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Effects of Vasopressin V1b Receptor Deficiency on Adrenocorticotropin Release from Anterior Pituitary Cells in Response to Oxytocin Stimulation

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Oxytocin (OT) is one of the secretagogues for stress-induced ACTH release. OT-induced ACTH release is reported to be mediated by the vasopressin V1b receptor in the rat pituitary gland, which contains both OT and V1b receptors. We examined OT-induced ACTH release using primary cultures of anterior pituitary cells from wild-type ($V1bR^{+/+}$) and V1b receptor knockout ($V1bR^{-/-}$) mice. OT stimulated similar levels of ACTH release from pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. OT-induced ACTH release was significantly inhibited by the selective V1b receptor antagonist SSR149415 and the OT receptor antagonist CL-14-26 in $V1bR^{+/+}$ mice. In addition, cotreatment with SSR149415 at 10^{-6} M and CL-14-26 at 10^{-6} M inhibited OT-induced ACTH release to the control level in $V1bR^{+/+}$ mice. In $V1bR^{-/-}$ mice, OT-induced ACTH release was significantly inhibited by CL-14-26 at 10^{-8} M and completely inhibited at 10^{-7} M. These results indicate that OT induces the ACTH response via OT and V1b receptors in $V1bR^{+/+}$ mice but via only OT receptors in $V1bR^{-/-}$ mice. The gene expression level of the OT receptor was significantly higher in the anterior pituitary gland of $V1bR^{-/-}$ mice than in that of $V1bR^{+/+}$ mice, suggesting that the OT receptor is up-regulated to compensate for ACTH release under conditions of V1b receptor deficiency. (*Endocrinology* 149: 4883–4891, 2008)

ACTH PRODUCED IN THE anterior pituitary gland is a stress response hormone that stimulates glucocorticoid release from the adrenal cortex. It is known that CRH and [Arg^8]-vasopressin (AVP), both of which are synthesized in the hypothalamic paraventricular nucleus, regulate ACTH secretion in response to stress (1–4). In addition to CRH and AVP, oxytocin (OT) produced in the paraventricular nucleus can act as an ACTH secretagogue (5, 6). Thus, several factors, including CRH, AVP, and OT, can regulate ACTH release from the anterior pituitary gland and contribute to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is essential for resisting harmful stress (2, 4, 7).

The AVP and OT receptors, which include the V1a, V1b, V2, and OT receptors and are coupled with G proteins, belong to the same receptor subfamily (8). Of these, the V1b receptor, which is dominantly expressed in the pituitary gland (9–11) and is localized in proopiomelanocortin-positive cells (12), has been cloned. Recent studies with the V1b receptor-selective antagonist SSR149415 showed that the V1b receptor was involved in AVP-induced ACTH release *in vitro* as well as *in vivo* (13, 14).

To analyze the functional role of the V1b receptor *in vivo*, we generated V1b receptor knockout ($V1bR^{-/-}$) mice. We found that the basal plasma ACTH level was decreased and that the ACTH response to stress stimulation in a forced swimming test was impaired in mutant mice (14). Moreover, the ACTH response to insulin-induced hypoglycemia was suppressed in mice lacking the V1b receptor (15). These findings indicate that the V1b receptor plays a crucial role in HPA axis activity under stress as well as basal conditions, by regulating ACTH release (14–17), and that other factors, including CRH, are unable to compensate for the ACTH release under such conditions. On the other hand, plasma ACTH and corticosterone levels were the same in control and mutant mice under stresses such as acute restraint or intruder stress (15, 17), suggesting that other factors could be involved in case of V1b receptor deficiency, consequently compensating for the stress-responsive ACTH release.

AVP stimulates ACTH release via the V1b receptor, which is predominantly expressed in the anterior pituitary gland (9, 11). The OT receptor is also expressed in the anterior pituitary gland (18, 19). However, the expression of the OT receptor gene is rarely detected in corticotroph cells, and most OT receptors in the anterior pituitary gland are present in lactotroph cells (19), where OT plays an important role in stimulating prolactin secretion (20). In addition, OT has been involved in stimulating ACTH release from the rat anterior pituitary gland; this release is mediated by the V1b receptor

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Abbreviations: AVP, [Arg^8]-vasopressin; DIG, digoxigenin; DNase, deoxyribonuclease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPA, hypothalamic-pituitary-adrenal; LE, Long-Evans; OT, oxytocin; PB, phosphate buffer; PLSD, projected least significant difference.

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but not the OT receptor (21). To investigate the mechanisms underlying OT-induced ACTH release, we examined OT-induced ACTH release using primary cultures of anterior pituitary cells from $V1bR^{+/+}$ and $V1bR^{-/-}$ mice.

Materials and Methods

Materials

DMEM and SuperScript III reverse transcriptase were purchased from Invitrogen Corp. (Carlsbad, CA). AVP and OT were from the Peptide Institute (Osaka, Japan). SSR149415, a specific antagonist for the V1b receptor, was donated by Sanofi-Aventis (Montpelier, France). $d(CH_2)_5[Tyr(Me)^{2,}\ Thr^{4,}\ Tyr\text{-}NH_2{}^{9}]OVT$ (CL-14-26), a specific antagonist for the OT receptor (22, 23), was a generous gift from Dr. Maurice Manning (University of Toledo College of Medicine, Toledo, OH). Collagenase S-1 was from Nitta (Osaka, Japan). Isogen was from Nippon Gene (Tokyo, Japan). Ex *Taq* polymerase and SYBR Premix Ex *Taq* were from Takara (Tokyo, Japan). The ACTH RIA kit was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan). The antibody against ACTH and reagent used for the ACTH RIA were from Mitsubishi Chemical Medience (Tokyo, Japan). All other chemicals were purchased from Wako (Tokyo, Japan).

