4-2013

Localizations of 11β-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptors in the rat hypothalamus

Remi Wilson

Localizations of 11β-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptors in the rat hypothalamus

by

Remi Wilson

Undergraduate honors thesis under the direction of

Dr. Ryoichi Teruyama

Department of Biological Sciences

Submitted to the LSU Honors College in partial fulfillment of
the Upper Division Honors Program.

April, 2013

Louisiana State University
& Agricultural and Mechanical College
Baton Rouge, Louisiana

Abstract

Mineralocorticoid receptor (MR) was found in vasopressin (VP) and oxytocin (OT) synthesizing magnocellular cells in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) in the hypothalamus. Aldosterone exerts its biological effect via MR; however, glucocorticoids also have high binding affinity to MR and substantially higher concentrations than aldosterone concentrations in the brain. Therefore, glucocorticoid, instead of aldosterone, could activate MR in the brain. An enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), converts glucocorticoids into inactive metabolites that increases aldosterone selectivity. The presence of 11β-HSD2 has been identified in the hypothalamus; however, the specific cell type(s) that express this enzyme is/are not known. The present study, which used immunocytochemical techniques, was conducted to elucidate whether 11β-HSD2 and MR are colocalized in VP and OT synthesizing magnocellular cells. Double immunofluorescence confocal microscopy demonstrated that MR and 11β-HSD2 immunoreactivities were found in both VP- and OT-immunoreactive magnocellular cells. Co-localization of MR and 11β-HSD2 in VP and OT synthesizing magnocellular cells suggest that aldosterone, not glucocorticoids, directly affects the activity of VP- and OT-neurons through MR.

Introduction

Vasopressin (VP) and oxytocin (OT) are peptide hormones secreted from the posterior pituitary. The importance of VP is to conserve water by reducing urine output by binding to VP receptors in the distal tubules of the kidney to promote water reabsorption. The secretion of VP increases in response to hyperosmolality, hypovolemia, and hypotension, which, in turn, produces antidiuretic and pressor effects (Sladek, 2000). In addition to the well-known effects of

OT such as contraction of the uterus and mammary gland during parturition and milk letdown, respectively, OT also plays a significant role in cardiovascular homeostasis. Plasma OT increases with hyperosmolality and hypernatremia (Huang, Lee, & Sjoquist, 1995) and induces natriuresis (sodium excretion) (Conrad, Gellai, North, & Valtin, 1986).

Aldosterone is secreted from the adrenal cortex and is regulated by hypotension sensed by the concentration of K^+ ions in extracellular fluids (Connell & Davies, 2005). Aldosterone through the mineralocorticoid receptor (MR) plays an important role in regulating electrolyte-water balance (Conrad, et al., 1986). In the kidney, the binding of aldosterone to MR stimulates reabsorption of Na^+ (Benos, Awayda, Ismailov, & Johnson, 1995), thereby, increasing water retention and blood pressure (Gomez-Sanchez, 1986; Gomez-Sanchez, Fort, & Gomez-Sanchez, 1990).

Our previous study has identified MR in VP- and OT-neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) in the hypothalamus (Teruyama, Sakuraba, Wilson, Wandrey, & Armstrong, 2012). Despite these findings, aldosterone target sites in the brain have been difficult to understand because it is unclear whether aldosterone or glucocorticoids activate MR in VP- and OT-neurons. MR has roughly the same affinity for glucocorticoids and aldosterone (Sheppard & Funder, 1987). Moreover, because glucocorticoids have a significantly higher concentration than aldosterone in the brain (Geerling, Kawata, & Loewy, 2006), glucocorticoids may occupy the majority of MR in the brain due to competitive binding. To promote the binding of aldosterone to MR, the enzyme, 11β -hydroxysteroid dehydrogenase type 2 (11β-HSD2), in the immediate vicinity of MR, converts glucocorticoids into inactive metabolites. Therefore, the presence of 11β-HSD2 indicates the MR is activated by aldosterone rather than glucocorticoid in the cells. The enzyme, 11β-HSD2 has been identified in

the hypothalamus, but the specific cell type(s) that express this enzyme is/are unknown. Therefore, the objective of the present study is to find out whether VP and OT synthesizing magnocellular cells are aldosterone sensitive. For the first time we show that MR and 11β-HSD2 are co-localized within VP- and OT-neurons using immunocytochemistry to indicate that these magnocellular cells are aldosterone sensitive.

