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Neurosci Lett. Author manuscript; available in PMC 2009 October 27.

Published in final edited form as:

Neurosci Lett. 2007 October 22; 426(3): 160–165. doi:10.1016/j.neulet.2007.08.066.

Role of neuronal nitric oxide in the regulation of vasopressin expression and release in response to inhibition of catecholamine synthesis and dehydration

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Abstract

We used neuronal nitric oxide synthase (nNOS) gene knockout mice to study the effects of catecholamines and neuronal nitric oxide on vasopressin expression in the hypothalamic neurosecretory centers. nNOS gene deletion did not change the level of vasopressin mRNA in the supraoptic or paraventricular nuclei. In contrast, vasopressin immunoreactivity was lower in nNOS deficient mice than in wild-type animals. Dehydration increased vasopressin mRNA levels and decreased vasopressin immunoreactivity in both wild-type and nNOS knockout mice, but these responses were more marked in the nNOS knockout mice. Treatment with α -mpt, a pharmacologic inhibitor of catecholamine synthesis, resulted in increased vasopressin mRNA levels in wild-type mice and in reduced vasopressin immunoreactivity in both wild-type and nNOS knockout mice. From these results, we conclude: (1) neuronal nitric oxide suppresses vasopressin expression under basal conditions and during activation of the vasopressin-ergic system by dehydration; (2) catecholamines limit vasopressin expression; (3) nNOS is required for the effects of catecholamines on vasopressin expression.

It is well known that vasopressin (VP) plays a major role in the regulation of body water and osmolality (1). VP is primarily synthesized by magnocellular neurons in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. It is then transported down the axons to the posterior neurohypophysis, where it is released into the systemic circulation. The biosynthesis and release of VP is regulated by plasma osmotic pressure and blood pressure or blood volume (2). Catecholamines influence VP synthesis and release by mechanisms that are still not fully understood. Catecholaminergic neurons make synaptic contacts with VP-ergic neurons in the hypothalamus (3) and all three types of adrenoreceptors (α 1, α 2, β) are found on SON and PVN neurons (4). Catecholamines have been reported both to activate and inhibit VP synthesis and release (5,6). Nitric oxide (NO) has also been reported to modulate VP

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synthesis and release, both in a stimulatory and an inhibitory manner (7). Based on this published data, we hypothesize that NO and catecholamines can both modulate VP expression, especially when VP synthesis is upregulated by dehydration. In this paper, we study the interaction between catecholamines and neuronal NO in VP synthesis and expression, using neuronal nitric oxide synthase (nNOS) gene knockout mice.

All procedures were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. WT (C57BL/6J) mice (Jackson Labs) and nNOS knockout mice lacking exon 2 (8) were housed under diurnal lighting conditions and allowed food and water ad libitum. We used 20 adult male WT and 20 nNOS knockout mice, 10-12 weeks of age, body weight 20-30 g. WT and nNOS knockout mice were divided into four groups of five animals. Mice in the first group (controls) had free access to food and water. Mice in the second group were deprived of water for 5 days. Mice in the third group received daily intraperitoneal injections of α -mpt, a blocker of catecholamine synthesis (9), for five days at a dose of 100 mg/kg body weight. Mice in the fourth group were deprived of water for five days, and received daily intraperitoneal injections of α -mpt.

The hypothalamic areas were dissected, fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Paraffin tissue blocks were cut into 8 μ m-thick frontal sections for in situ hybridization and 5 μ m-thick frontal sections for immunohistochemistry. The sections containing the SON and PVN, and zona inserta (ZI) were sequentially processed for the in situ hybridization and immunostaining procedures.

The VP probe was generated from a 229-bp Dra/Pst cDNA fragment. It binds to the 3'-end of the VP mRNA. This fragment was inserted into a pGEM1 vector (gift of Prof. H. Gainer, NINDS, NIH). The plasmid was linearized by HindIII and used as template for synthesis of antisense mRNA probe. Digoxigenin-labeled probe was prepared from VP cDNA templates using digoxigenin-UTP (Boehringer Mannheim). Hybridization was performed with digoxigenin-labeled VP riboprobes using standard protocols as previously described (10).