Animals

V1b receptor knockout ($V1bR^{-/-}$) mice were generated by gene targeting and homologous recombination to eliminate the first exon of the gene, as described previously (14). Male mice 10–12 wk of age were used in this study. Non-V1b receptor-deficient mice littermates ($V1bR^{+/+}$) were used as age-matched controls. The genetic background of control and knockout mice was maintained as a mixture of 129Sv and C57BL/6J. All animals were housed in microisolator cages in a pathogen-free barrier facility and placed on a 12-h light, 12-h dark cycle with *ad libitum* access to food and water. All experiments were performed under the approved guidelines for the Care and Use of Laboratory Animals of the National Research Institute for Child Health and Development.

Primary culture of anterior pituitary cells

Anterior pituitary cells were cultured as described with some modifications (14). Briefly, mice were killed by cervical dislocation, and the anterior pituitary gland, except for the posterior pituitary gland, was removed and immediately placed in buffer A [137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM HEPES (pH 7.3), 50 U/ml penicillin, and 50 μg/ml streptomycin]. The collected anterior pituitary glands were treated with 0.4% collagenase in buffer A containing 10 mM glucose, 0.1% BSA, and 190 U/ml deoxyribonuclease (DNase) I for 2 h at 37 C. After incubation and suspension, the digested cells were washed with DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in DMEM medium and filtered through a 100-μm nylon mesh (Cell Strainer; BD Falcon, Bedford, MA). We used 20 mice per cell preparation and obtained approximately 4.0×10^5 cells per pituitary. Six individual assays with cells prepared from pituitaries were performed to calculate the mean and SEM. The cells were seeded in poly-L-lysine-coated 24-well plates (3.5×10^5 cells per well) and cultured at 37 C in 5% CO_2 and 95% air for 5 d, changing the medium at 2-d intervals.

Measurement of ACTH release from anterior pituitary cells

Cultured anterior pituitary cells were washed with an assay medium [DMEM containing 0.1% BSA and 20 mM HEPES (pH 7.3)] and incubated with the same medium for 1 h at 37 C. After preincubation the cells were incubated with and without OT and/or AVP. Antagonists were added 5 min before stimulation with OT. The conditioned medium was collected after 3 h incubation and stored at −80 C until the assay. The ACTH concentration in the conditioned medium was measured by RIA. Each value for ACTH release was calculated from the results of an experiment based on six individual assays.

Total RNA extraction and RT-PCR

Mice were killed by cervical decapitation. The anterior pituitary glands, except for the posterior pituitary gland, were isolated from two mice, and total RNA was extracted using Isogen. Aliquots of total RNA were treated with ribonuclease-free DNase and reverse transcribed to first-strand cDNA using SuperScript III reverse transcriptase. RT-PCR was then performed using Ex *Taq* polymerase as follows: 95 C for 5 min as a denaturing step; 28 cycles of 30 sec at 95 C, 30 sec at 65 C, and 1 min at 72 C as an amplification step; and 72 C for 10 min as an extension step. The primers used for the V1b receptor, OT receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were exon spanning as follows: V1b receptor, forward 5′-CATTGTGCTGGCCTACATTG-3′ and reverse 5′-TGGTGAAAGCCACATTGGTAG-3′; OT receptor, forward 5′-AGGAGCTGTTCTCAACCATC-3′ and reverse 5′-TGCAAACCAATCAATAGGCAC-3′; and GAPDH, forward 5′-AGGTCATCCATGACAACTTTG-3′ and reverse 5′-TTCAGCTCTGGGATGACCTT-3′. The PCR products were electrophoresed and visualized on a 1.5% agarose gel stained with ethidium bromide.

Similarly, total RNA was extracted from pituitaries of AVP-deficient Brattleboro and control Long-Evans (LE) rats at 16 wk of age, and used for RT-PCR analysis. The primers used for the V1b receptor, OT receptor, and GAPDH were exon spanning as follows: V1b receptor, forward 5′-TGCATCTGTTCGTGCTGCAC-3′ and reverse 5′-AGGAGGTCAGAGCCCTGGAAG-3′; OT receptor, forward 5′-TTCTTCGTGCAGATGTGGAG-3′ and reverse 5′-AAGCGCTGCACGAGTTCGTG-3′; and GAPDH, forward 5′-AGGTCATCCATGACAACTTTG-3′ and reverse 5′-TTCAGCTCTGGGATGACCTT-3′. The reactions were prepared in 20 μl reaction volumes, and cycle-sequencing reactions were performed as follows: 1 cycle at 95 C for 5 min as a denaturing step; 30 cycles of 30 sec at 95 C, 30 sec at 58 C, and 1 min at 72 C as an amplification step; and 72 C for 10 min as an extension step.

Real-time PCR

As describe previously, total RNA was extracted from the anterior pituitary glands using Isogen. Aliquots of total RNA were treated with ribonuclease-free DNase and reverse transcribed to first-strand cDNA using SuperScript III reverse transcriptase. A quantitative real-time PCR assay with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) was performed using SYBR Premix Ex *Taq* containing SYBR Green I. We used the *GAPDH* gene to normalize the cDNA to quantify OT receptor expression levels among the samples. The primers were used as described previously. The reactions were prepared in reaction volumes of 20 μl, and cycle-sequencing reactions were performed for 1 cycle of 10 sec at 95 C, 40 cycles of amplification for 5 sec at 95 C and 30 sec at 60 C.

In situ hybridization and immunohistochemistry

Sense and antisense cRNA probes corresponding to 770 bases (nucleotides 1103–1872) of the mouse OT receptor cDNA (GenBank NM_001081147), which contain the 3′-noncoding region, were generated. Nucleotides 1103–1872 of the mouse OT receptor cDNA were amplified by PCR using cDNA derived from the pituitary gland as a template. The PCR products were subcloned into a pGEM-T easy vector (Promega Corp., Madison, WI) according to the manufacturer's protocol. Probes were synthesized from this cDNA template with SP6 (sense) or T7 (antisense) RNA polymerases (Roche, Basel, Switzerland) and digoxigenin (DIG) RNA labeling mix (Roche).

