_{gki} = e^*_{gki}$.
(3) Compute the bootstrap statistics $F^*_{g,b}$ for $g = 1, 2, \ldots, G$, where b is the bth resampling.
(4) Repeat steps (2) and (3) $B = 100{,}000$ times. The bootstrap-based raw *p*-values are

$$p^*_g = \frac{1}{B}\sum_{b=1}^{B} I(F^*_{g,b} \geq F_g), \quad g = 1, 2, \ldots, G,$$

where $I(\cdot)$ is the indicator function, which equals 1 if the condition in the parenthesis is true and 0 otherwise. This algorithm was implemented in a C^{++} program. R package *mutittest* was used for *p*-value adjustment that accounts for the multiplicity problem. Statistically differentially expressed genes were identified based on the Benjamini and Hochberg's false discovery rate (FDR) [32]. The 99% basic bootstrap confidence intervals (CIs) for the REV of each condition were computed as $\left(2^{\{\hat{\mu}_{gk} - y^*_{(0.995*(B+1))}\}}, 2^{\{\hat{\mu}_{gk} - y^*_{(0.005*(B+1))}\}}\right)$, where $\hat{\mu}_{gk}$ and y^*_{gki} are defined as above, and $y^*_{(n)}$ is the nth ordered value of $\{y^*_{gki}\}$. Hierachical clustering analysis was performed using *GeneCluster* and *TreeView* [33].

2.4. Real-time RT-PCR analysis of gene expression

The SuperScript™ III First-Strand Synthesis System was used to synthesize first-strand cDNA from total RNAs (100 ng of RNA was used for each reaction) according to the manufacturer's instruction (Invitrogen). The RNAs used for real-time RT-PCR were the same as those used in the microarray experiment. For each reaction, 2 μl of cDNA product was used for SYBR Green-based real-time PCR in an ABI 7300 real-time thermocycler according to the manufacturer's instruction (Applied Biosystems). The primer sequences are listed in Table 1. Samples were run in duplicate. The resulting threshold cycle (C_T) values were acquired using software provided by Applied Biosystems. The $\Delta\Delta C_T$ method [34] was used to determine the REV of genes of interest for treatment vs. vehicle control. For example, the REV of Hcrt to B2m (beta-2 microglobulin, an endogenous control) in the DHT treatment group, denoted as $\Delta C_{T\text{-DHT}}$, equals Hcrt $C_{T\text{-DHT}}$ minus B2m $C_{T\text{-DHT}}$ in the DHT treatment group ($\Delta C_{T\text{-DHT}}$ = Hcrt $C_{T\text{-DHT}}$ − B2m $C_{T\text{-DHT}}$); and the REV of Hcrt to B2m for the vehicle control group, $\Delta C_{T\text{-VEH}}$ = Hcrt $C_{T\text{-VEH}}$ − B2m $C_{T\text{-VEH}}$. Thus, the REV of Hcrt for the DHT treatment to the vehicle control, $\Delta\Delta C_T = \Delta C_{T\text{-DHT}} - \Delta C_{T\text{-VEH}}$. In terms of fold induction, the REV of Hcrt for the treatment to the control is $2^{-\Delta\Delta C_T}$.

2.5. Immunohistochemical (IHC) analysis of prepro-Hcrt (pHcrt) level in the lateral hypothalamus

To evaluate the effects of DHEA and DHT treatments on brain pHcrt levels, OVX CF-1 female mice were randomly divided among three groups ($n = 5$) and treated as described earlier. Three hours after the last injection, mice were anesthetized with sodium pentobarbital and perfused with ice-cold PBS followed by 4% formaldehyde in PBS. The brains were post-fixed for 24 h in the same fixative and 30 μm frozen sections were cut on a rotary microtome. Sections were treated with 0.15% Trition X-100/PBS for 40 min, then 10% fetal bovine serum/PBS for 30 min. Free-floating sections were incubated for 48 h at 4 °C in 1% BSA/PBS containing a rabbit polyclonal pHcrt antiserum (1 μg/ml; Abcam, Cambridge, MA). The sections were washed and placed in biotinylated goat antirabbit IgG solution (1:400 Vector Labs, Burlingame, CA) for 2 h, washed in PBS, and then incubated in avidin–biotin–peroxidase complex solution (prepared according to the manufacturer's instructions) for 2 h. Nickel (0.16%) intensified diaminobenzadine (1 mg/ml) was used as the chromogen. After IHC staining, sections were mounted on gelatin-treated glass slides, air dried, dehydrated through a graded ethanol series, cleaned in Hemo D, and coverslipped with Permount medium.