To visualize digoxigenin-staining, sections were rinsed twice in buffer 1 (100 mM Tris-HCl/150 mM NaCl, pH 7.5) for 10 min, followed by 30 min incubation in modified blocking solution (buffer 1 containing 0.5 % bovine serum albumin and 1 % blocking reagent, Boehringer Mannheim). They were then incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antiserum (Boehringer Mannheim; 1:500 in modified blocking solution). Sections were consecutively rinsed twice in buffer 1 for 15 min, and in buffer 2 (100 mM Tris-HCl/100 mM NaCl/10 mM MgCl₂, pH 9.5) for 3 min. Finally, sections were incubated overnight at RT in the dark in buffer 2 containing 0.34 mg/ml nitroblue tetrazolium salt and 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (NBT/BCIP; Boehringer Mannheim), rinsed in buffer 3 (100 mM Tris-HCl/1 mM EDTA) and coverslipped with carbonate buffered glycerol (0.5 M carbonate buffer, pH 8.6/50 % glycerol).

After deparaffinization and rehydration, slides containing 5 μ m-thick frontal sections of hypothalamic region were incubated in peroxidase blocking solution (3% H₂O₂ in PBS) for 15 min. Staining of experimental and control slides was performed simultaneously in the same solutions. The peroxidase-antiperoxidase complex (PAP) method was used for VP (1:100) and tyrosine hydroxylase (TH, 1:500) immunostaining. After peroxidase blocking, the slides were rinsed in PBS, incubated in 10% sucrose in PBS, washed in PBS and placed in blocking buffer (2% normal goat serum in PBS) for 1 hour. The sections were incubated successively with: primary rabbit antibody against VP or TH overnight; goat anti-rabbit IgG (1:100) for 1 h; and PAP complex (1:100) for 1 hour. Finally, the sections (without additional counterstaining) were dehydrated and mounted with coverslips. All immunohistochemical reactions were

carried out in parallel with reactions lacking primary antibody to ensure the specificity of the observed staining.

Digoxigenin-labeled VP-mRNA level and VP-immunoreactivity in neurosecretory cells were quantified by optical density measurements using a digital video analysis system (VideoTest) (11). 5-6 slices of SON and the magnocellular part of PVN were analyzed from each mouse. The number of mice in each experimental group was five. VP-immunoreactivity was measured at 550 nm. Optical density of digoxigenin-labeled VP-mRNA was estimated at 650 nm in adjacent slices. Optical density was calculated as gray level of immunoreactive brain tissue field minus background grey level. Optical density of background was estimated using a non-immunoreactive brain tissue field in the same section. The results are presented in relative units of optical density at μm^2 . Measurement of VP mRNA in hypothalamic neurons reflects VP synthesis. This data, taken together with VP-immunoreactivity (VP content) allows us to estimate VP release.

The data are expressed as the mean \pm SEM. t-test was used to determine significance among groups. A value of $P < 0.05$ was considered to be statistically significant.

In this paper, we study the interaction between NO and catecholamines, and the involvement of this crosstalk in the regulation of VP expression. nNOS knockout mice are useful tools to define the roles of neuronal NO under stress conditions like water deprivation or depressed catecholamine innervation. While there are pharmacologic inhibitors of nNOS, they are not absolutely specific and have been shown to inhibit the other NOS isoforms (12). In the current study, we used nNOS knockout mice to determine whether long-term NO deficiency would affect VP expression, and how this condition would modulate the effects of dehydration or α -mpt. nNOS knockout mice show normal arterial blood pressure and renal blood flow compared with WT mice (13). Importantly, expression of neurophysin, the carrier protein of vasopressin/oxytocin, is not altered (14).

We found that nNOS gene deletion did not change the optical density of labeled VP mRNA in the SON or PVN (Fig. 1A, 2A). In contrast, VP immunoreactivity was lower in nNOS deficient mice than in WT animals (Fig. 1B, 2B). There is substantial evidence that NO is involved in the regulation of VP synthesis and release (15). Dehydration increases NO synthase activity in the hypothalamus and pituitary (16,17). nNOS expression in the SON and PVN is upregulated by non-osmotic hypovolemia (17). Blockade of NO synthesis increases vasopressin secretion (7). We believe that increased VP mRNA levels and decreased VP immunoreactivity are consistent with increased release of VP from the cell bodies. This claim and methodology is validated by publications in the literature demonstrating that increased mRNA levels for VP, oxytocin and some other hypothalamic neuropeptides indicates increased synthesis. Furthermore, decreased immunoreactivity in the cell bodies suggests increased release into the hypophyseal portal system (10,18-21).