Mice were killed with diethyl ether, and the pituitary glands were immediately removed and immersed in 4% paraformaldehyde in 0.07 M phosphate buffer (PB) overnight at 4 C, followed by immersion in a sucrose solution (30% in PB) overnight at 4 C. The pituitary glands were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and frozen on dry ice. They were sectioned at a thickness of 10 μm, collected on Matsunami adhesive silane-coated slides (Matsunami Ltd., Osaka, Japan), and then treated with 0.5 μg/ml proteinase K for 30 min at 37 C after washing with PBS. They were fixed with 4% paraformaldehyde in PB for 10 min and treated with 0.2 M HCl for 10 min, and then treated with 0.25% acetic anhydride in 0.1 M triethanol amine for 10 min. Hybridization was performed overnight at 42 C with 1000 ng/ml of the probe in 3× standard saline solution, 50% formamide, 0.12 M PB, 1× Denhardt's solution, 125 μg/ml tRNA, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. After washing the probe, the sections were incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:1000 in 0.1 M Tris-HCl

buffer (pH 7.4) containing 0.15 M NaCl and 0.01% Tween 20. After washing, visible signals were detected with a 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride substrate system (Dako Corp., Carpinteria, CA). For double staining with *in situ* hybridization and immunohistochemistry, after incubation with an alkaline phosphatase-conjugated anti-DIG antibody, the sections were treated with the 2-hydroxy-3-naphthoic-acid-2′-phenyl-anilide phosphate Fluorescent Detection Set (Roche) according to the manufacturer's protocol. Next, they were incubated with an anti-ACTH antibody (adrenocorticotrophic hormone, mouse monoclonal antibody, 1:200; BIOMOL International, L.P., Plymouth Meeting, PA). The sections were then incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using the unpaired Student's *t* test, one-way ANOVA, followed by a *post hoc* comparison using Fisher's protected least significant difference (PLSD), and two-way ANOVA, using StatView software, version 5.0 (Concepts, Inc., Berkeley, CA). Differences between groups were considered statistically significant at the level of $P < 0.05$.

Results

OT-induced ACTH release from anterior pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice

We evaluated ACTH release from anterior pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice by stimulation with OT, AVP, or CRH. CRH was used as a positive control because it is known to be a potent stimulator (14). CRH at 10^{-8} M induced an 8-fold increase in ACTH release above baseline in cells from $V1bR^{+/+}$ and $V1bR^{-/-}$ mice (Fig. 1A). There was no significant difference in CRH-stimulated ACTH release between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice (187.2 ± 9.9 ng/ml in $V1bR^{+/+}$ mice, n = 6; 212.5 ± 25.6 ng/ml in $V1bR^{-/-}$ mice, n = 6; $P = 0.39$ by unpaired Student's *t* test), indicating that anterior pituitary cells from $V1bR^{-/-}$ as well as $V1bR^{+/+}$ mice were intact and able to release ACTH. As reported previously (14), AVP stimulated ACTH release in cells from $V1bR^{+/+}$ mice, whereas AVP did not induce significant ACTH release in cells from $V1bR^{-/-}$ mice (90.3 ± 12.7 ng/ml at 10^{-7} M AVP in $V1bR^{+/+}$ mice, n = 6, $P < 0.0001$ in comparison with control; 39.0 ± 8.3 ng/ml at 10^{-7} M AVP in $V1bR^{-/-}$ mice, n = 6, $P = 0.44$ in comparison with the control) (Fig. 1, A and B). OT also induced ACTH release from anterior pituitary cells of $V1bR^{+/+}$ mice in a dose-dependent manner, although the increase in ACTH release induced by stimulation with OT at 10^{-7} M was smaller than that with AVP at 10^{-7} M or CRH at 10^{-8} M[23.7 ± 2.2 ng/ml at 0 M OT, n = 6; 39.2 ± 5.5 ng/ml at 10^{-9} M OT, n = 6; 48.2 ± 4.5 ng/ml at 10^{-8} M OT, n = 6; and 64.8 ± 6.3 ng/ml at 10^{-7} M OT, n = 6; $P < 0.05$ and $P < 0.0001$ for each concentration of OT and AVP (10^{-7} M) and OT and CRH (10^{-8} M), respectively; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD] (Fig. 1A). This result indicates that OT is a potent stimulator of ACTH release but that OT is less potent than AVP or CRH. Similar to the results in $V1bR^{+/+}$ mice, OT also stimulated ACTH release in a dose-dependent manner in $V1bR^{-/-}$ mice (24.9 ± 3.3 ng/ml at 0 M OT, n = 6; 46.4 ± 5.2 ng/ml at 10^{-9} M OT, n = 6; 52.4 ± 7.1 ng/ml at 10^{-8} M OT, n = 6; and 65.6 ± 5.9 ng/ml at 10^{-7} M OT, n = 6; $P < 0.0001$ in comparison with each concentration of OT and 10^{-8} M CRH; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD) (Fig. 1B). There were no significant differences in OT-induced ACTH release between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice ($P = 0.75$ for $V1bR^{+/+}$ *vs.* $V1bR^{-/-}$ at 0 M OT, $P = 0.37$ at 10^{-9} M OT, $P = 0.63$ at 10^{-8} M OT, and $P = 0.93$ at 10^{-7} M OT by the unpaired Student's *t* test; $P = 0.65$ for $V1bR^{+/+}$ *vs.* $V1bR^{-/-}$ by two-way ANOVA).