2.6. Image analysis

Immunoreactive pHcrt signals in brain sections were quantified according to previously published methods, with minor modifications [27]. Briefly, sections were matched between treatment groups according to Franklin and Paxinos [35]. Hcrt immunohistochemical staining was only visible in the lateral hypothalamus (LH). Images were captured with a MagnaFire CCD digital camera connected to a Dell computer with an Optonic IEEE-1394 PCI card, using the 10× objective of a Nikon TE2000 microscope. Measurements of staining intensity were made after the images were thresholded by density slicing to the same value. The mean background density for each image was determined after density slicing the background area and was used as a correction factor. Data generated by this approach include the total area of stained particles and mean particle density. These parameters were used to calculate the integrated particle density (IPD), defined as the total area of stained particles × (mean particle density − mean background density). The IPD provided a semi-quantitative index of average staining intensity within the LH region and was used as the unit of analysis.

Table 1
Primer sequences for the real-time quantitative RT-PCR.

Gene name and GeneBank accession #	Sequence (5′ → 3′)	Position in the exons	Amplicon length (bp)
Pmch	Fwd: AAGAATTCAAAGAACACAGGCTCC	1/2	112
(AK020723)	Rev: TCCGTAGCCTTCCCAGCTG	2	
Hcrt	Fwd: CCTTCTACAAAGGTTCCCTGGG	1/2	108
(NM_010410)	Rev: CCTCTGCCCGACTGCTGT	2	
Prkcd	Fwd: CTTTGGCAAGGTGCTGCTG	10/11	121
(NM_011103)	Rev: TACCATGGTGGAGAAGCGG	11	
B2m	Fwd: TCTGGTGCTTGTCTCACTGACC	1	102
(NM_009735)	Rev: AGTATGTTCGGCTTCCCATTCT	2	

The positions of the primers in the exons are annotated based on the sequences obtained from "Ensembl Mouse ExonView" (http://www.ensembl.org/). Fwd, forward primer; Rev, reverse primer.

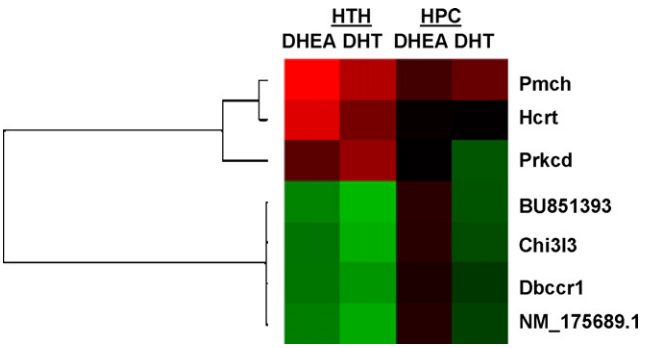

Fig. 1. Hierarchical clustering analysis of differentially expressed genes. The Pearson correlation coefficient is used as the similarity metric. Genes with similar expression profiles are clustered to the immediate vicinity of each other. Red, green, and black represent expression levels that are up-regulated, down-regulated, and not changed, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.7. RIA of serum DHEA, DHT, and estradiol (E2)

Trunk blood samples ($n = 12$) were collected into microtainer serum separator tubes (BD Biosciences, NJ). Samples were allowed to sit at room temperature for 10 min then centrifuged at 2500 × *g* for 15 min. Serum was stored at −72 °C until use. On the day of assay, serum was thawed at 4 °C. Samples from three animals within each group were pooled and divided for the determination of DHEA, DHT and E2 concentrations. Assays were performed according to the manufacturer's instructions (DSL, TX) with duplicate determinations for each sample. Data were analyzed and mean serum concentrations interpolated from the standard curve using GraphPad 5 (GraphPad, CA).