Our results suggest that NO plays a role in suppressing the release of VP. This may occur not only from axonal terminals in the neurohypophysis, but also from neuronal cell bodies. Decreased immunoreactivity and increased mRNA level might represent enhanced release, decreased translation or increased protein degradation. Further studies with measurement of plasma VP levels need to be done to clarify effects on VP release.

To assess the effect of dehydration or α -mpt on VP mRNA contents, we measured optical density of digoxigenin-labeled VP mRNA in SON of WT and nNOS knockout mice. As seen in Figure 1A, in the SON of WT animals, blockade of catecholamine synthesis with α -mpt, dehydration with α -mpt treatment and dehydration (Fig. 1A, 3A, 3B), all increase VP mRNA expression compared with control animals. In nNOS knockout mice, dehydration increased VP mRNA synthesis (Figure 1A, 3C, 3D), but α -mpt treatment did not (Figure 1A).

Administration of α -mpt to nNOS knockout mice during dehydration caused a decrease in VP mRNA level as compared with dehydration alone, but the level was still significantly higher than in untreated nNOS knockout mice (Figure 1A).

In WT mice, treatment with α -mpt, dehydration, and treatment with α -mpt with dehydration all increase VP mRNA levels in PVN (Figure 1A and 2A). Treatment with α -mpt caused some increase in VP mRNA level in nNOS knockout mice, but less than that seen in WT mice. In contrast, dehydration caused a significantly higher increase in VP mRNA in nNOS knockout mice than in WT mice. Finally, α -mpt treatment of dehydrated nNOS knockout mice caused a decrease in VP mRNA level as compared with dehydration alone.

We also measured VP immunoreactivity in the SON and PVN of WT and nNOS knockout mice (Figure 1B, 2B). In WT mice, blockade of catecholamines synthesis with α -mpt (Figure 1B, 2B), dehydration (Figure 1B, 2B, 4A, 4B), and dehydration with α -mpt treatment (Figure 1B, 2B) all moderately reduce VP immunoreactivity. nNOS knockout mice showed less immunoreactive VP in the SON and PVN than in WT mice under control conditions and in all experimental groups. Dehydration caused a marked decrease in VP immunoreactivity in nNOS knockout mice (Figure 1B, 2B, 4C, 4D). Treatment with α -mpt also reduced VP immunoreactivity, but to a lesser extent (Figure 1B, 2B). Finally, the combination of α -mpt treatment and dehydration did not lower VP immunoreactivity any further in nNOS knockout mice (Figure 1B, 2B).

The role of NO in VP content is controversial. NO has been shown both to stimulate or to inhibit VP release (22). Central inhibition of NOS with L-NAME showed decrease in plasma level of VP (23). Our data directly confirm that neuronal NO modulates the VP response to osmotic stimulation. Dehydration increases VP mRNA levels in the PVN and SON of both WT and nNOS knockout mice. This response is more marked in nNOS knockout mice, suggesting that nNOS normally blunts the response to dehydration.

In addition to VP synthesis, NO also appears to play a role in VP release, both under basal conditions, and with dehydration. The decrease in VP immunoreactivity with dehydration, reflecting VP release from nerve terminals, was more marked in nNOS knockout mice. Based on published data (18-21), we interpret the decreased immunoreactivity as a reflection of increased secretion level of VP from the cell bodies. This suggests that nNOS-derived NO blunts the release of VP. In its absence, VP release occurs more readily.

To identify expression of catecholamines, we measured TH immunoreactivity in ZI in WT and nNOS knockout mice. The ZI mainly contains dopaminergic neurons, and is one of the main regions that innervates PVN with dopamine (24). Our results demonstrate that nNOS deficiency led to significant decrease of catecholamines content in the cell bodies (Fig. 5).