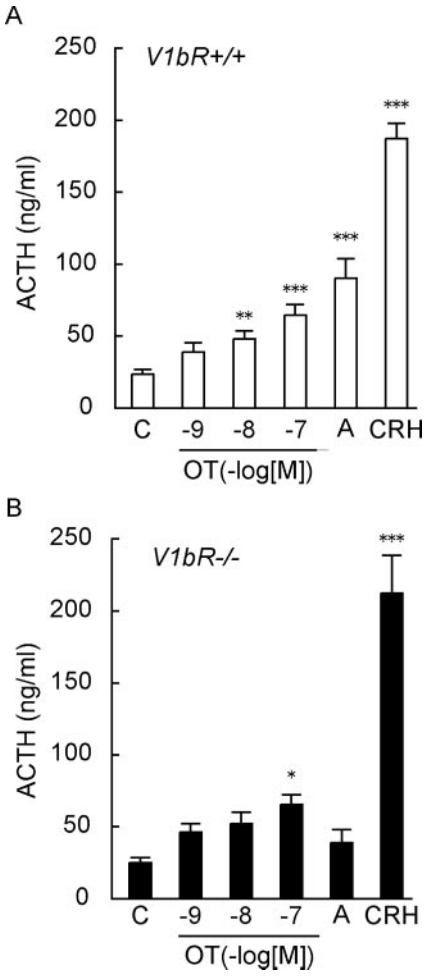

FIG. 1. OT-induced ACTH release from primary cultures of anterior pituitary cells from $V1bR^{+/+}$ (A) and $V1bR^{-/-}$ (B) mice. Values are mean ± SEM (n = 6 in $V1bR^{+/+}$ and $V1bR^{-/-}$ mice). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control for each genotype; *post hoc* comparison using Fisher's PLSD. A, AVP stimulation at 10^{-7} M; C, control (0 M OT); CRH, stimulation with 10^{-8} M CRH as a positive control; OT, OT stimulation at 10^{-9} M (−9), 10^{-8} M (−8), and 10^{-7} M (−7).

Involvement of the OT receptor in OT-induced ACTH release

It has been reported that OT can stimulate ACTH release via the V1b receptor in the rat anterior pituitary gland (21). However, our study with $V1bR^{-/-}$ mice showed that OT-induced ACTH release was observed in cells from $V1bR^{-/-}$ mice (Fig. 1B), suggesting that OT induces ACTH release via other receptors in $V1bR^{-/-}$ mice. Therefore, we examined the subtypes of AVP receptors involved in OT-induced ACTH release in mice with receptor-specific antagonists. Because we had found that OT and V1b receptors, but not V1a and V2 receptors, were expressed in the mouse pituitary gland

(24), we used two selective antagonists for this analysis: SSR149415 for the V1b receptor and CL-14-26 for the OT receptor. SSR149415 (10^{-6} M) and CL-14-26 (10^{-6} M) significantly inhibited OT-induced ACTH release in $V1bR^{+/+}$ mice (35.2 ± 2.6 ng/ml at 10^{-6} M SSR149415, n = 6; 37.1 ± 3.7 ng/ml at 10^{-6} M of CL-14-26, n = 6; $P = 0.01$ for 10^{-8} M OT *vs.* 10^{-6} M SSR149415 and $P = 0.02$ for 10^{-8} M OT *vs.* 10^{-6} M CL-14-26; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD) (Fig. 2A). The inhibition rates were about 53 and 45% of the stimulation value (10^{-8} M of OT) for the SSR149415 and CL-14-26 treatments, respectively. The ACTH response to OT stimulation at the concentration of 10^{-8} M was not completely inhibited by SSR149415 and CL-14-26 at concentrations higher than 10^{-6} M (data not shown).

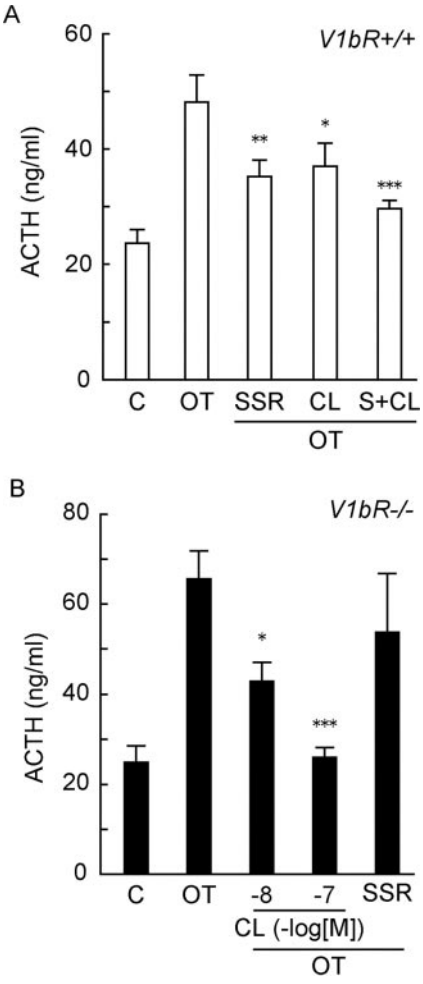

FIG. 2. Inhibitory effects on OT-induced ACTH release from pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice by antagonists for V1b and OT receptors. A, Effect of single treatment with SSR149415, or CL-14-26 or cotreatment with both SSR149415 and CL-14-26 on OT-induced ACTH release in $V1bR^{+/+}$ mice. Values are mean ± SEM (n = 6). C, Control (0 M OT); CL, 10^{-6} M CL-14-26; OT, OT stimulation at 10^{-8} M; S+CL, cotreatment with 10^{-6} M SSR149415 plus 10^{-6} M CL-14-26; SSR, 10^{-6} M SSR149415. B, Effect of single treatment with SSR149415 or CL-14-26 on OT-induced ACTH release in $V1bR^{-/-}$ mice. Values are mean ± SEM (n = 6). C, Control (0 M OT); CL, 10^{-8} or 10^{-7} M CL-14-26; OT, OT stimulation at 10^{-7} M; SSR, 10^{-6} M SSR149415. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* vehicle (OT stimulation without antagonist); *post hoc* comparison using Fisher's PLSD.

