Hypocretin/orexin increases the expression of steroidogenic enzymes in human adrenocortical NCI H295R cells

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1Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, Lübeck, Germany; 2Warwick Medical School, Warwick University, Coventry, United Kingdom; and 3Carl Gustav Carus University Hospital, Medical Clinic III, University of Dresden, Dresden, Germany

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Wenzel J, Grabinski N, Knopp CA, Dendorfer A, Ramanjaneya M, Randeva HS, Ehrhart-Bornstein M, Dominika P, Jöhren O. Hypocretin/orexin increases the expression of steroidogenic enzymes in human adrenocortical NCI H295R cells. Am J Physiol Regul Integr Comp Physiol 297: R1601–R1609, 2009. First published September 30, 2009; doi:10.1152/ajpregu.91034.2008.—Hypocretins/orexins act through two receptor subtypes: OX1 and OX2. Outside the brain, orexin receptors are expressed in adrenal glands, where orexins stimulate the release of glucocorticoids. To further address the regulation of steroidogenesis, we analyzed the effect of orexins on the expression of steroidogenic enzymes in human adrenocortical National Cancer Institute (NCI) H295R cells by qPCR. In NCI H295R cells, OX2 receptors were highly expressed, as they were in human adrenal glands. After treatment of NCI H295R cells with orexin A for 12–24 h, the cortisol synthesis rate was significantly increased, whereas 30 min of treatment showed no effect. While CYP11B1 and CYP11B2 mRNA levels were increased already at earlier time points, the expression of HSD3B2 and CYP21 mRNA was significantly up-regulated after treatment with orexin A for 12 h. Likewise, orexin B increased CYP21 and HSD3B2 mRNA levels showing, however, a lower potency compared with orexin A. The mRNA levels of CYP11A1 and CYP17 were unaffected by orexin A. OX2 receptor mRNA levels were down-regulated after 12 and 24 h of orexin A treatment. Orexin A increased intracellular Ca2+ but not cAMP concentrations in NCI H295R cells. Furthermore, inhibition of PKC and MAPK kinase/ERK kinase (MEK1/2) prevented the increase of HSD3B2 expression by orexin A. Accordingly, orexin A treatment of NCI H295R cells markedly enhanced ERK1/2 phosphorylation that was prevented by PKC and, in part, PKA inhibition. In conclusion, orexins may influence adrenal steroidogenesis by differential regulation of the expression of steroidogenic enzymes involving Ca2+, as well as PKC-ERK1/2 signaling.

hypocretin-1 and -2; OX1 and OX2 receptor; real-time reverse transcriptase-polymerase chain reaction; cortisol

Hypocretins/orexins are produced by neurons within the lateral hypothalamus and are involved in the central control of feeding and arousal but also regulate endocrine functions (7, 41, 44, 45). Orexins act at two subtypes of G protein-coupled receptors, named OX1 and OX2 receptors. Orexin A, also referred to as hypocretin-1, binds OX1 and OX2 receptors with the same affinity, whereas Orexin B (hypocretin-2) has a significantly lower affinity for the OX1 receptor (41). Orexinergic neurons project to various brain regions, such as the cerebral cortex, hippocampus, locus coeruleus, and the brain stem (6, 35), where orexin receptors are expressed (27). In addition, peripheral actions of orexins have been described in the gastrointestinal tract but also on adrenocortical cells (22, 26), and orexin receptors were detected in peripheral organs, including the gut and pancreas (22, 32, 33). There is also strong evidence for the expression of orexin receptor subtypes in adrenal glands of various species, including rats and humans (13). Furthermore, orexins were shown to stimulate glucocorticoid secretion from adrenocortical cells in various species (25, 26, 29, 31, 47, 49). Little, however, is known regarding the mechanisms involved in the effects of orexins leading to an increased secretion of glucocorticoids from adrenal glands.