3. Results

3.1. Significance analysis of gene expression

The ANOVA model without normality assumption on random errors was applied to identify differentially expressed genes (see Section 2). A gene is said to be differentially expressed if any log-ratio is statistically significantly different from zero in any of the four conditions. Differentially expressed genes were determined based on the false discovery rate that account for the multiple testing problem [32]. With an FDR set to 0.05, five genes showed statistically significant alterations in expression in response to DHEA and/or DHT treatments (Table 2). These five genes include three annotated genes, Pmch, Hcrt and Chi3l3 (chitinase 3-like 3), and two unannotated genes BU851393 and NM_175689.1 (GeneBank accession numbers). Using an FDR of 0.063, another two genes, protein kinase C delta (Prkcd) and deleted in bladder cancer chromosome region candidate 1 (Dbccr1) were also differentially expressed. The DHEA- and DHT-induced fold changes and their basic bootstrap 99% confidence intervals are shown in Table 2. Pmch, Hcrt and Prkcd were up-regulated in the hypothalamus by both DHEA and DHT treatment (Table 2 and Fig. 1). Because MCH and Hcrt neurons are predominantly distributed in the hypothalamus [36,37], it is not surprising to find that the effects of DHEA and DHT on the expression of these genes were more potent in the hypothalamus compared to hippocampus (Table 2). In addition, it is noteworthy that DHT down-regulated Prkcd in the hippocampus. The other four genes, Chi3l3, Dbccr1 and the two unannotated genes, were down-regulated by DHEA and DHT treatments in the hypothalamus (Table 2 and Fig. 1). These genes were not differentially expressed in the hippocampus as inferred from the 99% confidence intervals. Fig. 1 displays the hierarchical clustering result for the seven differentially expressed genes using Pearson correlation coefficient as the similarity metric [33]. Genes with similar expression profiles clustered in the immediate vicinity of one another (e.g. Hcrt, Pmch and Prkcd).

Table 3
Relative gene expression (in fold change) for treatments vs. vehicle control based on the real-time RT-PCR experiment.

Gene Name	DHEA/Veh HTH	DHT/Veh HTH	DHEA/Veh HPC	DHT/Veh HPC
Pmch	4.41	2.19	1.57	1.83
Hcrt	3.07	1.53	1.19	1.38
Prkcd	2.12	2.94	1.17	0.55

3.2. Relative quantitation of gene expression using real-time RT-PCR

Two-step real-time RT-PCR was used to confirm changes in the expression of three genes (Pmch, Hcrt and Prkcd) using the same RNA sample employed in the microarray experiment. Beta-2 microglobulin (B2m) was used as an endogenous control because the microarray experiment showed that its expression levels were relatively constant across the four conditions (2 treatments × 2 brain regions). To reduce possible non-specific amplification of genomic DNA, the forward primers for the three target genes were designed to cross exon and intron boundaries, and the two primers for B2m were located in two adjacent exons (Table 1). Real-time PCR was performed using SYBR Green I dye as the detection reagent. The amplification plots of the four genes from the mouse hypothalamus and hippocampus are shown in Figs. 2 and 3. For the hypothalamus, the three target genes have lower threshold cycles in samples from DHEA- and DHT-treated animals than in those from controls, indicating that the amounts of target mRNAs were greater in the former than the latter (Fig. 2a–c). In the hippocampus, the Pmch gene has a smaller threshold cycle in DHEA- and DHT-treated mice (Fig. 3a), and the Prkcd gene has a lower threshold cycle following DHT treatment (Fig. 3c). The differences in threshold cycles for the other genes are trivial (Figs. 2d and 3b and d). Applying the $\Delta\Delta C_T$ method, the changes in gene expression were calculated and shown in Table 3. The real-time RT-PCR results are consistent with the microarray experiment results (Table 2), although in general the changes in gene expression are slightly larger in the real-time RT-PCR experiment than in the microarray experiment. For example,

Table 2
Relative gene expression (in fold change) for the treatments to vehicle control for the seven differentially expressed genes.