Methods and Materials

Animals

Male Wister-Kyoto rats were used (180-210 g; Harlan Laboratories, Indianapolis). The rats were housed in a room on a 12:12-h light-dark cycle, with access to food and water available ad libitum. The Institutional Animal Care and Use Committees at Louisiana State University approved all protocols.

Tissue Process for Immunohistochemistry

Rats were deeply anesthetized with ketamine/xlyazine and perfused with perfusion buffer followed by 4% paraformaldehyde. After decapitation, the heads were soaked overnight in 4% paraformaldehyde solution. The following day, the brains were extracted and left to soak in 0.1M phosphate buffer with 20% sucrose overnight for cryoprotection. The brains were sectioned at 40 μm using a freezing microtome (SM2010R, Leica) and used for all immunocytochemical stainings.

Immunohistochemistry of MR and 11ß-HSD2

For immunocytochemical localization of MR, the brain sections were incubated with the

monoclonal MR antibody (MRN3 3F10-s: developed by Gomez-Sanchez and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242) at 1:100 dilution. For immunocytochemical localization of 11ß-HSD2, the sheep polyclonal antibody against 11ß-HSD2 (catalog #AB1296, Millipore, Temecula, CA) was used at 1:40K dilution. These brain sections were subsequently incubated with biotinylated goat anti-mouse IgG (for MRN3) or rabbit anti-sheep IgG (for 11βHSD2) secondary antibody and processed for avidin-biotin complex staining method according to the manufacture's recommendation (Vectastain ABC system, Vector Lab., Burlingame, CA). Finally, immunogens were visualized with 3,3-diaminobenzidine/ (DAB). All antibodies and reagents are diluted in phosphate buffered saline with 0.5% Triton x-100 (PBST). Brain sections were washed 5 min with PBST 3 times between each step. Brain sections were then mounted on gelatin coated slides, approximately 6 sections per slide, and air-dried overnight. The following day, sections were dehydrated in serial concentrations of ethanol and cleared in xylene. Lastly, slides were cover-slipped using paramount and observed under the microscope.

Double Immunofluorscence Microscopy

MR and VP or OT in the SON and PVN

Rabbit polyclonal antibodies to VP (Rob-VP) and OT (Rob-OT), provided by Alan Robinson, UCLA, were used to detect VP- and OT neurons. One set of brain sections were incubated with Rob-VP at 1:20,000 in PBST overnight. Other set were incubated with Rob-OT at 1:10,000 in PBST overnight. Subsequently, both sets of brain sections were incubated with goat anti-rabbit IgG conjugated with DyLight 594 fluorescence marker at 1:200 dilution overnight.

Both sets of brain sections were then incubated in the same MR antibody at the same dilution as described above. Finally, these sections were treated with goat anti-mouse IgG DyLight 488 fluorescense marker at 1:200 dilution overnight.

MR and 11β-HSD2 in the SON and PVN

Brain sections were treated with MR antibody and secondary antibody as described above. Subsequently, the brain sections were incubated with 11β-HSD2 primary antibody at 1;40,000 dilution for 2 day followed by incubation with goat anti-sheep IgG DyLlight 594 fluorescence marker at 1:200 dilution overnight.

All sections were washed 3 times for 5 minutes in PBST after each staining procedure. Finally, all brain sections were mounted, preferably 2 sections per slide, and cover-slipped with 1,4-diazabicyclo[2.2.2]octane (DABCO). All fluorescence stains were viewed using a Leica TCS-SP2 spectral confocal microscope. The optical section thickness was 1 μm. The photomicrographs were generated in stacks of 3-5 sections using ImageJ software (NIH).

