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**Sustained elevated levels of circulating vasopressin selectively stimulate the proliferation of kidney tubular cells via the activation of V<sub>2</sub> receptors.**

**Running title: Vasopressin stimulates kidney cell proliferation**

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**Abstract**

The hypothalamic hormone vasopressin (AVP) has known mitogenic effects on various cell types. This study was designed to determine whether sustained elevated levels of circulating AVP could influence cell proliferation within adult tissues known to express different AVP receptors, including the pituitary, adrenal gland, liver and kidney. Plasmatic AVP was chronically increased by submitting animals to prolonged hyperosmotic stimulation or by implanting them with a AVP-containing osmotic minipump. After several days of either treatment, increased cell proliferation was only detected within the kidney. This kidney cell proliferation was not affected by the administration of selective  $V_{1a}$  or  $V_{1b}$  receptors antagonists, but was either inhibited or mimicked by the administration of a selective  $V_2$  receptor antagonist or agonist, respectively. Kidney proliferative cells mostly concerned a subpopulation of differentiated tubular cells known to express the  $V_2$  receptors and were associated with the phosphorylation of extracellular signal-regulated kinase. These data indicate that in the adult rat, sustained elevated levels of circulating AVP stimulates the proliferation of a sub-population of kidney tubular cells expressing the  $V_2$  receptor, providing the first illustration of a mitogenic effect of AVP via the activation of the  $V_2$  receptor subtype.

## Introduction

Vasopressin (AVP) is a nonapeptide, synthesized by hypothalamic neurons and released into the blood by axons terminating in the median eminence and neurohypophysis. Secreted AVP exerts its biological effects through its binding to three subtypes of AVP receptors, the  $V_{1a}$ ,  $V_{1b}$  and  $V_2$ , all members of the G protein-coupled superfamily of receptors. AVP is mainly known as an antidiuretic and vasoconstrictor hormone via its effects on renal  $V_2$  receptor ( $V_2$ -R) (1) and  $V_{1a}$  receptor ( $V_{1a}$ -R) of arteriolar vascular smooth muscle cells (2). AVP also stimulates hypophyseal corticotroph cell secretion and regulates stress responses and anxiety via  $V_{1b}$  receptors ( $V_{1b}$ -R) (3, 4). Besides its classical hormonal effects, evidence has been provided indicating that AVP is also a potent proliferating factor for a variety of cell types expressing, in particular the  $V_{1a}$ -R, including fibroblasts (5), intestinal epithelial cells (6), adrenal gland glomerulosa cells (7), hepatocytes (8), and kidney mesangial cells (9).

The secretion of hypothalamic AVP is finely regulated by peripheral and central receptors that are sensitive to modifications of the volume and/or osmolality of extracellular fluids. Therefore, with the exception of transient rises following volumic or osmotic modifications (10), the levels of circulating AVP remain very low in adult mammals under basal physiological conditions (less than 4 pg/ml). However, it is known that levels of circulating AVP can be chronically increased under pathological conditions (more than 8 pg/ml), such as the syndrome of inappropriate antidiuresis (SIADH; (11, 12), renal insufficiency (13-15), uncontrolled diabetes mellitus (16, 17) and major depression (18, 19). To date, the data supporting a mitogenic role of AVP mostly derived from *in vitro* studies (5), (6). In the present study we investigate whether *in vivo*, prolonged stimulation of AVP receptors by sustained elevated levels of circulating AVP may influence cell proliferation within adult organs expressing these receptors. For this, high levels of circulating AVP were maintained over several days by submitting adult rats to prolonged hyperosmotic stimulation or by

implanting them with a AVP-containing Alzet osmotic minipump. The rate of cell proliferation was examined within the pituitary expressing  $V_{1b}$ -R (20), the adrenal gland expressing both  $V_{1a}$ -R and  $V_{1b}$ -R (21), the liver expressing  $V_{1a}$ -R (22), and the kidney expressing  $V_{1a}$ -R,  $V_2$ -R (23) and perhaps  $V_{1b}$ -R (24). Our data show that under these conditions, increased cell proliferation was only detected in the kidney. This proliferative response involved  $V_2$ -R stimulation and mainly affected differentiated tubular kidney cells localized to specific medullary regions.

## Materials and Methods

### Materials

Arginin-vasopressin (AVP) and desmopressin (dDAVP) were purchased from Bachem (Basel, Switzerland). AVP receptor antagonists (SR49059, SR149415, and SR121463) were kindly provided by Sanofi-Aventis. Alzet osmotic minipumps were purchased from Charles River Laboratories (l'Arbresle, France), the bromodeoxyuridine (BrdU), Cremophore and DMSO from Sigma (Lisle d'Abeau Chesne, France), and the vasopressin direct radioimmunoassay kit from Bühlmann Laboratories (Schönenbuch, Basel, Switzerland).

### Animals

Animals used were adult (2 month-old) male Sprague Dawley rats (Janvier, France). They were housed in light (12h dark and 12h light) and temperature (21°C) controlled rooms and had free access to standard dry food and either tap water. All animals were treated in accordance with the principles of laboratory animal care published by the French Ethical Committee.

### Hyperosmotic stimulation

Animals received 2% saline (680 mosmol) as drinking fluid during 1 (n = 5), 2 (n = 5), 3 (n = 10) and 6 (n = 10) days.

### Administration of vasopressin and/or receptors agonists and antagonists

1) acute administration of exogenous AVP or dDAVP: Animals received one intraperitoneal (IP) injection of 0.5 ml

saline either alone (n = 5) or containing 13 µg of AVP (n = 8) or dDAVP (n = 8).

2) chronic administration of exogenous AVP or dDAVP: rats were treated by repeated IP injections of increasing doses of AVP (twice daily injections of 20 (n = 5) and 60 µg (n = 5) AVP for 3 days), or were subcutaneously implanted with an Alzet 7-day osmotic pump filled with 100 µl phosphate-buffered saline (PBS) alone (n = 5) or containing AVP or dDAVP, infusing 0.4 (n = 4), 1.3 (n = 4) or 13 µg (n = 8) of peptide per day.

3) administration of vasopressin receptor antagonists: osmotically stimulated rats were treated with specific antagonists of  $V_{1a}$ -R (SR49059, n = 4),  $V_{1b}$ -R (SR149415, n = 4), or  $V_2$ -R (SR121463, n = 5) receptors during 3 days. AVP-infused rats (n = 5) were treated with a specific antagonist of  $V_2$ -R (SR121463) during 3 days. Since these antagonists are poorly water soluble, they were dissolved in saline containing 5% DMSO + 7.5% cremophore (vol/vol) and administrated via two daily IP injections (at 1mg/kg) for 3 days.

#### Plasmatic vasopressin assay

The levels of plasma AVP were determined by collecting the trunk blood after decapitation of conscious animals. To prevent manipulation-induced stress, animals were manipulated two times per day for 4 days before the sacrifice. Blood samples were centrifuged at 2000 g in prechilled tubes containing EDTA for 15 min at 4°C. The plasma concentration of AVP was determined by the direct Bühlman radioimmunoassay kit (Bühlman Laboratories, Basel, Switzerland).

#### Western Blot analysis

Rat kidney tissues from the cortex, outer medulla and inner medulla were carefully dissected and treated for immunoblot analysis as previously described (25). Phosphorylated extracellular signal-regulated kinases (ERK) were revealed by using a 1:2000 dilution of mouse monoclonal anti-phospho-ERK antibody (phospho-p44/42 MAPK Thr202/Tyr204 from Cell Signaling Technology, Inc). After stripping, total ERK proteins were revealed by using a 1:750 dilution of anti ERK2 (C14): sc-154 antibody from Santa Cruz

Biotechnology. Blots were then probed with anti-mouse (Goat anti-mouse IgG HRP: sc2005 from Santa Cruz Biotechnology, Inc.) or anti-rabbit (ECL Rabbit IgG, HRP from Amersham Biosciences) secondary antibodies and visualized by using Enhanced Chemiluminescence detection reagent (Amersham Biosciences).

#### Immunocytochemistry

After deep anesthesia induced by Equithesin, animals were perfused through the ascending aorta with PBS, pH 7.4, followed by 400 ml of a solution of 4% paraformaldehyde 0.1 M phosphate buffer, pH 7. The adrenal glands, the liver and the kidneys were dissected and fixed by immersion overnight in the same fixative. They were then cut with a vibratome into 50-µm thick sections that were carefully rinsed in PBS and subsequently treated for multiple fluorescence labeling.

The vibratome sections were incubated for 48 hours at 4°C with one or two primary antibodies (see Table 1 for details on the different antibodies used in this study). After rinsing in PBS, sections were incubated for 4 hours at 4°C with the corresponding secondary antibodies conjugated with Alexa-488 (Molecular Probes, Eugene, USA) or Cy3 (Jackson Laboratories, USA). The primary and secondary antibodies were diluted in PBS containing 1% normal goat or donkey serum and 0.1% Triton X-100.

