Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei

Jia-Da Li,1 Katherine J. Burton,1 Chengkang Zhang,1 Shuang-Bao Hu,2 and Qun-Yong Zhou1
1Department of Pharmacology, University of California, Irvine, California; and 2Clinical Neuroendocrinology Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

Submitted 30 May 2008; accepted in final form 24 November 2008

Li JD, Burton KJ, Zhang C, Hu SB, Zhou QY. Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei. Am J Physiol Regul Integr Comp Physiol 296: R824–R830, 2009.—The suprachiasmatic nuclei (SCN) of the anterior hypothalamus play an important role in the generation of circadian rhythms. SCN neurons coordinate daily cycles of behavior and physiology for mammals. A network of transcriptional and translational feedback loops underlies the operating molecular mechanism for circadian oscillation within the SCN neurons. It remains unclear how timing information is transmitted from SCN neurons to eventually evoke circadian rhythms. Intercellular communication between the SCN and its target neurons is critical for the generation of circadian rhythms. At the molecular level, neuropeptides encoded by clock-controlled genes have been indicated as important output mediators. Arginine vasopressin (AVP) is the product of one such clock-controlled gene. Previous studies have demonstrated a circadian rhythm of AVP levels in the cerebrospinal fluid and the SCN. The physiological effects of AVP are mediated by three types of AVP receptors, designated as V1a, V1b, and V2. In this study, we report that V1a mRNA levels displayed a circadian rhythm in the SCN, peaking during night hours. The circadian rhythmicity of locomotor activity was significantly reduced in V1a-deficient (V1a−/−) mice (50–75% reduction in the power of fast Fourier transformation). However, the light masking and light-induced phase shift effects are intact in V1a−/− mice. Whereas the expression of clock core genes was unaltered, the circadian amplitude of prokineticin 2 (PK2) mRNA oscillation was attenuated in the SCN of V1a−/− mice (~50% reduction in the peak levels). In vitro experiments demonstrated that AVP, acting through V1a receptor, was able to enhance the transcriptional activity of the PK2 promoter. These studies thus indicate that AVP-V1a signaling plays an important role in the generation of overt circadian rhythms.

prokineticin

ALMOST ALL ORGANISMS from bacteria to mammals exhibit circadian (~24 h) physiology and behavior to adapt to the environmental changes imposed by the daily revolutions of the planet Earth. In mammals, the endogenous pacemaker that drives circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN coordinates daily cycles of behavior and physiology, such as the rhythm of activity and rest (23, 31, 32). A network of transcriptional and translational feedback loops has been demonstrated as the operating machinery for circadian oscillation within the SCN neurons (31, 32). Some so-called clock-controlled genes (CCGs) are also rhythmically regulated using the same transcriptional machinery that controls the clock feedback loops. The peptides encoded by three CCGs [prokineticin 2 (PK2), cardiothrophin-like cytokine, and arginine vasopressin (AVP)] have been particularly implicated as critical SCN output molecules that link the SCN and its efferent targets (6, 17, 19). AVP is one of the first neurotransmitters discovered in the SCN (5, 37, 41). The concentrations of AVP in the cerebrospinal fluid (CSF) vary in a circadian cycle, with morning levels approximately five times higher than those of night hours (30). Previous studies have demonstrated that the circadian variation of AVP levels in the CSF originates from the AVP content in the SCN (16, 34, 38, 40). Recent molecular biology studies revealed that the transcription of AVP gene in the SCN is rhythmically regulated by the same positive and negative elements that control the core molecular loops (17). The CLOCK/BMAL1 complex positively activates the expression of AVP gene, which is suppressed by periods (Per) and cryptochromes (Cry). The vasopressinergic fibers have been observed in the SCN as well as some of the SCN targets, including the paraventricular nuclei and the dorsomedial hypothalamic nuclei (11). Thus AVP was thought to not only function as an output factor but also regulate the activity of SCN neurons.

There are three types of AVP receptors, designated as V1a, V1b, and V2 (9, 22, 25). Among them, only V1a and V1b receptor subtypes are expressed in the central nervous system. The V1b receptor is located primarily in the pituitary and discrete areas of the brain, including the amygdala, whereas V1a receptor is expressed in many areas throughout the brain. Importantly, V1a receptor is expressed in the SCN and its target area, indicating that it may be the major mediator for the circadian function of AVP. To understand the role of V1a receptor in the circadian regulation, we examined the circadian locomotor activity and the expression of clock genes in V1a−/− mice.