We used α -mpt to block catecholamine synthesis. Under the conditions we used, α -mpt reduces catecholamines levels to 14% of control levels (9). Treatment with α -mpt resulted in increased VP mRNA (greater synthesis) levels in SON and PVN of WT mice (Fig. 1A,2A). However the effect was absent or markedly blunted in nNOS knockout mice, where synthesis of catecholamines was significantly diminished (Fig. 5). These results suggest that nNOS is required for the effects of catecholamines synthesis blockade. In this regard, hypothalamic nNOS expression is increased by α -mpt in rats (25). On the other hand, CA modulates VP release from hypothalamo-hypophyseal system (5,6). Therefore, the lack of effect of CA in nNOS knockout mice indicates that catecholamine acts through NOS to modulate VP production. nNOS is main isoform modulating catecholamine secretion and release (26,27). eNOS can also modulate catecholamine secretion and release (27,28), but there is as yet no evidence for a role for iNOS in catecholamine regulation.

We demonstrated that α -mpt treatment reduces VP immunoreactivity in both WT and nNOS knockout mice in PVN and SON neurons. These results suggest that catecholamine synthesis tonically blunts VP release, so that when catecholamine synthesis is blocked by α -mpt, there is more VP release.

In WT mice, dehydration and α -mpt treatment reduce VP immunoreactivity in the SON more than dehydration alone. However, VP mRNA levels were comparable in these two groups. We conclude that catecholamine depression enhances the effects of water deprivation on VP release. This suggests that catecholamines may limit VP release, even under conditions of dehydration, a possible protective mechanism to prevent depletion of VP under conditions that stimulate release.

Dehydration increases VP mRNA levels. Decreased VP immunoreactivity was more marked in nNOS knockout mice. Simultaneous treatment with α -mpt did not change VP protein or mRNA content in nNOS knockout animals, confirming a role for nNOS as a dominant regulator of the effects of water deprivation.

In conclusion, we show that deletion of the nNOS gene is associated with decreased VP protein content in the hypothalamic neurons, suggesting increased release of VP from cell bodies. Neuronal nitric oxide suppresses VP release under basal conditions and during activation of the VP-ergic system by dehydration. Inhibition of catecholamine synthesis results in enhanced VP release, suggesting that catecholamines limit VP release under basal and osmotic stimulation conditions. nNOS is required for the effects of catecholamines on VP synthesis. Thus, nNOS plays important roles in basal VP release, VP responses to dehydration, and the effects of catecholamines on VP synthesis.

Acknowledgments

This work was supported by PHS NS033335, NS048426, and HL057818 from the NIH, and RFBR 05-04-48099-a from the Russian Foundation for Fundamental Research.