Results

Immunohistochemical Localization of MR and 11β-HSD2 in the SON and the PVN

Prominent immunoreactivities to MR and 11β-HSD2 were found in magnocellular cells of the SON and PVN. In the hypothalamus, 11β-HSD2 immunoreactive cells were found only in the SON and PVN (Fig. 1, A). Using a high power objective lens, it was clear that immunoreactive products filled the entire cell bodies of neurons in the SON and PVN. There were numerous cell nuclei that were filled dark with immunoreactive products throughout the entire brain section (Fig. 1, A). However, there were no cells, other than neurons in the SON and

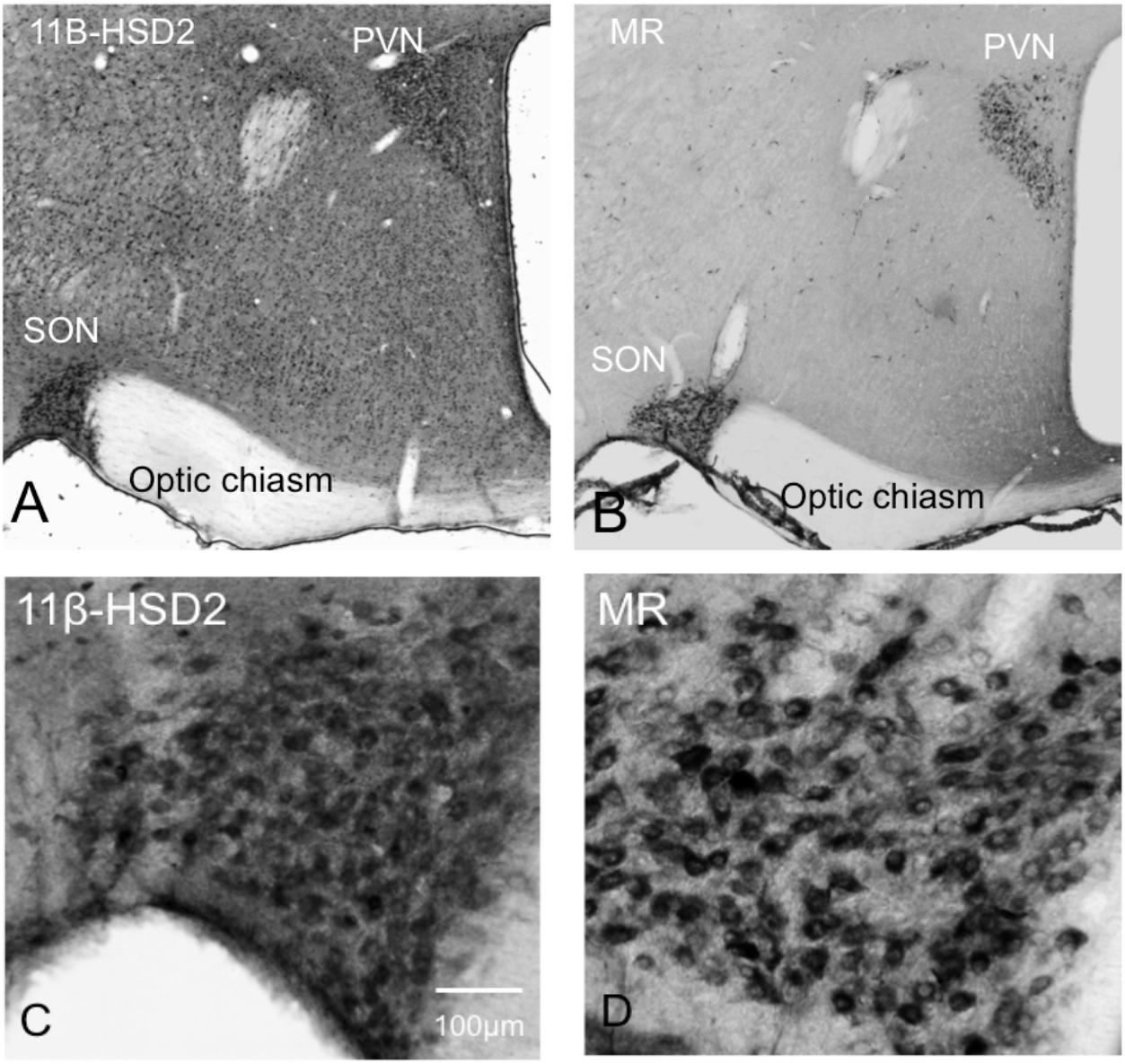

Fig. 1. Prominent immunoreactivities to MR and11β-HSD2 were found in the cytoplasm of magnocellur cells in SON and PVN in the hypothalamus. *A*: Immunoreactive products were visualized in the nuclei of the cells (black dots); however, only in the SON and PVN intense immunoreactivity of the cell body was visualized. *B:* MR immunoreactivity was only associated with the SON and PVN. *C*: A higher power images of the SON in A: the 11-βHSD2 immunoreactive products filled the magnocellular neurons in the SON. *D*: A higher power image of the SON in B: the MR immunoreactivites are clearly demonstrated in the mganocelular neurons in the SON.