#### Detection of proliferative cells

Thymidine analog 5-bromodeoxyuridine (BrdU) was administered at the end of the different treatments (hyperosmotic stimuli, administrations of AVP and related molecules): animals received two IP injection of BrdU (100 mg/kg in 0.5ml of 0.01N sodium hydroxide solution) 16 and 5 hours before their fixation. Sections treated for the immunodetection of BrdU were incubated in 2N HCl for 30 minutes at room temperature, carefully rinsed in PBS, and incubated for 48 hours at 4°C with a mouse monoclonal antibody, anti-BrdU, either alone or in combination with another primary antibody.

#### Imaging and quantification

Labeled sections were rinsed in PBS,

mounted in Mowiol and observed under a Biorad MRC 1024 confocal laser scanning microscope equipped with a krypton/argon mixed gas laser. Two laser lines emitting at 488 nm and 568 nm were used for exciting the Alexa-488 or Cy3-conjugated secondary markers. The background noise of each confocal image was reduced by averaging four image inputs. The organization of the immunostained structures was studied 1) on single confocal images or 2) on bi-dimensional reconstructed images obtained by collecting 5 to 10 consecutive confocal images 1  $\mu$ m apart through the whole vibratome section thickness, and by projecting on the same plane. Unaltered digitalized images were transferred to a PC type computer and Adobe Photoshop was used to prepare final figures.

Ten  $\mu$ m thick reconstructed images obtained by using the x10 or x 20 objectives were used to evaluate the numerical density of BrdU-labeled nuclei within the different organs considered. For this, Image Tool analysis software (University of Texas Health Science Center) was used to count the labeled nuclei detected in squared areas (side: 250 or 500  $\mu$ m) centered on different regions of the organs, with 4 sections per animal and 4 animals per experimental group. The proportion of proliferative cells expressing V<sub>2</sub>-R was evaluated on sections immunostained for BrdU and for both uromucoid (UR) and aquaporin 2 (AQ2). The number of double-labeled cells was pooled from at least 4 sections per rat and 4 rats per experiment. Data were statistically compared by using the non parametric test of Mann and Whitney.

## Results

### **Increasing circulating AVP exclusively stimulates cell proliferation within the kidney**

#### Effects of prolonged osmotic stimulus

We provided rats with a 2% saline drinking solution, known to produce a strong hyperosmotic stimulus that maintains increased synthesis and release of endogenous AVP for several days without marked physiological alteration (26). In a

first step, we looked for possible proliferative effects of a 6 days osmotic stimulation on several organs known to express large amounts of AVP receptors, including the pituitary, the adrenal gland (zona glomerulosa), the liver, and the kidney. In control (normally hydrated) rats, only scarce BrdU-immunostained (IS) cells were detected throughout the four organs examined (Fig. 1 A-D). In 6 days osmotically stimulated rats, the distribution and numerical density of the proliferative (BrdU-IS) cells detected throughout the pituitary, adrenal cortex and liver was not modified as compared with the controls (Fig. 1 E-G), whereas a strong increase of cell proliferation was detected within the kidney medullary regions (Fig. 1 H).

In a second step, we focused on the kidney proliferative response to osmotic stimulation. Adult rats were submitted to osmotic stimuli from 1 to 6 days, and the rate of cell proliferation was examined in four anatomo-functional regions of the rat kidney: 1) the cortex (CX) that contains convoluted tubules and the glomerules, 2) the outer strip of the outer medulla (OSOM), that contains loop of Henle tubules 3) the inner strip of the outer medulla (ISOM), that contains a mixture of loop of Henle tubules and collecting ducts, and 4) the inner medulla (IM) that essentially contains collecting ducts (Fig. 2).

AVP radioimmunoassay confirmed that increased levels of plasmatic AVP (from 300% to 400% of controls) were achieved in rats osmotically-stimulated from 1 to 6 days (Fig. 2 lower panel). After 1 and 2 days of osmotic stimulation, we detected no change in the number of BrdU-immunostained (IS) nuclei within the different kidney regions as compared with controls (Fig. 2 B, C). In contrast, after 3 and 6 days of stimulation, we observed an increase in cell proliferation throughout the whole kidney, but with marked regional differences: BrdU-IS nuclei were preferentially localized to the (ISOM) and, to a lesser extent, the outer border of the IM, though they were markedly scarce within the cortical glomeruli and the deep IM (Fig. 2 D, E). As for the 6 days stimulation (Fig. 1), we detected no significant change in the rate of cell

proliferation compared to control after 1, 2 or 3 days of stimulation in the pituitary, adrenal cortex and liver (data not shown). These data indicate that prolonged hyperosmotic stimulation selectively increases cell proliferation within specific kidney regions of the adult rat.

#### Effects of exogenous vasopressin administration

Since prolonged hyperosmotic stimulus may generate osmotic stress that influences kidney cell proliferation (27, 28), we induced a prolonged rise in circulating AVP without hyperosmotic stimulus. Normally hydrated rats were implanted with an alzet osmotic minipump to infuse either PBS (control rats) or AVP (0.4, 1.3 or 13  $\mu$ g AVP per day) for 3 or 6 days. Measurements of plasmatic AVP indicated that increasing levels of circulating AVP were associated with the increasing doses of infused AVP (Fig. 3 lower panel). As compared with controls, the kidneys of rats infused with AVP contained numerous proliferative cells after 3 and 6 days, mostly localized to the ISOM and the outer border of the IM (Fig. 3 A-D and lower panel). As observed following prolonged hyperosmotic stimulation, no change in the rate of cell proliferation was detected in the pituitary, adrenal cortex or liver of the AVP-infused rats (data not shown).

#### **The proliferative response of kidney cells to increased levels of circulating AVP involves the stimulation of $V_2$ receptors**

AVP exerts its biological effects via its binding to three types of receptors, the  $V_{1a}$ ,  $V_{1b}$  and  $V_2$  receptors. The kidney has been shown to express different AVP receptor subtypes including both  $V_2$ -R and  $V_{1a}$ -R (29), and perhaps  $V_{1b}$ -R (30). We therefore wished to investigate which of these subtypes is involved in the proliferative response to hyperosmotic stimulus or AVP infusion.

#### The proliferative response of kidney cells to hyperosmotic stimulus or AVP infusion is antagonized by the administration of a selective $V_2$ -R antagonist

Rats osmotically-stimulated or AVP-infused were treated twice daily with IP injections of non-peptidic antagonists of  $V_{1a}$ -R (SR49059),  $V_{1b}$ -R (SR149415), or  $V_2$ -R (SR121463), or of vehicle alone. We detected no significant change in the pattern of kidney cell proliferation in osmotically-stimulated rats treated with an antagonist to  $V_{1a}$ -R (Fig. 4 B) or  $V_{1b}$ -R (Fig. 4 C), compared with osmotically-stimulated controls (Fig. 4 A). In contrast, in both osmotically-stimulated and AVP-infused rats treated with the  $V_2$ -R antagonist, the rate of cell proliferation was markedly decreased compared with controls within the ISOM and the outer IM, (Fig. 4 D, F). These data strongly suggest that the proliferative responses of kidney cells to either hyperosmotic stimulus or AVP infusion involved the stimulation of the  $V_2$ -R.

#### The administration of a $V_2$ -R agonist mimics the effects of hyperosmotic stimulation or AVP infusion

We next examined whether the kidney cell proliferation induced by hyperosmotic stimulation or AVP-infusion could be reproduced by the administration of the specific  $V_2$ -R agonist dDAVP to normally hydrated rats. To verify the pharmacological specificity of dDAVP *in vivo*, rats were fixed 1 hour after an acute injection of 13  $\mu$ g of either dDAVP or AVP, and the early gene product c-Fos was looked for in the kidney (containing cells expressing either  $V_{1a}$ -R or  $V_2$ -R), and in the adrenal gland cortex (containing cells expressing  $V_{1a}$ -R but not  $V_2$ -R) (21). The administration of AVP induced a strong accumulation of c-Fos in both kidney and adrenal cells, whereas dDAVP only induced the accumulation of c-Fos in kidney cells and in fewer numbers to that observed with AVP (see supplemental Fig. 1). Adult rats were then implanted with an Alzet osmotic minipump containing either PBS (controls) or dDAVP (delivering 0.4, 1.3 or 13  $\mu$ g dDAVP/day), and were fixed after 3 or 6 days as described above. Urine osmolarity was consistently increased after 6 days in rats infused with dDAVP at 0.4  $\mu$ g / day ( $1920 \pm 60$  mosmol,  $n = 3$ ), 1.3  $\mu$ g / day ( $2100 \pm 72$  mosmol,  $n = 3$ ), or 13  $\mu$ g ( $2320 \pm 150$  mosmol/l,  $n = 6$ ) as compared

with control rats ( $1095 \pm 95$  mosmol/l,  $n = 6$ ), attesting for the effective stimulation of kidney  $V_2$ -R. Whatever the dose of dDAVP infused and the duration of the infusion, we detected no change in the rate of cell proliferation within the adrenal gland, the pituitary or the liver (not shown) compared with controls, in contrast to a large increase observed within the kidney. In all the dDAVP-infused rats examined, such a proliferative response was very similar to what was observed in osmotically-stimulated or AVP-infused rats: high concentrations of proliferative (BrdU-IS) cells were preferentially localized to the ISOM and the outer portions of the IM (Fig. 5 and lower panel). For the same doses of dDAVP and AVP infused, such a kidney proliferative response was always stronger in the dDAVP-infused rats (compare Figs. 3 and 5). These data indicate that a strong proliferation of kidney cells can be induced by the administration of dDAVP to normally hydrated rats, suggesting that the proliferative response of kidney cells to hyperosmotic stimulation or exogenous AVP administration mainly results from the stimulation of the  $V_2$  receptor subtype.