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Supraoptic Nucleus

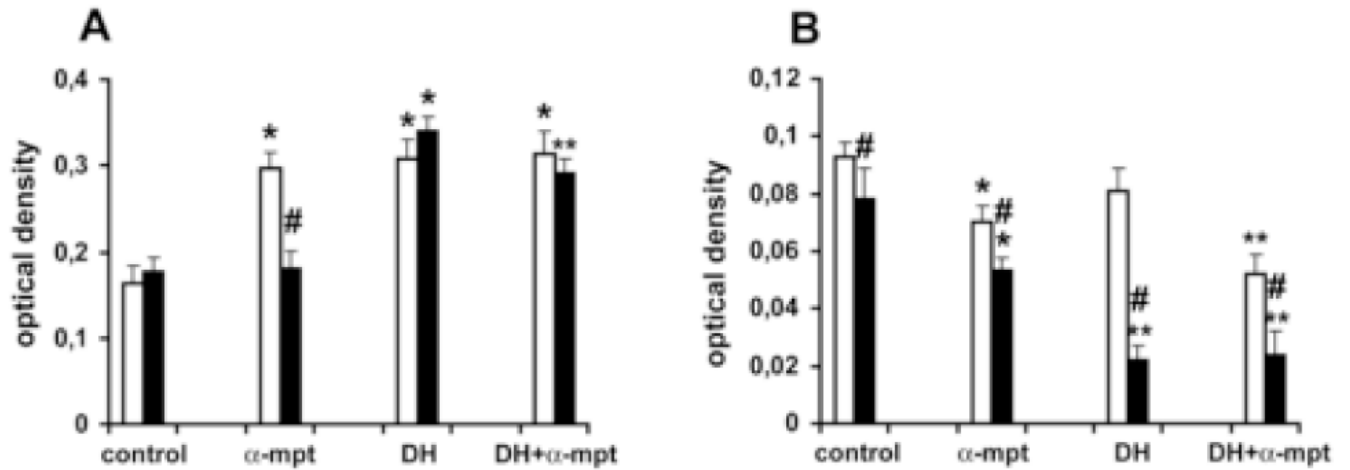


Figure 1.

α -mpt treatment, dehydration, and α -mpt treatment during dehydration increase VP mRNA content and diminish VP immunoreactivity in VP magnocellular neurons of hypothalamic SON. **A.** The content of VP RNA; **B.** VP-immunoreactivity in the SON in WT (white columns) and nNOS knockout mice (black columns). X axis: control – intact mice; α -mpt – injection of catecholamine synthesis blockator α -mpt (100 mg/kg); DH –dehydrated mice; DH+ α -mpt - α -mpt injection (100 mg/kg) during dehydration.

Y axis: optical density (conventional units/ μm^2)

* - $p < 0.05$ compared to control;

** - $p < 0.05$ compared to control and *;

- $p < 0.05$ as compared with WT mice.

Paraventricular Nucleus

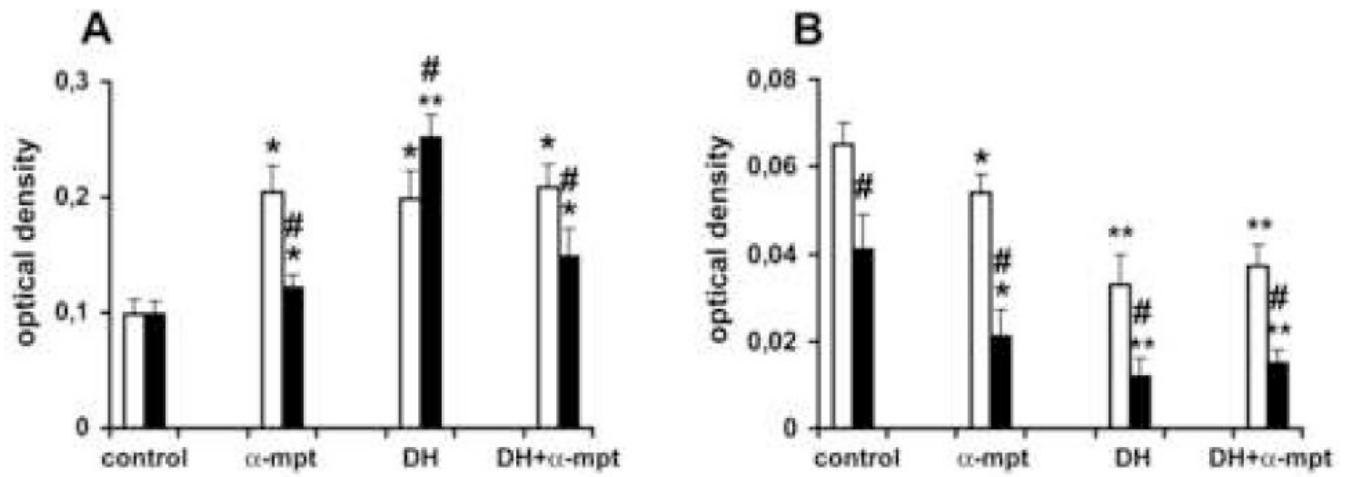


Figure 2. Effects of α -mpt treatment, dehydration, and α -mpt treatment during dehydration on VP expression in PVN of hypothalamus. **A.** The content of VP RNA; **B.** VP-immunoreactivity in the PVN in WT (white columns) and nNOS knockout mice (black columns). X and Y axes, *, **, # are same as that for Figure 1.

