Estradiol and Testosterone Regulate Arginine-Vasopressin Expression in SH-SY5Y Human Female Neuroblastoma Cells Through Estrogen Receptors-α and -β

Daniela Grassi, Maria Jose Bellini, Estefania Acaz-Fonseca, GianCarlo Panzica, and Luis M. Garcia-Segura

Instituto Cajal (D.G., M.J.B., E.A.-F., L.M.G.-S.), Consejo Suerior de Investigaciones Científicas, 28002 Madrid, Spain; Department of Neuroscience (D.G., G.P.), University of Turin, 10126 Turin, Italy; and Neuroscience Institute Cavalieri-Ottolenghi (G.P.), Orbassano, 10043 Turin, Italy

The expression of arginine-vasopressin (AVP) is regulated by estradiol and testosterone (T) in different neuronal populations by mechanisms that are not yet fully understood. Estrogen receptors (ERs) have been shown to participate in the regulation of AVP neurons by estradiol. In addition, there is evidence of the participation of ERβ in the regulation of AVP expression exerted by T via its metabolite 5α-dihydrotestosterone (5α-DHT) and its further conversion in the androgen metabolite and ERβ ligand 3β-diol. In this study we have explored the role of ERs in the regulation exerted by estradiol and T on AVP expression, using the human neuroblastoma cell line SH-SY5Y. Estradiol treatment increased AVP mRNA levels in SH-SY5Y cells in comparison with cells treated with vehicle. The stimulatory effect of estradiol on AVP expression was imitated by the ERα agonist 4,4′,4′,-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol and blocked by the ER antagonist, ICI 182,780, and the ERα antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1hpyrazoledihydrochloride. In contrast, the ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile reduced AVP expression, whereas the ERβ antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol enhanced the action of estradiol on AVP expression. T increased AVP expression in SH-SY5Y cells by a mechanism that was dependent on aromatase but not on 5α-reductase activity. The T effect was not affected by blocking the androgen receptor, was not imitated by the T metabolite 5α-DHT, and was blocked by the ERα antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1hpyrazoledihydrochloride. In contrast, 5α-DHT had a similar effect as the ERβ agonists 2,3-bis(4-hydroxyphenyl)-propionitrile and 3β-diol, reducing AVP expression. These findings suggest that estradiol and T regulate AVP expression in SH-SY5Y cells through ERs, exerting a stimulatory action via ERα and an inhibitory action via ERβ. **(*Endocrinology* 154: 2092–2100, 2013)**

Arginine-vasopressin (AVP) is expressed by different neuronal populations in the brain (1–3) and is involved in different functions, including the control of water homeostasis (4–7), blood pressure (8–10), thermoregulation (11–13), social behavior (3, 14, 15), and stress response (16–19). Synaptic activity regulates the firing of magnocellular AVP neurons (20), and in particular approximately 60% of the total number of synapses in the supraoptic (SON) and paraventricular (PVN) nuclei use γ-aminobutyric acid as neurotransmitter (21). In addition, several endocrine factors, including gonadal hormones, may participate in the reg-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received November 16, 2012. Accepted April 2, 2013.
First Published Online April 12, 2013

Abbreviations: AR, androgen receptor; AVP, arginine-vasopressin; 5α-DHT, 5α-dihydrotestosterone; 3β-diol, 5α-androstane-3β,17β-diol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; ER, estrogen receptor; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1hpyrazoledihydrochloride; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol; PPT, 4,4′,4′,-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; PVN, paraventricular nucleus; SON, supraoptic nucleus; T, testosterone.

ulation of AVP neurons and AVP expression. Both estradiol and T have been shown to regulate AVP expression in the brain (22–24). In particular, estradiol is able to regulate AVP expression in the PVN of male mice (25), and it increases AVP plasma levels in ovariectomized female rats (22, 26), whereas T regulates AVP expression in the SON (27) and in the bed nucleus of the stria terminalis (28) of male rats.

The mechanisms involved in the regulation of AVP expression by T and estradiol are not fully clarified. Estrogen receptors (ERs) have been shown to participate in the regulation of AVP neurons by estradiol (29–32). ERβ is expressed in AVP neurons of the SON and the PVN (33–35), whereas ERα is expressed in the medial preoptic area (31), lateral septum, and medial amygdala (32), important targets for the parvocellular AVP system. Moreover, ERα seems to be involved in the regulation of AVP expression in the SON and PVN of female rats (30, 36), probably through a mechanism mediated by afferent neurons projecting to the SON and PVN and expressing ERα (7, 30, 37, 38). However, there is also evidence of T participation in the ERβ regulation of AVP expression via its metabolite 5α-dihydrotestosterone (5α-DHT) and its further conversion in 3β-diol, which is a ligand of ERβ (39, 40).

In this study we explored the role of ERs in the regulation exerted by estradiol and T on AVP expression, using the human neuroblastoma cell line SH-SY5Y. These cells express ERs (41) and aromatase (42). In the present study, we determined that SH-SY5Y also expresses AVP and is therefore a good model to assess the role of ERs in the action of T and estradiol on AVP expression. Our findings indicate that both estradiol and T exert stimulatory and inhibitory actions on AVP mRNA levels acting through ERα and ERβ, respectively.

Materials and Methods

Cell culture

The human SH-SY5Y neuroblastoma cell line was used in this study. SH-SY5Y cells are genetically female. The line was purchased from American Type Culture Collection (Manassas, Virginia) and was cultured in DMEM-F12 supplemented with 10% fetal bovine serum. Cells were passaged twice per week and were not used after 10 passages.

Treatments

After 12 hours of serum starvation using serum-free medium, cells were incubated with fresh serum-free medium containing vehicle (ethanol); 17β-estradiol (from 10^{-10} to 10^{-7} M; Sigma, Madrid, Spain); ICI 182,780 (10^{-9} M; Sigma); 4,4′,4′,-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; from 10^{-10} to 10^{-7} M; Tocris, Madrid, Spain); 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1hpyrazoledihydrochloride (MPP; from 10^{-9} to 10^{-6} M; Tocris); 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; from 10^{-10} to 10^{-7} M; Tocris); 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; from 10^{-9} to 10^{-6} M; Tocris); T (from 10^{-9} to 10^{-5} M; Sigma); 5α-DHT (from 10^{-10} to 10^{-6} M; Sigma); 3β-diol (5α-androstane-3β,17β-diol (from 10^{-9} to 10^{-6} M; Sigma); flutamide (from 10^{-9} to 10^{-5} M; Sigma); letrozole (10^{-10} to 10^{-7} M, Novartis, Basel, Switzerland); and/or finasteride (10^{-10} to 10^{-6} M; Sigma).