Thus, each antagonist did not reduce the OT-induced ACTH release to the control (0 M OT) level ($P = 0.01$ and $P = 0.002$ for the control *vs.* SSR149415- and CL-14-26-treated OT stimulation, respectively; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD). However, cotreatment with SSR149415 and CL-14-26 inhibited OT-induced ACTH release to the control level in $V1bR^{+/+}$ mice (29.7 ± 1.2 ng/ml for cotreatment with SSR149415 and CL-14-26, n = 6; $P = 0.23$ for 10^{-6} M SSR149415 *vs.* cotreatment, $P = 0.11$ for 10^{-6} M CL-14-26 *vs.* cotreatment, and $P = 0.14$ for the control *vs.* cotreatment; one-way ANOVA, followed by *post hoc* comparison using Fisher's PLSD) (Fig. 2A). These results suggest that both the OT receptor and V1b receptor are involved in OT-induced ACTH release in $V1bR^{+/+}$ mice. Next, we examined the effects of SSR149415 and CL-14-26 on OT-induced ACTH release in $V1bR^{-/-}$ mice. CL-14-26 at 10^{-8} M significantly inhibited OT-induced ACTH release, and at 10^{-7} M it almost completely inhibited ACTH release (42.8 ± 4.0 ng/ml at 10^{-8} M CL-14-26, n = 6; 26.0 ± 1.2 ng/ml at 10^{-7} M CL-14-26, n = 6; $P = 0.02$ for 10^{-7} M OT *vs.* 10^{-8} M CL-14-26, $P = 0.0003$ for 10^{-7} M OT *vs.* 10^{-7} M CL-14-26, $P = 0.06$ for the control *vs.* 10^{-8} M CL-14-26, and $P = 0.91$ for the control *vs.* 10^{-7} M CL-14-26; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD) (Fig. 2B). The V1b receptor-selective antagonist SSR149415 did not significantly inhibit OT-induced ACTH release at the concentration of 10^{-6} M in $V1bR^{-/-}$ mice (53.8 ± 12.8 ng/ml at 10^{-6} M SSR149415, n = 6, $P = 0.22$ for 10^{-7} M OT *vs.* 10^{-6} M SSR149415; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD). This indicates that OT stimulated ACTH release via the OT receptor in $V1bR^{-/-}$ mice.

We next examined the ACTH response to costimulation with OT and AVP in cells from $V1bR^{+/+}$ and $V1bR^{-/-}$ mice because AVP stimulates ACTH release via the V1b receptor, and OT stimulates via the OT and V1b receptors. OT at the concentration of 10^{-7} M induced an increase in ACTH release in $V1bR^{+/+}$ mice, and costimulation with AVP (10^{-7} M) plus OT (10^{-7} M) induced a greater increase in ACTH release compared with only OT stimulation (87.4 ± 5.7 ng/ml for costimulation with AVP plus OT, n = 6; $P = 0.003$ for 10^{-7} M OT *vs.* costimulation with AVP plus OT; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD) (Fig. 3). In $V1bR^{-/-}$ mice, OT at 10^{-7} M significantly stimulated ACTH release, but there was no significant increase in ACTH release when the cells were costimulated with AVP (10^{-7} M) plus OT (10^{-7} M) compared with OT stimulation alone (69.8 ± 5.0 ng/ml for costimulation with AVP plus OT, n = 6; $P = 0.55$ for 10^{-7} M OT *vs.* costimulation with AVP plus OT; one-way ANOVA followed by *post hoc* comparison using Fisher's PLSD). When $V1bR^{+/+}$ and $V1bR^{-/-}$ mice were compared, the response to costimulation with OT and AVP in $V1bR^{-/-}$ mice was weaker than that in $V1bR^{+/+}$ mice ($P = 0.04$ for costimulation of AVP and OT in $V1bR^{+/+}$ *vs.* $V1bR^{-/-}$; unpaired Student's *t* test) (Fig. 3).

Expression levels of OT receptor mRNA in $V1bR^{-/-}$ mice

Because the levels of OT-induced ACTH release were similar between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, we investigated the expression levels of OT receptor mRNA in $V1bR^{-/-}$ mice by

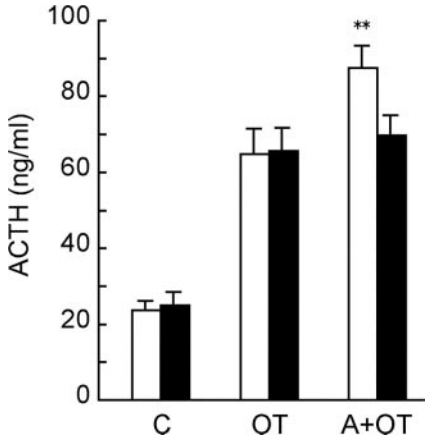

FIG. 3. ACTH release induced by single stimulation with OT or co-stimulation with OT plus AVP in pituitary cells from $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. *Open and closed bars* show $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, respectively. Values are mean ± SEM (n = 6 in $V1bR^{+/+}$ mice and n = 6 in $V1bR^{-/-}$ mice). **, $P < 0.01$ *vs.* OT in $V1bR^{+/+}$ mice; *post hoc* comparison using Fisher's PLSD. A+OT, Costimulation with 10^{-7} M AVP and 10^{-7} M OT; C, control (0 M OT); OT, OT stimulation at 10^{-7} M.

RT-PCR and the quantitative real-time PCR method. Conventional RT-PCR showed that the V1b and OT receptors were both detected in the anterior pituitary gland from $V1bR^{+/+}$ mice, as reported previously (24), and that the expression level of the OT receptor was lower than that of the V1b receptor in $V1bR^{+/+}$ mice (Fig. 4A). GAPDH was used as an internal standard for the PCR. Expression of the V1b receptor was not detected in $V1bR^{-/-}$ mice, but the OT receptor was expressed in $V1bR^{-/-}$ mice. The signal of the OT receptor was stronger in $V1bR^{-/-}$ mice than $V1bR^{+/+}$ mice, whereas GAPDH signals were similar for the two genotypes, indicating that the expression of the OT receptor was increased in $V1bR^{-/-}$ mice. Next, we performed a real-time PCR assay to assess the expression levels of the OT receptor quantitatively. Results of the real-time PCR experiment indicated that the expression level of the OT receptor mRNA in $V1bR^{-/-}$ mice was approximately 2.6-fold higher than that in $V1bR^{+/+}$ mice ($P = 0.01$ by unpaired Student's *t* test) (Fig. 4B).