Gene name	BH adjusted *p*-value	DHEA/Veh HTH	DHT/Veh HTH	DHEA/Veh HPC	DHT/Veh HPC
Pmch	<0.001	3.50 (2.72, 4.47)	2.02 (1.57, 2.59)	1.30 (1.01, 1.66)	1.50 (1.17, 1.92)
Hcrt	<0.001	2.41 (1.89, 3.10)	1.59 (1.25, 2.05)	1.03 (0.81, 1.32)	1.01 (0.79, 1.30)
Prkcd	0.063	1.43 (1.11, 1.85)	1.81 (1.40, 2.33)	1.01 (0.79, 1.31)	0.71 (0.55, 0.91)
DBCCR1	0.063	0.63 (0.49, 0.80)	0.55 (0.43, 0.71)	1.12 (0.88, 1.43)	0.80 (0.63, 1.03)
Chi3l3	0.029	0.63 (0.49, 0.81)	0.50 (0.39, 0.65)	1.17 (0.91, 1.51)	0.74 (0.58, 0.96)
BU851393	<0.001	0.59 (0.46, 0.76)	0.48 (0.38, 0.62)	1.18 (0.92, 1.52)	0.72 (0.56, 0.92)
NM_175689.1	<0.001	0.61 (0.47, 0.78)	0.51 (0.40, 0.66)	1.16 (0.90, 1.49)	0.77 (0.60, 0.99)

The numbers in the parentheses are the 99% basic bootstrap confidence intervals (CIs).

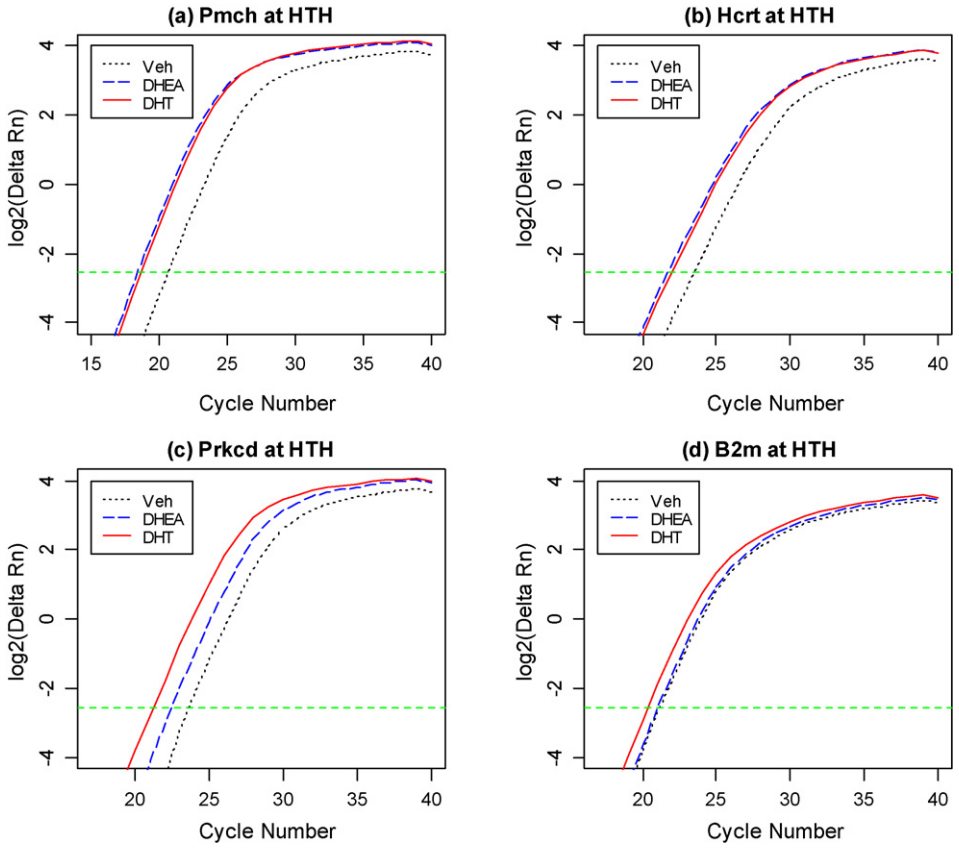

Fig. 2. Amplification plots of Pmch, Hcrt, Prkcd and B2m genes in the mouse hypothalamus (HTH). The cDNAs of the four genes were amplified by using the ABI 7300 real-time thermocycler (Applied Biosystems). Delta Rn is the fluorescence values of SYBR Green I dye at each cycle subtracted from the fluorescence values of the background.

