Original Paper

The Pulsatility of ACTH Secretion in the Rat Anterior Pituitary Cell Perifusion System

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Key Words

Anterior Pituitary • ACTH • Pulsatility • CRH • AVP

Abstract

Aims: This study aimed to examine the physiological mechanism whereby the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) exert their influence on adrenocorticotrophic hormone (ACTH) secretion in pituitary cells. **Methods:** Anterior pituitary cells were harvested from male rats and placed in the perifusion system. Cells were perfused with serum-free medium for 6 hours before fraction collection. After 30-minute of baseline collection, perifusion media was changed to expose the cells to CRH with or without AVP. ACTH concentration in each fraction was measured using enzyme immunoassay chemiluminescent kit. **Results:** The lowest physiological concentration of CRH (10 pM) or AVP (10 pM) was not able to induce a marked increase in ACTH secretion. Higher concentration of CRH (30 pM) or AVP (100 pM) in the physiological range caused sustained elevation of ACTH secretion ($P < 0.001$), while the secretion remained at similar levels for up to 1 hour with continuous stimulation. Perifusion with 10 pM AVP and 10 pM or 30 pM CRH caused a 2.38-fold and 2.99-fold increase in pulsatile ACTH secretion in pituitary cells, respectively. The duration of pulsatility caused by perifusion with 10 pM AVP and 30 pM CRH was close to that observed under physiological condition. **Conclusions:** By using the rat anterior pituitary cell perifusion system, we found that CRH and AVP potentiate the effect of each other on ACTH secretion, but AVP was a less potent agonist than CRH. The data suggest that CRH and AVP are essential for the pulsatility of ACTH, and AVP acts like a switch of the pulsatility.

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Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is an important component of the endocrine system. It is involved in controlling stress response and has an important physiological role in regulating digestion, the immune system, emotions and moods, sexual behavior and energy storage and consumption. The hypothalamus synthesizes corticotropin-

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releasing hormone (CRH) as well as arginine vasopressin (AVP), which are released into the pituitary portal circulation and reach corticotroph cells in the pituitary gland, where they stimulate the secretion of adrenocorticotrophic hormone (ACTH) into the peripheral circulation [1, 2]. The main target of ACTH action is the zona fasciculata of the adrenal cortex, where it stimulates the production and secretion of glucocorticoids. When the glucocorticoids concentration reaches sufficiently high levels, it will in turn inhibit the HPA axis activity by restraining the expression and secretion of CRH and AVP in the hypothalamus and inhibiting ACTH secretion and the transcription of its precursor protein, proopiomelanocortin (POMC). Glucocorticoids are important for normal metabolic activity and essential for adaption to stress [3].

In this study, a rat pituitary cell perfusion system was developed to study the key role of the pituitary gland in the HPA axis. Hypothalamic CRH and AVP released into the pituitary portal circulation gain access to receptors in the anterior pituitary corticotroph, where they stimulate ACTH secretion. CRH is a potent stimulant of ACTH secretion [4] and POMC transcription *in vitro* and *in vivo* [5]. The corticotroph of *crhr1* gene knockout mice is not able to synthesize and release ACTH [6]. In contrast, AVP, on its own, is a weaker stimulator of ACTH secretion, but it markedly potentiates the stimulatory effect of CRH on ACTH secretion [7, 8]. The number of AVP receptor 1B (V1b) is closely related with the response to stress [9]. In contrast to CRH, AVP does not stimulate POMC transcription on its own or in combination with CRH. Moreover, *in vivo* and *in vitro* studies have shown an inhibitory effect of AVP on POMC transcription [10].