To confirm the RT-PCR results, we performed *in situ* hybridization analysis using DIG-labeled cRNA probes from the 3′ noncoding region of OT receptor cDNA. A positive signal was detected in both $V1bR^{+/+}$ and $V1bR^{-/-}$ mice with an antisense probe for the OT receptor (Fig. 5, A–D). The signals in the pituitary gland from $V1bR^{-/-}$ mice tended to be higher than those in the pituitary gland from $V1bR^{+/+}$ mice. No positive signals were observed in the study with *in situ* hybridization using the sense probe (Fig. 5, E and F), implicating that signals detected with the antisense probe in the *in situ* hybridization analysis were specific to the OT receptor mRNA in this study. These results indicate that the OT receptor is expressed in the anterior pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, and could be up-regulated under the V1b receptor-deficient condition. In addition, our combined study with *in situ* hybridization for the OT receptor and immunohistochemical analysis for ACTH showed that some ACTH-positive cells were present among OT receptor-expressing cells in the mouse anterior pituitary gland (Fig. 5, G and H), implicating that the mouse corticotrophs expressed the OT receptor.

Furthermore, we analyze the expression levels of V1b and OT receptors in the pituitaries of AVP-deficient Brattleboro rats to investigate whether AVP receptor expression was different under the AVP-deficient condition. RT-PCR analysis of RNA from pituitaries of Brattleboro rats showed that the expression levels of the V1b and OT receptors were increased in the pituitary of Brattleboro rats compared with those in control LE rats. These results suggest that the receptors are up-regulated to compensate for AVP signaling under the AVP-deficient condition (Fig. 4C).

Discussion

Various hormones, such as CRH and AVP, induce ACTH secretion from the anterior pituitary glands (25). CRH is a well-known and potent factor involved in ACTH secretion (26). Inhibition of the CRH/CRH receptor type 1 signal resulted in decreased ACTH secretion under stress conditions

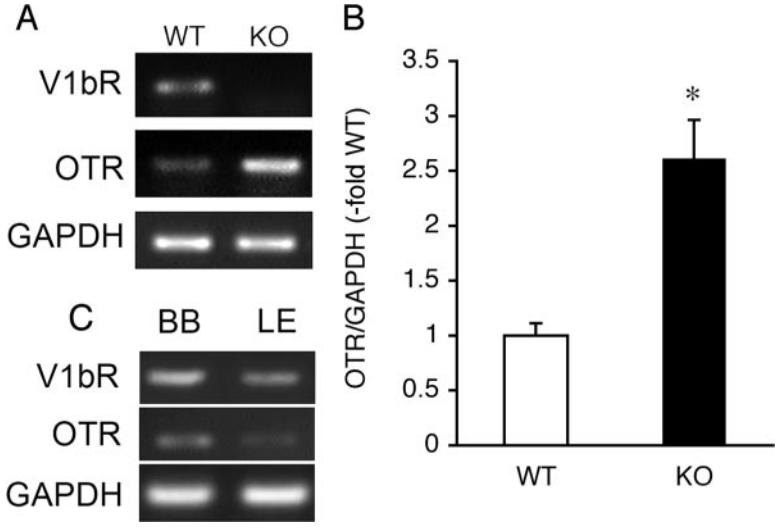

FIG. 4. Expression level of OT receptor (OTR) mRNA by RT-PCR in anterior pituitary glands of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. A, RNA was isolated from anterior pituitary glands of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, and expression levels of V1b and OT receptor mRNAs were analyzed by RT-PCR. B, Quantitative analysis of OT receptor mRNA levels by real-time PCR. Expression levels of OT receptor and GAPDH were measured and were expressed as the ratio of OT receptor to GAPDH. Values are mean ± SEM (n = 3 in each genotype). *, $P < 0.05$ *vs.* $V1bR^{+/+}$ mice; unpaired Student's *t* test. C, RNA was isolated from pituitary glands of Brattleboro (BB) and control LE rats, and mRNA expression of V1b and OT receptors was analyzed by RT-PCR. KO, $V1bR^{-/-}$ mice; LE, control LE rats; V1bR, V1b receptor; WT, $V1bR^{+/+}$ mice.