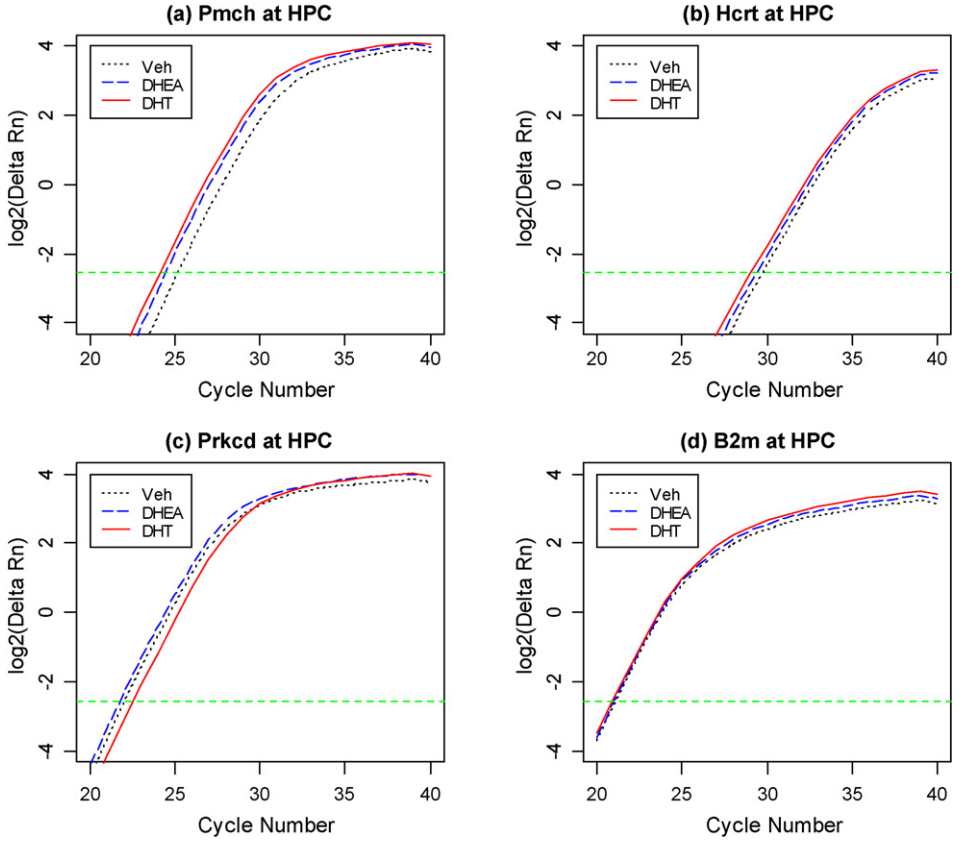

Fig. 3. Amplification plots of Pmch, Hcrt, Prkcd and B2m genes in the mouse hippocampus (HPC). The cDNAs of the four genes were amplified by using the ABI 7300 real-time thermocycler (Applied Biosystems). Delta Rn is the fluorescence values of SYBR Green I dye at each cycle subtracted from the fluorescence values of the background.

Table 4
Serum DHEA, DHT, and E2 concentrations (pg/ml) after 7 days of treatment with either DHEA (1 mg), DHT (100 μg) or VEH. Values shown are mean ± S.E.M.

Steroid concentration	Treatment		
	VEH	DHEA	DHT
DHEA	1499.66 ± 75.32	66310.81 ± 11219	1740.81 ± 102.11
DHT	37.30 ± 3.96	74.11 ± 6.62	267.76 ± 46.23
E2	ND[a]	ND	ND

[a] Observed values were below lower limit of detection (4.7 pg/ml).

Hcrt was up-regulated 2.41-fold by DHEA in the hypothalamus in the microarray experiment, but 3.07-fold in the real-time RT-PCR experiment. Correspondingly, Prkcd was down-regulated 0.71-fold by DHT in the hippocampus in the microarray experiment, but 0.55-fold in the real-time RT-PCR experiment. This is commonly the case; real-time RT-PCR is more sensitive and quantitative than microarray-based methods.

3.3. Immunohistochemical (IHC) analysis of prepro-Hcrt levels

Because products of gene expression can be regulated at levels other than transcription, we assessed whether the expression of the Hcrt gene was regulated at the protein level by DHEA and DHT treatments. pHcrt was immunohistochemically stained and the resulting images were analyzed using Scion Image software. pHcrt signals were observed exclusively in the lateral hypothalamus (Fig. 4), mainly in neuronal perikarya and processes, consistent with the earlier reports. Both DHEA and DHT significantly increased pHcrt levels compared to vehicle treatment ($p < 0.01$, Newman–Keuls multiple comparison test). There was no significant difference in IPD between the DHEA and DHT treatments (Fig. 4).