The synthesis of mineralocorticoids, glucocorticoids, and androgens in distinct cortical zones of the human adrenal gland is achieved by the selective expression of some steroid-synthesizing enzymes, such as aldosterone synthase (CYP11B2) in the zona glomerulosa and 11β-hydroxylase (CYP11B1) in the zona glomerulosa and fasciculata, while other enzymes, such as cholesterol side chain cleavage cytochrome P-450 (CYP11A1), 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (HSD3B2), and 21-hydroxylase (CYP21), are more generally expressed (34). In the present study, we have investigated the effect of orexins on the secretion of cortisol and on the expression of the involved

Table 1. Nucleotide sequences of PCR primer directed against human cDNA as published in the NCBI GenBank database

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>PCR Product (bp)</th>
<th>GeneBank Accession #</th>
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<td>OX2</td>
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<tr>
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<td>127</td>
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<tr>
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<td>AS</td>
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<td>108</td>
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<td>CTTCAATGACCCACCTGCTGCT</td>
<td>133</td>
<td>NM_000198</td>
</tr>
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S, sense; AS, antisense. Primers were tested for unique nucleotide sequences using BLAST.

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enzymes in the human adrenocortical cell line National Cancer Institute (NCI) H295R. This cell line expresses enzymes of all three zones of the human adrenal cortex and synthesizes all particular steroids (36).

**MATERIALS AND METHODS**

**Cell culture and treatment.** NCI H295R cells were maintained in a 1:1 mixture of DMEM and Ham’s F-12 medium (PAA Laboratories), supplemented with FBS (2%, Biochrom AG), hydrocortisone (3.623 ng/ml; Sigma), penicillin/streptomycin (100 µg/ml; PAA Laboratories), apo-transferrin (1 µg/ml; Sigma), sodium selenite (5 ng/ml, Sigma), β-estradiol (2.724 ng/l, Sigma), and insulin (0.379 µg/ml, Sigma). Before treatment with orexin A or B, ANG II (all Bachem), or forskolin (Tocris), cells were incubated for 24 h in medium without supplements.

The PKA-specific inhibitor KT5720, the PKC inhibitor GF109203X, and U0126, a specific inhibitor of MEK (an upstream molecule of MAPK), were obtained from Tocris. SB202190, a specific inhibitor of p38-MAPK, JNK inhibitor II (SP-600125), myristoylated PKA inhibitor amide 1–22 (MIP), and the PKC inhibitor 2-[1-[2-(1-Methylpyrrolidino)ethyl]-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (bis) were purchased from Calbiochem (Merck). All substances were dissolved in DMSO (Sigma) and stored at −20°C.

**RNA isolation and cDNA synthesis.** After lysis of NCI H295R cells, RNA was extracted on an ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer’s instructions. The amount of total RNA was determined by optical density at 260 nm. First-strand cDNA was synthesized in 5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 U/µl RNasin, 0.5 µg oligo-(dT)₁₅ primer and 15 U AMV reverse transcriptase (Promega). After the reaction was completed, samples were stored at −20°C until PCR. Adult adrenal glands from humans were obtained at the time of nephrectomy for renal carcinoma, as approved by the Ethics Committee of the Technical University of Dresden, and written informed consent was obtained by the participants (17). RNA was isolated using the RNeasy kit (Qiagen).

**Real-time quantitative RT-PCR.** Sense and antisense oligonucleotide primers for amplification of mRNAs of human OX1 and OX2 receptor, the housekeeping gene peptidylprolyl isomerase A (PPIA), and the steroidogenic enzymes CYP11A, HSD3B2, CYP17, CYP21, CYP11B1, and CYP11B2 were designed on the basis of published cDNA sequences (NCBI GenBank), as shown in Table 1 using the PrimerExpress software (Applied Biosystems). Oligonucleotide primers were obtained from Invitrogen.

Real-time quantitative PCR (qPCR) was performed as described previously (12, 15) with 2 µl of first-strand cDNA reaction in the presence of 50 mM MgCl₂, 5 mM dNTPs, specific buffer, 0.65 U Hot GoldStar polymerase, the fluorescence dye SYBR green, and the appropriate sense and antisense primers (50–900 nM). The PCR was...
carried out on the 7000 Sequence Detection System of Applied Biosystems. Each sample was analyzed along with standards and no template controls. Product purity was regularly confirmed for each sample by dissociation curve analysis. Amplification products were also analyzed by agarose gel electrophoresis.

