Supporting Information

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SI Materials and Methods

Materials. DMEM, FBS, penicillin, streptomycin, glutamine, fungizone, G418, and PBS were from Wisent. Cell culture plates and dishes were all purchased from BD Biosciences. Platelet-activating factor was purchased from Cayman. Arginine-8 vasopressin (AVP) and 1,10-phenanthroline were from Sigma Chemical, whereas EGF, insulin-like growth factor (IGF), and cholera toxin (CTX) were from Calbiochem. ECL was obtained from PerkinElmer. All of the inhibitors for protein kinases were from Calbiochem, whereas marimastat was from Tocris Bioscience. Antibodies recognizing ERK1/2 (rabbit polyclonal K-23) and their phosphorylated forms (P-ERK; mouse monoclonal E-4) as well as anti-myc 9E10, anti-HA 12CA5, anti–IGF1-Rα (1H7), anti–IGF1-Rβ (C-20), anti-EGF receptor (EGFR; 528), anti-EGFR (1005), and anti–β-arrestin2 (H9) IgGs were all from Santa Cruz Biotechnology, whereas antibodies recognizing the IGF receptor phosphorylated at tyrosine 1131, c-Src phosphorylated at tyrosine 416 (2101), and β-arrestin-1/2 (D24H9) were purchased from Cell Signaling Technology. Anti-Src (GD11) was purchased from Upstate Biotechnology. Anti-mouse and anti-rabbit HRP-conjugated IgG were from GE Healthcare. The Cy3 donkey anti-rabbit antibody was from Jackson ImmunoResearch.

Expression Vectors. The plasmids encoding rat ERK2-GFP (1) and β-arrestin-1 deleted in its C tail from amino acid 319 to amino acid 418 (β-arr Δ318–419) were gifts from K. DeFea (University of California, Riverside, CA) and Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA), respectively. Plasmid encoding HA-tagged IGFR dominant negative mutant (IGFR1-972) (2) was provided by Doug Yee (University of Minnesota, Minneapolis, MN), whereas plasmid encoding the V2 vasopressin receptor (V2R; Ala6) mutant was provided by the laboratory of Marc Caron (Duke University, Durham, NC).

Cell Culture and Transfections. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.1 UI/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL fungizone. Stable cell lines were generated by transfection of the appropriate plasmids using the calcium phosphate precipitation method, and neomycin-resistant cells were selected in the presence of G418 (450 μg/mL). Resistant clones were screened for V2R expression by radioligand binding. Platelet-activating factor receptor-stable cell line was provided by Stéphane Laporte (McGill University, Montréal, Québec). Transient transfections were performed using the FuGENE 6 Transfection Reagent (Roche Diagnostics) according to the manufacturer's protocol, and cells were harvested 48 h after transfection. The previously described siRNAs for β-arrestin-1 and -2 (3) were purchased from QIAGEN and transfected at a final concentration of 100 pM (50 pM β-arrestin-1 siRNA and 50 pM β-arrestin-2 siRNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitation. HEK293 cells endogenously expressing the IGFR and EGFR and transfected with either forms of myc-tagged β-arrestin were treated or not with 100 ng/mL IGF1, 10 ng/mL EGF, or 1 μM AVP and harvested in lysis buffer containing 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and protease inhibitor mixtures. Lysates were then subjected to immunoprecipitation O/N at 4 °C using a monoclonal antibody specific to the α-subunit of IGFR, an antibody specific to the N terminus of EGFR, or an anti-myc antibody (Santa Cruz), and proteins were resolved by SDS/PAGE. Western blot analysis was performed using anti-myc or anti–IGF-1Rβ antibodies (Santa Cruz). The phosphorylated IGFR was detected by a phosphospecific antibody directed against the phosphorylated tyrosine 1131 after its immunoprecipitation with the monoclonal anti–IGF-1Rα antibody. The nitrocellulose membranes were stripped and probed again using a polyclonal antibody specific to the β-subunit of the IGFR (Santa Cruz) to confirm equal expression of the IGFR in each sample. The expression of myc-β-arrestins was detected by probing the immunoblot with an anti-myc antibody. All immunoblots were visualized by ECL.

Animals. Animals used were adults (2 mo) male Sprague–Dawley rats (Janvier). They were housed in light- (12-h dark and 12-h light cycles) and temperature- (21 °C) controlled rooms and had free access to standard dry food and tap water. All animals were treated in accordance with the French Ethical Committee principles under the supervision of an authorized investigator (G.G.).