3.4. Plasma RIA of DHEA, DHT, and E2

The RIA results are shown in Table 4. Compared to values in VEH animals, DHEA treatment produced a nearly 45-fold increase in serum DHEA to over 66,000 pg/ml, no detectable change in E2, and a 1-fold increase in DHT to 74 pg/ml. The DHT treatment produced a 7.2-fold increase in serum DHT, no significant change in DHEA, and no detectable change in E2 compared to VEH controls.

4. Discussion

In the current study, we identified seven genes with significantly altered expression in response to DHEA and DHT treatment in mouse hypothalamus and hippocampus using DNA microarrays, confirmed the changes in Hcrt, Pmch and Prkcd transcript levels using real-time RT-PCR and, in the case of Hcrt, also by immunohistochemistry. The findings represent the first demonstration of DHEA-induced changes in gene expression in the CNS and, given the similarities in the effects between DHEA and DHT, extend previous studies that had strongly suggested AR as a transcription factor mediating potential genomic effects of DHEA. Broadly speaking, the regulated genes are involved in energy homeostasis, appetite, alertness (Hcrt and Pmch), and apoptosis/cell survival (Prkcd, Dbccr1 and Chi3l3), indicating that a range of functional consequences may result from changes in their expression. The observed genomic effects, then, may help provide a molecular basis for the diverse effects of DHEA reported in humans and rodents.

Both Hcrt and Pmch were up-regulated in the hypothalamus in response to DHEA and DHT treatments. Increased activities of the peptide products of these genes, orexin and MCH, are generally linked to better appetite, energy homeostasis, and normal sleep [38–44]. Improvement in sense of well-being, enhanced libido, and remittance of depressive symptoms might be expected to accompany these effects [45,15]. Results of studies in animals and patients with adrenal insufficiency support this suggestion. In humans, for example, salivary DHEA was elevated in the first three hours post-awakening, a period defined by heightened alertness [46]. Women with androgen deficiency treated with oral DHEA (20–30 mg/day) for 6 months showed significant improvements in alertness, initiative, and stamina according to partner questionnaires [47]. Reduced libido has been described in women with lower DHEA levels vs. comparably aged controls [48]. In patients with adrenal insufficiency, glucocortoicoid and mineralocorticoid replacement leaves impairments in well-being and it has been suggested that addition of DHEA may be needed to address this aspect of the disease [4,49,8]. In some patient populations with major and minor depression, DHEA replacement reduced depressive symptoms [50,51].

The MCH receptor type 1 (MCHR1) has been suggested as a therapeutic target for the treatment of anxiety and stress. Inhibitors of MCHR1 function reportedly had anxiolytic and anti-depressant effects in rodent models [52,53], but not all workers have replicated these findings [54] and our data do not support them. Also, administration of MCH in non-stressed animals leads to increased sexual behavior and improved appetite [55–57]. Further, DHEA to date has not been described as anxiogenic and when administrated to humans appear either to be beneficial or without significant effect (e.g. see review by von Muhlen et al. [19]).

The observed changes in gene expression show that DHEA affects cells in the CNS. How DHEA acts there is still an open question. Most past studies focused on effects mediated by membrane receptors. The parallel up-regulation of Hcrt and Pmch by DHEA or DHT treatment suggests that DHEA may act within neurons by activating the androgen receptor, which is abundant in hypothalamus and hippocampus [24,25,27,28]. This is consistent with the fact that DHEA promotes androgen-regulated gene activity in a CAT reporter system, induces nuclear accumulation of AR in transfected CV-1 cells, and competes with DHT for AR binding in a cell-free assay system [24–26]. Orexin expression is androgen-sensitive and shows down-regulation after castration in rats [58]. Studies on the steroidal regulation of Pmch are lacking in mammals, but in goldfish both testosterone up-regulates Pmch mRNA in mediobasal hypothalamus [59]. It seems reasonable to infer that androgens, including DHEA, play a fundamental role in maintaining Hcrt and Pmch expression through which multiple biological functions are regulated or influenced.