A deconvolution analysis of frequent sampling reveals that ACTH is secreted in pulses, with 40 ± 1.5 pulses measured per 24 hours [11], which is not entirely due to pulsatile CRH release. Light mediates the circadian rhythm of ACTH secretion by activating the transcription of the *PER1* gene, which can alter the specific structure of chromatin and the accessibility to genes destined for transcription [12]. AVP may be responsible for the modulation of ACTH pulses, a study has revealed a circadian rhythm in AVP RNA expression and the loss of the circadian rhythm of AVP RNA expression was shown in transgenic CLOCK knockout mice [13]. Here in this study, we proposed that physiological concentration of CRH and AVP are able to stimulate pituitary cells *in vitro* to secrete ACTH in a pulsatility, this also suggested that the pulsatility of ACTH secretion is not controlled by hypothalamus.

Materials and Methods

Animals

Male Sprague Dawley rats weighing approximately 250 g were exposed to light for 16 hours per day over five days before use. Euthanasia was performed by decapitation after CO₂ sedation, and pituitary glands were rapidly removed and collected in ice-cold M199 medium (Gibco).

Cell culture

Pituitary glands were minced and transferred to a 50 ml tube (BD Falcon) and washed with buffer 1 (calcium- and magnesium-free Dulbecco's PBS, containing 2 mM L-glutamine, 1.26 mM CaCl₂ and 0.3% BSA). After a 15-minutes incubation in a water bath at 37°C with shaking (60 rpm) in 10 ml trypsin solution (buffer 1 containing 4 mg/mL trypsin). Next, 10 ml of buffer 1 containing 10 µg of deoxyribonuclease I (Sigma) were added and the tube was kept on ice for 1 minute. The supernatant was replaced with 5 ml of buffer 1 containing trypsin inhibitor (2–2.5 mg/mL), and the tissue was incubated at 37°C for 5 minutes. After a two steps wash with 2 mM EDTA for 5 minutes and 1 mM EDTA for 10 minutes, pituitary cells were mechanically dispersed by repeated gentle pipetting in buffer 1 until all cells were released. Cells were collected by centrifugation at 200 × g for 10 minutes and re-suspended in serum-free M199 at a density of 1.5 million cells/mL with Cytodex I micro-carriers (GE Healthcare). Cells were transferred to a 37°C cell incubator under 5% CO₂-95% air. Before the addition of the serum and antibiotics, cells were gently shaken every 30 minutes for 2 hours to avoid clamping.

Perfusion

Rat anterior pituitary cells cultured on Cytodex beads were transferred into 0.5 ml perfusion chambers (Acusyst-S Cellex Biosciences) and then were perfused for 6 hours with basic perfusion medium (M199 medium containing 0.1% BSA) at 37 °C. Every 5-minute fraction was collected as a sample in 5 ml polypropylene tubes containing 50 µl collection medium (500 mM EDTA and 4000 kIU/ml aprotinin). Before the treatments, six samples (30 minutes) were collected as baseline control, the samples were immediately placed in dry ice and then stored in -80°C.

ACTH measurement

The ACTH concentration was measured using the ACTH chemiluminescence ELISA kit (Phoenix Pharmaceuticals Corporation). The standard curve was adjusted as follows: 10 ng/ml, 33 ng/ml, 100 ng/ml, 330 ng/ml. Samples were thawed out and diluted with basic perfusion medium. We followed the protocol provided by the manufacturer of the kit to calculate the ACTH concentration.

Statistical analysis

The data are presented as the mean ± SEM. The statistical significance of the differences between the groups was determined by an independent *t*-test or *one/two*-way analysis of variance (ANOVA), followed by a Fisher's protected least-significant difference post-hoc test unless otherwise indicated. Statistical significance was set at $p < 0.05$.

Results

Effects of CRH or AVP pulses on ACTH secretion in column perfused rat anterior pituitary cells

In this study, rat anterior pituitary cells were kept in perfusion columns perfused with the estimated basal pituitary portal circulation levels of CRH (Bachem) [14, 15] and AVP (Bachem) [15]. Neither the lowest physiological concentration of CRH (10 pM) nor that of AVP (10 pM) was able to cause noticeable increase in ACTH secretion. However, increasing concentration of AVP significantly stimulated ACTH secretion ($p < 0.001$, 1.94 ± 0.08 -fold by 100 pM and 2.36 ± 0.14 -fold by 1 nM). Thirty picomolars of CRH increased ACTH secretion by 1.99 ± 0.14 -fold, whereas a higher concentration of 100 pM increased it by 6.99 ± 0.22 -fold, but secretion started to decline 15 minutes after continuous perfusion. (Fig. 1).