Animal Treatment. Rats (200 g) were implanted with an Alzet 7d-osmotic pump filled with 100 mL PBS alone or containing desmopressin (dDAVP) infusing from 0.013 to 1.3 μg peptide per rat per day for 3 d. Because marimastat was poorly water-soluble, it was dissolved in saline containing DMSO 5% (vol/vol) and Cremophor 7.5% (vol/vol) and was administered by two i.p. injections per day (3.6 mg/rat per day) for 3 d. The tyrphostin AG1024 in saline was injected intraperitoneally two times per day (0.30 mg/d) for 3 d. Immunohistological preparation of the tissues was performed as previously described (4). Briefly, after a deep anesthesia induced by Equithsein, animals were perfused through the ascending aorta with PBS (pH 7.4) followed by a solution of 4% paraformaldehyde (vol/vol) in 0.1 M phosphate buffer (pH 7). The kidneys were dissected and fixed by immersion overnight in the same fixative.

Imaging and Quantification of Animal Tissue Sections. Phospho-ERK immunofluorescence images were obtained in kidney slices using an ImagerZ1 epifluorescence microscope. On the same slice, series of images corresponding to the papilla, medulla, and cortex were acquired successively, and the full kidney sections were obtained after reconstitution with the MosaiX software. For quantification, labeled sections were rinsed in PBS, mounted in Mowiol, and observed using a Zeiss LSM510 Meta confocal microscope. The fluorescence was detected by exciting the preparation with the helium/neon laser at 543 nm and collecting the emission with an LP 560-nm filter. The immunofluorescence was quantified using the ImageJ software, and positive cells were detected on each section (450 × 450-μm field, Airy 1, optical slice thickness = 800 nm) in three regions of the kidney (papilla, medulla, and cortex). Results are expressed as the mean ± SEM of the number of p-ERK–positive cells obtained from three different animals for which three distinct slices were analyzed.

Data Analysis. Immunoreactivity was determined by densitometric analysis of the films using Quantity One Software. Statistical analysis and curve fitting were done using Prism 3.01 (GraphPad Software). Statistical significance of the differences was assessed using one-way ANOVA and posthoc Bonferroni test.

1. DeFea KA, et al. (2000) Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148: 1267–1281.
2. Sachdev D, Hartell JS, Lee AV, Zhang X, Yee D (2004) A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells. *J Biol Chem* 279:5017–5024.
3. Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ (2003) Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci USA* 100:1740–1744.
4. Alonso G, et al. (2009) Sustained elevated levels of circulating vasopressin selectively stimulate the proliferation of kidney tubular cells via the activation of V2 receptors. *Endocrinology* 150:239–250.

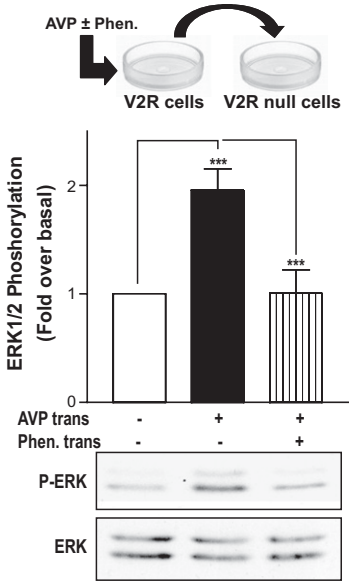

Fig. S1. Serum-starved V2R null cells were incubated for 5 min at 37 °C with the transferred supernatant from V2R-expressing cells previously incubated or not for 30 min with 500 μM 1,10-phenanthroline (Phen) and stimulated or not with AVP. MAPK activity was detected by Western blot analysis using a phosphospecific anti-ERK1/2 antibody (P-ERK). Expression levels of the MAPK were controlled using antibodies directed against the total kinase population (ERK). Data presented in the bar graph are expressed as fold increase of P-ERK/ERK ratio compared with basal conditions. Addition of the supernatant from AVP-treated, V2R-expressing cells to V2R-null cells increased ERK1/2 activity significantly above the level observed in cells treated with supernatant from non-stimulated V2R-expressing cells. The release of a transactivating factor in the extracellular medium is prevented by the metalloproteinase inhibitor phenanthroline, confirming the role of metalloproteinases. Data represent the mean ± SEM of at least four independent experiments. ***$P < 0.001$.