#### **Proliferative kidney cells induced by $V_2$ -R stimulation mainly correspond to $V_2$ -R-expressing cells**

Within the kidney,  $V_2$ -R is essentially expressed by principal cells of the renal collecting ducts (23) and by the thick ascending limb of Henle's loop (TAL) tubular cells (29). Since the immunocytochemical detection of  $V_2$ -R could not be performed (due to the absence of efficient antibodies), we identified the  $V_2$ -R expressing kidney cells by the detection of kidney cell-type-specific markers, i.e. aquaporin 2 (AQ2) for collecting ducts principal cells (31) and uromucoid (UR) for TAL cells (32). In 3 and 6 day dDAVP-infused rats, the examination of kidney sections immunostained for BrdU and for either UR or AQ2, indicated a predominant localization of BrdU-IS nuclei in kidney regions containing either UR- and AQ2-IS cells (the cortex and the ISOM), or AQ2-IS cells only (the outer layer of the IM) (Fig. 6 A-C). The examination of double

immunostained kidney sections indicated that BrdU-IS nuclei frequently associated with either UR-IS or AQ2-IS cells throughout the different kidney regions, preferentially associating with UR-IS cells in the ISOM and with AQ2-IS cells in the outer layer of the IM (Fig. 6 D-I). To determine the proportion of proliferative cells corresponding to  $V_2$ -R expressing cells in these kidney regions, kidney sections of 3 and 6 day dDAVP-infused rats were double immunostained for BrdU and for both UR and AQ2 (Fig. 6 J): the quantitative analysis of these double immunostained sections indicated that more than 70% of the BrdU-IS nuclei detected throughout the kidney regions were associated with UR- or AQ2-IS cells. In all dDAVP-infused rats examined, both types of immunostained tubules appeared more numerous and exhibited increased diameters (more than 2 fold) compared with controls (data not shown). Moreover, a comparison of the kidney weight between 6 day dDAVP-infused and control rats further revealed a moderate but significant increase following prolonged dDAVP infusion ( $1.81 \pm 0.07$  g vs.  $1.47 \pm 0.06$  g,  $n = 6$ ,  $p < 0.01$ ). Together these data indicate that the proliferative effects of  $V_2$ -R stimulation mostly correspond with the proliferation of differentiated tubular cells expressing the  $V_2$ -R.

#### **The cell proliferation induced by $V_2$ -R stimulation is associated with the activation of the ERK pathway**

ERK is known to be involved in a large number of signaling pathways initiated by mitogens (33). We then examined whether the induction of the proliferation process by  $V_2$ -R stimulation correlated with the activation of ERK1/2 within the kidney cells. Western-blot analysis was first used to compare the levels of phosphorylated ERK (P-ERK) in the different kidney regions of control rats to those found in rats infused with dDAVP for 3 days. Compared with controls, P-ERK labeling was only increased in the outer medullary kidney regions of dDAVP-infused rats, with no change in total ERK (Fig. 7). We then investigated any direct association between the ERK activation induced by dDAVP



administration and kidney cell proliferation. For this, kidney sections of control or 3 day dDAVP-infused rats were treated for P-ERK immunostaining. We detected only faint immunostaining for P-ERK throughout the different kidney regions in control rats, in contrast to strong cellular immunostaining within the ISOM and the outer portions of the IM in dDAVP-infused rats (Fig. 8 A, B). The examination of kidney sections double immunostained for P-ERK and BrdU indicated a predominant localization of both immunostainings within the same kidney regions (Fig. 8 C, D), with intense P-ERK immunostaining frequently associated with the cytoplasm and the nucleus of BrdU-IS cells, although some did appear P-ERK-negative (Fig. 8 E). We made similar observations in kidney sections of osmotically-stimulated or AVP-infused rats (data not shown). These data strongly suggest that the proliferative effects of prolonged  $V_2$ -R stimulation relates to the activation of the ERK pathway and to its accumulation within both the cytoplasm and nucleus of  $V_2$ -R expressing cells located within the ISOM and the outer IM.

## Discussion

The concept that AVP plays a mitogenic role was principally developed from a series of *in vitro* studies on a variety of cell types. A prominent finding of the present study is that the proliferative effects induced *in vivo* by increasing circulating AVP in adult rats do not agree with these previous studies carried out *in vitro*. Indeed, under *in vivo* experimental conditions that increase the levels of circulating AVP, we detected no proliferative effect on several cell types that have previously been reported to proliferate *in vitro* following AVP application (including the adrenal glomerulosa cells (7), the hepatocytes (8) and the renal mesangial cells (9)). However, we did detect an unexpected proliferative response on tubular cells of specific medullar kidney regions. Although the reasons for such discrepant responses to AVP stimulation remain to be elucidated, our data reinforce the idea that the proliferative ability of a given cell type and

its regulation can be markedly modified by *in vitro* conditions (34). We would like to draw attention, however, to the possible drawbacks introduced by the *in vivo* approach used in the present study. Firstly, a prolonged hyperosmotic stimulus generates stressful conditions that may interfere with cell proliferation, independently of an augmented secretion of AVP. However, the fact that similar proliferative responses were induced by either osmotic stimuli or exogenous AVP administration strongly suggests a major role of circulating AVP. Secondly, since AVP is a potent vasoconstrictor, it would be reasonable to assume that the proliferative response detected within the kidney results from local tissue lesions due to vasoconstriction and/or hypertension. In a complementary study, we demonstrated the absence of kidney hypoxia and/or lesion under the experimental conditions used in the present study to raise the levels of circulating AVP (i.e. prolonged hyperosmotic stimulus and infusion of exogenous AVP). Kidney hypoxia and lesion were only induced by repeated acute injections of high (20 and 60  $\mu$ g) AVP doses (see supplemental Fig. 2). This agrees with previous studies indicating that *in vivo*, vasoconstriction of renal arterioles can only be induced by high, non-physiological doses (nanomolar range) of AVP (35), whereas low physiological doses (picomolar range) are efficient *in vitro* (36). One possible explanation is the attenuation of AVP-induced vasoconstriction by nitric oxide release *in vivo* (37). In addition, we found that the patterns of cell proliferation associated with prolonged hyperosmotic stimulation or AVP infusion (i.e. absence of cell lesion markers and proliferation of differentiated tubular cells of the ISOM and outer IM) markedly differ from those associated with kidney hypoxic lesions (i.e. tissue infiltration by activated macrophages and proliferation of precursor-like cells of the OSOM) (supplemental Fig. 2). Lastly, we found that no kidney cell proliferative response could be detected in rats implanted with an osmotic pump infusing high doses of angiotensin (a potent pressor agent, 13  $\mu$ g/day for 3 days) or bradykinin (a potent vasodilator agent, 13  $\mu$ g/day for 3 days) (see supplemental fig3). It can thus

reasonably be assumed that the proliferation of kidney cells detected in osmotically-stimulated or AVP-infused rats at least partly results from a direct activation of AVP receptors.

Since the three identified AVP receptor subtypes ( $V_{1a}$ ,  $V_{1b}$ , and  $V_2$ ) have been described in the kidney, the effects of sustained elevated levels of circulating AVP levels on the proliferation of kidney cells described here could be attributed to the stimulation of any of these receptors. Two findings however, strongly suggest that this mitogenic effect mainly results from the activation of  $V_2$  receptors: kidney cell proliferation was either inhibited by the administration of a selective antagonist for  $V_2$ -R, or mimicked by the administration of dDAVP, a selective  $V_2$ -R agonist. Moreover, this effect seems to be direct since most of the kidney proliferative cells corresponded to cells expressing the  $V_2$ -R, i.e. cells of the thick ascending limb of Henle's loop (TAL) or of the collecting duct (immunostained for UR or AQ2, respectively).