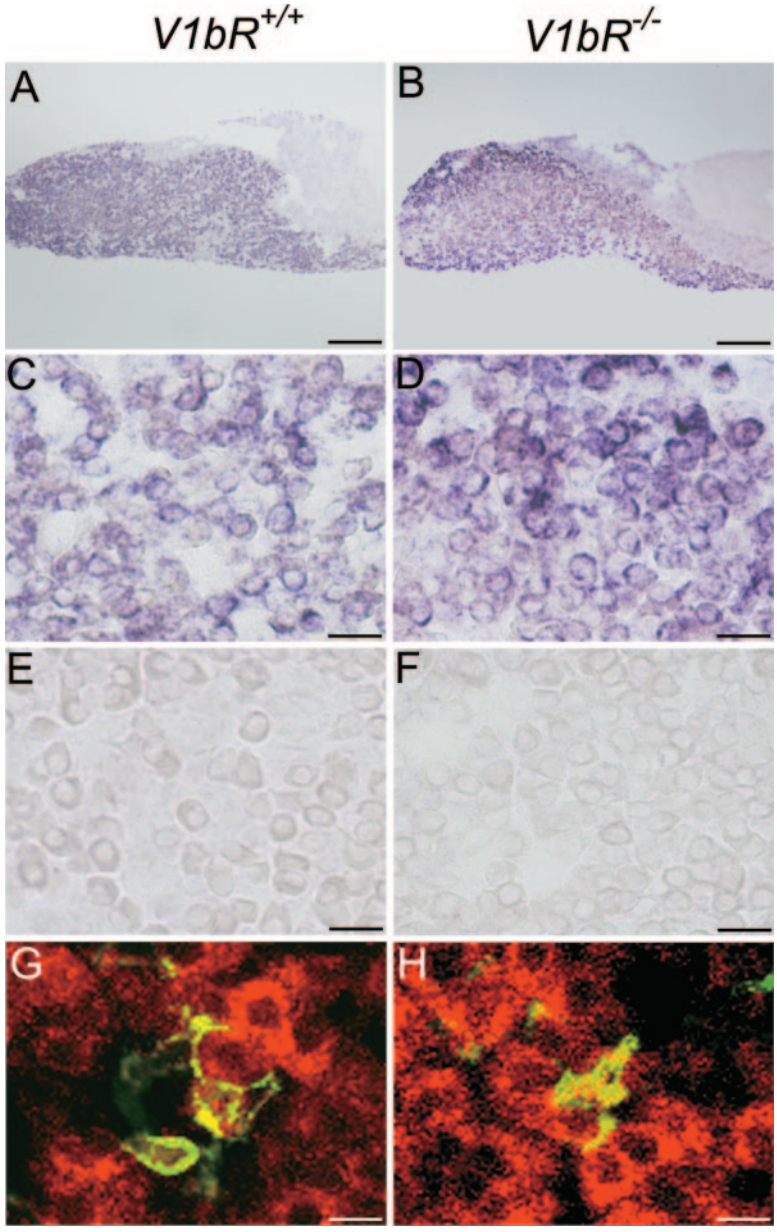

FIG. 5. OT receptor mRNA expression in the anterior pituitary gland of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. A and B, *In situ* hybridization using the antisense probe for OT receptor mRNA in the anterior pituitary gland of $V1bR^{+/+}$ (A) and $V1bR^{-/-}$ (B) mice. C and D, High-magnification image of *in situ* hybridization of $V1bR^{+/+}$ (C) and $V1bR^{-/-}$ (D) mice. E and F, *In situ* hybridization using the sense probe for OT receptor mRNA in anterior pituitary gland of $V1bR^{+/+}$ (E) and $V1bR^{-/-}$ (F) mice. G and H, Double-staining analysis for OT receptor mRNA and ACTH protein in the anterior pituitary gland of $V1bR^{+/+}$ (G) and $V1bR^{-/-}$ (H) mice. Positive cells for OT receptor mRNA are shown in *red*, and positive cells for ACTH protein are shown in *green*. *Scale bar*, A and B, 200 μm; C–F, 20 μm; and G and H, 10 μm.

without altering the basal ACTH level (27). In contrast, inhibition of the AVP/V1b receptor signal resulted in decreased ACTH levels under both stress and basal conditions (14), suggesting that the AVP/V1b receptor pathway is involved in regulating the HPA axis under both conditions, whereas CRH/CRH receptor type 1 plays a crucial role only under stress conditions. In addition, OT can induce ACTH release from cultured anterior pituitary cells, although the effects of a deficiency in the OT/OT receptor signal on the plasma concentration of ACTH have not been clarified. We observed that OT at the concentration of 10^{-9} M stimulated ACTH release in mouse anterior pituitary cells. When OT is released into the hypophyseal portal vessels from nerve terminals, the OT concentration in these portal vessels is approximately 2–12 ng/ml (6, 28). Therefore, it is possible that OT can induce ACTH release from the anterior pituitary gland of $V1bR^{-/-}$ mice as well as $V1bR^{+/+}$ mice under physiological conditions. In the present study, OT potently stimulated ACTH release from anterior pituitary cells, but the potency of OT was weaker than that of AVP or CRH. OT at 10^{-8} and 10^{-7} M induced 2.0- and 2.7-fold increases in ACTH release, respectively, whereas AVP at 10^{-7} M and CRH at 10^{-8} M induced 3.8- and 7.9-fold increases over baseline, respectively. In addition, our present and previous studies showed that AVP stimulation at 10^{-7} M induced an increase of approximately 2.7-fold in ACTH release, whereas CRH at 10^{-8} M induced an increase of 4-fold over baseline (14). Although the experimental conditions, such as the cell numbers used in the ACTH assay, differ between the present and previous studies (14), our studies suggest that the rank order of potency for ACTH release from mouse cultured pituitary cells is CRH > AVP > OT at the same concentration of each ligand. Similar findings were obtained in rat anterior pituitary cells (29).

Because OT could induce ACTH release from anterior pituitary cells of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, we investigated which receptor was involved in OT-induced ACTH release in experiments using antagonists. We used OT at the concentration of 10^{-8} M in the inhibition test with antagonists in $V1bR^{+/+}$ mice (Fig. 2A), and at 10^{-7} M in the inhibition test in $V1bR^{-/-}$ mice (Fig. 2B), as well as in an experiment to determine ACTH release induced by a single stimulation with OT or costimulation with OT plus AVP in pituitary cells from $V1bR^{+/+}$ and $V1bR^{-/-}$ mice (Fig. 3). We initially used OT at 10^{-7} M in the inhibition test in $V1bR^{+/+}$ mice, and found that the partial inhibition of OT-induced ACTH by SSR149415 and CL-14-26 was similar at this concentration to the inhibition obtained with 10^{-8} M OT; however, the cotreatment with SSR149415 (10^{-6} M) plus CL-14-26 (10^{-6} M) did not completely inhibit the OT-induced ACTH response at this OT concentration (data not shown). Because the response was not inhibited by greater concentrations of antagonists than 10^{-6} M, receptors other than the OT and V1b receptors could be involved in OT-induced ACTH release, or that the OT concentration of 10^{-7} M was extremely high for the antagonists could not completely inhibit the OT-induced ACTH release. Because V1a and V2 receptors are not expressed at the anterior pituitary gland, and the response to 10^{-8} M OT was completely inhibited by both antagonists, we considered that the 10^{-7} M concentration of OT was too high for the inhibition test in $V1bR^{+/+}$ mice and that the 10^{-8} M concentration seemed more appropriate for the experiment. Therefore, 10^{-8} M OT was used in the experiment, which indicated that OT and V1b receptors, but not V1a and V2 receptors, mediated OT-induced ACTH release in the mouse anterior pituitary gland. In addition, costimulation with AVP plus OT induced a further increase in ACTH release from anterior pituitary cells of $V1bR^{+/+}$ mice compared with OT stimulation alone. On the other hand, although the ACTH response induced by OT in anterior pituitary cells was similar in $V1bR^{-/-}$ and $V1bR^{+/+}$ mice, no further increase was observed in $V1bR^{-/-}$ mice on costimulation with AVP plus OT. These results indicate that, in anterior pituitary cells from $V1bR^{+/+}$ mice, V1b receptors were present in sufficient quantity to bind the additional AVP, whereas in cells from $V1bR^{-/-}$ mice, OT receptors were unable to bind the additional AVP. In general, our results suggest that OT stimulates ACTH release via the OT receptor in addition to the V1b receptor under normal conditions and via the OT receptor under V1b receptor-deficient conditions in mice.