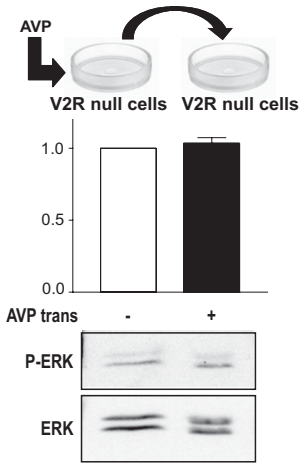

Fig. S2. Serum-starved V2R-null cells were incubated for 5 min at 37 °C with the transferred supernatant from V2R-null cells previously stimulated or not with 1 μM AVP for 5 min. ERK1/2 phosphorylation was detected as described in Fig. 1*A*, and data are expressed as fold increase of P-ERK/ERK ratio compared with basal conditions. No activation of ERK1/2 was observed whether the supernatant of V2R-null cells was stimulated or not by AVP before the transfer.

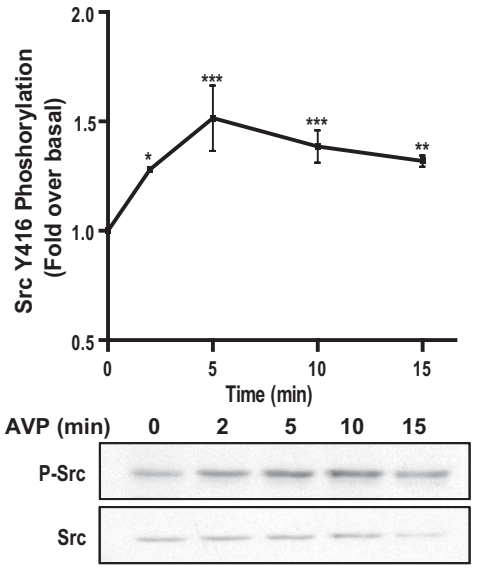

Fig. S3. Serum-starved HEK293 cells stably expressing V2R were stimulated at 37 °C with 1 μM AVP for the indicated times, and Src phosphorylation at Y416 was detected and quantified using phosphospecific anti-Src (P-Src) and anti-Src GD11 (Src) antibodies. AVP promoted a time-dependent increase in Src phosphorylation that peaked at 5 min. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

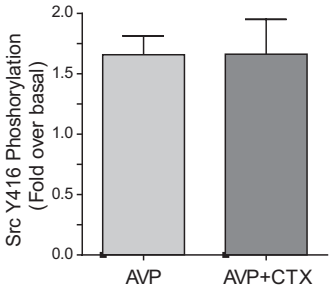

Fig. S4. Serum-starved HEK293 cells stably expressing V2R were pretreated or not with 300 ng/mL CTX for 16 h at 37 °C before 1 μM AVP stimulation for 5 min. c-Src phosphorylation at Y416 was detected and quantified using phosphospecific anti-Src (P-Src) and anti-Src GD11 (Src) antibodies. Data are expressed as fold increase of P-Src/Src ratio compared with basal conditions. Prolonged CTX treatment did not affect the AVP-promoted increase in Src Y416 phosphorylation, indicating that V2R-mediated Src activation is Gs-independent. Data shown represent the mean ± SEM of three independent experiments.

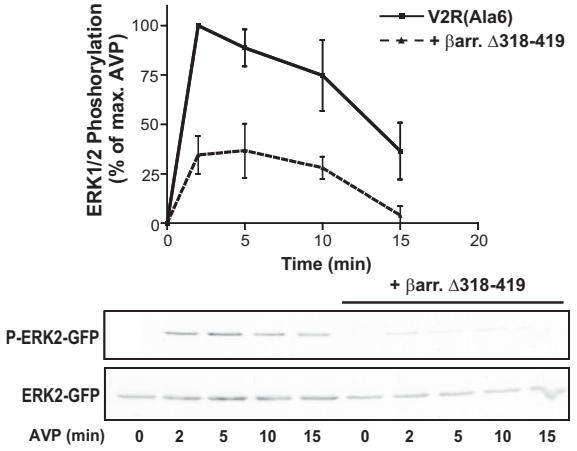

Fig. S5. HEK293 cells stably expressing V2R(Ala6) were cotransfected with ERK2-GFP and either β-arrestin-1 Δ318-419 (dotted line) or pcDNA3.1 (solid line) and serum-starved before stimulation at 37 °C with 1 μM AVP for the indicated times. MAPK activity was detected by Western blot analysis using phospho-specific anti-ERK1/2 antibody (P-ERK2–GFP). Expression levels of the MAPK were controlled using anti-ERK1/2 antibody directed against the total kinase population (ERK2-GFP). Data are expressed as a percentage of P-ERK–GFP/ERK-GFP of the level observed in AVP-stimulated conditions. The dominant negative β-arrestin Δ318–419 inhibited the V2R(Ala6)-stimulated MAPK activity.