The other genes significantly regulated by DHEA – Prkcd, Dbccr1, and Chi3l3 – are implicated in cell survival. Prkcd mRNA and protein were up-regulated in several brain regions after focal brain ischemia, a change associated with neuronal death [60,61]. Over-expression of Prkcd-induced apoptosis, while inhibition of Prkcd or expression of a dominant negative mutant of Prkcd inhibited apoptosis [62–64]. Dbccr1 is a potential tumor suppressor gene [65]. Over-expression of Dbccr1 in bladder tumor cells suppressed cell proliferation and even caused cell death [66,67]. Chitinase-3l3 increases with aging, after neuronal injury, and during remodeling [68]. In response to DHEA and DHT treatment, Prkcd was up-regulated in hypthalamus, while Dbccr1 and Chi3l3 were down-regulated. Interestingly, Prkcd was down-regulated by DHT in the hippocampus. The absence of regional consistency in the expression of Prkcd is not unusual but it makes interpretation of these findings difficult. It appears reasonable, then, to suggest that cell type influences the function of Prkcd. For example, while several investigations have shown that increased expression of this kinase is associated with apoptosis and cell death [62,63,69], a recent study demonstrated that Prkcd was a positive modulator of cell survival and proliferation in murine mammary cells [70]. The functional importance of the change in Dbccr1 expression is unclear. In the CNS, lowered expression was found in astrocytomas [71], but

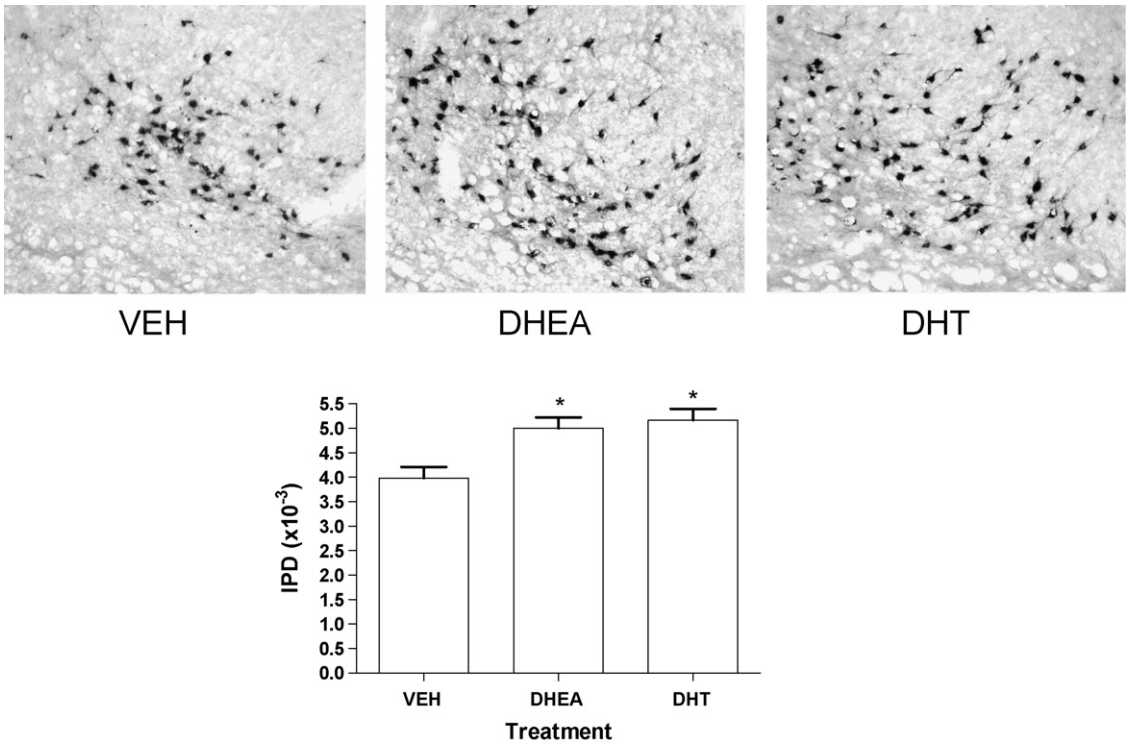

Fig. 4. Immunohistochemical (IHC) analysis for effects of DHEA and DHT on Hcrt levels. GDX CF-1 female mice were injected i.p. daily with 1 mg DHEA or 100 μg DHT for 7 consecutive days. Brain sections were incubated with Hcrt antiserum (1 μg/mL) and further processed to allow color development. Hcrt signals in lateral hypothalamas were imaged, semi-quantified using Scion Image Beta 4.0.2, and statistically analyzed with Graphpad software. Representative Hcrt images for DHEA, DHT and vehicle groups are shown, bar value represents mean IPD ± S.E.M. *Significantly different.