Copy number calculations were based on the cycle threshold method (11). Known amounts of specific cDNA fragments were used as standards (12, 15). The threshold cycle number (CT) for each sample was calculated using the 7000 sequence detection system software with an automatic baseline setting and a fluorescence threshold ($R_n$) of 0.2. No-template control samples typically did not surpass the $R_n$ value of 0.2 below 35 cycles. Copy numbers were corrected to PPIA mRNA levels. The mRNA levels of this housekeeping gene correlated with total RNA concentration of the samples used, and no differences were observed between treatment groups. Because CYP11B1 and CYP11B2 transcripts are highly similar, the specificity of PCR was confirmed by restriction analysis.

Calcium measurements. NCI H295R cells were grown to confluence and plated in 96-well black-walled tissue culture plates 24 h prior to the experiment. Relative levels of intracellular $Ca^{2+}$ were measured using the Fluo-4 NW calcium assay kit (Molecular Probes, Invitrogen) according to the manufacturer’s protocol. Cells were loaded with fluo-4 AM in the presence of 2.5 mM probenecid in HBSS. Orexin A was automatically injected, and fluorescence was measured using a Fluostar Optima fluorescence plate reader (with an excitation at 485 nm and emission at 520 nm) at room temperature.

Fig. 3. mRNA Expression of the steroidogenic enzymes CYP11A (A), CYP17 (B), HSD3B2 (C), CYP21 (D), CYP11B1 (E), and CYP11B2 (F) in NCI H295R cells treated with 1 μM orexin A for various time periods. Results are presented as means ± SE; $n = 8$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with untreated cells.

Fig. 4. Comparison of the effect of 1 μM orexin A on the relative mRNA levels of steroidogenic enzymes with that of 1 μM ANG II and 50 μM forskolin after 12 h of treatments. Results are presented as means ± SE.
Cyclic AMP radioimmunoassay. NCI H295R cells were grown in 96-well tissue culture plates and incubated with saline, forskolin, or orexin A for 30 min. Cyclic AMP (cAMP) was determined using a cAMP [125I] RIA kit (Perkin-Elmer), according to the manufacturer’s instructions.

Cortisol ELISA. Levels of cortisol in culture supernatants were measured using the high-sensitivity salivary cortisol enzyme immunoassay kit (Salimetrics). Cortisol levels were normalized to total protein amount determined according to Lowry. The interassay and intra-assay variation coefficient was less than 8%.

Western blotting. Protein lysates were prepared by adding of Laemmli buffer to cells followed by sonication and boiling. Samples were separated by SDS-PAGE (10% resolving gel) and transferred to PVDF membranes. Membranes were incubated with primary antibody for phosho ERK1/2 or phospo p38 (Cell Signalling Technology) raised in rabbit at a 1:1,000 dilution in TBS-0.1% Tween (TBST), and 5% BSA overnight at 4°C. Membranes were washed, incubated with secondary anti-rabbit horseradish peroxidase-conjugated antibody (1:2,000) for 1 hour at room temperature, and washed for 60 min with TBST. Antibody complexes were visualized using ECL Plus, chemiluminescence detection kit. Densities were measured using a scanning densitometer coupled to Scion Image software (Scion). Membranes were stripped and reprobed for total ERK1/2 or p38 with antibody raised in rabbit at 1:2,000 dilution in TBST 5% BSA overnight at 4°C and measured as above.

Statistics. Data are presented as means ± SE and were analyzed using the GraphPad Prism software. Differences between treatment groups were estimated by one- or two-way ANOVA, dependent on the data types; both followed by Bonferroni’s post test. If there were only two groups, a t-test was used. For determining outliers, we used the Grubb’s test for outliers. P < 0.05 indicated statistically significant differences.