The mitogenic effects of AVP that have been previously described on various cell types, generally involved the mobilization of intracellular calcium and the activation of several kinases via  $V_{1a}$ -R stimulation (38). The molecular mechanisms triggering kidney cell proliferation via  $V_2$ -R activation remain to be established. The stimulation of  $V_2$ -R induces a strong accumulation of cAMP within kidney tubular cells through the positive coupling to the stimulatory Gs protein (39). Although both positive and negative interactions between cAMP and ERK have been reported in various systems (40), it is generally admitted that increased levels of cAMP inhibit both the activation of ERK and the proliferation of normal kidney cells (41). This appears inconsistent with the present finding of an association between kidney cell proliferation induced by  $V_2$ -R stimulation and ERK phosphorylation. It is noteworthy that a proliferative response of kidney cells could only be detected after at least 3 days of hyperosmotic stimulation or of AVP or dDAVP infusions. It can therefore be reasonably assumed that prolonged stimulation of the kidney  $V_2$  receptors

progressively alters the associated intracellular signaling pathways. Yamaguchi et al. previously reported the conversion of kidney cells normally exhibiting growth inhibition by cAMP into cells in which growth is stimulated by cAMP, by altering their calcium metabolism (42, 43). A first possibility is thus that prolonged stimulation alters calcium metabolism within a subpopulation of  $V_2$ -R-expressing kidney cells, converting them to a cAMP-dependent proliferative phenotype.

Interestingly, we have shown that the proliferative response to  $V_2$ -R stimulation concerns a subpopulation of the  $V_2$ -R-expressing kidney cells, mostly localized to the ISOM and the outer IM that contain numerous TAL cells. Upon prolonged  $V_2$ -R activation, TAL cells have been shown to secrete prostaglandins that eventually inhibit the  $V_2$ -R-dependent increase in intracellular cAMP (44, 45). A second possibility is that locally secreted prostaglandins modulate intracellular cAMP contents. Ongoing studies based on the inhibition of prostaglandins synthesis in AVP- or dDAVP-infused rats should clarify the role played by locally secreted prostaglandins in ERK activation and cell proliferation.

High levels of plasma AVP, comparable to those obtained here following hyperosmotic stimuli or infusion of the lowest AVP doses, have been reported in a number of pathologies including SIADH (11, 12), renal insufficiency (13-15), uncontrolled diabetes mellitus (16, 17) and major depression (18, 19). To our knowledge, it is not known whether these pathological conditions have any affect on kidney cell proliferation. By contrast, the abnormal proliferation of tubular kidney cells is a hallmark of the polycystic kidney diseases (PKD), a group of genetic disorders causing dramatic renal failure and death both in children and adults (46, 47). Interestingly, AVP levels have been reported to increase in patients and in animal models with PKD, possibly in an attempt to compensate for reduced concentrating capacity of the lesioned kidney (48). Moreover, the involvement of circulating AVP in PKD has been supported by a series of recent studies demonstrating a

limited progression of PKD following the administration of V<sub>2</sub> receptor antagonists (49, 50) or decreasing circulating AVP levels (51, 52). Since the kidney cell types that were found to proliferate following prolonged hyperosmotic stimulation of AVP or dDAVP infusions, i.e. TAL and collecting ducts tubular cells, are those kidney cells that form cysts in PKD (53), it can reasonably be assumed that elevated levels of circulating AVP may play a key role in PKD. In agreement with previous studies (54, 55), we observed that the proliferation of kidney cells induced by the longest infusions of dDAVP (6 days) was consistently associated with marked hypertrophy of tubular cells in the TAL and collecting ducts of the ISOM region, and with a moderate ( $+23 \pm 5$  %), but significant increase in kidney weight (data not shown). However, whatever the experimental conditions used in this study, kidney cell proliferation was never associated with the formation of renal cysts. It can thus be assumed that, although they do not represent determinant factors for the development of PKD, pathological deregulation of endogenous AVP secretion, or prolonged therapeutic administration of AVP or dDAVP may constitute aggravating factors to this renal disease.

In conclusion, the present findings provide new insight into the mitogenic roles of vasopressin *in vivo*. In adult rats, sustained elevated levels of circulating AVP induce strong proliferation of a specific population of kidney tubular cells expressing the V<sub>2</sub>-R. Although the molecular mechanisms involved remain to be elucidated, these findings provide a

unexpected explanation of the role played by V<sub>2</sub> receptors in the development and/or progression of PKD.

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Abbreviations:

AQ2: aquaporin 2; AVP: arginin vasopressin; BrdU: bromodeoxyuridine; CX: cortex; dDAVP: desmopressin; DMSO: dimethyl sulfoxide; ED1: rat activated macrophages antigen; ERK: extracellular signal-regulated kinase; IM: inner medulla; IP: intraperitoneal; IS: immunostained  
ISOM: inner strip of the outer medulla; OM: outer medulla; OSOM: outer strip of the outer medulla; PBS: phosphate buffer saline; PKD: polycystic kidney disease; SDS: sodium dodecyl sulphate; TAL: thick ascending limb of Henle's loop; UA: uranyl acetate; UR: uromucoid; AVP: vasopressin; V-R: vasopressin receptor

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## Figures legend

Fig. 1: Effects of osmotic stimulus on cell proliferation within organs expressing vasopressin receptors.

Detection of BrdU-immunostained (IS) nuclei within pituitary, adrenal gland, liver and kidney of normally hydrated controls (A-D) and 6 days osmotically-stimulated (E-H) rats.

Lower panel: quantitative analysis of the numerical density of BrdU-IS nuclei within the different organs. Data represent the means  $\pm$  SD of the number of BrdU-IS nuclei counted within a 500  $\mu$ m side squared field centered on specific organs regions (shown in Fig. A-D). \*\* $P < 0.01$ . Scale bar = 250  $\mu$ m.

Fig. 2: Effects of hyperosmotic stimuli on kidney cell proliferation.

A-E: montages of adjacent images showing the distribution of BrdU-immunostained (IS) nuclei throughout the different kidney regions of rats normally hydrated (Ct), osmotically-stimulated by drinking a 2% saline solution during 1 (os 1d), 2 (os 2d), 3 (os 3d) and 6 days (os 6d). Note that BrdU-IS nuclei are scarce within the cortical glomeruli (arrows in D and E).

Lower panel: quantitative analysis of the numerical density of BrdU-IS nuclei within the different kidney regions of rats normally hydrated rats (Ct) or osmotically-stimulated (os 1, 2, 3 and 6d). Data represent the means  $\pm$  SD of the number of BrdU-IS nuclei counted within a 500  $\mu$ m side squared field (shown in Fig. 1 A) centered on the different kidney regions of three animals. \* $P < 0.05$ ; \*\* $P < 0.01$ . Inset shows the plasmatic AVP concentrations in normally hydrated rats (Ct) and rats osmotically-stimulated for 1 to 6 days (os 1d to 6d).

CX: cortex; g: glomerulus; IMi: internal portion of the internal medulla; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla. Scale bar = 250  $\mu$ m

Fig. 3: Effects of exogenous AVP infusion on kidney cell proliferation.

A-D: montages of adjacent images showing the distribution of BrdU-immunostained (IS) nuclei throughout the different kidney regions of rats normally hydrated rats implanted with an osmotic pump infusing either PBS (Ct), or increasing doses of AVP (0.4, 1.3, and 13  $\mu$ g/day) during 6 days. Note that in all the AVP-infused rats, the number of BrdU-IS nuclei appear to increase throughout the medullary kidney regions as compared with controls, whereas the glomeruli appear devoid of BrdU-IS nuclei (arrows in D).

Lower panel: quantitative analysis of the numerical density of BrdU-IS nuclei within the different kidney regions. Data represent the means  $\pm$  SD of the number of BrdU-IS nuclei counted within a 500  $\mu$ m side squared field (shown in Fig. 1 A) centered on the different kidney regions of three animals. \* $P < 0.05$ ; \*\* $P < 0.01$ . Inset shows the plasmatic AVP concentrations in rats infused with PBS (Ct) and with the different AVP doses during 6 days.

CX: cortex; g: glomerulus; IMi: internal portion of the internal medulla; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla. Scale bar = 250  $\mu$ m.

Fig. 4: Effects of selective AVP-receptor antagonists administration on kidney cell proliferation induced by hyperosmotic stimulus or AVP infusion.

A-F: montages of adjacent images showing the distribution of BrdU-immunostained (IS) nuclei throughout the different kidney regions of rats osmotically-stimulated during 3 days and treated with the vehicle (os 3d + veh = control) or with an antagonist to  $V_{1a}$ -R (os 3d +  $V_{1a}$ -R ant),  $V_{1b}$ -R (os 3d +  $V_{1b}$ -R ant) or  $V_2$ -R (os 3d +  $V_2$ -R ant), or normally hydrated rats AVP-infused during 3 days (13  $\mu$ g/ day) and treated with either the vehicle (AVP 3d + veh = control) or with an antagonist to  $V_2$ -R (AVP 3d +  $V_2$ -R ant).