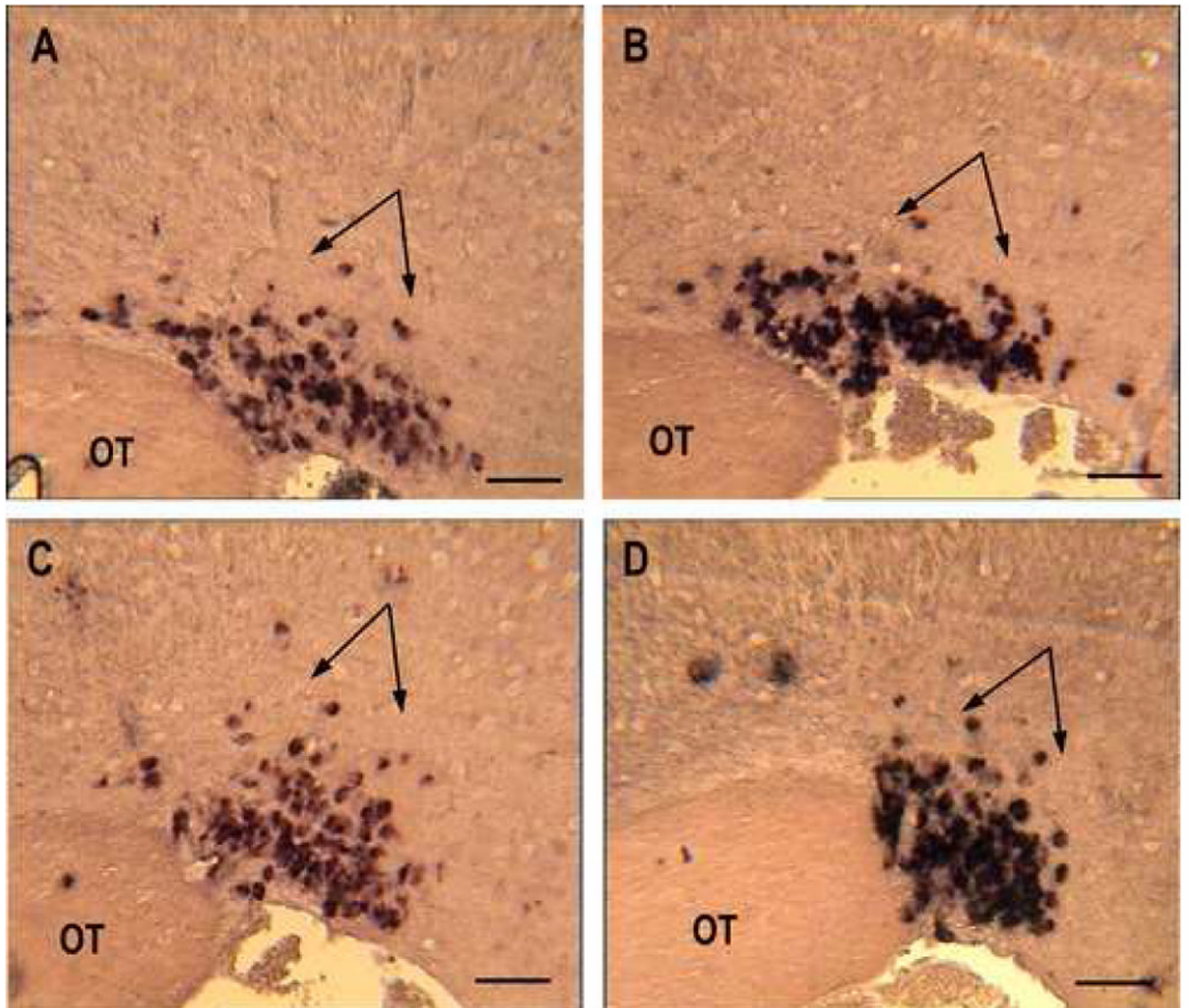


Figure 3. In situ hybridization of VP mRNA with digoxigenin-labeled antisense VP-RNA probe in the SON of nNOS deficient and WT mice. **A, C** – digoxigenin-positive VP mRNA in control group of WT and nNOS knockout mice respectively; and in dehydrated animals: **B** – WT mice, **D** – nNOS knockout mice. Hybridization with sense VP-RNA probe was negative (not shown). Arrows indicate localization of the SON. OT is optical tract. Scale bar is 100 μ m.