RNA isolation and quantitative real-time PCR

After 24 hours, cells were lysed and total RNA was extracted using the illustra RNAspin mini-RNA isolation kit (GE Healthcare, Buckinghamshire, United Kingdom) as per the manufacturer's instructions to measure AVP, ERα, and ERβ mRNA levels. 18S rRNA was used as the control housekeeping gene. Deoxyribonuclease digestion was carried out on a column using the same kit. The first cDNA strand was synthesized using all RNA extracted by reverse transcription in a final volume of 15 μL using the RevertAidTM H Minus first-strand cDNA synthesis kit (MBI Fermentas, Bath, United Kingdom) according to the supplied protocol. After reverse transcription, the cDNA was diluted 1:4 and 5 μL were amplified by real-time PCR in 20 μL using SYBR Green master mix (Applied Biosystems, Foster City, California) in an ABI Prism 7500 sequence detector (Applied Biosystems), with conventional Applied Biosystems cycling parameters (40 cycles of 95°C, 15 seconds, and 60°C, 1 minute).

Primers for human AVP, human androgen receptor (AR), and human 18S were designed using Primer Express 1.0 (Applied Biosystems) and Amplify software. Primers for human ERα and human ERβ were used as previous described in our group (43). See Table 1 for transcript references and primer sequences.

After amplification, the size of the quantitative real-time PCR products was verified by electrophoresis on 2% (wt/vol) ethidium bromide-stained agarose gel, and the identity of PCR products was ascertained by sequencing. Bands were excised and

Table 1. Transcript References and Primer Sequences Used for Quantitative Real-Time-PCR

Transcript	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
AVP (NM_000490.4)	TGTGTGCACCAGGATGCCT	TCAGCTCCAGGTCGGACAT
18S (NR_003286.1)	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG
ERα (NM_000125.1)	GCTTCGATGATGGGCTTACTGA	ATGCGGAACCGAGATGATGT
ERβ *(AF051427)	AGATTCCCGGCTTTGTGG	GCTTCCGGCTGCTGTCA
AR transcript 1 (NM_000044.3)	CCCATCCCCACGCTCGCATC	CATGCAGGCTCGCCAGGTCC

cDNA was purified using the QIAquick PCR purification kit (QIAGEN Gmbg, Hilden, Germany). One hundred nanograms of AVP, AR, ERα, and ERβ mRNA samples were sequenced (Automatic Sequencing Center, Consejo Suerior de Investigaciones Científicas, Madrid, Spain) with the same sense and antisense primers. The obtained sequence was aligned with the expected sequence obtained from the GenBank. In addition, dissociation curve analysis was also performed after each PCR to ensure that a single product and no primer-dimers were present. All reactions were performed in triplicate. The relative mRNA expression level was calculated using the ΔΔCT method, using ribosomal 18S RNA as the endogenous RNA control to correct for the amount of total RNA used in each PCR reaction.

Statistical analysis

Data were analyzed via 1-way ANOVA, followed, when appropriate, by a post hoc analysis with the Bonferroni's test. The SPSS 17.0 software (SPSS Inc, Chicago, Illinois) was used for calculating probability values. $P < .05$ was considered statistically significant. Data are represented as the mean ± SEM. Results of the statistical analyses are shown in the figure legends.

Results

Characterization of AVP, ER, and AR expression in SH-SY5Y neuronal cells

SH-SY5Y cells were initially tested for the expression of AVP, ERs and AR. Real-time PCR indicated the expression of AVP, ERα, and ERβ mRNA in this cell line (Figure 1). In contrast, we did not detect AR mRNA in our cells (as positive control we used the prostate adenocarcinome cell line LNCaP). Sequence analysis by automatic sequencing with the same sense and antisense primers revealed coincidence with human AVP, ERα, ERβ, and AR mRNA sequences, respectively.

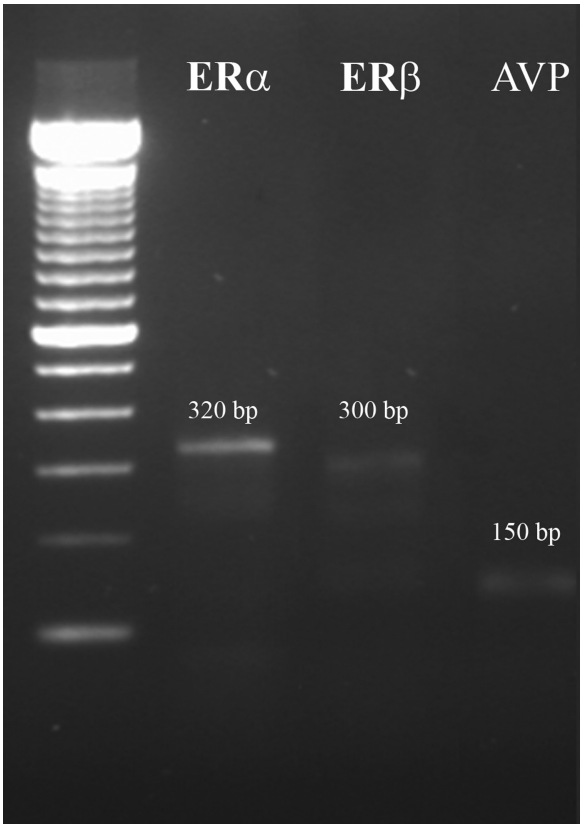

Figure 1. PCR analysis of ERα, ERβ, and AVP in SH-SY5Y human neuroblastoma cells.