MATERIALS AND METHODS

Animals. The generation of V1a−/− mice by homologous recombination has been described previously (12). V1a−/− mice and their littermate wild-type (WT) mice with 50% C57BL/6 × 50% 129/Sv mix background or 94% C57BL/6 background were used. Mixed female and male mice (~:1) from 3–5 mo of age were used. Before behavioral experiments, mice of the same sex were group-housed (3–5 animals per cage) under controlled conditions [temperature, 20 ± 2°C; relative humidity, 50–60%; 12:12-h light-dark (LD) cycle, lights on at 7:00 AM and lights off at 7:00 PM] and had free access to food and water. All procedures regarding the care and use of animals were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Address for reprint requests and other correspondence: Q.-Y. Zhou, Dept. of Pharmacology, Univ. of California, Irvine, CA 92697 (e-mail: qzhou@uci.edu).

http://www.ajpregu.org

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Locomotor behavior. Mice (16 WT and 16 V1a−/− mice with mixed background; 6 WT and 7 V1a−/− mice with 94% C57BL/6 background) were individually housed within cages equipped with running wheels or infrared motion sensors and were allowed free access to food and water. Their locomotor activities were recorded as revolutions per 5-min interval. Mice were entrained to an initial LD cycle (light intensity ~150 lux, lights on at 7:00 AM and lights off at 7:00 PM). After 2–3 wk of activity recording in 12:12-h LD conditions, the mice were placed in constant darkness (DD) with a dim red light (~5 lux) for ~4 wk. Another group of mice (6 WT and 6 V1a−/− mice with mixed background) in LD were also exposed to a light treatment (white light, 60 min, ~150 lux) during their activity period [zeitgeber time (ZT) 15; ZT is measured in hours after the light has been turned on in a LD cycle] to measure negative masking behavior, i.e., light-induced suppression of activity. The number of revolutions during this light treatment was compared with the number recorded during the same phase on the previous day. The light-induced phase shift was also performed on this group of mice at the 15th day in DD. Animals in their home cages were moved to another room and exposed to a 10-min pulse of white light (~150 lux) at circadian time (CT) 16, at which CT12 was designated as activity onset. Light-induced phase-shift amplitude was derived from regression lines drawn through the activity onset of at least 7 days immediately before the day of stimulation and 7 days after reestablishment of steady-state circadian period after stimulation.

The free-run period and fast Fourier transformation (FFT) were analyzed using ClockLab software (Actimetrics, Evanston, IL) in the MatLab environment. The free-run period was measured by a χ² periodogram from days 10 through 25 under DD. The daily revolution and FFT were determined by analyzing the activity of the last 10 days under LD and days 10–25 under DD. FFT circadian amplitude values represent the peak relative amplitude in the circadian range (18–30 h) normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in activity onset, we calculated a linear regression to 15 normalized to a total variance of 100%.

In situ hybridization. Mice were killed via cervical dislocation, and brains were quickly frozen and stored at −80°C until use. In situ hybridization was carried out on coronal sections (16 μm) as described by Winzer-Serhan et al. (43). Antisense and sense cRNA probes were generated by in vitro transcription in the presence of 35S-labeled UTP (1,200 Ci/ml) overnight at 60°C. After completion of the wash steps, slides were air-dried and apposed to Kodak BioMax MR films for 4 days. The specific hybridization signals were quantified using a video-based computer image analysis system (MCID, Imaging Research, UK). A calibration curve of optical density versus radioactivity [disintegrations per minute (dpm) per mg tissue wet weight] was constructed using 14C standards. Specific hybridization signals in the SCN were obtained by subtracting background values obtained from adjacent brain areas that have no hybridization signal. Data were normalized with respect to the differences between signal intensities in equal areas of SCN. The detection threshold for the in situ hybridization was at the range of 10–20 copies of mRNA per cell (43).

Luciferase assay. A 2.8-kb 5′-flanking region of the mouse PK2 gene was subcloned into a pGL3-Basic vector (Promega, Madison, WI) to generate the PK2.8-Luc plasmid (6). PK2.8-Luc- and V1a-expressing plasmids were transfected into human embryonic kidney (HEK-293) cells using Lipofectamine (Invitrogen, Carlsbad, CA). At 24 h after transfection, cells were treated with various concentrations of AVP (0–50 nM) in DMEM supplemented with 0.5% fetal bovine serum and incubated for another 24 h. Cells were lysed, and the firefly luciferase activity was assayed with a Sirius luminometer (Berthold, Bad Wildbad, Germany). Luciferase activities were normalized to protein concentration. Protein concentration was determined using a Bio-Rad protein assay, with a detection level of 1 μg of protein.