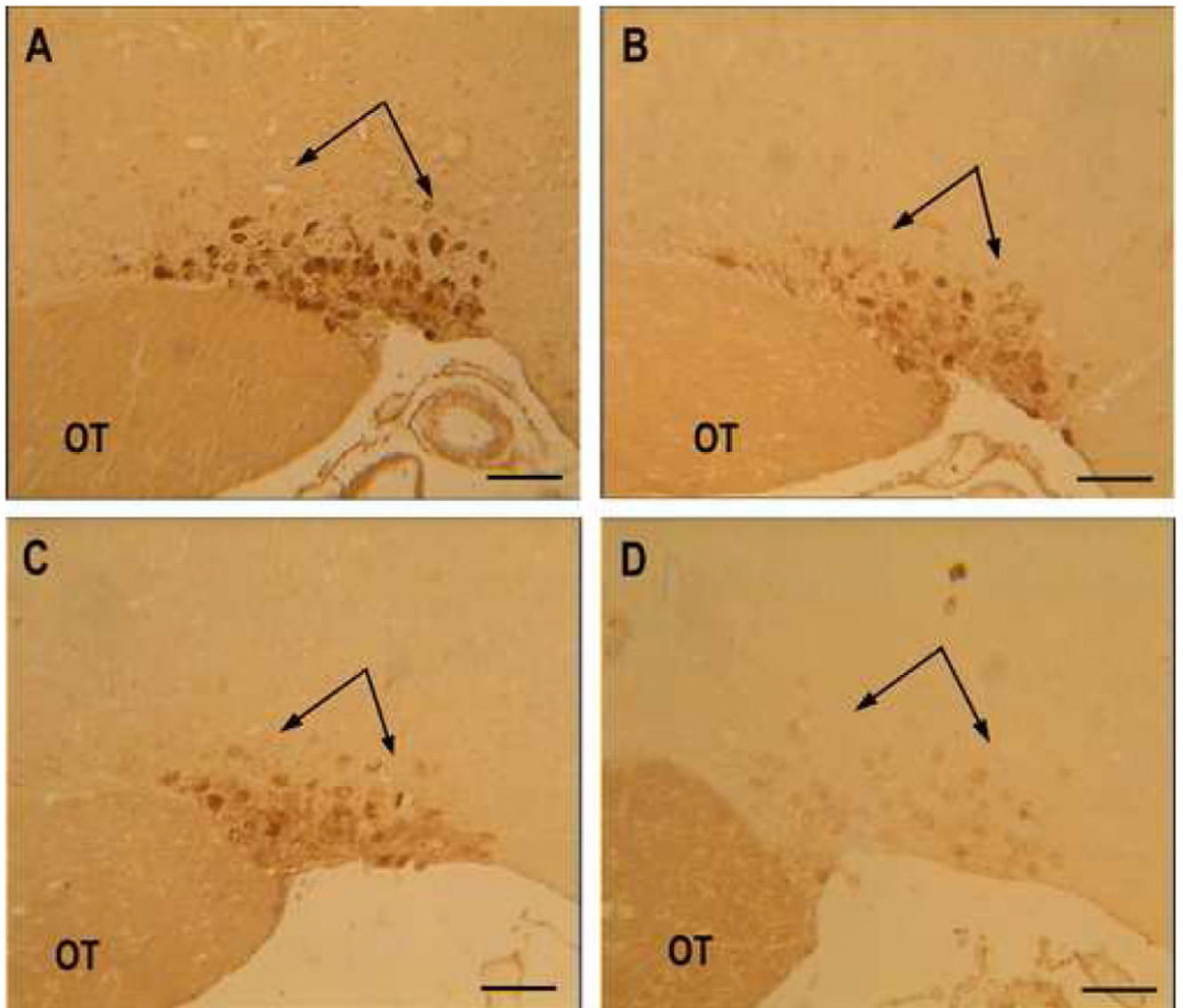


Figure 4. Effect of dehydration on functional activity of VP-ergic cells in the SON. Cytoplasmic stain of VP in neurons of the SON in control WT (A) and nNOS knockout mice (C); and dehydrated animals: B – WT, and D – nNOS knockout mice. Arrows indicate location of SON. Detection of VP was performed without additional counterstaining. OT is optical tract. Scale bar is 100 μm .