AVP mRNA levels were up-regulated by estradiol in SH-SY5Y neuronal cells by a mechanism blocked by an ERα antagonist and enhanced by an ERβ antagonist

SH-SY5Y cells were treated with either vehicle or estradiol for 24 hours. Estradiol treatment at concentrations of 10^{-7} and 10^{-8} M did not significantly affect AVP mRNA levels. However, a significant increase in AVP mRNA levels was detected when the cells were treated with 10^{-9} or 10^{-10} M estradiol. The maximal effect was observed at 10^{-9} M (Figure 2A). Treatment with ER antagonists MPP, PHTPP, and ICI 182,780 alone, did not affect AVP mRNA levels (data not shown). However, the effect of estradiol was blocked by the ERα antagonist MPP (Figure 2B) and by the ER antagonist ICI 182,780 (Figure 2C) but not by the ERβ antagonist PHTPP (Figure 2E). In addition, the combination of estradiol with the ERα antagonist MPP at 10^{-7} M resulted in significantly lower AVP mRNA levels compared with control values. In contrast, the combination of estradiol with the ERβ antagonist PHTPP at 10^{-7} M resulted in significantly higher AVP mRNA levels compared with estradiol values (Figure 2, B and D). A possible interpretation of these results is that estradiol is acting on both ERs, enhancing or decreasing AVP mRNA levels through ERα or ERβ, respectively. To test this hypothesis, in the next experiment, cells were treated with an ERα or an ERβ agonist.

AVP mRNA levels were up-regulated by an ERα agonist and down-regulated by an ERβ agonist in SH-SY5Y neuronal cells

As shown in Figure 2E, treatment of SH-SY5Y cells with the ERα agonist PPT, at 10^{-8} M, resulted in a significant increase in AVP mRNA levels compared with control values. In contrast, the treatment with the ERβ agonist DPN, at 10^{-7} M, resulted in a significant decrease in AVP mRNA levels (Figure 2F). The enhancement of AVP mRNA levels by PPT was stronger than the decrease in

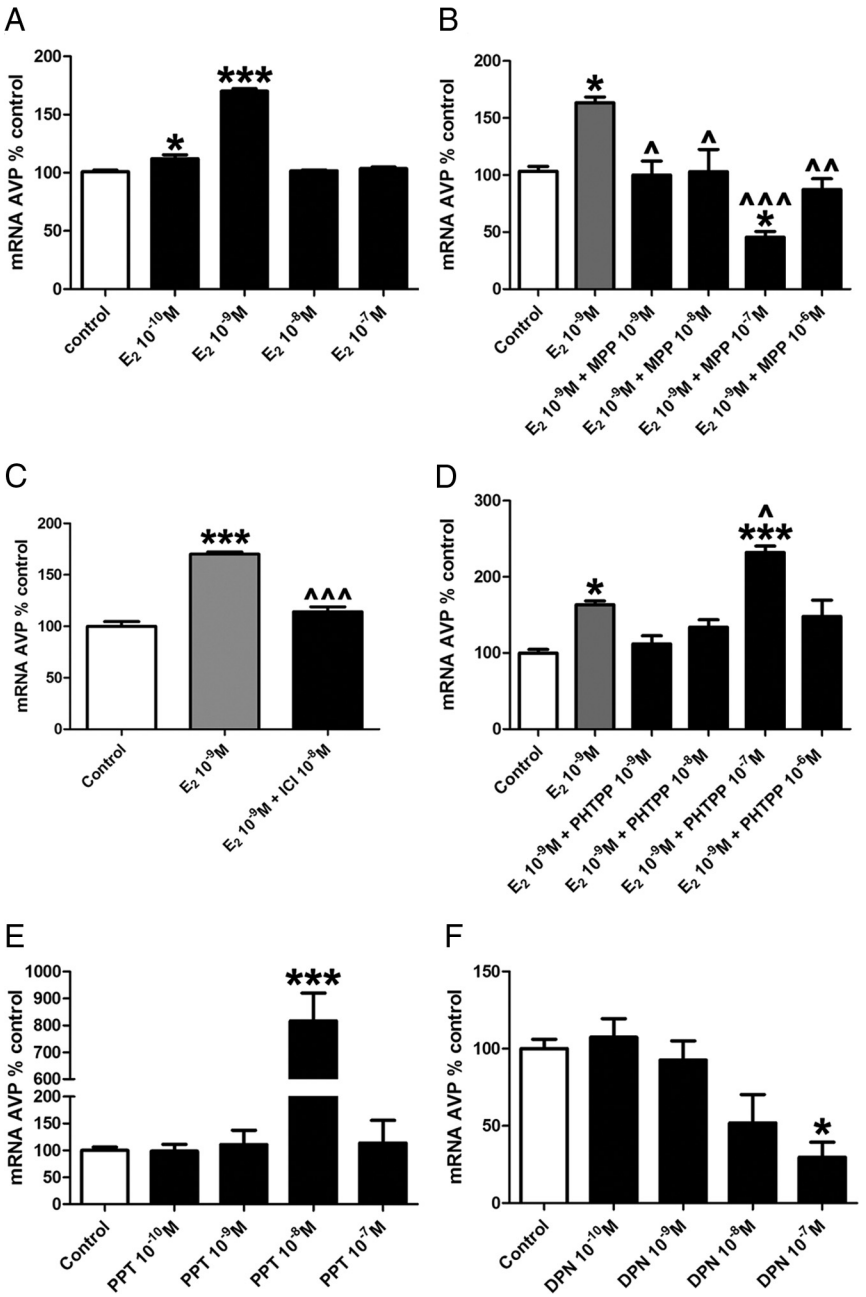

Figure 2. AVP mRNA levels in SH-SY5Y human neuroblastoma cells. A, Cells were treated for 24 hours with vehicle (control) or different doses of 17β-estradiol (E2). ANOVA: $F_{(4, 19)} = 221.3$; $P < .0001$. B, Cells were treated for 24 hours with vehicle, 10^{-9} M E2, or 10^{-9} M E2 and different doses of the ERα antagonist MPP. ANOVA: $F_{(5, 17)} = 12.53.3$; $P = .0002$. C, Cells were treated for 24 hours with vehicle, 10^{-9} M E2, or 10^{-9} M E2 and the ER antagonist ICI 182,780 (ICI; 10^{-8} M). ANOVA: $F_{(4, 14)} = 175.9$; $P < .001$. D, Cells were treated for 24 hours with vehicle, 10^{-9} M E2, or 10^{-9} M E2 and different doses of the ERβ antagonist PHTPP. ANOVA: $F_{(5, 17)} = 16.84$; $P < .0001$. E, Cells were treated for 24 hours with vehicle or different doses of the ERα agonist PPT. ANOVA: $F_{(4, 21)} = 25.03$; $P < .0001$. F, Cells were treated for 24 hours with vehicle or different doses of the ERβ agonist DPN. ANOVA: $F_{(4, 23)} = 6.78$; $P < .0014$. Data are represented as the mean ± SEM. Results of the Bonferroni's test: significant differences of *, $P < .05$ and ***, $P < .001$ vs the control value; ^, $P < .05$, ^^, $P < .01$, and ^^^, $P < .001$ vs the estradiol value.