Fig. 1. Effects of CRH or AVP pulses on ACTH secretion in anterior pituitary cells. Cells were perfused with serum-free medium before the stimulation. After 30 minutes of collection of the baseline samples ($n=6$), cells received another 30-minutes stimulation of perfusion medium with different concentrations of AVP (A) or CRH (B). The data represent the average of the ACTH values with or without stimulus, expressed as mean ± SEM. **, $p < 0.001$.

Effect of AVP on ACTH secretion stimulated by 30 pM CRH in anterior pituitary cells

To test the dose-response effect of AVP on ACTH secretion stimulated by 30 pM CRH, rat anterior pituitary cells were perfused with different doses of AVP at 30-minute-interval. Consistent with the previous data, with a combination of 30 pM CRH and 10 pM AVP produced about 2.80-fold increase in ACTH secretion. A hundred picomolars AVP caused a 3.99-fold increase and the highest concentration 1 nM increase ACTH secretion by 6.40-fold. (Fig. 2).

Effect of the combination of CRH and 10pM AVP on ACTH secretion in anterior pituitary cells

To determine the effect of the lowest physiological concentration of AVP on the CRH-stimulated ACTH secretion, rat pituitary cells were subjected to continuous perfusion with 10 pM AVP together with CRH. The results, shown in Fig. 3, revealed that a combination of 10 pM CRH and 10 pM AVP produced a 2.38 ± 0.09 -fold increase in ACTH secretion, while the higher concentration of CRH (30 pM) in combination with 10 pM AVP increased the ACTH secretion by 2.99 ± 0.10 -fold.

Effect of AVP on CRH-stimulated ACTH secretion in anterior pituitary cells

Noteworthy, we found that a pulsatile ACTH secretion in anterior pituitary cells receiving constant 10 pM CRH and 10 pM AVP, which is consistent with the reported physiological condition [14, 15]. We repeated this experiments, and found the duration of the pulsatility

Fig. 2. Effect of AVP on ACTH secretion stimulated by 30 pM CRH in anterior pituitary cells. Cells were perfused with serum-free medium before the stimulation. After collection of the baseline samples ($n = 6$), cells received constant stimulation with perfusion medium containing 30 pM CRH. Three 10-minute pulses of AVP were added 30 minutes later, at 30-minutes interval. The time periods of the perfusion with CRH and AVP are shown by the clear-dotted and gray-dotted boxes.

Fig. 3. Effect of the combination of CRH and 10 pM AVP on ACTH secretion in anterior pituitary cells. Cells were perfused with serum-free medium before stimulation. After 30 minutes of collection of the baseline samples ($n=6$), cells received another 30-minutes stimulation with perfusion medium containing different concentrations of CRH and 10 pM AVP. The data represent the average of the ACTH values with or without stimulus, expressed as mean $\pm$ SEM. **, $p < 0.001$.

Fig. 4. Effect of AVP on CRH-stimulated ACTH secretion in anterior pituitary cells. Cells were perfused with serum-free medium before stimulation. After 30 minutes of collection of the baseline samples (n=6), cells received constant stimulation with perfusion medium containing CRH, with or without 10 pM AVP. The time periods of the perfusion with CRH and AVP are shown by the clear box.