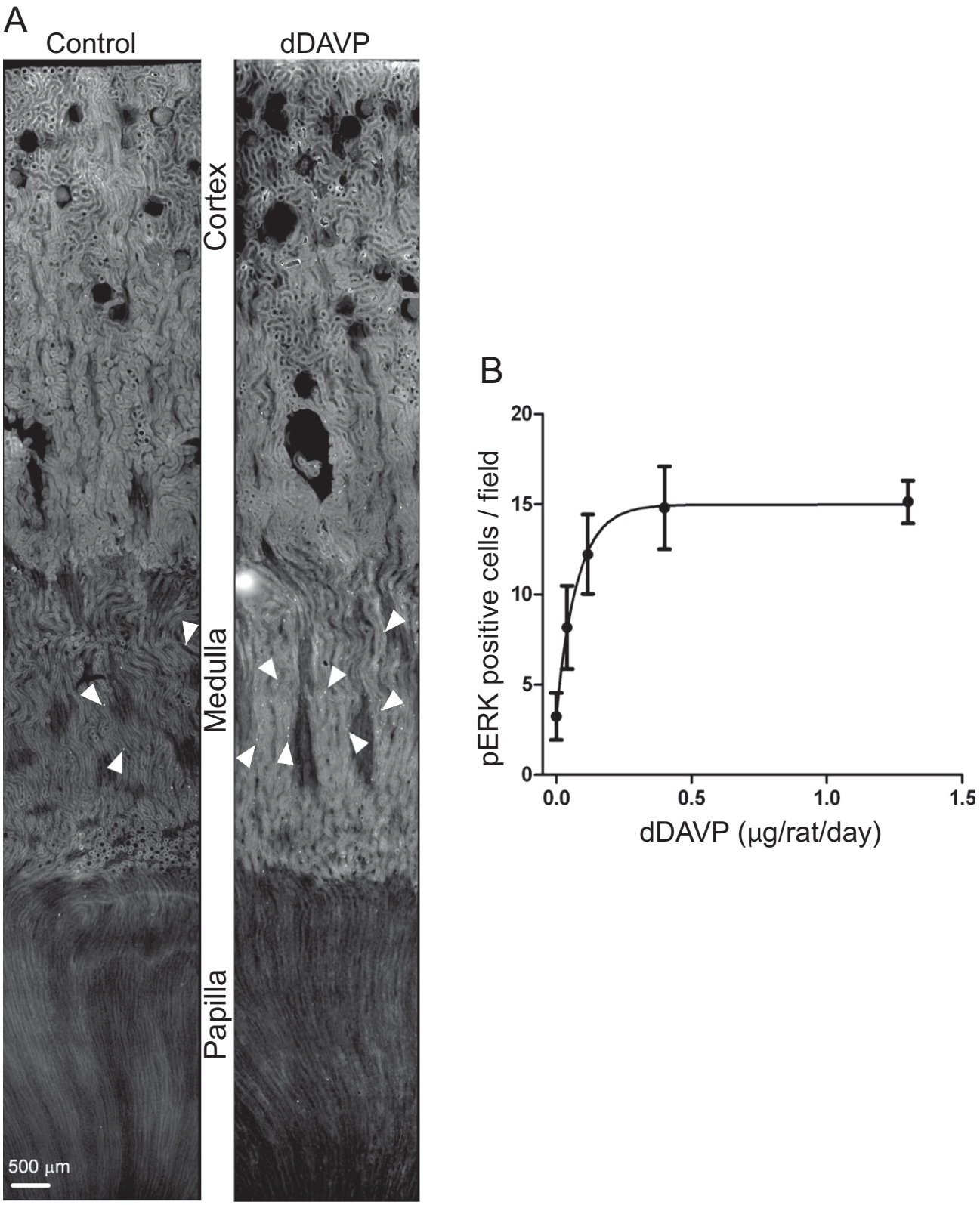

Fig. S6. Phospho-ERK1/2 activity was assessed in kidney slices from rat treated or not with dDAVP. (*A*) The full kidney section was constructed by imaging successively the different regions (papilla, medulla, and cortex) of the kidney slice after acquisition of individual epifluorescence images (air objective = 10×) and reconstitution. The slices were obtained from rats treated for 3 d with vehicle (*Left*) or 1.3 μg/rat per day dDAVP (*Right*). White arrowheads point to examples of phospho-ERK1/2–positive cells. (*B*) Quantification of the number of pERK1/2-positive cells detected in a 450-μm-side square field of the kidney medulla as a function of increasing amount of dDAVP administered for 3 d. Three animals per dose were used for each experiment, and the data shown represent the mean ± SEM of three independent experiments. dDAVP induced a dose-dependent increase in ERK1/2 activity that reached a maximum at 0.45 μg/rat per day.