AVP mRNA levels caused by DPN. This finding further indicates that ERα and ERβ have opposite effects on AVP mRNA levels in SH-SY5Y cells.

AVP mRNA levels in SH-SY5Y neuronal cells were up-regulated by T by a mechanism independent of its conversion to 5α-DHT and the consequent activation of AR

T, at 10^{-8} M, significantly increased AVP mRNA in SH-SY5Y cells compared with control cells (Figure 3A). One possible mechanism of action of the hormone is by its conversion in 5α-DHT and the activation of AR. Although we did not detect AR mRNA in our cells, previous studies have reported that SH-SY5Y cells metabolize T in 5α-DHT (44) and express AR (45). Thus, we explored this possible mechanism of regulation. The T-induced AVP mRNA increase was not affected when cells were treated with finasteride, an inhibitor of 5α-reductase, the enzyme that converts T in 5α-DHT (Figure 3B). Furthermore, the action of T was not affected when cells were treated with the AR antagonist flutamide (Figure 3C). Finasteride and flutamide alone did not significantly affect AVP mRNA levels (data not shown).

To further exclude the possible implication of AR in the action of T, SH-SY5Y cells were treated with the AR agonist 5α-DHT. 5α-DHT treatment, at concentrations ranging from 10^{-7} to 10^{-9} M, not only did not imitate the effect of T but also even had an opposite effect, resulting in a significant decrease in AVP mRNA levels compared with control values (Figure 3D). The effect of 5α-DHT was not affected by flutamide (Figure 3E). These findings suggest that T and 5α-DHT regulate AVP mRNA levels in SH-SY5Y cells in opposite ways and by mechanisms that are independent of AR.

AVP mRNA levels in SH-SY5Y neuronal cells were down-regulated by the 5α-DHT metabolite 3β-diol

In the previous experiments, we observed that both 5α-DHT and the ERβ agonist DPN exerted an inhibitory ac-