Lower panel: quantitative analysis of the density of BrdU-IS nuclei within the different kidney regions. Data represent the means  $\pm$  SD of the number of BrdU-labeled nuclei counted within a 500  $\mu$ m side squared field centered on the different kidney regions of three animals (see Fig. 1 A). \* $P < 0.05$ ; \*\* $P < 0.01$ .



CX: cortex; IMi: inner portion of the inner medulla; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla. Scale bar = 250  $\mu$ m.

Fig. 5: Effects of dDAVP infusion on kidney cell proliferation.

A-D: montages of adjacent images showing the distribution of BrdU-IS nuclei within the different kidney regions of normally hydrated rats implanted with an osmotic pump infusing either PBS (Ct) or different doses of dDAVP (0.4, 1, 3 and 13  $\mu$ g/day) during 6 days (dDAVP 6d).

Lower panel: quantitative analysis of the density of BrdU-IS nuclei within the different kidney regions. Data represent the means  $\pm$  SD of the number of BrdU-labeled nuclei counted within a 500  $\mu$ m side squared field centered on the different kidney regions of three animals (see Fig. 1 A). \*P<0.05; \*\*P<0.01

CX: cortex; IMi: internal portion of the internal medulla; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla. Scale bar = 250  $\mu$ m.

Fig. 6. Identification of proliferative kidney cells in 3 days dDAVP-infused rats.

A-C: montages of adjacent images of kidney sections immunostained for BrdU (A), uromucoid (UR, B), and aquaporin 2 (AQ2, C). D-J: colored images of superimposed double immunostainings for BrdU and either UR or AQ2 showing that BrdU-labeled nuclei are associated with either UR- or AQ2-labeled cells of the OSOM (D, E), the ISOM (F, G) and the outer portion of the IM (I). J: colored image of superimposed double immunostaining for BrdU (red) and both UR and AQ2 (blue) showing that a majority of the BrdU-labeled cells detected in the ISOM are associated with either UR- or AQ2-labeled cells. The frames in A illustrate the anatomical localization of the kidney areas shown in D-J.

AQ2: aquaporin 2; IMi: inner portion of the internal medulla; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla; UR: uromucoid. Scale bar = 250  $\mu$ m in C (applies for A-C); 50  $\mu$ m in J (applies for D-J).

Fig. 7. Western blotting analysis of phosphorylated ERK (P-ERK) and total ERK in different kidney regions of rats infused with saline (Ct) or dDAVP (dDAVP) during 3 days. The ERK phosphorylation signal of each kidney region was normalized to the relative amount of corresponding total ERK and expressed as the percentage of P-ERK signal detected in dDAVP-infused vs the corresponding control rats. Results are the means  $\pm$  SEM of 4 independent experiments. \* = p<0.05 (Mann-Whitney test).

CX: cortex; OM: outer medulla; IM: inner medulla

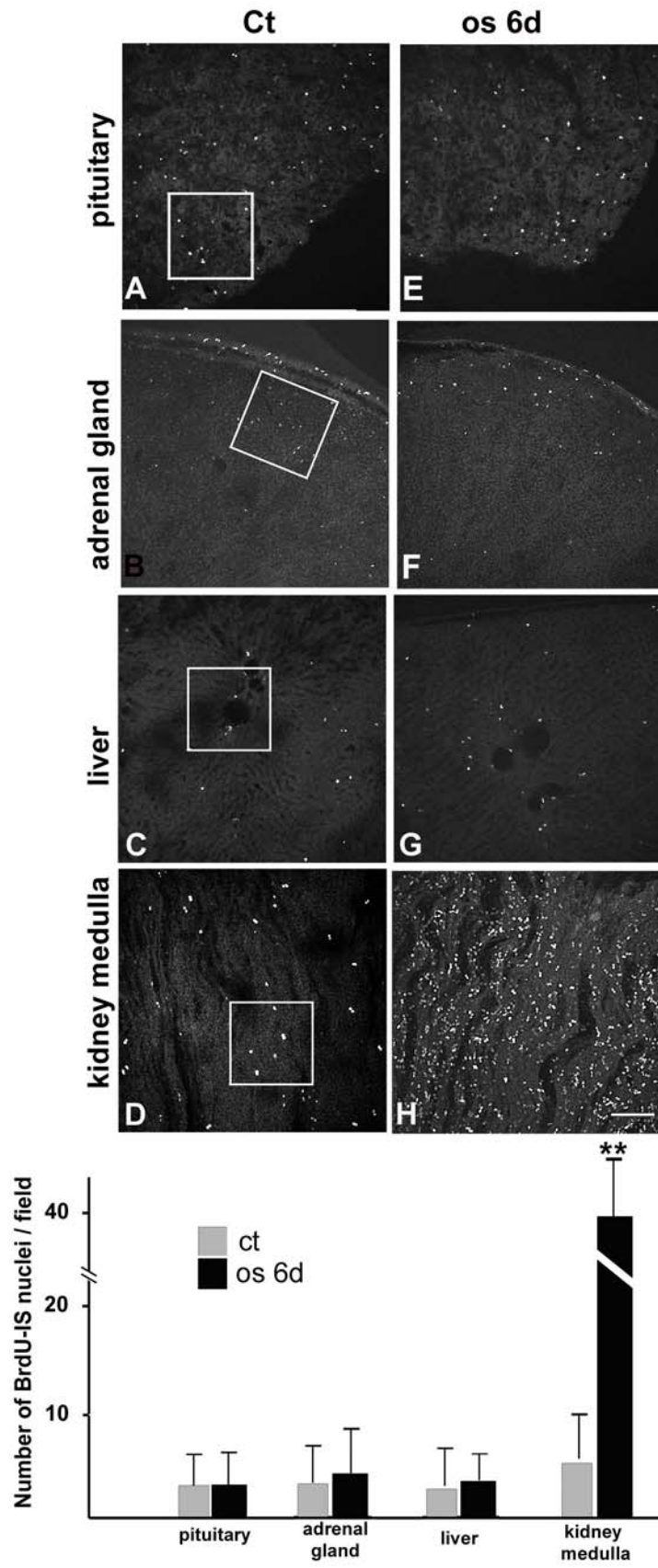
Fig. 8. Cellular localization of phospho-ERK.

A-B: montages of adjacent images of kidney sections from normally hydrated rats infused during 3 days with saline (control) (A), or dDAVP (dDAVP 3d) (B), immunostained for P-ERK. Note that P-ERK immunostaining that is faint throughout the kidney of the control rat, is highly increased following dDAVP-infusion, with a preferential localization to the ISOM and the outer portion of the IM. C-E: kidney sections from 3 days dDAVP-infused rats. C-D: Paired images of a section double immunostained for P-ERK and BrdU showing a close anatomical association of both immunostainings at the limit between the ISOM and IM regions. E: color image of superimposed double immunostaining showing that P-ERK and BrdU immunostainings are frequently colocalized within the nucleus of double labeled cells (yellow color, arrows). The frames in B illustrate the anatomical localization of the kidney areas shown in C-D.