Table 1. ACTH secretion and pulsatility induced by CRH and AVP in the rat anterior pituitary perfusion system. Cells were perfused with serum-free medium before the addition of CRH and AVP. "-" represented no addition, and "/" no fold change or pulsatility. Fold change was expressed as the ACTH values under the stimulations divided by the basal line samples collected before treatment

CRH (pM)	AVP (pM)	ACTH (Fold Change)	Duration (min)
10	-	/	/
-	10	/	/
10	10	2.38 ± 0.09	51.25 ± 1.875 (n=8)
30	-	1.99 ± 0.14	/
30	10	2.80 ± 0.17	43.75 ± 1.275 (n=6)
30	100	3.99 ± 0.29	/
30	1000	6.40 ± 0.33	/
100	-	6.99 ± 0.22	/
-	100	1.94 ± 0.08	/
-	1000	2.36 ± 0.14	/

was 51.25 ± 1.875 minutes (n=8). When we compared the patterns of ACTH secretion in anterior pituitary cells perfused with 30 pM CRH, with or without 10 pM AVP, we again found the same pulsatile ACTH secretion in the column with 30 pM CRH and 10 pM AVP. The duration of the pulsatility was 43.75 ± 1.275 minutes (n=6). The ACTH secretion in the column perfused with constant 30 pM CRH was stable, with little pulsatility. (Fig. 4).

Discussion

In this study, a rat anterior pituitary cell perfusion system is successfully established *in vitro*. In this system, the physiological concentration of CRH and AVP are able to induce a pulsatile secretion of ACTH, and the duration of the pulsatility is very similar to that observed *in vivo*, the data obtained from the rat anterior pituitary perfusion system was shown in Table 1. This system will be helpful for studying the HPA axis activity and ACTH secretion.

It is generally recognized that CRH as well as AVP are synthesized by the neurons located in the dorsomedial parvocellular subdivision of the paraventricular nucleus (PVN) of the hypothalamus. A large body of evidence shows that AVP plays a role in regulating

pituitary corticoreoph [1, 16, 17]. A number of studies have shown that inhibition of vasopressinergic activity by the use of VP antibody or receptor antagonists can decrease ACTH and corticosterone responses to acute stress. Hypothalamic CRH and AVP released into the pituitary portal circulation gain access to receptors in the anterior pituitary corticotroph cells, where they stimulate ACTH secretion. Unlike VP secreted into the peripheral circulation from magnocellular neurons, AVP responsible for corticotroph regulation is secreted from parvocellular axon terminals in the external zone of the median eminence [16]. In all species studied, including rat, mouse and human, a proportion of CRH-producing neurons express AVP, about 50% in basal conditions. And this proportion increases substantially during stress, especially chronic and prolonged adrenalectomy [18, 19]. AVP is co-released with CRH and both contribute to ACTH secretion and activation of the HPA axis.

Using rat anterior pituitary cells perfused with the estimated basal pituitary portal circulation levels of CRH and AVP, the present study demonstrates that the pituitary corticotroph is sensitive to CRH and AVP pulses in the range of basal free circulation levels of these hormones. Preliminary experiments in a previous study indicated that 5-minute increasing pulses of CRH resulted in concentration-dependent ACTH secretion with a threshold of 30 pM [20]. In this study, the lowest physiological levels of CRH, 10 pM was not able to produce a marked increase in ACTH secretion (Fig. 1). Actually, thirty picomolar of CRH increased ACTH by 2-fold, and the ACTH secretion was maintained stable for more than 2 hours with a continuous perfusion. A higher concentration of 100 pM caused a 7-fold increase, but the secretion started to decline 15 minutes later (data not shown), and this may be due to the desensitization of the receptors under treatment with the stress level of hormone.

Continuous perfusion with 10 pM AVP for up to 30 minutes did not alter ACTH secretion. Higher concentrations of AVP, specifically 100 pM and 1 nM, caused a 1.94-fold and 2.36-fold increase in ACTH secretion, respectively. The increased ACTH was stable and lasted for more than 1 hour. These results indicated that AVP was a less potent agonist compared with CRH, agreed with the published papers [1, 7, 8, 16].

An experiment was designed to test the effect of AVP on ACTH secretion stimulated by 30 pM CRH. In the cells perfused with continuous 30 pM CRH, 5-minute increasing pulses of AVP resulted in concentration-dependent ACTH secretory peaks. This finding is consistent with Prickett's findings that the AVP pulse acts as an efficient stimulus on ACTH secretion in normal equine anterior pituitary cells *in vitro* when pre-incubated with CRH [21].