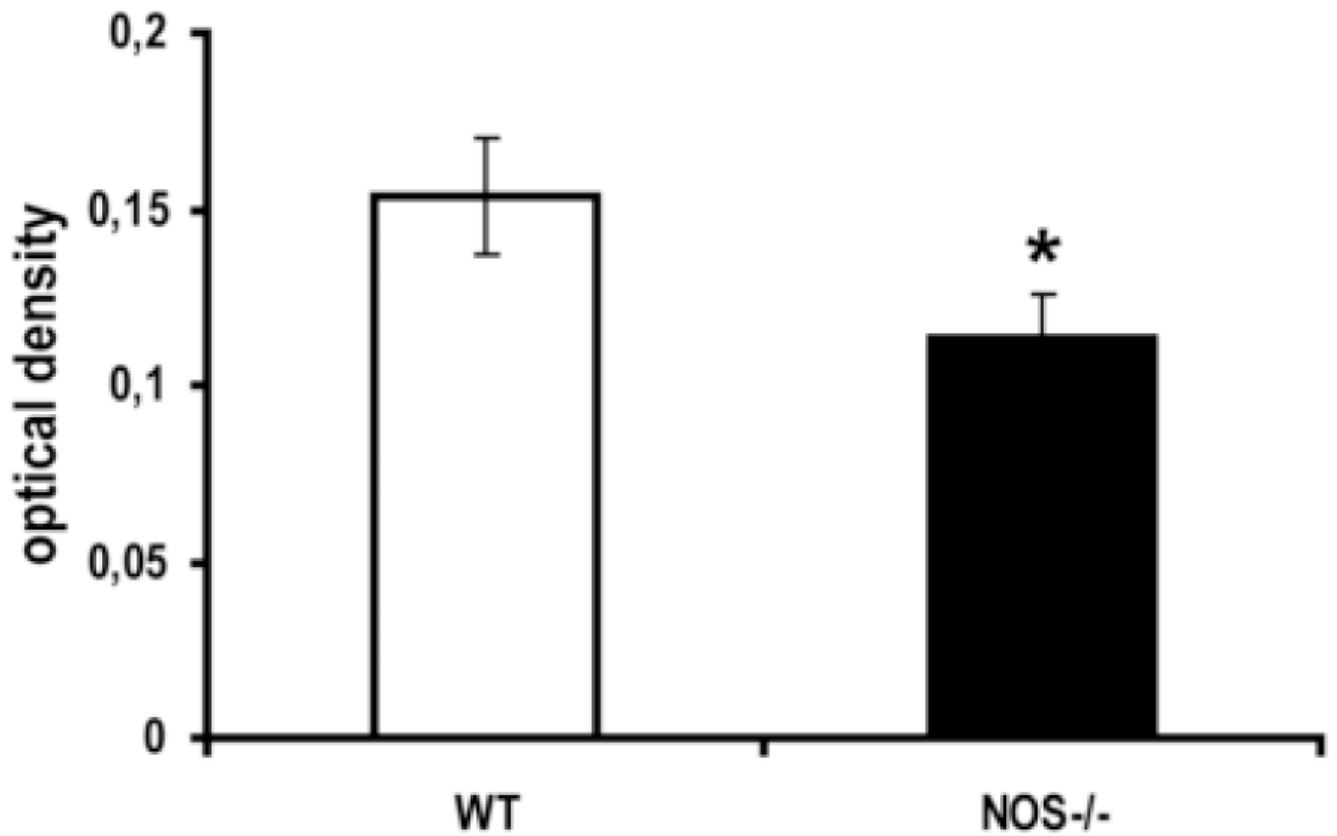


Figure 5. nNOS deficiency leads to decrease of TH protein content in ZI of mouse hypothalamus. Data for optical density is presented as arbitrary units per μm^2 . Axis X: WT (white columns) – intact WT mice; nNOS-/- (black columns) – nNOS knockout intact mice.
* - $p < 0.05$ compared to WT mice.