PVN that contain cytoplasmic immunoreacitivities (Fig. 1, C). Therefore, this observation suggests these are a result of nonspecific binding of the nuclei.

The immunoreactivity to MR is clearly shown to associate in the SON and PVN (Fig. 1,

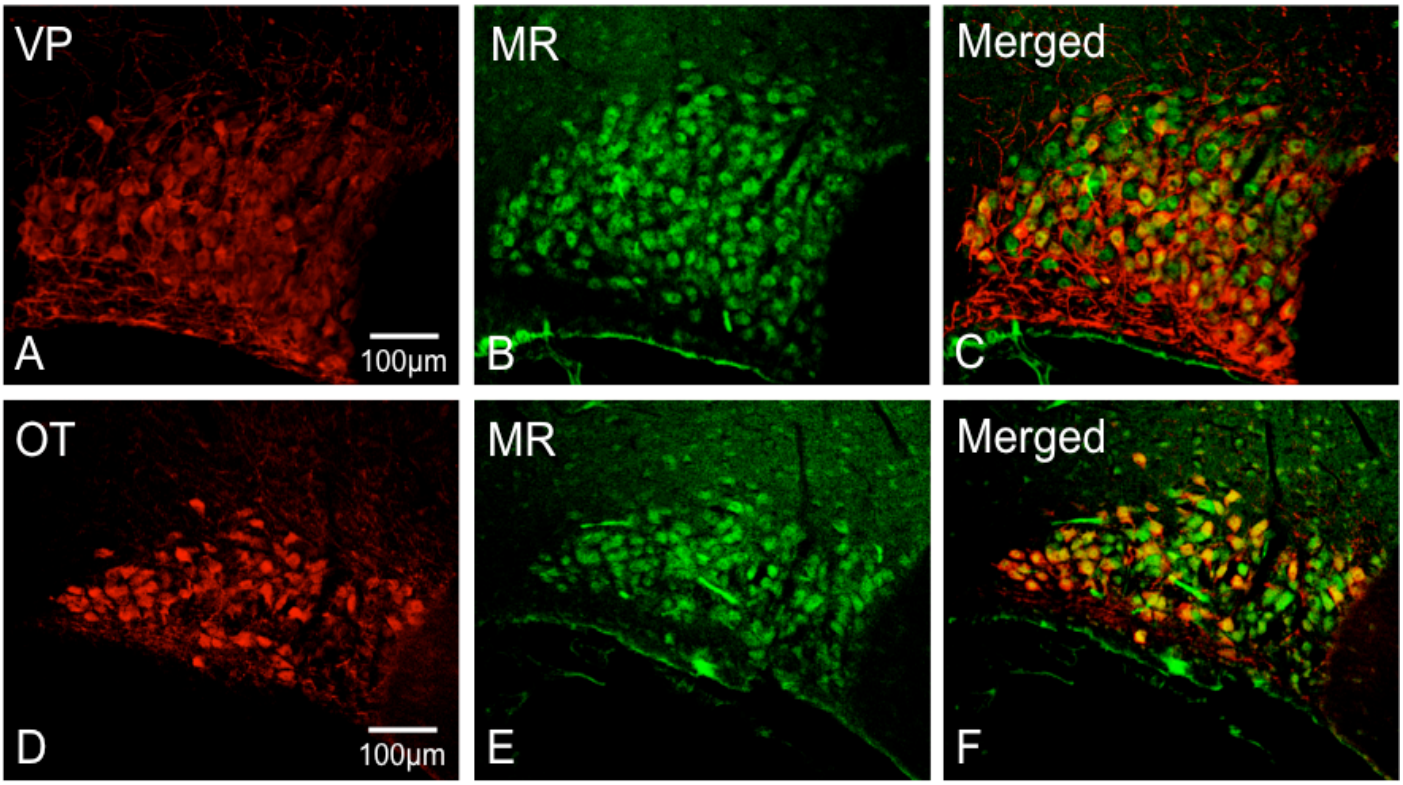

Fig. 2. Double Immunofluorescence Photomicrographs of MR- and VP- and OT-immunoreactivities in the SON. *A* and *D*: VP- and OT-immunoreactivity labeled with DyLight 594 (red)-conjugated secondary antibody. *B and E*: MR immunoreactivity labeled with DyLight 488 (green)-conjugated secondary antibody. MR immunoreactivity is confined largerly to the cell body of magnocellular cells. *C* and *F*: The merged images revealed that MR immunoreactivity is colocalized (yellow) with both VP- and OT-immunoreactivities in magnocellular cells in the SON. Note all VP- and OT-immunoreactive neurons are immunoreactive to MR in the SON.

B). Using a high power objective lens, the immunoreactivity of MR was also visualized in the cytoplasm of magnocellular cells (Fig.1, D). Vasopressin- and oxytocin-synthesizing magnocellular cells are the only cell types in the SON; therefore, this observation suggests that MR immunoreactivity is localized in magnocellular cells.

Double Immunocytochemical Localization of MR, Vasopressin, and Oxytocin in the SON and the PVN

The immunoreactivity to MR is colocalized with either VP- or OT-immunoreactivity in the magnocellular neurons in the SON. The colocalization is designated by the yellow fluorescence of the merged image (Fig, 2, C and F). Most importantly, all VP- and OT-