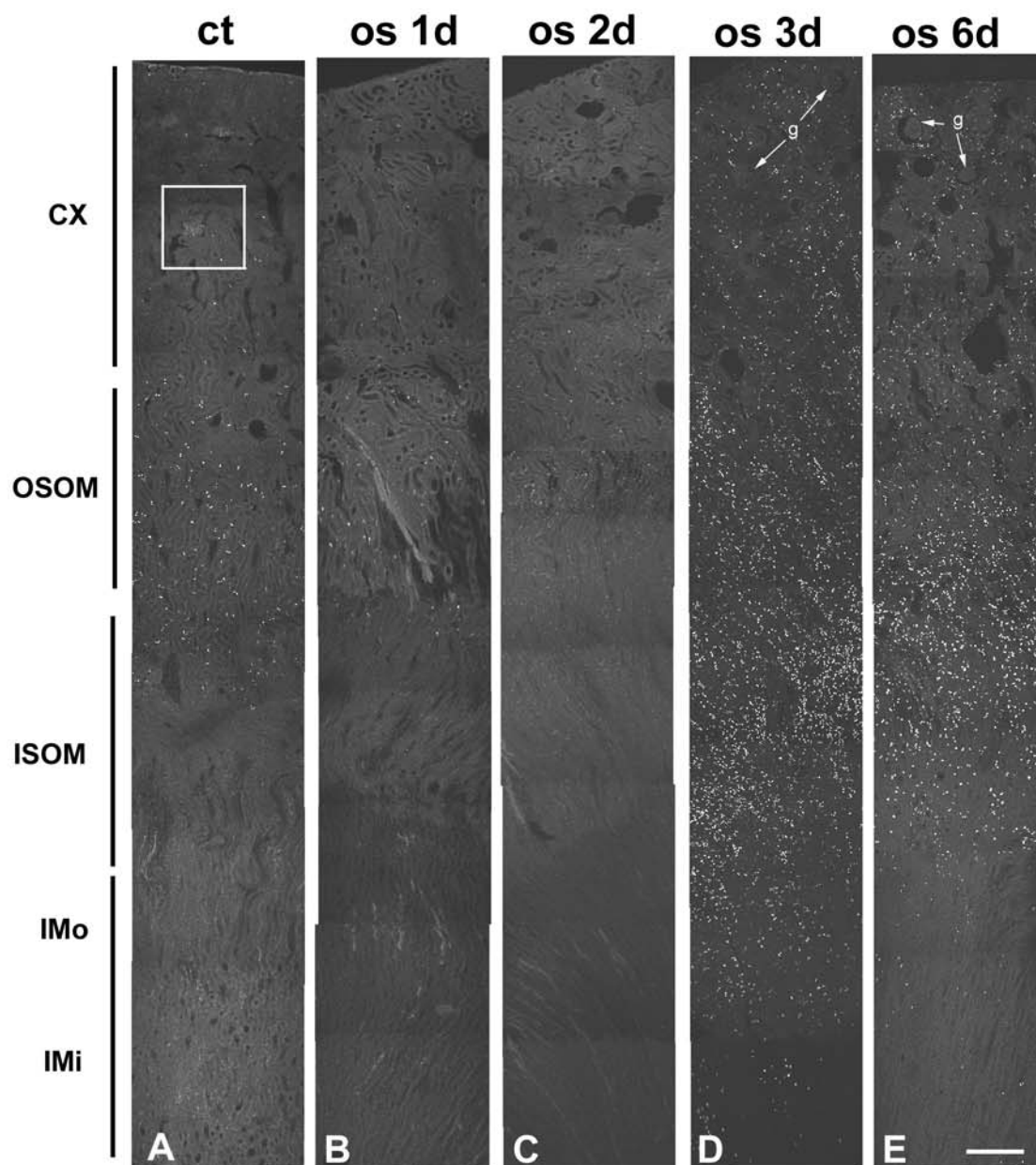
AQ2: aquaporin 2; CX: cortex; IMo: outer portion of the internal medulla; ISOM: inner strip of the outer medulla; OSOM: outer strip of the outer medulla; UR: uromucoid. Scale bar = 250  $\mu$ m in B (applies for A-B); 100  $\mu$ m in D (applies for C-E); 25  $\mu$ m in F.

**Table 1.** Antibodies used for immunochemistry

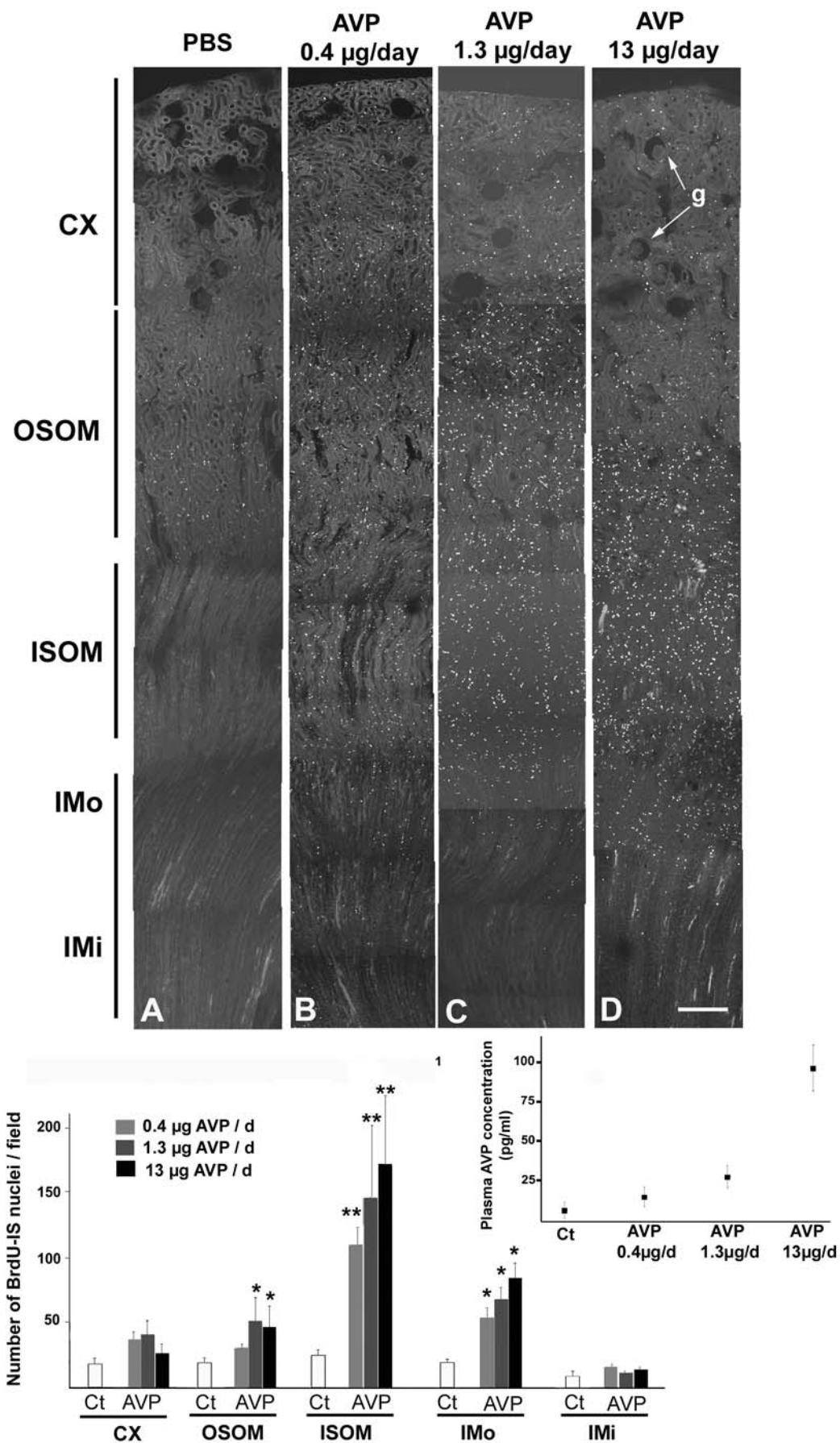
<b>Antibody</b>	<b>Species</b>	<b>Working dilution</b>	<b>Vendor or product or</b>	
Bromodeoxyuridine (BrdU)	Mouse IgG	1: 500	Novo Castra (Newcastle, UK)	
Aquaporin 2 (AQ2)	Goat IgG	1:500	Santa Cruz Biotechnoly (Santa Cruz, USA)	
Aquaporin 2 (AQ2)	Rabbit IgG	1:500	Abcys (Paris, France)	
Uromucoid (UR)	Sheep IgG	1:200	AbCys SA (Paris, France)	
Vimentin (VIM)	Mouse IgM	1:500	Sigma (StLouis, USA)	
Phosphorylated extracellular signal-regulated kinase (P-ERK)	Rabbit IgG	1:500	Biosource (Camarillo, USA)	
ED1	Mouse IgG	1:2000	Chemicon (Temecula, USA)	