Statistical analyses. A repeated-measures ANOVA followed by unpaired t-test was used to analyze the data for differences between genotypes and time points. All statistical analyses was performed using Prism 4.4 (GraphPad Software, La Jolla, CA).

RESULTS

Circadian rhythm of V1a mRNA in wild-type and Bmal1−/− mice. We quantified the V1a transcript level in the SCN under constant darkness through in situ hybridization. As shown in Fig. 1A, V1a mRNA in the SCN displayed a robust circadian rhythm under constant darkness [F = 16.93, P < 0.0001, 1-way ANOVA], consistent with previous reports (28, 39). Peak level at CT17 was ~2.4 times that of the nadir level at CT5. In the V1a−/− mice, no V1a transcript was detected in the SCN at any time point examined, confirming the targeted allele

Fig. 1. Arginine vasopressin (AVP) receptor V1a mRNA level in the suprachiasmatic nuclei (SCN) shows a circadian rhythm. A: quantitative analysis of V1a mRNA signals in the SCN of wild-type (WT) and V1a−/− mice sampled every 4 h beginning at 49 h under constant darkness. Values are means ± SE; n = 3 mice. *P < 0.05; **P < 0.01; post hoc Dunnett’s t-test. B: representative in situ hybridization images of V1a mRNA signals from WT or V1a−/− mice killed at circadian time (CT) 17. C: the circadian rhythm of V1a mRNA in the SCN was attenuated in Bmal1−/− mice. Values are means ± SE; n = 3 mice per genotype per time point. ZT, zeitgeber time. *P < 0.05; post hoc Bonferroni t-test.
as a null mutation (Fig. 1, A and B). Furthermore, under LD conditions, the rhythm of V1a mRNA was significantly attenuated in mice deficient in a clock core gene, Bmal1 [F(1) = 8.92, P < 0.02, genotype × time, 2-way ANOVA] (Fig. 1C). Thus the oscillation of V1a mRNA in the SCN seems under the control of a clockwork oscillator. Because the BMAL1/CLOCK heterodimer had no effect on the transcriptional activity of V1a promoter (data not shown), BMAL1 may regulate the transcription of V1a gene in the SCN indirectly. Apparently, the LD cycle also affects the oscillation of V1a expression in the SCN, because it was reduced but not abolished in the SCN of Bmal1−/− mice.

V1a−/− mice showed attenuated circadian rhythms in locomotor activities. To address the function of V1a in the regulation of circadian rhythms, we first monitored the wheel-running activity of V1a−/− mice and their WT littermate controls. Animals were housed individually in cages equipped with running wheels. After continuous monitoring of wheel-running activities for 2–3 wk under a 12:12-h LD schedule, mice were switched to constant darkness (DD) for 4 wk. Under LD conditions, both WT and V1a−/− mice entrained to LD cycles and showed no significant difference in daily counts or amplitude of locomotor rhythmicity shown by FFT power (Fig. 2A and Table 1). However, the FFT power of V1a−/− mice under DD was significantly attenuated compared with the WT controls (Fig. 2A and Table 1). V1a−/− mice exhibited an expansion in duration of their daily activity bout (α). There was a significant genotypic difference on the duration of activity (α) after 25 days under DD (Table 1). As a result, 4 of the 16 V1a−/− mice became arrhythmic after 4 wk under DD. Moreover, the cycle-to-cycle variability in the onset of the daily activity bout was significantly increased in the V1a−/− mice. The V1a−/− mice showed about threefold higher cycle-to-cycle variability than WT controls in the phase of activity onset (Table 1). However, there were no significant differences in the daily counts, free-running period, or phase angle (Table 1).

Home cage activity of the mice was also monitored by infrared motion sensors. Consistent with the wheel-running activity, no significant differences were observed in LD conditions (Fig. 2B and Table 1). However, the daily activity periods expanded gradually when V1a−/− mice were subjected to DD conditions, whereas WT mice still displayed consolidated activity patterns. At day 25 under DD, the activity periods of V1a−/− mice were significantly longer than those of WT mice (Table 1). Some of the V1a−/− mice (4/16) became arrhythmic gradually (FFT power < 0.01) as the activity...
distributed uniformly during the 24-h span (Fig. 2B). As a result, the mean FFT power of V1a−/− mice under DD was only 30% of that for WT mice (Table 1). Interestingly, the daily activity of V1a−/− mice under DD was also significantly lower than that of WT mice (Table 1), whereas the daily activity under LD did not show a marked genotypic difference.