to the best of our knowledge, specific functions in non-cancerous neural cells have not been defined. Down-regulation of Chi3l3 is of interest because of the anti-aging effect of chronic DHEA replacement. Decreased activation would be consistent with this effect as well as with the neuroprotective effect of DHEA. Interestingly, DHEA, T, and other androgens protect against excitatory amino acid-induced neuronal injury or death *in vitro* and *in vivo* [72,73]. For example, DHEA and T attenuated NMDA- and AMPA-induced neurotoxicity in rat hippocampal neurons and reduced oxidative brain damage induced by chronic hyperglycemia or ischemic insult in rat brain [11,74,75,70]. DHEA also protected against neuronal damage induced by serum deprivation and cell death-induced *in vitro* by hydrogen peroxide [76,72]. Androgens and AR are central in these effects because flutamide, a non-steroidal anti-androgen that specifically binds to AR, prevented neuroprotection [73].

One wonders whether significant metabolism of DHEA into other androgens or estrogens is a necessary step for activity in brain cells. The current RIA data support the view that it is not obligatory in the CNS, although it has been suggested that this may be important in the periphery [17,77]. Metabolism of DHEA to other major steroids was minimal following exogenous treatment. Plasma DHEA levels were some 45-fold higher in treated animals compared to controls after a 1 mg per day dose for 7 days. There was no detectable change in circulating E2 and the increase in DHT compared to VEH animals was less than 0.06% compared to that seen in DHEA. Although we cannot exclude the possibility of local metabolism in the brain that was not detected by the serum RIAs, it seems unlikely that such effects occurred. In cultured GT1-7 cells and embryonic rat hippocampal neurons, the major metabolites of DHEA are 7α-OH-DHEA and 7β-OH-DHEA, which are essentially devoid of androgenic or estrogenic activity [24,25,78,79,26,80]. On the other hand, BV2 microglia are capable of metabolizing DHEA to Δ^5-adiol, which can exert weak androgenic or estrogenic activity [81]. Androstenedione (Adione) and androstenediol have been detected as DHEA metabolites in astrocytes, but the yield is significantly influenced by cell density [82,83]. When cells were plated at low density, Adione accounted for about 10% of the total metabolites of DHEA. At high density, however, 7α-OH-DHEA was the major metabolite and Adione was almost undetectable [82]. Other studies *in vitro* demonstrated that DHEA has androgenic activity even in the presence of trilostane, a known 3β-hydroxysteroid dehydrogenase inhibitor that blocks conversion of DHEA to more potent androgens [24,25]. These findings are consistent with the hypothesis that DHEA acts directly on the androgenic signaling pathway.

It is noteworthy that far fewer genes seem to be affected by DHEA in the brain than in prostate or LnCAP cells [84,85]. In studies of the latter, the DHEA dose used was 3–6× higher than the one we employed. In addition, peripheral androgen-dependent tissues such as the prostate and seminal vesicles, as well as LnCAP cells, are particularly rich in AR compared to brain cells and are likely to be far more responsive to androgenic stimulation.

DHEA treatment for 1 week produced significant changes in gene expression in hypothalamus and hippocampus. The significantly affected genes include hypocretin (Hcrt), pro-melanin-concentrating hormone, protein kinase C delta, deleted in bladder cancer chromosome region candidate 1, and chitinase 3-like 3, which collectively are directly implicated in the regulation of appetite, energy utilization, alertness, apoptosis, and cell survival. The observed neurogenomic effects may help explain the diverse benefits attributed to replacement therapy with DHEA. In addition, the findings advance our understanding of the genomic mechanism of action of DHEA in the CNS and strongly support a central role for the androgen receptor in the production of the effects. Finally, the results have significant implications for the clinical use of DHEA because they provide new insights into processes that appear to mediate the diverse CNS effects attributed to this steroid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.01.015.

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