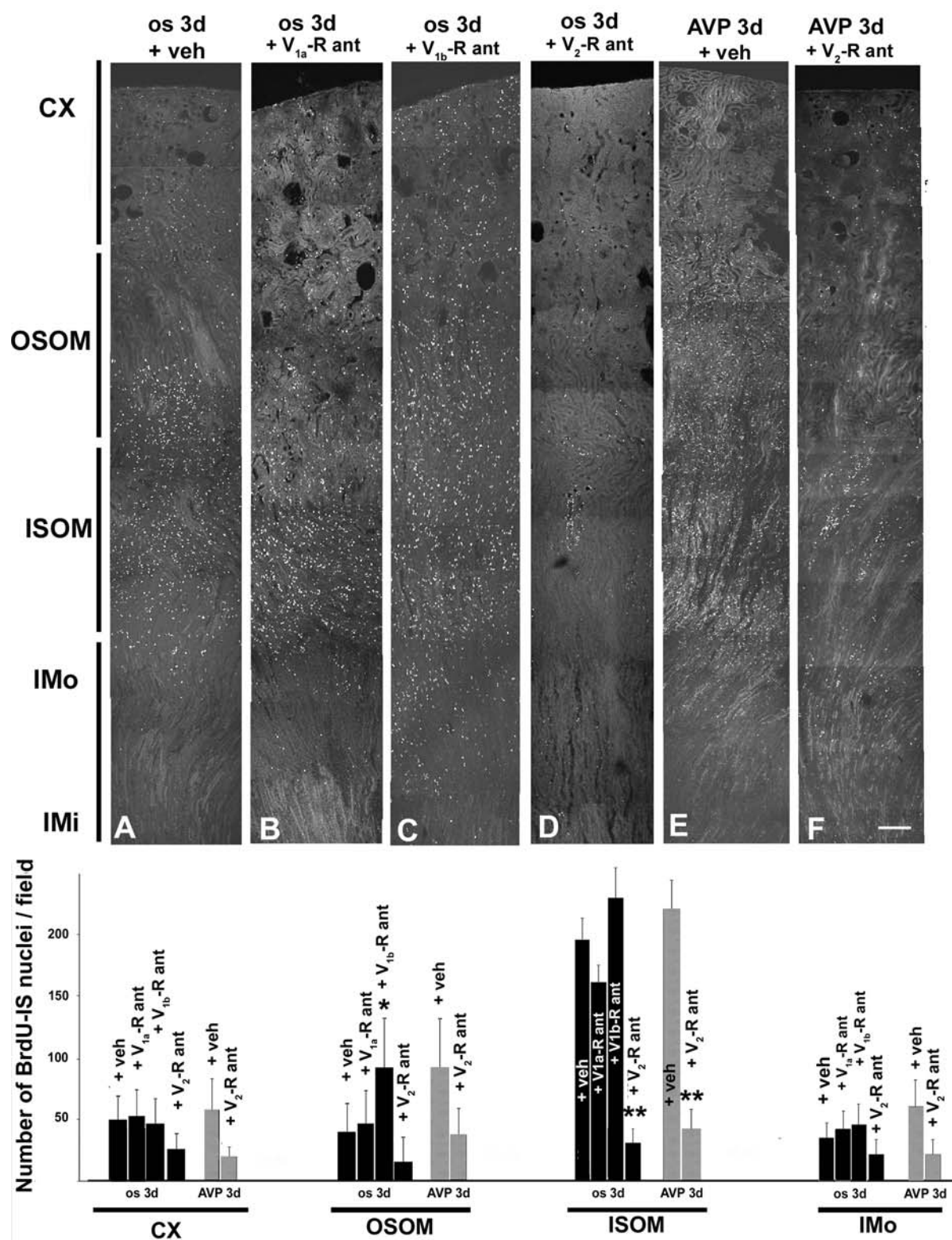
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**

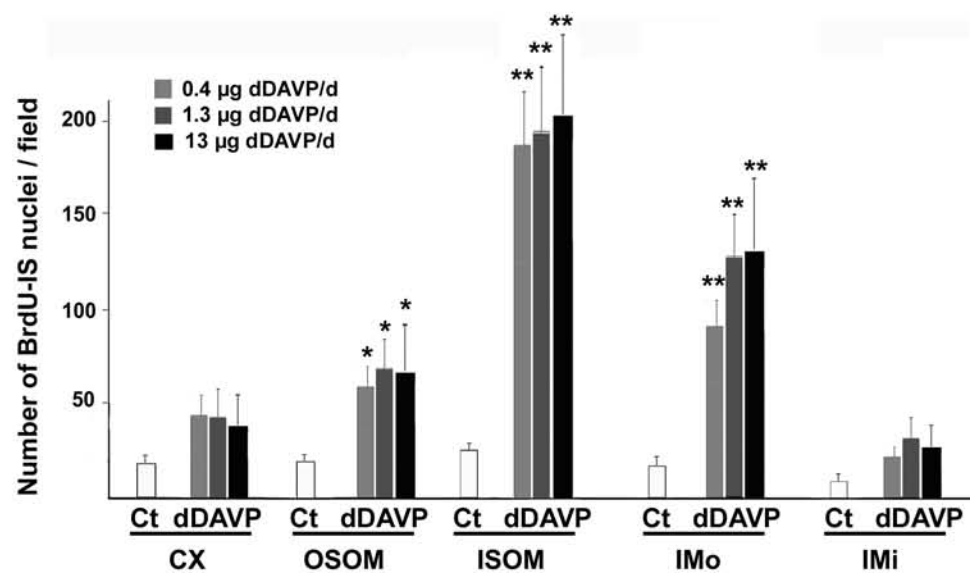
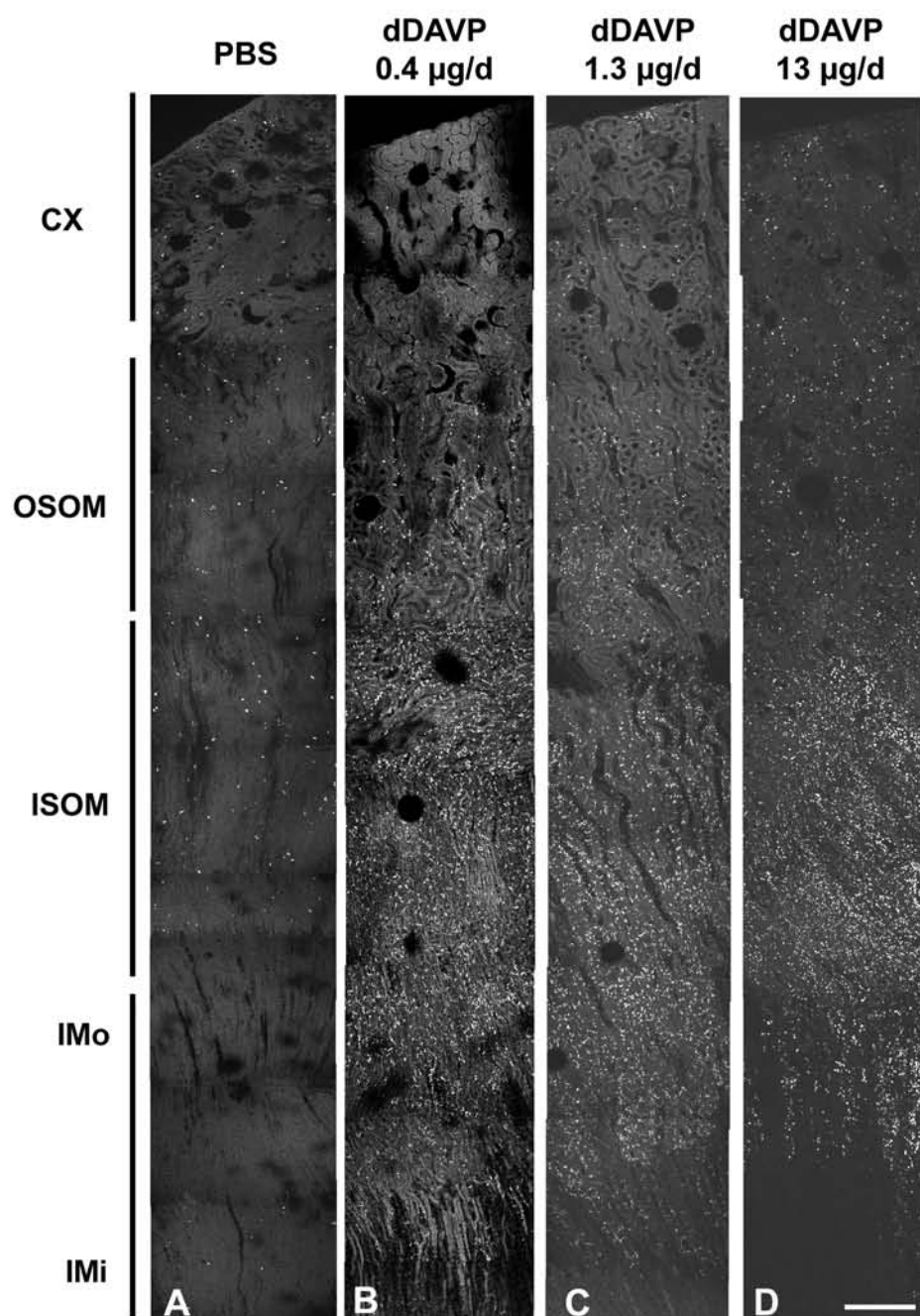
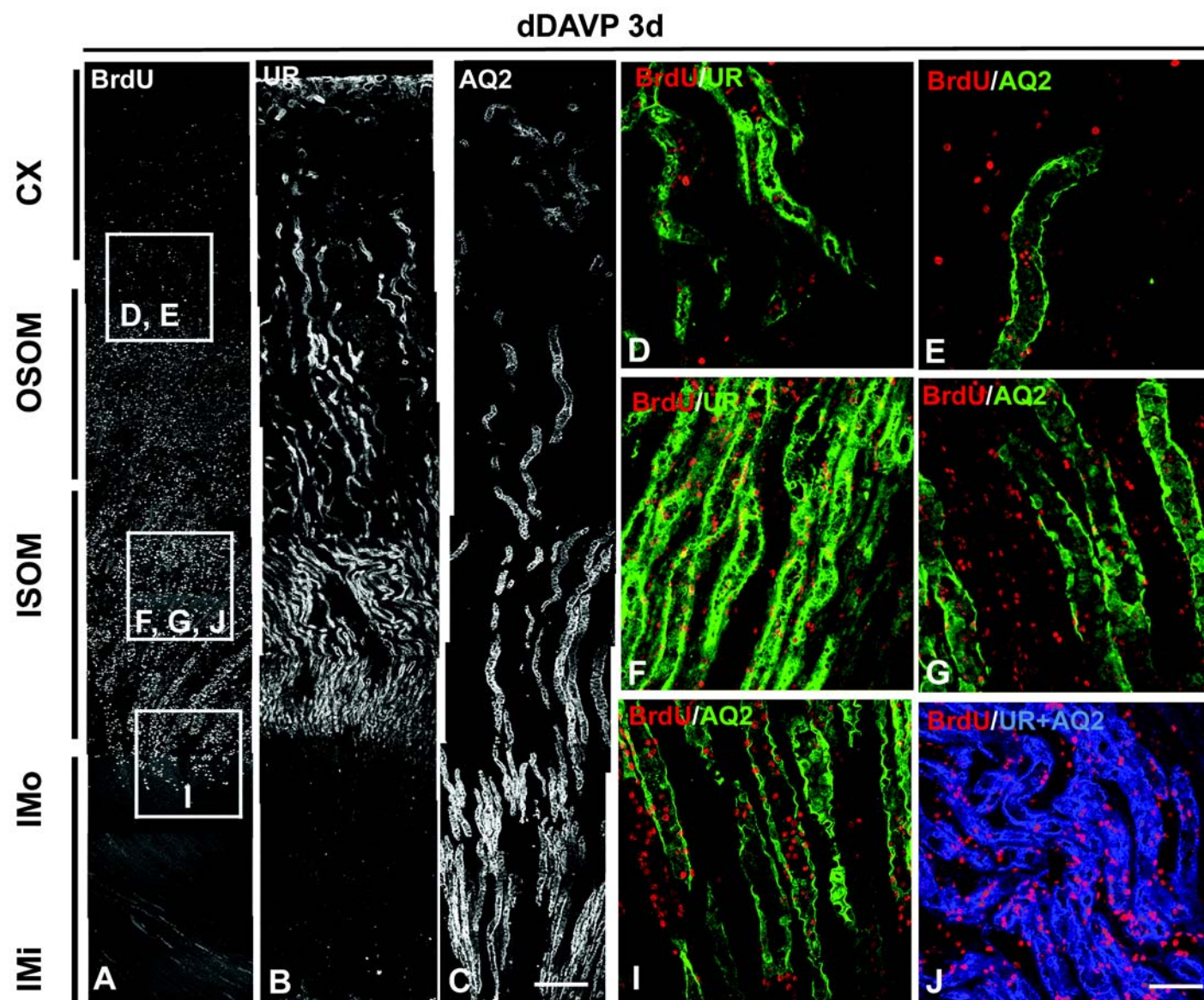