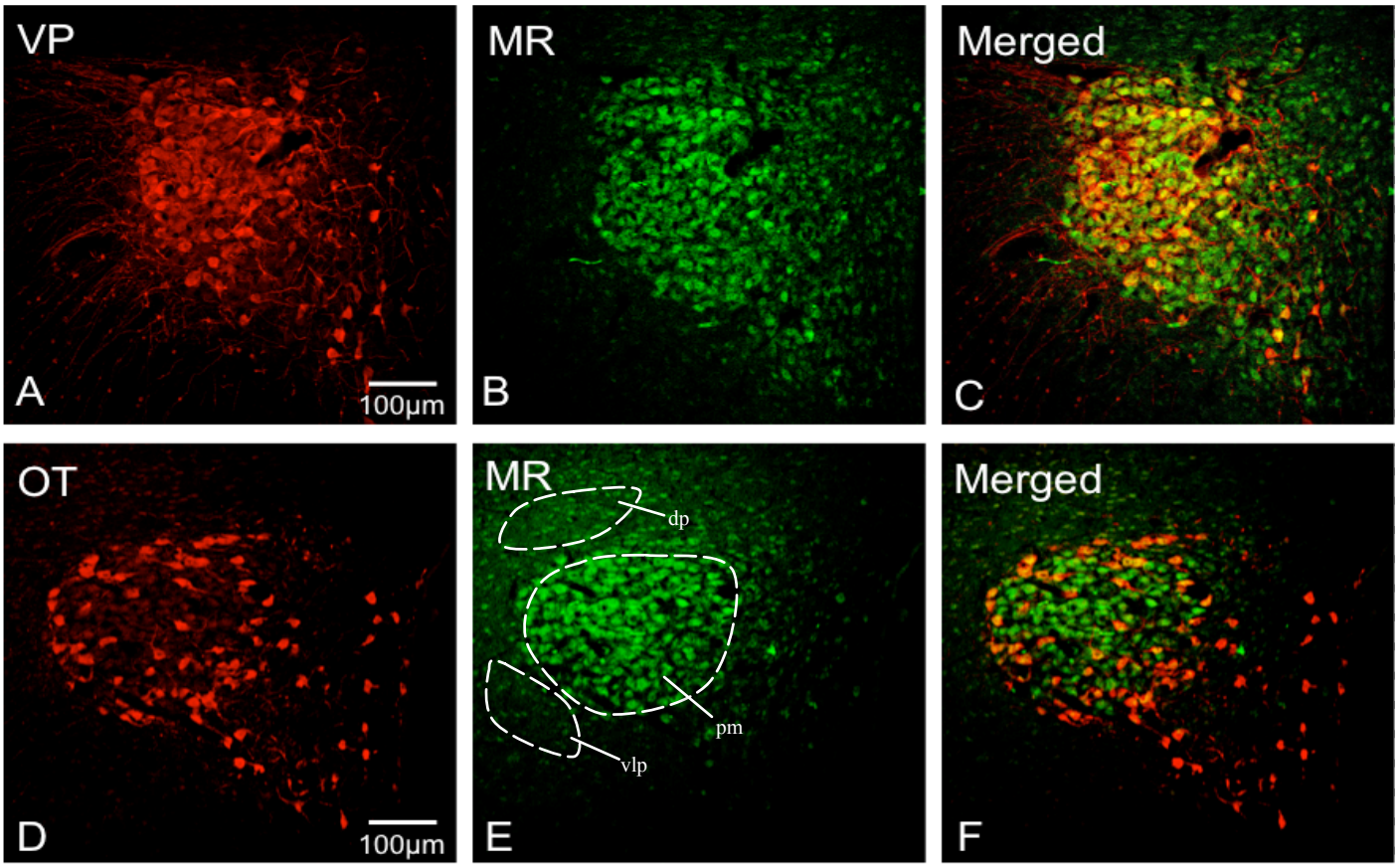

Fig. 3. Double Immunofluorescence Photomicrographs of MR- and VP- and OT-immunoreactivities in the PVN. *A* and *D*: VP- and OT-immunoreactivity, labeled with DyLight 594 (red)-conjugated secondary antibody, is located in a cluster in the posterior magnocellular region (pm). *B and E*: MR immunoreactivity labeled with DyLight 488 (green)-conjugated secondary antibody, is also visualized in the posterior magnocelluar region. *E*: Considerable MR immunoreactivity was also observed in the parvocellular cells in dorsal (dp), ventrolateral (vlp), and posterior parvocellular regions. Note these regions were not immunoreactive to VP or OT. *C* and *F*: the merged images shows that MR immunoreactivity is colocalized (yellow) with both VP- and OT-immunoreactivities within magnocellular cells in the PVN.

immunoreactive neurons are immunoreactive to MR in the SON (Fig. 2). In the PVN, MR immunoreactivity was located in a cluster of VP-immunoreactive neurons in the posterior magnocellular region (Fig. 3, A). OT immunoreactive neurons are spatially distributed throughout the PVN and appear to have a smaller number of colocalized immunoreactivity (Fig. 3, F). Another cell type, parvocellular neurons, is also present in the dorsal, ventrolateral, and posterior parvocellular subregions of the PVN (Fig. 3, E). These parvocellular neurons were also immunoreactive to MR, but not to VP or OT.