RESULTS

Expression of OX1 and OX2 receptor mRNA in NCI H295R cells. Analysis of orexin receptor mRNA levels in untreated NCI H295R cells and in human adrenal glands by qPCR revealed high amounts of OX2 receptors in both NCI H295R cells and adult human adrenal glands (Fig. 1). In contrast to OX2 receptors, OX1 receptor-specific DNA fragments were amplified at very low levels reaching the threshold value of 0.2, only at high cycle numbers. The specificity of the PCR was confirmed by dissociation curves analysis and gel electrophoresis (Fig. 1B) of the PCR products.

Effects of orexin A on cortisol synthesis. Treatment of NCI H295R cells for 30 min with 1 μM orexin A showed no effect on the concentration of cortisol in the supernatant (Fig. 2A). However, after a 24-h treatment of NCI H295R cells with orexin A, cortisol concentrations in supernatants of treated cells were about twofold higher compared with untreated control cells (Fig. 2B). No differences were found between the...
treatment groups after 3 and 6 h of treatment. Accordingly, the cortisol synthesis rate (as ng cortisol per hour) within the first 6 h of treatment showed no significant difference between treated and untreated cells, but the synthesis rate within 12 and 24 h of treatment with orexin A was significantly higher than the synthesis rate of the control group (Fig. 2C).

**Effect of orexin A on the expression of steroidogenic enzymes.** The mRNA levels of all essential steroidogenic enzymes were determined by qPCR in NCI H295R cells after treatment with 1 μM orexin A for various time periods compared with untreated control cells (Fig. 3) but also to cells treated with ANG II or forskolin (Fig. 4). While we found no difference in the mRNA levels of CYP11A and CYP17 at any time point analyzed (Fig. 3, A and B), orexin A induced a significant increase of mRNA levels of both CYP21 and HSD3B2 after 12 h of treatment (Fig. 3, C and D). Furthermore, the mRNA levels of CYP11B1 and CYP11B2 were significantly increased after treatment with orexin A also at earlier time points (Fig. 3, E and F). Compared with the effect of ANG II or forskolin, the effects of orexin A on CYP11B1 on CYP11B2 were relatively low (Fig. 4). The increase of HSD3B2 mRNA levels by orexin A was similar to that of ANG II.

**Effect of orexin A on OX2 receptor mRNA.** OX2 receptor mRNA levels in NCI H295R cells were decreased after 6, 12, and 24 h of treatment with orexin A (Fig. 5).

**Dose-dependent effect of orexin A and B.** Dose-response curves for the effect of both orexin A and B, on the expression of HSD3B2 and CYP21 in NCI H295R cells were generated after 12 h of treatment (Fig. 6). The effect of orexin A on the mRNA levels of HSD3B2 and CYP21 was more potent than that of orexin B (EC50: 27 nM and 19 nM compared with 244 nM and 157 nM, respectively).

**Effect of orexin A on cAMP and intracellular Ca2+.** To characterize possible intracellular signal pathways, we analyzed the effect of orexin A on cAMP production in NCI H295R cells. Although forskolin significantly increased cAMP levels, no effect was observed by orexin A (Fig. 7A). In addition, neither KT 5720 (Fig. 7B) nor H-89 (10 \(^{-5}\) M), both PKA inhibitors, affected the increase in HSD3B2 mRNA levels produced by orexin A. On the contrary, orexin A dose dependently produced a significant increase in concentrations of intracellular Ca2+ in NCI H295R cells (Fig. 7, C and D).

**Signal transduction pathways involved in HSD3B2 regulation by orexin A.** Since orexin A increased intracellular Ca2+, we assessed whether PKC is involved in the orexin-mediated increases of HSD3B2 mRNA. We found a significant reduction of HSD3B2 mRNA after orexin A by the PKC inhibitor GF 109203X (Fig. 8A, \(P < 0.001\)). As the stimulation of the mitogen-activated protein (MAP) kinase isoforms ERK1/2 by orexins was shown recently (39), we addressed the involvement of MAP kinases in the effect of orexin on HSD3B2 expression. The effect of orexin A on HSD3B2 mRNA levels was abolished by the MAPK kinase/ERK kinase (MEK1/2) inhibitor U0126 (Fig. 8B). On the other hand, the p38 MAPK inhibitor SB202190 and JNK inhibitor II did not affect the orexin-mediated increase of HSD3B2 mRNA, indicating the selective involvement of ERK1/2 MAP kinases (Fig. 8, C and D).