Fig. 5





**Fig. 6**



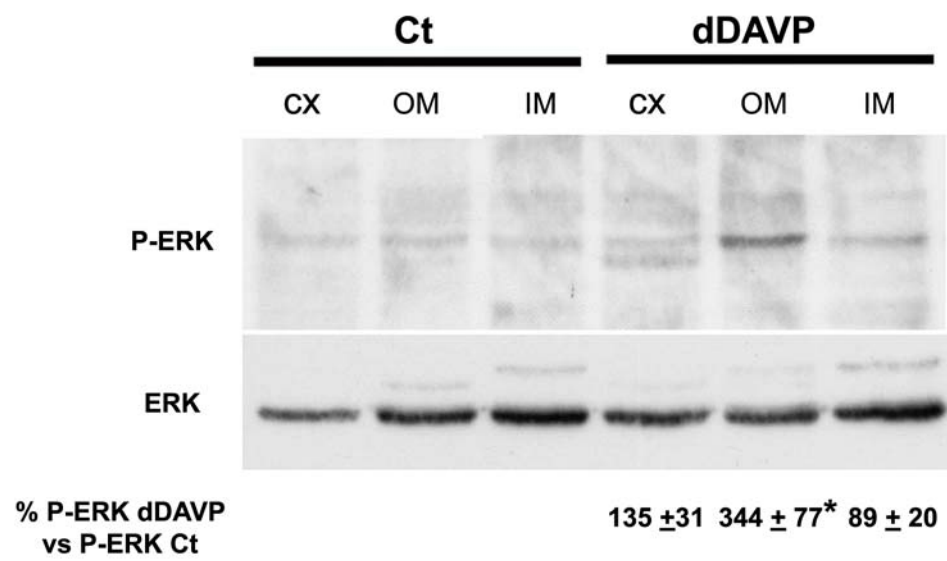


Fig. 7

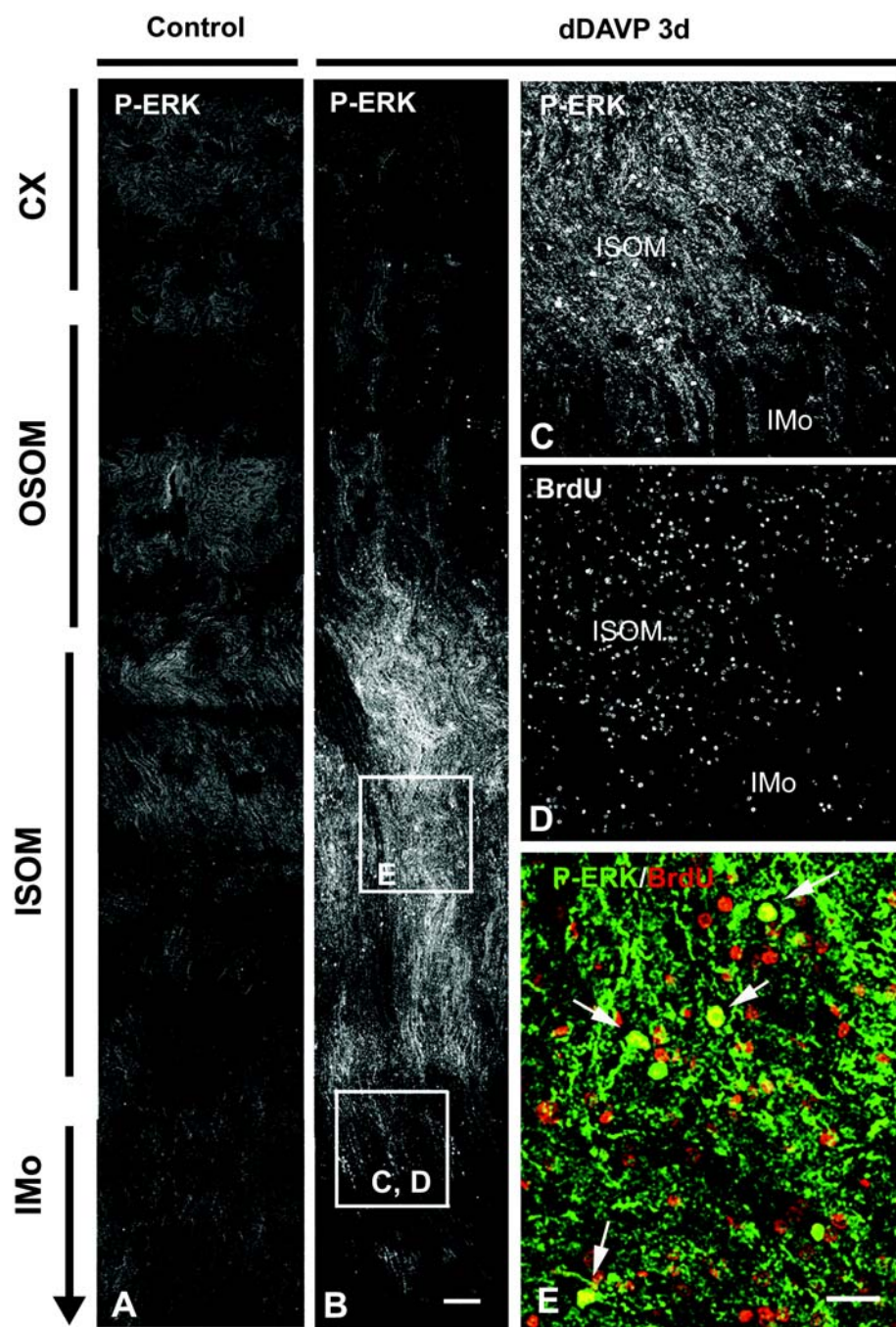


Fig. 8