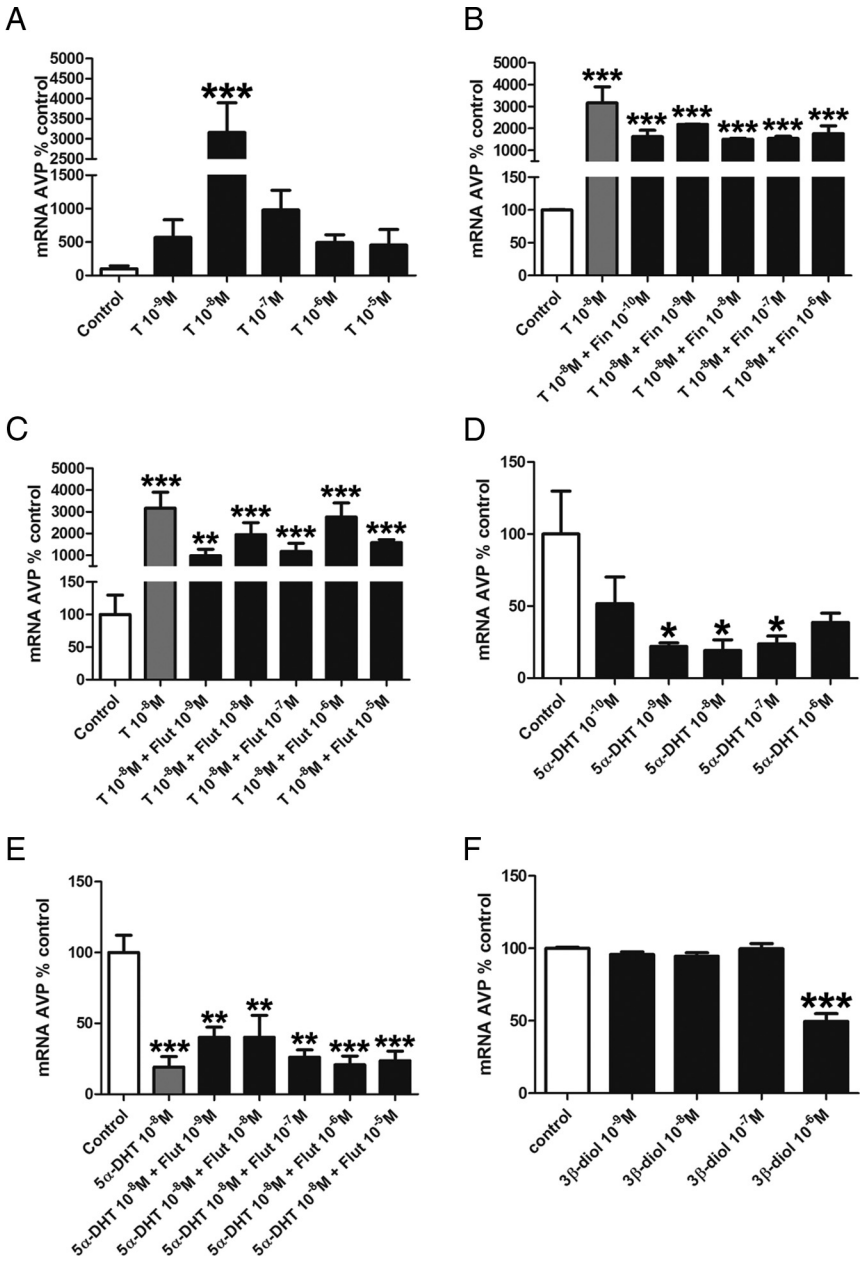

Figure 3. AVP mRNA levels in SH-SY5Y human neuroblastoma cells. A, Cells were treated for 24 hours with vehicle (control) or different doses of T. ANOVA: $F_{(5, 17)} = 9.648$; $P = .0007$. B, Cells were treated for 24 hours with vehicle, 10^{-8} M T, or 10^{-8} M T and different doses of the 5α-reductase inhibitor finasteride (Fin). ANOVA: $F_{(6, 20)} = 60.14$; $P = .0001$. C, Cells were treated for 24 hours with vehicle, 10^{-8} M T, or 10^{-8} M T and different doses of the androgen receptor antagonist flutamide (Flut). ANOVA: $F_{(6, 20)} = 15.60$; $P < .0001$. D, Cells were treated for 24 hours with vehicle or with different doses of 5α-DHT. ANOVA: $F_{(5, 18)} = 4.495$; $P < .0134$. E, Cells were treated for 24 hours with vehicle, 10^{-8} M 5α-DHT, or 10^{-8} M 5α-DHT and different doses of the of the androgen receptor antagonist flutamide (Flut). ANOVA: $F_{(6, 21)} = 9.402$; $P < .0002$. F, Cells were treated for 24 hours with vehicle or different doses of the ERβ agonist 3βdiol. ANOVA: $F_{(4, 18)} = 48.18$; $P < .0001$. Data are represented as the mean ± SEM. Results of the Bonferroni's test: significant differences of *, $P < .05$, **, $P < .01$, and ***, $P < .001$ vs the control value.

tion on AVP mRNA levels in SH-SY5Y cells. Therefore, a possible mechanism for the action of 5α-DHT is the activation of ERβ after its conversion in its metabolite 3β-diol. Thus, we treated SH-SY5Y cells with 3β-diol to determine whether this steroid regulates AVP mRNA levels. 3β-Diol significantly reduced AVP mRNA levels but only at high concentration (10^{-6} M).

AVP mRNA levels in SH-SY5Y neuronal cells were up-regulated by T by a mechanism blocked by an aromatase inhibitor and an ERα antagonist

We had previously observed that our SH-SY5Y cell line expresses the enzyme aromatase (42), which converts T in estradiol. Therefore, T may potentially affect AVP mRNA levels by the activation of ERs after its conversion into estradiol. To test this hypothesis, SH-SY5Y cells were treated with T in the presence of letrozole, an aromatase inhibitor. Under these conditions, T not only did not increase but also even resulted in a significant decrease in AVP mRNA levels (Figure 4A). This finding suggested that T up-regulates AVP mRNA levels by its conversion in estradiol. To further test this possibility, SH-SY5Y cells were treated with T in the presence or absence of ER antagonists. Interestingly, the up-regulation of AVP mRNA levels by T was blocked by the ERα antagonist MPP and by the ER antagonist ICI 182,780 (Figure 4B). The combination of T with the ERα antagonist MPP resulted in a significant decrease of AVP mRNA levels compared with control values, suggesting a possible inhibitory action of ERβ. However, the ERβ antagonist PHTPP did not affect the up-regulation of AVP mRNA levels by T (Figure 4B). These findings suggest that T up-regulated AVP mRNA levels in SH-SY5Y cells after its conversion to estradiol and the consequent activation of ERα.

Discussion

AVP is synthesized by different neuronal populations of the central nervous system. AVP synthesized by the mag-

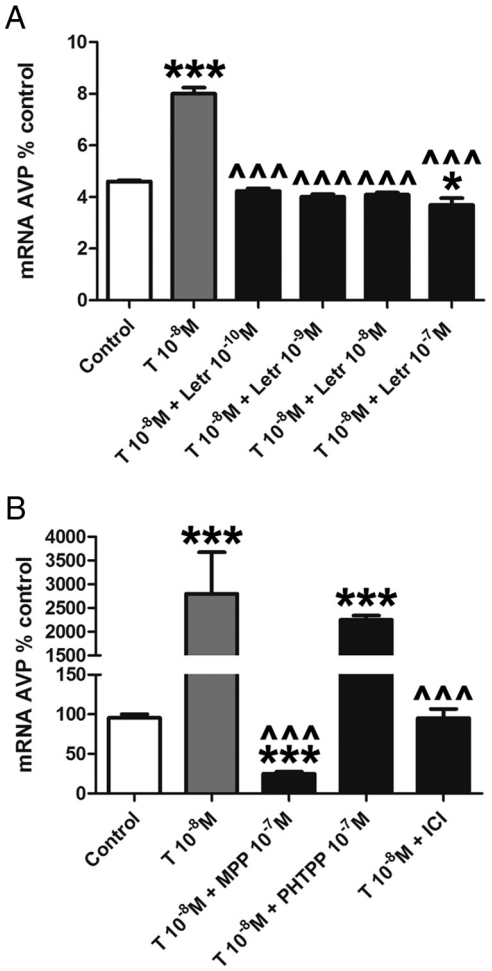

Figure 4. AVP mRNA levels in SH-SY5Y human neuroblastoma cells. A, Cells were treated for 24 hours with vehicle (control), 10^{-8} M T, or 10^{-8} M T and different doses of the aromatase inhibitor letrozole (Letr). ANOVA: $F_{(5, 21)} = 85.76$; $P < .0001$. B, Cells were treated for 24 hours with vehicle, 10^{-8} M T, 10^{-8} M T and 10^{-7} M MPP (ERα antagonist), 10^{-8} M T and 10^{-7} M PHTPP (ERβ antagonist), and 10^{-8} M T and 10^{-8} M ICI 182,780 (ICI; ER antagonist). Results of the Bonferroni's test: significant differences of *, $P < .05$ and ***, $P < .001$ vs the control value; ^^^, $P < .001$ vs the T value.

nocellular neurons of the paraventricular and supraoptic nuclei (46) is released in the neurohypophysis and exert hormonal actions, regulating water balance. AVP synthesized by other neuronal groups, such as the bed nucleus of the stria terminalis, medial amygdala, suprachiasmatic nucleus, and the parvocellular neurons from the paraventricular nucleus (47) act as a neuromodulator and participates in the regulation of circadian rhythms, cardiovascular function, the stress response, social recognition, social behavior, and attachment (1, 48–51). These behaviors and physiological processes are modulated by estradiol and T, which also regulate AVP expression in the brain by mechanisms not fully elucidated. In this study, we have used SH-SY5Y human neuroblastoma cells to assess the role of ERs on the regulation of AVP expression. We have first shown that SH-SY5Y cells express AVP, ERα, and ERβ, therefore being an adequate cellular model for our aims.