It is known that the genetic background affects the circadian behavior of mice. To rule out this possible confounding effect, we backcrossed V1a+/− mice with C57BL/6 mice for four sequential generations (N4), and N4 V1a+/− mice were mated to obtain N4F1 V1a−/− and their WT controls. These N4F1 V1a−/− mice are predicted to have a ~94% C57BL/6 background. We measured the circadian locomotor phenotypes of N4F1 V1a−/− mice. The defect of N4F1 V1a−/− mice in circadian locomotor rhythm was quite similar to that obtained from the mixed C57/129 background. As shown in Table 1, N4F1 V1a−/− mice were normal under entrained LD conditions but displayed significantly damped circadian rhythms and a longer free-running period under DD. Two of seven N4F1 V1a−/− mice gradually became arrhythmic under DD. Together, these data unequivocally indicate that a deficiency in the V1a gene results in attenuation of circadian locomotor rhythms.

Light masking and light-induced phase shift. Since the V1a−/− mice behave normally under LD but not DD conditions, we next examined the direct influence of light on locomotor activity in these mice. When mice were maintained in an LD cycle, we found no differences in the ability of light (white light, 1 h, ZT15) to acutely suppress (e.g., mask) wheel-running activity during an animal’s activity period (WT, 99.84 ± 0.07%; V1a−/−, 99.97 ± 0.02%; P = 0.165, n = 6 mice/genotype).

We also compared the phase shifts generated by the exposure of a brief light pulse to WT and V1a−/− littermates at

Fig. 3. Analysis of clock genes in the SCN. The V1a−/− mice and WT controls were killed every 4 h beginning at 49 h under constant darkness. In situ hybridization on coronal brain sections was performed with corresponding 35S-labeled probes. The expression of PK2 in the SCN displayed significant genotypic differences [F(1) = 12.72, P < 0.01]. Each data point represents the mean ± SE of 3 mice. *P < 0.05; **P < 0.01; post hoc Bonferroni t-test.
CT16 (white light, ~150 lux, 10 min). In this treatment, we did not see any significant differences between WT and V1a<sup>−/−</sup> mice (WT, 1.23 ± 0.44 h, n = 6; V1a<sup>−/−</sup>, 1.25 ± 0.21 h, n = 4; P = 0.97). However, this result should be interpreted with caution, because V1a<sup>−/−</sup> mice showed greater cycle-to-cycle variability than WT mice under DD conditions (Table 1).

Altered circadian rhythms of CCG expression in the SCN. To determine whether attenuated activity rhythms were caused by altered clock gene expression, we quantified gene expression in the SCN by in situ hybridization on brain sections of WT and V1a<sup>−/−</sup> mice. Samples were collected every 4 h after mice spent 49 h under DD conditions. As shown in Fig. 3, Per1, Per2, and Rev-erb-a expression showed similar patterns in WT and V1a<sup>−/−</sup> mice. Bmal1 expression was slightly shifted in V1a<sup>−/−</sup> mice, and vasopressin peak levels were slightly attenuated in V1a<sup>−/−</sup> mice. However, these differences were not significant. Interestingly, the circadian amplitude of PK2 mRNA was significantly attenuated in V1a<sup>−/−</sup> mice [F(1) = 12.72, P < 0.01, 2-way ANOVA]. Particularly, the values of PK2 mRNA at CT1 and CT5 were about one-half that in V1a<sup>−/−</sup> mice compared with WT controls (Fig. 3).

Vasopressin enhanced PK2 promoter activity through V1a in vitro. We have previously shown that activation of PKR2 by PK2 dose-dependently stimulates the CLOCK/BMAL1-mediated transcription of the 2.8-kb PK2 promoter (6). Since the signaling of PKR2 and V1a are similar (3, 20), the attenuated circadian amplitude of PK2 expression in the SCN of V1a<sup>−/−</sup> mice prompted us to investigate the effects of V1a receptor signaling on the activity of PK2 promoter. As shown in Fig. 4, vasopressin enhanced the luciferase activity in a dose-dependent manner when V1a and PK2.8-Luc were cotransfected into HEK-293 cells [F = 31.68, P < 0.0001, 1-way ANOVA].

DISCUSSION

The role of AVP signaling in the regulation of circadian rhythms has been studied with a variety of approaches, including behavior models, electrophysiology, histology, and pharmacology (4, 8, 10, 14–16, 18, 27, 33, 35, 42). However, the involvement of the AVP receptor subtypes in the regulation of circadian rhythms is still poorly understood. In this report, we examined the role of V1a receptor in the regulation of circadian rhythm. V1a receptor deficiency led to attenuation in circadian locomotor rhythms. Furthermore, our results revealed that V1a signaling is important in the maintenance of high amplitude of PK2 mRNA oscillation in the SCN, indicating that SCN output molecules also appear to function within the SCN to coordinate the expression of CCGs.