To determine the effect of CRH on AVP-stimulated ACTH secretion, rat anterior pituitary cells received continuous perfusion with 10 pM AVP and CRH. These results showed that even the combination of the lowest physiological levels of CRH (10 pM) and AVP, caused a 2.38-fold increase, similar to the effect of the stimulation by 1 nM AVP (2.36-fold).

The finding of a pulsatility in ACTH secretion by anterior pituitary cells receiving constant 10 pM AVP with either 10 pM or 30 pM CRH represents a very exciting discovery. Also, as we all known, ACTH released by the pituitary stimulates glucocorticoid secretion, and when it reaches sufficiently high levels it inhibits the pituitary corticotroph thus leading to the decrease of ACTH secretion, and the consequent decrease in glucocorticoid secretion. The group of Lightman in Bristol showed that low doses of CRH generates pulsatile corticosterone secretion in rats [22]. This finding is consistent with our results indicating that pulsatile corticosterone secretion resulted in ACTH pulsatility. Engler et al. also showed in 1990 that hypothalamic disconnection of the pituitary in sheep does not suppress the ultradian rhythm of corticosterone [23].

The duration of the pulsatility caused by 10 pM CRH and 10 pM AVP was about 50-55 minutes ($n = 8$), and the duration of that caused by 30 pM CRH and 10 pM AVP was 40-45 minutes ($n = 3$). The latter was close to the physiological conditions reported by Veldhuis [11]. This indicated that the physiological levels of CRH and AVP were around 30 pM and 10 pM, respectively.

The generator of the ACTH pulsatility remains unclear. Studies using Brattleboro rats or vasopressin receptor gene knockout mice indicated that the ACTH secretion was unaltered in the absence of VP [24-26], but it was generally accepted that VP plays a role mediating

ACTH responses [27-29]. As shown in Figure 4B, the cells in the column perfused with 30 pM CRH and 10pM AVP were more pulsatile compare to the cells in the column with 30 pM CRH alone.

It has been shown that the actions of AVP was mediated by plasma membrane receptor [30, 31], and the receptor present in pituitary corticotroph is V1b receptor [32-35]. Aguilera's study showed that the regulation of V1b receptor plays an important role in the sensitivity of ACTH responses, maintaining the corticotroph responsiveness even in the presence of elevated glucocorticoid levels, but the expression of CRH and pituitary type 1 CRH receptor were not changed or even reduced in conditions of high HPA activation [1]. A reported electrophysiological study supported that the circadian rhythmicity in the suprachiasmatic nuclei (SCN) neurons is independent of the afferent inputs from other brain areas [36]. Further study on single neuronal activity in the SCN revealed that the dorsomedial part of the SCN is an intrinsic oscillating pacemaker [37]. Additionally, the dorsomedial part of SCN is also the main location of AVP neurons [12]. Moreover, the circadian rhythm persists despite a continuous infusion of CRH [38] or AVP, suggesting that CRH or AVP alone is not the mediator of ACTH pulses. Actually, a study discovered a circadian rhythm in AVP RNA expression and the loss of the circadian rhythm of AVP RNA expression was shown in the transgenic CLOCK knockout mice [13].

In this study, a rat anterior pituitary cells perfusion model was used to examine the ACTH secretory patterns regulated by CRH and AVP. CRH and AVP in the physiological range cause marked increase in ACTH secretion, and potentiate the effect of each other on ACTH secretion. Under constant perfusion with a combination of low levels of AVP and CRH, cells were able to produce pulsatile ACTH secretion. Accordingly, we can draw the conclusion that CRH and AVP are essential for the ACTH pulsatility, and AVP acts like a switch of the pulsatility. However, the underlying mechanisms need to be further investigated.

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Disclosure Statement

The authors have nothing to disclose.