Our findings indicate that estradiol increases AVP expression in SH-SY5Y cells. This effect of estradiol was imitated by an ERα agonist, PPT, and was blocked by both an ER antagonist, ICI 182,780, and an ERα antagonist, MPP. In contrast, an ERβ agonist, DPN, reduced AVP expression in SH-SY5Y cells. In addition, the ERα antagonist MPP caused a significant reduction in AVP mRNA levels, whereas the ERβ antagonist PHTPP enhanced the action of estradiol on AVP expression. These findings suggest that estradiol is acting on both ERs to regulate AVP expression with opposite effects, enhancing AVP expression via ERα and decreasing AVP expression via ERβ. The action on ERα predominates at low concentrations of the hormone (10^{-9} to 10^{-10} M), and the result is a net increase in AVP expression after estradiol treatment. However, at higher concentrations (10^{-8} to 10^{-7} M), estradiol no longer increases AVP expression. This may in part reflect the activation of ERβ, which compensates the transcriptional action of ERα. Our findings are in agreement with previous studies showing that estradiol induces transcriptional activation of the AVP gene in SK-N-SH cells transfected with ERα, whereas the hormone reduces transcriptional activation of the AVP gene in SK-N-SH cells transfected with ERβ (52). Although the stimulatory effect of ERα on the AVP promoter involves estrogen response elements, the action of ERβ at activator protein-1 sites (52, 53) may be involved in the inhibitory effect of ERβ on the AVP promoter.

The different effect of ERα and ERβ on AVP expression is of physiological relevance. For instance, estradiol decreases AVP transcript levels in male mice PVN, a region in which the main ER form is ERβ, and this action is abolished in ERβ knockout mice (25). Our present findings, showing that the activation of ERβ decreases AVP mRNA levels, are in agreement with this observation. Our results are also relevant for the estrogenic regulation of AVP in other brain regions such as the bed nucleus of the stria terminalis, the medial nucleus of the amygdala, and the periventricular preoptic nucleus, in which both ERα and ERβ are expressed (54).

In male and female rodents, the in vivo expression of AVP is down-regulated by gonadectomy and up-regulated by T in the bed nucleus of the stria terminalis and medial amygdala (28, 55–57). Our present findings indicate that T up-regulates AVP expression in SH-SY5Y cells. SH-SY5Y cells express the enzyme 5α-reductase and are therefore able to convert T in the AR agonist 5α-DHT (44). Although we have not been able to detect AR mRNA in our cells, a previous study has reported the expression of

AR in the SH-SY5Y cell line (45). Therefore, to fully exclude the possible implication of AR in the action of T, we used the 5α-reductase inhibitor finasteride, the AR antagonist flutamide, and the AR agonist 5α-DHT. The effect of T on AVP expression was not affected by these treatments, indicating that AR is not involved in the regulation of AVP expression by T in the SH-SY5Y cells.

SH-SY5Y cells express aromatase (42); therefore, T may be converted to estradiol in these cells. Interestingly, the effect of T on AVP expression in SH-SY5Y cells was blocked by an aromatase inhibitor and by an ERα antagonist. This suggests that T regulates AVP expression in SH-SY5Y cells by its conversion to estradiol and the activation of ERα. In contrast to T, 5α-DHT decreased AVP expression in SH-SY5Y cells. This effect of 5α-DHT was not affected by flutamide and therefore seems to be independent from AR. It is known that 5α-DHT can be converted to the androgen metabolite 3β-diol, which is an agonist of ERβ (58–61). Interestingly, the ERβ agonist DPN had a similar effect to 5α-DHT in reducing AVP expression. Therefore, one of the possible mechanisms involved in the regulation of AVP expression by 5α-DHT is the conversion to the androgen metabolite 3β-diol and the activation of ERβ (39, 40). Indeed, we detected that 3β-diol decreased AVP mRNA levels in SH-SY5Y cells.

A question raised by our findings is the interpretation of the results obtained with the ER antagonist ICI 182,780. In the cells treated with estradiol or T and ICI 182,780, AVP mRNA levels were not different from control values. This is in contrast with the effects of 10^{-7} M MPP and 10^{-7} M PHTPP in the presence of estradiol or T, decreasing and increasing AVP expression, respectively. One possible interpretation is that ICI 182,780 blocked the ERα-induced increase and the ERβ-induced decrease in AVP mRNA levels, resulting in a net zero effect. This suggests that in other systems the differential regulation by ERα and ERβ may not be fully appreciated by using ICI 182,780.

In summary, our findings indicate that estradiol regulates AVP expression in the SH-SY5Y cells through both ERs, exerting a stimulatory action via ERα and an inhibitory action via ERβ. In addition, the regulation of AVP expression in the SH-SY5Y cells by T is independent of AR and involves, as for estradiol, a stimulatory action via ERα and an inhibitory action via ERβ. These data suggest that distinct AVP neuronal populations coexpressing different relative levels of ERα and ERβ may be differentially regulated by gonadal hormones.

Acknowledgments

We thank Dr Gema Moreno-Bueno for her gift of the LNCaP cell line.

Address all correspondence and requests for reprints to: Luis M. Garcia-Segura, Instituto Cajal, Consejo Suerior de Investigaciones Científicas, Avenida Doctor Arce 37, E-28002 Madrid, Spain. E-mail: lmgs@cajal.csic.es.

This work was supported by the Fondazione Cavalieri Ottolenghi, University of Torino; Fondazione San Paolo (Progetto Neuroscienze, Grant PF-2009.1180, to G.P.); and Ministerio de Economía y Competividad, Spain (Grant BFU2011-30217-C03-01).

Disclosure Summary: The authors have nothing to declare.