**Effect of orexin A on ERK1/2 phosphorylation.** Orexin A rapidly stimulated the phosphorylation of ERK1/2 and p38.
The effect on ERK1/2 phosphorylation was prevented by PKC inhibition and reduced by PKA inhibition. In contrast, p38 phosphorylation by orexin A was not attenuated by inhibition of PKA or PKC.

**DISCUSSION**

Various results indicate a relationship between the hypocretin/orexin system and steroid hormone action and regulation at various levels of the hypothalamus-pituitary-adrenal axis (3, 15, 16, 42, 43, 46). Here, we describe the effect of orexins on the expression of the various steroidogenic enzymes in human adrenocortical cells that was associated with an increased production of cortisol. Furthermore, we found a strong increase in intracellular Ca²⁺ after stimulation of NCI H295 cells with orexin and evidence for an involvement of PKC and ERK1/2 activation in the stimulation of HSD3B2 expression by orexin. In addition, the high mRNA levels of OX₂ receptors compared with much lower levels of OX₁ receptors in both NCI H295R cells and human adrenals confirmed the predominance of OX₂ receptors in the rat and human adrenal cortex (15, 40).

Orexins were shown previously to enhance cortisol release from human and porcine adrenocortical cells within 30 or 60 min of treatment (29, 31). In NCI H295R cells, we found the highest cortisol synthesis rate after 12–24 h of treatment with orexin A, possibly because of a stimulation of the expression of cortisol synthesizing enzymes. Using qPCR, we investigated all steroidogenic enzymes in NCI H295R cells after stimulation with orexins for 3, 6, 12, and 24 h and found increased mRNA levels of CYP11B1 and CYP11B2 already at the early time points, while HSD3B2 and CYP21 mRNA levels were significantly increased after prolonged treatment for 12 h and CYP11A, as well as CYP17, were unaffected by orexins. As raised levels of HSD3B2 and CYP21 direct the steroid synthesis from androgens to an enhanced production of mineralocorticoids and glucocorticoids (34), our data could explain the increased cortisol secretion observed after prolonged treatment with orexin A. As NCIH295R cells are relatively insensitive to ACTH (37), a characteristic also found in our cells (unpublished data), we verified their capacity to react to ANG II and forskolin. Compared with the effect of ANG II or forskolin on CYP11B1 and CYP11B expression, the effect of orexin A on these enzymes was low, whereas the effect of orexin A on HSD3B2 mRNA was comparable to that of ANG II.

The selective upregulation of HSD3B2 and CYP21 mRNA after prolonged stimulation by orexins indicates the existence of a specific pathway for the transcriptional regulation. Bird et al. (4) described a similar regulation of HSD3B2 and CYP21 in NCI H295R cells, conversely to the regulation of CYP17 and CYP11A, caused by PKG agonists. Comparable to the effects of ANG II on NCI H295R cells (4), prolonged treatment with orexins may selectively enhance the expression of CYP21 and HSD3B2 via Ca²⁺ signaling and PKC activation, as shown by our present results. The coupling of orexin receptor subtypes to...
the Gq/G11 protein with subsequent Ca\(^{2+}\) mobilization and PKC activation is well established and also evident in NCI H295R cells (38, 41, 48). In contrast, some studies showed the increase of cortisol secretion by orexins in adrenocortical cells by activation of PKA via raised levels of cAMP within 30 – 60 min (29, 31). We were unable to detect such a rapid effect of orexin on cortisol production in NCI H295R cells. Thus, both the PKA and PKC pathway may be involved in the regulation of the adrenal cortex through orexins possibly dependent on the duration of stimulation by orexins and the type or source of cells investigated. Our data further suggest the direct involvement of ERK1/2 MAP kinases in the orexin-stimulated HSD3B2 expression. A PKA-independent activation of ERK1/2 MAP kinase pathway was identified in NCI H295R cells recently (23), and orexins were shown to Ca\(^{2+}\) dependently phosphorylate ERK1/2 MAP kinases not only via OX1 but also OX2 receptors (1). Here, we show phosphorylation of ERK1/2 by orexin A in NCI H295R cells that was abolished by inhibition of PKC and in part by PKA inhibition in agreement with our recent data (38). Orexins were found to enhance the expression of steroidogenic acute regulatory protein in NCI H295R cells via activation of ERK1/2 MAP kinases (39).