References

- 1 Aguilera G: Regulation of pituitary ACTH secretion during chronic stress. *Front Neuroendocrinol* 1994;15:321-350.
- 2 Antoni FA: Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocr Rev* 1986;7:351-378.
- 3 Kabir NN, Kazi JU: Grb10 is a dual regulator of receptor tyrosine kinase signaling. *Mol Biol Rep* 2014;41:1985-1992.
- 4 Vale W, Rivier C, Brown MR, Spiess J, Koob G, Swanson L, Bilezikjian L, Bloom F, Rivier J: Chemical and biological characterization of corticotropin releasing factor. *Recent Prog Horm Res* 1983;39:245-270.
- 5 Autelitano DJ, Lundblad JR, Blum M, Roberts JL: Hormonal regulation of POMC gene expression. *Annu Rev Physiol* 1989;51:715-726.

- 6 Bale TL, Vale WW: CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* 2004;44:525-557.
- 7 Abou-Samra AB, Harwood JP, Manganiello VC, Catt KJ, Aguilera G: Phorbol 12-myristate 13-acetate and vasopressin potentiate the effect of corticotropin-releasing factor on cyclic AMP production in rat anterior pituitary cells. Mechanisms of action. *J Biol Chem* 1987;262:1129-1136.
- 8 Gillies GE, Linton EA, Lowry PJ: Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* 1982;299:355-357.
- 9 Aguilera G, Pham Q, Rabadan-Diehl C: Regulation of pituitary vasopressin receptors during chronic stress: relationship to corticotroph responsiveness. *J Neuroendocrinol* 1994;6:299-304.
- 10 Levin N, Blum M, Roberts JL: Modulation of basal and corticotropin-releasing factor-stimulated proopiomelanocortin gene expression by vasopressin in rat anterior pituitary. *Endocrinology* 1989;125:2957-2966.
- 11 Veldhuis JD, Iranmanesh A, Johnson ML, Lizarralde G: Amplitude, but not frequency, modulation of adrenocorticotropin secretory bursts gives rise to the nyctohemeral rhythm of the corticotrophic axis in man. *J Clin Endocrinol Metab* 1990;71:452-463.
- 12 Yamase K, Takahashi S, Nomura K, Haruta K, Kawashima S: Circadian changes in arginine vasopressin level in the suprachiasmatic nuclei in the rat. *Neurosci Lett* 1991;130:255-258.
- 13 Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, Reppert SM: A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 1999;96:57-68.
- 14 Plotsky PM, Vale W: Hemorrhage-induced secretion of corticotropin-releasing factor-like immunoreactivity into the rat hypophysial portal circulation and its inhibition by glucocorticoids. *Endocrinology* 1984;114:164-169.
- 15 Fink G, Robinson IC, Tannahill LA: Effects of adrenalectomy and glucocorticoids on the peptides CRF-41, AVP and oxytocin in rat hypophysial portal blood. *J Physiol* 1988;401:329-345.
- 16 Antoni FA: Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Front Neuroendocrinol* 1993;14:76-122.
- 17 Mc CS, Brobeck JR: Evidence for a role of the supraopticohypophyseal system in regulation of adrenocorticotrophin secretion. *Proc Soc Exp Biol Med* 1954;87:318-324.
- 18 Whitnall MH: Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Prog Neurobiol* 1993;40:573-629.
- 19 de Goeij DC, Kvetnansky R, Whitnall MH, Jezova D, Berkenbosch F, Tilders FJ: Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. *Neuroendocrinology* 1991;53:150-159.
- 20 Deng Q, Riquelme D, Trinh L, Low MJ, Tomic M, Stojilkovic S, Aguilera G: Rapid Glucocorticoid Feedback Inhibition of ACTH Secretion Involves Ligand-Dependent Membrane Association of Glucocorticoid Receptors. *Endocrinology* 2015;156:3215-3227.
- 21 Prickett TCR, Inder WJ, Evans MJ, Donald RA: Interleukin-1 potentiates basal and AVP-stimulated ACTH secretion in vitro--the role of CRH pre-incubation. *Horm Metab Res* 2000;32:350-354.
- 22 Walker JJ, Spiga F, Waite E, Zhao Z, Kershaw Y, Terry JR, Lightman SL: The origin of glucocorticoid hormone oscillations. *PLoS Biol* 2012;10:e1001341.
- 23 Engler D, Pham T, Liu JP, Fullerton MJ, Clarke IJ, Funder JW: Studies of the regulation of the hypothalamic-pituitary-adrenal axis in sheep with hypothalamic-pituitary disconnection. II. Evidence for in vivo ultradian hypersecretion of proopiomelanocortin peptides by the isolated anterior and intermediate pituitary. *Endocrinology* 1990;127:1956-1966.
- 24 Wersinger SR, Ginns EI, O'Carroll AM, Lolait SJ, Young WS, 3rd: Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. *Mol Psychiatry* 2002;7:975-984.
- 25 Makara GB, Mergl Z, Zelena D: The role of vasopressin in hypothalamo-pituitary-adrenal axis activation during stress: an assessment of the evidence. *Ann N Y Acad Sci* 2004;1018:151-161.
- 26 Tanoue A, Ito S, Honda K, Oshikawa S, Kitagawa Y, Koshimizu TA, Mori T, Tsujimoto G: The vasopressin V1b receptor critically regulates hypothalamic-pituitary-adrenal axis activity under both stress and resting conditions. *J Clin Invest* 2004;113:302-309.
- 27 Rivier J, Rivier C, Vale W: Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. *Science* 1984;224:889-891.