References

1. **De Vries GJ, Miller MA.** Anatomy and function of extrahypothalamic vasopressin systems in the brain. *Prog Brain Res*. 1999;119:3–20.
2. **Kalsbeek A, Palm IF, Buijs RM.** Central vasopressin systems and steroid hormones. *Prog Brain Res*. 2002;139:57–73.
3. **De Vries GJ, Panzica GC.** Sexual differentiation of central vasopressin and vasotocin systems in vertebrates: different mechanisms, similar endpoints. *Neuroscience*. 2006;138:947–955.
4. **Voisin DL, Bourque CW.** Integration of sodium and osmosensory signals in vasopressin neurons. *Trends Neurosci*. 2002;25:199–205.
5. **Leng G, Mason WT, Dyer RG.** The supraoptic nucleus as an osmoreceptor. *Neuroendocrinology*. 1982;34:75–82.
6. **Ramieri G, Panzica GC.** Comparative neuroanatomical aspects of the salt and water balance in birds and mammals. *J Endocrinol Invest*. 1989;12:59–74.
7. **Voisin DL, Simonian SX, Herbison AE.** Identification of estrogen receptor-containing neurons projecting to the rat supraoptic nucleus. *Neuroscience*. 1997;78:215–228.
8. **Altura BM, Altura BT.** Vascular smooth muscle and neurohypophyseal hormones. *Fed Proc*. 1977;36:1853–1860.
9. **Aoyagi T, Koshimizu TA, Tanoue A.** Vasopressin regulation of blood pressure and volume: findings from V1a receptor-deficient mice. *Kidney Int*. 2009;76:1035–1039.
10. **Fujiwara Y, Tanoue A, Tsujimoto G, Koshimizu TA.** The roles of V1a vasopressin receptors in blood pressure homeostasis: a review of studies on V1a receptor knockout mice. *Clin Exp Nephrol*. 2012;16:30–34.
11. **Sudbury JR, Ciura S, Sharif-Naeini R, Bourque CW.** Osmotic and thermal control of magnocellular neurosecretory neurons—role of an N-terminal variant of trpv1. *Eur J Neurosci*. 2010;32:2022–2030.
12. **Li JD, Hu WP, Zhou QY.** The circadian output signals from the suprachiasmatic nuclei. *Prog Brain Res*. 2012;199:119–127.
13. **Tang Y, Yang YL, Wang N, Shen ZL, Zhang J, Hu HY.** Effects of arginine vasopressin on firing activity and thermosensitivity of rat PO/AH area neurons. *Neuroscience*. 2012;219:10–22.
14. **Compaan JC, Buijs RM, Pool CW, De Ruiter AJ, Koolhaas JM.** Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. *Brain Res Bull*. 1993;30:1–6.
15. **Carter CS.** Neuroendocrine perspectives on social attachment and love. *Psychoneuroendocrinology*. 1998;23:779–818.
16. **Herman JP.** In situ hybridization analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids. *J Comp Neurol*. 1995;363:15–27.
17. **Jezova D, Skultetyova I, Tokarev DI, Bakos, P, Vigas M.** Vasopressin and oxytocin in stress. *Ann NY Acad Sci*. 1995;771:192–203.