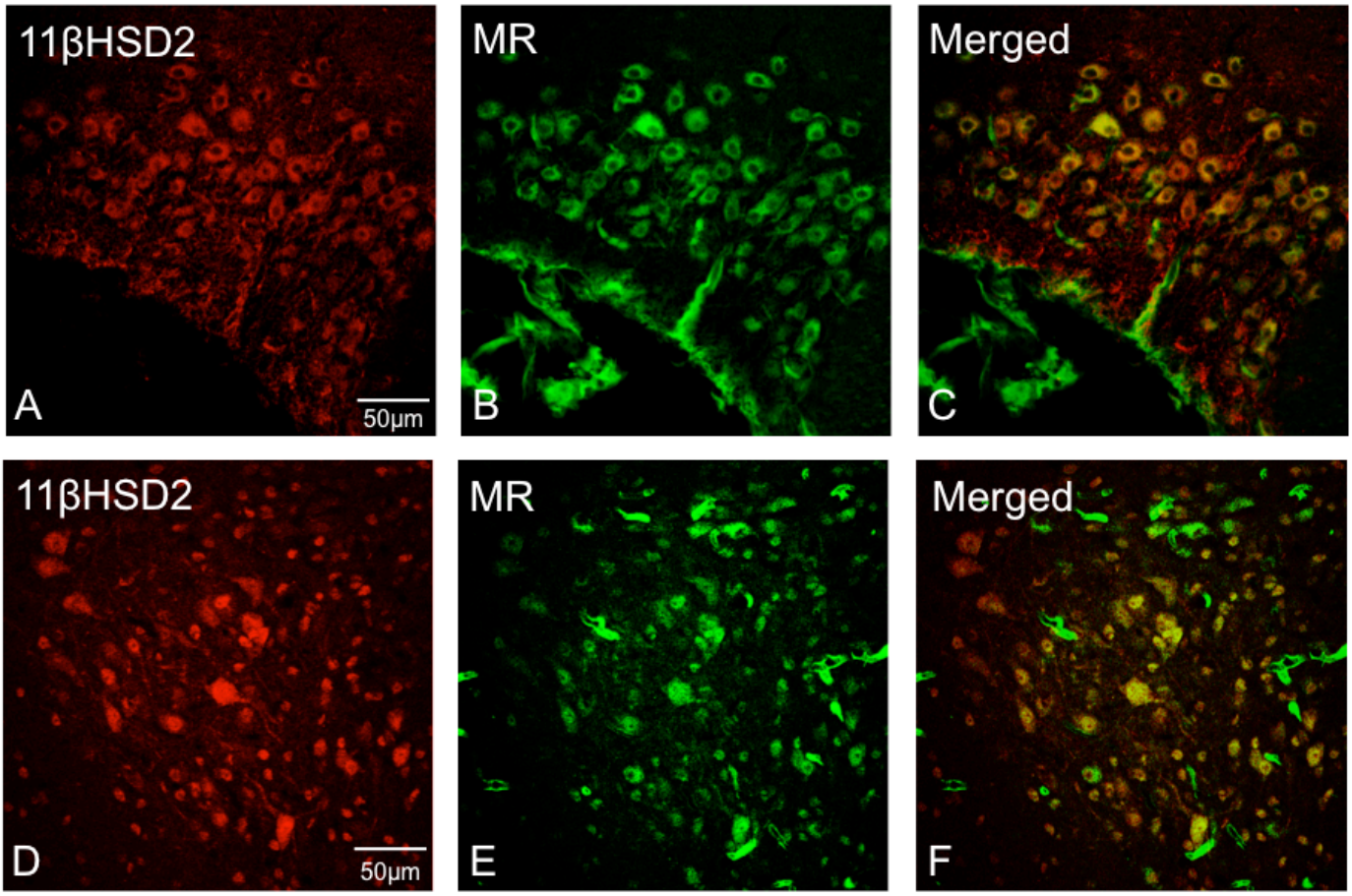

Fig. 4. Double Immunofluorescence Photomicrographs of MR- and 11β-HSD2-immunoreactivities in the SON (A-C) and the PVN (D-F). *A and D*: 11β-HSD2 immunoreactivity labeled with DyLight 594 (red). 11β-HSD2 immunoreactivity is largely confined to the cell body of magnocellular cells. *B and E*: MR immunoreactivity labeled with DyLight 488 (green). MR immunoreactivity is also confined largely to the cell body *B*: Note that magnocellular cells is the only cell type in the SON. *C and F*: merged images revealed that MR- and 11β-HSD2-immunoreactivities are colocalized in magnocellular neurons in the SON and PVN, indicated by the yellow fluorescence.

Double Immunocytochemical Localization of MR and 11β-HSD2

The immunoreactivity to 11β-HSD2 was largely confined to the cell body of magnocellular cells in the SON and PVN (Fig. 4, A and D). Most of the cells immunoreactive to 11β-HSD2 were shown to colocalize with MR immunoreactive cells. These cells were visualized by yellow fluorescent immunoreactivity in the merged image (Fig. 4, C and F). In the PVN, immunoreactivity to 11β-HSD2 was observed in the posterior magnocelluar region (Fig. 4, D).

Discussion

Based on the colocalized immunoreactivity of MR and 11β-HSD2 in magnocellular cells in the SON and PVN, we conclude that MR and 11β-HSD2 proteins are colocalized within VP- and OT-neurons in the hypothalamus. The colocalization of MR and 11β-HSD2 proteins within these magnocellular cells is important because now we know that magnocellular cells elicit a response due to aldosterone binding to MR. Therefore, VP- and OT-neurons are aldosterone sensitive.