- 28 Olsen L, Knigge U, Warberg J: Gastrin-releasing peptide stimulation of corticotropin secretion in male rats. *Endocrinology* 1992;130:2710-2716.
- 29 Suda T, Nakano Y, Tozawa F, Sumitomo T, Sato Y, Yamada M, Demura H: The role of corticotropin-releasing factor and vasopressin in hypoglycemia-induced proopiomelanocortin gene expression in the rat anterior pituitary gland. *Brain Res* 1992;579:303-308.
- 30 Jard S, Barberis C, Audigier S, Tribollet E: Neurohypophyseal hormone receptor systems in brain and periphery. *Prog Brain Res* 1987;72:173-187.
- 31 Peter J, Burbach H, Adan RA, Lolait SJ, van Leeuwen FW, Mezey E, Palkovits M, Barberis C: Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cell Mol Neurobiol* 1995;15:573-595.
- 32 Seibold A, Brabet P, Rosenthal W, Birnbaumer M: Structure and chromosomal localization of the human antidiuretic hormone receptor gene. *Am J Hum Genet* 1992;51:1078-1083.
- 33 Sugimoto T, Saito M, Mochizuki S, Watanabe Y, Hashimoto S, Kawashima H: Molecular cloning and functional expression of a cDNA encoding the human V1b vasopressin receptor. *J Biol Chem* 1994;269:27088-27092.
- 34 Lolait SJ, O'Carroll AM, Brownstein MJ: Molecular biology of vasopressin receptors. *Ann N Y Acad Sci* 1995;771:273-292.
- 35 Lolait SJ, O'Carroll AM, Mahan LC, Felder CC, Button DC, Young WS, 3rd, Mezey E, Brownstein MJ: Extrahypothalamic expression of the rat V1b vasopressin receptor gene. *Proc Natl Acad Sci USA* 1995;92:6783-6787.
- 36 Inouye ST, Kawamura H: Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci USA* 1979;76:5962-5966.
- 37 Shibata S, Liou S, Ueki S, Oomura Y: Influence of environmental light-dark cycle and enucleation on activity of suprachiasmatic neurons in slice preparations. *Brain Res* 1984;302:75-81.
- 38 Ur E, Capstick C, McLoughlin L, Checkley S, Besser GM, Grossman A: Continuous administration of human corticotropin-releasing hormone in the absence of glucocorticoid feedback in man. *Neuroendocrinology* 1995;61:191-197.