AVP-deficient Brattleboro rats serve as excellent models for studying the role of AVP in circadian rhythms. These rats display attenuated rhythms in a variety of circadian parameters, including body temperature, hormone synthesis, SCN neural firing, and sleep-wakefulness (8, 14, 26, 27, 29, 33, 42). Recent studies from common voles further support an important role of AVP in circadian locomotor rhythm. Arrhythmic voles exhibit constantly high populations of AVP-immunoreactive neurons in the SCN (16). Conversely, AVP release in cultured SCN neurons is attenuated in the arrhythmic voles (15). However, contradictory results also exist indicating that AVP is not essential for circadian locomotor behaviors. For instance, central infusion of either AVP or V1 receptor antagonist failed to produce significant effects on the patterns of wheel-running activity (1, 36). In this report, we present evidence that deficiency in V1a receptor results in attenuated oscillation of PK2 mRNA in the SCN as well as locomotor rhythms in mice. The interrupted communication between the SCN and its vasopressinergic effenter targets through V1a receptor may contribute to the damped locomotor rhythms in V1a<sup>−/−</sup> mice. However, because PK2 has been proposed to be an inhibitory factor for locomotor activity in rats (6), the reduced and compacted oscillation of PK2 expression in the SCN of V1a<sup>−/−</sup> mice may contribute mainly to the expansion of the activity period and attenuation in circadian rhythms.

It is estimated that nearly one-third of SCN neurons synthesize AVP and that a high proportion of the neurons (>40%) can be excited by AVP through the V1 receptors, suggesting that AVP may act as a feedback regulator within the nucleus (13, 14). Indeed, AVP-deficient Brattleboro rats showed a dampened amplitude of firing in the SCN during the subjective day, when AVP levels are high (14). The effect of AVP on the SCN gene expression has been reported, yet not pursued in detail, since AVP peak levels are attenuated (~20–34%) in Brattleboro rats (40, 44). In this study, we have shown that AVP, via the V1a receptor, positively regulated the expression of a clock-controlled output gene, PK2. V1a<sup>−/−</sup> mice displayed reduced PK2 mRNA levels during the subjective day, when AVP levels are high. Conversely, AVP, acting through the V1a receptor, was able to enhance the PK2 promoter activity in an in vitro luciferase assay. Interestingly, the peak value of AVP also showed a trend of reduction, yet not significant, in V1a<sup>−/−</sup> mice. These data suggest that AVP may specifically enhance the output signals in the SCN. The exact molecular mechanism of how AVP signaling may regulate the oscillatory amplitude of PK2 mRNA is still unclear. It is possible that AVP released locally within SCN or from the collateral output projections may regulate the expression of certain clock-related genes, such as PK2. Alternatively, it is also possible that the damped PK2 rhythm in the SCN of V1a<sup>−/−</sup> mice is due to the reduced electrophysiological activity of SCN neurons. AVP is known to positively regulate the firing activity of SCN neurons (21, 24). Recently, tetrodotoxin, an Na<sup>+</sup> channel blocker, has been shown to suppress the oscillation of PK2 in cultured SCN without affecting the oscillation of all clock core genes (2). The absence of AVP-V1a signaling may thus lead to reduced...
neuronal activity in the SCN that eventually results in attenuated peak PK2 expression levels in the SCN. Nevertheless, we cannot exclude the possibility that the attenuated PK2 expression amplitude in the SCN is caused by the absence of AVP-V1a signaling in some brain areas afferent to the SCN.

**Perspective and Significance**

In mammals, almost all physiological parameters display circadian rhythms, which are under the control of the master clock in the SCN. The SCN is enriched with a variety of neuropeptides. Several secreted factors encoded by clock-controlled genes (PK2, AVP, and cardiotrophin-like cytokine) have been implicated as output molecules that transmit circadian information from the SCN to its target areas. We presently report that disruption of AVP-V1a signaling results in damped circadian rhythms in locomotor activity. We also demonstrate that AVP as an output signal may also function within the SCN, regulating certain clock gene expression. Importantly, our data reveal an interaction between two putative output molecules, AVP and PK2. Nevertheless, animals with disruption in a single output pathway still display residual circadian rhythms, suggesting that these output factors may play some redundant or synergistic functions in eliciting the overall circadian rhythms. It will be of interest to decipher the interactions between these output pathways by analyzing double or triple knockout mice.

**ACKNOWLEDGMENTS**

We thank members of the Zhou laboratory for discussion and critical comments on the manuscript.

**GRANTS**

This work was supported in part by National Institute of Mental Health Grant MH67753.

**REFERENCES**