The release of VP and autonomic nervous system activity are the major mechanisms by which the central nervous system (CNS) influences blood pressure. VP neurons not only regulate the release of its hormone, but also affect the autonomic nervous system. Therefore, VP neurons play a pivotal role in the regulation of blood pressure.-Abnormally elevated plasma levels of VP and OT were observed in salt-sensitive hypertensive individuals, and the excess of VP is implicated in the pathogensis of many cardiovascular diseases. In a study, a high-salt diet or intracerebroventricular (ICV) infusion of the mineralocorticoid, aldosterone, causes hypertension in salt-sensitive Dahl (Dahl-SS) rats; however, ICV infusion of a specific mineralocorticoid receptor (MR) antagonist prevents the hypertension in these rats (Gomez-Sanchez, Fort, & Thwaites, 1992). Moreover, ICV infusion of glucocorticoid at an equimolar concentration as the hypertensinogenic dose of aldosterone did not alter blood pressure, in fact, it mitigated the increase in blood pressure produced by the ICV infusion of aldosterone (Gomez-Sanchez, Venkataraman, Thwaites, & Fort, 1990). These studies demonstrated that aldosterone activating MR in the CNS causes an increase in blood pressure (Gomez-Sanchez et al., 1990a). Considering the functions of VP and OT in cardiovascular homeostasis, the presence of MR and 11β-HSD2 in these cells strongly suggest that these neurons are, at least partly, responsible for aldosterone-mediated regulation of blood pressure. These findings contribute to understanding the etiology of

hypertension and future pharmacological interventions.

References:

Benos, D. J., Awayda, M. S., Ismailov, II, & Johnson, J. P. (1995). Structure and function of amiloride-sensitive Na+ channels. *J Membr Biol, 143*(1), 1-18.

Connell, J. M., & Davies, E. (2005). The new biology of aldosterone. [Review]. *The Journal of endocrinology, 186*(1), 1-20.

Conrad, K. P., Gellai, M., North, W. G., & Valtin, H. (1986). Influence of oxytocin on renal hemodynamics and electrolyte and water excretion. *Am J Physiol, 251*(2 Pt 2), F290-296.

Geerling, J. C., Kawata, M., & Loewy, A. D. (2006). Aldosterone-sensitive neurons in the rat central nervous system. *J Comp Neurol, 494*(3), 515-527.

Gomez-Sanchez, E. P. (1986). Intracerebroventricular infusion of aldosterone induces hypertension in rats. *Endocrinology, 118*(2), 819-823.

Gomez-Sanchez, E. P., Fort, C., & Thwaites, D. (1992). Central mineralocorticoid receptor antagonism blocks hypertension in Dahl S/JR rats. *Am J Physiol, 262*(1 Pt 1), E96-99.

Gomez-Sanchez, E. P., Fort, C. M., & Gomez-Sanchez, C. E. (1990). Intracerebroventricular infusion of RU28318 blocks aldosterone-salt hypertension. *Am J Physiol, 258*(3 Pt 1), E482-484.

Gomez-Sanchez, E. P., Venkataraman, M. T., Thwaites, D., & Fort, C. (1990). ICV infusion of corticosterone antagonizes ICV-aldosterone hypertension. *Am J Physiol, 258*(4 Pt 1), E649-653.

Huang, W., Lee, S. L., & Sjoquist, M. (1995). Natriuretic role of endogenous oxytocin in male rats infused with hypertonic NaCl. *Am J Physiol, 268*(3 Pt 2), R634-640.

Sheppard, K. E., & Funder, J. W. (1987). Equivalent affinity of aldosterone and corticosterone for type I receptors in kidney and hippocampus: direct binding studies. *J Steroid Biochem, 28*(6), 737-742.

Sladek, C. D. (2000). Antidiuretic Hormone: Synthesis and Release. In J. C. S. Fray (Ed.), *Handbook of Physiology Section 7* (Vol. Endocrinology Volume III: Hormonal Regulation of Water and Electrolyte Balance, pp. 436-495): Oxford University Press.

Teruyama, R., Sakuraba, M., Wilson, L. L., Wandrey, N. E., & Armstrong, W. E. (2012). Epithelial Na(+) sodium channels in magnocellular cells of the rat supraoptic and paraventricular nuclei. [In Vitro Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *American journal of physiology. Endocrinology and metabolism, 302*(3), E273-285.