18. **Kudielka BM, Kirschbaum C.** Sex differences in HPA axis responses to stress: a review. *Biol Psychol*. 2005;69:113–132.
19. **Lightman SL.** The neuroendocrinology of stress: a never ending story. *J Neuroendocrinol*. 2008;20:880–884.
20. **MacVicar BA, Andrew RD, Dudek FE, Hatton GI.** Synaptic inputs and action potentials of magnocellular neuropeptidergic cells: intracellular recording and staining in slices of rat hypothalamus. *Brain Res Bull*. 1982;8:87–93.
21. **Haam J, Popescu IR, Morton LA, et al.** GABA is excitatory in adult vasopressinergic neuroendocrine cells. *J Neurosci*. 2012;32:572–582.
22. **Skowsky WR, Swan L, Smith P.** Effects of sex steroid hormones on arginine vasopressin in intact and castrated male and female rats. *Endocrinology*. 1979;104:105–108.
23. **Crofton JT, Share L, Brooks DP.** Pressor responsiveness to and secretion of vasopressin during the estrous cycle. *Am J Physiol*. 1988; 255:1041–1045.
24. **Ota M, Crofton JT, Liu H, Festavan G, Share L.** Increased plasma osmolality stimulates peripheral and central vasopressin release in male and female rats. *Am J Physiol*. 1994;267:923–928.
25. **Nomura M, McKenna E, Korach KS, Pfaff DW, Ogawa S.** Estrogen receptor-β regulates transcript levels for oxytocin and arginine vasopressin in the hypothalamic paraventricular nucleus of male mice. *Brain Res Mol Brain Res*. 2002;109:84–94.
26. **Hartley DE, Dickson SL, Forsling ML.** Plasma vasopressin concentrations and Fos protein expression in the supraoptic nucleus following osmotic stimulation or hypovolaemia in the ovariectomized rat: effect of oestradiol replacement. *J Neuroendocrinol*. 2004;16: 191–197.
27. **O'Keefe JA, Crowley RS, Hrivnak P, Kim NB, Amico JA.** Effect of testosterone and its metabolites upon the level of vasopressin messenger ribonucleic acid in the hypothalamus of the hyperosmotically stimulated male rat. *Neuroendocrinology*. 1995;61: 405–411.
28. **De Vries GJ, Wang Z, Bullock NA, Numan S.** Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats. *J Neurosci*. 1994;14:1789–1794.
29. **Somponpun SJ, Sladek CD.** Role of estrogen receptor-β in regulation of vasopressin and oxytocin release in vitro. *Endocrinology*. 2002; 143:2899–2904.
30. **Sladek CD, Somponpun SJ.** Estrogen receptors: their roles in regulation of vasopressin release for maintenance of fluid and electrolyte homeostasis. *Front Neuroendocrinol*. 2008;29:114–127.
31. **Clipperton-Allen AE, Lee AW, Reyes A, et al.** Oxytocin, vasopressin and estrogen receptor gene expression in relation to social recognition in female mice. *Physiol Behav*. 2012;105:915–924.
32. **Scordalakes EM, Rissman EF.** Aggression and arginine vasopressin immunoreactivity regulation by androgen receptor and estrogen receptor alpha. *Genes Brain Behav*. 2004;3:20–26.
33. **Alves SE, Lopez V, McEwen BS, Weiland NG.** Differential colocalization of estrogen receptor β (ERβ) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. *Proc Natl Acad Sci USA*. 1998;95: 3281–3286.
34. **Somponpun SJ, Sladek CD.** Osmotic regulation of estrogen receptor-β in rat vasopressin and oxytocin neurons. *J. Neurosci*. 2003; 23:4261–4269.
35. **Suzuki S, Handa RJ.** Regulation of estrogen receptor-β expression in the female rat hypothalamus: differential effects of dexamethasone and estradiol. *Endocrinology*. 2004;145:3658–3670.
36. **Grassi D, Amorim MA, Garcia-Segura LM, Panzica G.** Estrogen receptor α is involved in the estrogenic regulation of arginine vasopressin immunoreactivity in the supraoptic and paraventricular nuclei of ovariectomized rats. *Neurosci Lett*. 2010;474: 135–139.
37. **Gruber KA, Wilkin LD, Johnson AK.** Neurohypophyseal hormone release and biosynthesis in rats with lesions of the anteroventral third ventricle (AV3V) region. *Brain Res*. 1986;378:115–119.
38. **Oliveira GR, Franci CR, Rodovalho GV, Franci JA, Morris M, Rocha MJ.** Alterations in the central vasopressin and oxytocin axis after lesion of a brain osmotic sensory region. *Brain Res Bull*. 2004; 63:515–520.
39. **Pak TR, Chung WC, Hinds LR, Handa RJ.** Estrogen receptor-β mediates dihydrotestosterone-induced stimulation of the arginine vasopressin promoter in neuronal cells. *Endocrinology*. 2007;148: 3371–3382.
40. **Pak TR, Chung WC, Hinds LR, Handa RJ.** Arginine vasopressin regulation in pre- and postpubertal male rats by the androgen metabolite 3β-diol. *Am J Physiol Endocrinol Metab*. 2009;296: E1409–E1413.
41. **Marwarha G, Rhen T, Schommer T, Ghribi O.** The oxysterol 27-hydroxycholesterol regulates α-synuclein and tyrosine hydroxylase expression levels in human neuroblastoma cells through modulation of liver X receptors and estrogen receptors—relevance to Parkinson's disease. *J Neurochem*. 2011;119:1119–1136.
42. **Yague JG, Muñoz A, de Monasterio-Schrader P, Defelipe J, Garcia-Segura LM, Azcoitia I.** Aromatase expression in the human temporal cortex. *Neuroscience*. 2006;138:389–401.
43. **Yague JG, Lavaque E, Carretero J, Azcoitia I, Garcia-Segura LM.** Aromatase, the enzyme responsible for estrogen biosynthesis, is expressed by human and rat glioblastomas. *Neurosci Lett*. 2004;368: 279–284.
44. **Melcangi RC, Maggi R, Martini L.** Testosterone and progesterone metabolism in the human neuroblastoma cell line SH-SY5Y. *J Steroid Biochem Mol Biol*. 1993;46:811–818.
45. **Butler R, Leigh PN, Gallo JM.** Androgen-induced up-regulation of tubulin isoforms in neuroblastoma cells. *J Neurochem*. 2001;78: 854–861.
46. **Swanson LW, Sawchenko PE.** Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Annu Rev Neurosci*. 1983;6:269–324.
47. **De Vries GJ, al-Shamma HA, Zhou L.** The sexually dimorphic vasopressin innervation of the brain as a model for steroid modulation of neuropeptide transmission. *Ann NY Acad Sci*. 1994;743:95–120.
48. **Pyner S.** Neurochemistry of the paraventricular nucleus of the hypothalamus: implications for cardiovascular regulation. *J Chem Neuroanat*. 2009;38:197–208.
49. **Insel TR.** The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. *Neuron*. 2010;65:768–779.
50. **Albers HE.** The regulation of social recognition, social communication and aggression:vasopressin in the social behavior neural network. *Horm Behav*. 2012;61:283–292.
51. **Bosch OJ, Neumann ID.** Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action. *Horm Behav*. 2012;61:293–303.
52. **Shapiro RA, Xu C, Dorsa DM.** Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor α and β. *Endocrinology*. 2000;141:4056–4064.
53. **Paech K, Webb P, Kuiper GG, et al.** Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science*. 1997;277: 1508–1510.
54. **Shughrue PJ, Scrimo PJ, Merchenthaler I.** Evidence for the colocalization of estrogen receptor-β mRNA and estrogen receptor-α immunoreactivity in neurons of the rat forebrain. *Endocrinology*. 1998;139:5267–5270.
55. **DeVries GJ, Buijs RM, VanLeeuwen FW, Caffé AR, Swaab DF.** The vasopressinergic innervation of the brain in normal and castrated rats. *J Comp Neurol*. 1985;233:236–254.
56. **Miller MA, Urban JH, Dorsa DM.** Steroid dependency of vasopressin neurons in the bed nucleus of the stria terminalis by in situ hybridization. *Endocrinology*. 1989;125:2335–2340.

57. **Brot MD, De Vries GJ, Dorsa DM.** Local implants of testosterone metabolites regulate vasopressin mRNA in sexually dimorphic nuclei of the rat brain. *Peptides*. 1993;14:933–940.
58. **Weihua Z, Lathe R, Warner M, Gustafsson JA.** An endocrine pathway in the prostate, ERβ, AR, 5α-androstane-3β,17β-diol, and CYP7B1, regulates prostate growth. *Proc Natl Acad Sci USA*. 2002; 99:13589–13594.
59. **Pak TR, Chung WC, Lund TD, Hinds LR, Clay CM, Handa RJ.** The androgen metabolite, 5α-androstane-3β, 17β-diol, is a potent modulator of estrogen receptor-β1-mediated gene transcription in neuronal cells. *Endocrinology*. 2005;146:147–155.
60. **Handa RJ, Pak TR, Kudwa AE, Lund TD, Hinds L.** An alternate pathway for androgen regulation of brain function: activation of estrogen receptor β by the metabolite of dihydrotestosterone, 5α-androstane-3β, 17β-diol. *Horm Behav*. 2008;53:741–752.
61. **Handa RJ, Sharma D, Uht R.** A role for the androgen metabolite, 5α androstane 3β, 17β diol (3β-diol) in the regulation of the hypothalamo-pituitary-adrenal axis. *Front Endocrinol*. 2011; 2:65.