Our findings of high levels of OX2 receptor mRNA in NCI H295R cells suggest that the effects of orexins on adrenocortical enzymes are mediated mainly via the OX2 receptor. The higher potency of orexin A to stimulate HSD3B2 and CYP21 mRNA expression, however, argues for an involvement of the OX1 receptor because of its lower affinity to orexin B. On the other hand, orexin B exhibits a noticeably shorter metabolic half-life than orexin A (21). Therefore, the attribution of unequal effects of orexin A and B to the different orexin receptor subtypes should be performed with care. Although we found a predominance of OX2 receptors in NCI H295R cells, both orexin receptor subtypes are clearly expressed in human adrenal cells, and OX1 receptors may also be involved in the regulation of steroidogenesis (13, 28, 40, 47). Interestingly, we found a down-regulation of OX2 receptors after orexin A treatment. This OX2 receptor auto-regulation may explain the observed maximal effect of orexin A on the expression of HSD3B2 and CYP21 after 12 h of treatment and the following reduced efficacy of orexin, and thereby it substantiates the involvement of this receptor subtype.

Orexin A has been detected in human and rat plasma (2, 15). The source of plasma orexin is not clarified although prepro-orexin mRNA was detected in peripheral organs such as testis, small intestine, gut, and pancreas (10, 15, 18, 22, 32, 33). Orexin-containing neuronal fibers may release orexins into the circulation from the median eminence and pituitary (5, 30), and the secretion of orexin A from pancreatic islets may significantly contribute to circulating orexins (33). In the adrenal, some orexin may be present (20, 40), but the failure to detect prepro-orexin mRNA in adrenals of rat and porcine makes an autocrine or paracrine action of orexins doubtful (15, 24, 31). Adrenal orexin receptors are regulated by changes of the metabolic state, such as food deprivation and diabetes (14, 19). Furthermore, low glucose stimulates the release of orexin A from the pancreas, and plasma orexin A is increased in fasted rats (33). In turn, orexin A increases blood glucose levels in rats (33). In addition, orexin A decreased plasma levels of leptin, and orexin receptors are functionally expressed in human adipose tissue (8, 9). Thus, circulating orexins appear to be involved in the regulation of energy homeostasis, and the effects on adrenal glucocorticoid synthesis may take an important part in such a regulation.

In summary, we have shown a differential regulation of steroidogenic enzymes by orexin in human adrenocortical cells that may cause an increased synthesis of cortisol. In addition, we revealed the auto-regulation of the OX2 receptor in re-

![Fig. 9. Effect of 1 μM orexin A on ERK1/2 (A) and p38 (B) phosphorylation. Cells were pretreated with PKA inhibitor (MIP, 1 μM) and PKC inhibitor (Bis, 2 μM) 2 h or 45 min, respectively, before stimulation with orexin A for 5 min. Western blots shown in A used an antibody against phosphorylated and total ERK1/2, which recognized bands with apparent molecular weights of 42 kDa for ERK1 and 44 kDa for ERK2. The corresponding antibodies for p38 detected bands at ~38 kDa (B). Densitometric analysis of the ratio of phosphorylated to total protein was calculated using Scion image software. Data are presented as means ± SE; n = 6. ***P < 0.001.](http://ajpregu.physiology.org/)
response to orexin A. The mechanisms by which orexins regulate the expression of HSD3B2 involve Ca\(^{2+}\) - and PKC signaling and ERK1/2 MAP kinase activation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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32. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Hellner HC, Sutcliffe JG.

33. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Hellner HC, Sutcliffe JG.