Because OT and V1b receptors belong to the same receptor family, AVP and OT have a modest affinity for OT and V1b receptors, respectively, and can bind to each other (10, 30). In the present study, OT induced ACTH release via the OT and V1b receptors, indicating that OT cross-reacted with the V1b receptor in the OT-induced ACTH response. Similar cross-reactivity with respect to AVP and OT in some peripheral tissues has been observed among AVP receptors. For example, AVP contributes to myometrium contraction through the OT receptor in the uterus, where the V1a and OT receptors are expressed (31–33). In addition, AVP and OT can induce glucagon secretion via both the V1b and OT receptor in the mouse pancreas (23). However, AVP induces ACTH release not via the OT receptor but via the V1b receptor (14, 15), whereas OT induces via both the OT and V1b receptors in the anterior pituitary gland. In the case of anterior pituitary cells, AVP cannot markedly stimulate prolactin release from lactotrophs (34), in which OT receptor transcripts are expressed (19). These findings indicate that AVP can bind to the V1b receptor and exert a physiological effect in the anterior pituitary gland, whereas OT exerts such an effect via both the OT and V1b receptors. Together, our findings suggest that the OT receptor could compensate for OT but not of AVP in the anterior pituitary gland in mice, and that the physiological function of the OT receptor is more evident, especially when that of the AVP receptor is abolished.

Apart from mice, the cross-reactivity of OT with the V1b receptor is observed in humans as well (9, 11). However, there is a species difference in the affinity of OT for the V1b receptor; the dissociation constant of OT for the V1b receptor in mice and humans are 103 ± 27 nM and 910 ± 390 nM (11), respectively. This difference indicates that the binding affinity of OT to the V1b receptor is lower in humans than mice according to the dissociation constant of an inhibitor values and that the V1b receptor-mediated ACTH release by OT could be a minor pathway in humans. Therefore, it is still controversial whether the V1b receptor plays an important role in OT-induced ACTH release in humans. Our results reveal that the OT receptor is involved in OT-induced ACTH release. Thus, it is likely that, in humans, the OT receptor rather than the V1b receptor is important for and contributes to OT-induced ACTH release.

Finally, we found that V1b and OT receptors were expressed in the mouse pituitary gland and that the V1b receptor was more predominantly expressed than the OT receptor, as reported previously (24). This was supported by the findings that the expression of the OT receptor was increased in $V1bR^{-/-}$ mice. Although the increased mRNA level of OT receptor did not necessarily correlate with the increased receptor level, the up-regulated OT receptor could lead to increased binding of OT to the receptors and consequent compensation of the ACTH response via the V1b receptor in mutant mice. Similar phenomena have been observed in other receptor-deficient mice. For instance, the α_{1D}-adrenergic receptor was up-regulated in the vas deferens in mice deficient in the α_{1A}-adrenergic receptor (35). Dopamine D1 and N-methyl-d-aspartic acid receptors were also up-regulated in the striatum and hippocampus of dopamine D4 receptor knockout mice (36). In addition, the OT receptor was able to compensate for loss of the V1b receptor in AVP-induced glucagon release from pancreatic islet cells of $V1bR^{-/-}$ mice (23). Thus, another subtype in the receptor family, or receptors in another family, were up-regulated and compensated for the function of the receptor when one receptor was deficient (23, 35, 36). Furthermore, we found that the expression levels of V1b and OT receptors were increased in the Brattleboro rat pituitary gland, suggesting that AVP receptors could be up-regulated under the AVP-deficient condition. Therefore, it is possible that receptor up-regulation may be evoked by a reduction in ligand signals.

In this study we found that the OT receptor was expressed in the mouse anterior pituitary cells by *in situ* hybridization analysis, and the expressions in $V1bR^{-/-}$ mice tended to be higher than those in $V1bR^{+/+}$ mice. These results suggest that

the OT receptor expression was increased in $V1bR^{-/-}$ mice, which is in agreement with the findings in the RT-PCR study. Furthermore, in the combined study with *in situ* hybridization for the OT receptor mRNA and immunohistochemistry for ACTH, we found that there were ACTH-positive cells, which are regarded as corticotrophs, in the OT receptor-positive cells. These findings implicate that corticotrophs also express the OT receptor gene in the mouse pituitary gland, whereas the OT receptor has been predominantly expressed in lactotrophs in the rat pituitary gland (19). Thus, the OT receptor could be involved in mediating the ACTH release from the mouse anterior pituitary cells, including corticotrophs, and be increased under the V1b receptor- or AVP-deficient condition to compensate the signals via the V1b receptor.

In summary, we examined the underlying mechanism of ACTH secretion when stimulated with AVP and/or OT, and found that OT induced ACTH release via V1b and OT receptors, whereas AVP induced only via V1b receptors. AVP plays a crucial role in regulating the HPA axis, but the physiological roles of OT in the regulation of the HPA axis remain to be clarified. Antagonists of V1b or OT receptors, as well as genetically modified mice, such as mice deficient in OT or V1b receptors, are useful in investigating the functions of these receptors in the HPA axis. Our findings in the present study suggest that long-term treatment with V1b receptor antagonists could affect signals via the OT receptor in the anterior pituitary gland, and that inhibition of V1b receptors or both V1b and OT receptors could affect ACTH release more profoundly than inhibition of OT receptors under basal and stress conditions, and could influence the HPA axis.

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