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Functional Characterization of the Arginine Vasotocin 4 Receptor (VT4R) in Sensory Circumventricular Organs of the Chicken Gallus gallus

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ABSTRACT

Past studies have shown that the avian vasotocin 4 receptor (VT4R), homologous to the mammalian arginine vasopressin receptor 1a (V1aR/AVPR1A) is involved in immobilization stress. It was not known, however, whether the receptor is also associated with osmotic stress, and if so, what brain regions may be involved. Four treatment groups of chicks were used for the study. One group was subjected to 1h immobilization stress and two groups were administered intraperitoneal injection of 3 M NaCl or 0.15 M NaCl. One additional group served as controls. After 1 h, blood samples were taken for the determination of plasma levels of arginine vasotocin and corticosterone by radioimmunoassay. Chick brains were sampled for immunohistochemistry utilizing an antibody, anti-VT4R, and for real time RT-PCR. Plasma corticosterone (CORT) concentrations were significantly increased in the immobilized group ($p < 0.01$) and hypertonic saline group ($p < 0.01$) compared with controls. Plasma arginine vasotocin (AVT) concentrations were significantly increased ($p < 0.01$) in hypertonic saline birds and immobilized birds compared with controls. Intense staining of the VT4R in the organum vasculosum of the lamina terminalis (OVLT) and subseptal organ (SSO) of both treatment groups showed marked morphological changes compared to controls. AT1AR mRNA, TRPV1 mRNA, and VT4R mRNA levels were increased in SSO in hypertonic saline birds, while these genes were increased in OVLT in acute immobilization stressed birds. The CRH-R1 mRNA genes were decreased in hypertonic saline birds, while increased in acute immobilization stressed birds. These results strongly suggest that physical stress affect the vasotocinergic system in the SSO to regulate the water balance through VT4R, while psychogenic stress causes change in VT4R expressed in the OVLT for the classical activation of the HPA. Taken together, results provide evidence that both osmotic challenge and psychological stress affect the vasotocinergic system via the VT4R in two avian sensory circumventricular organs.

Key Words: vasopressin receptor (V1aR), subfornical organ, organum vasculosum of lamina terminalis.

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DEDICATION

I dedicate this work to Jesus Christ for his blessings during my stay in the USA. . I also dedicate this thesis to Aman Desire Emmanuel, Aman Leslie Mariane, Akassi Rachel, and Benande Molme.

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INTRODUCTION

In the terminology utilized by Dr. Selye, stress describes an animal's defense mechanisms, and thus, a stress stimulus (stressor) as any situation that elicits defense responses (Selye, 1963). The integration of the autonomic nervous system and the hypothalamo-pituitary-adrenal axis (HPA) are activated in response to stress. The activation of the sympathetic nervous system in response to stress results in reflex-like "alarm" or "emergency**"** reaction or "fight or flight" responses (Cannon, 1929). This neurogenic system, consisting of postganglionic neurons and the adrenal medulla, causes the release of catecholamines: norepinephrine and epinephrine (adrenaline) as a result of acute stressors **(**Sturkie and Lin, 1968; Edens and Siegel, 1975; Siegel, 1980)**.** In addition, the neural input and the blood-borne stimuli from various stressors activate another system that responds to a stressor over a more prolonged time span. That system involves a neuroendocrine response and results in the production of hormones whose actions persist for much longer periods of time. Specifically, the neuroendocrine system comprises parvocellular neurons releasing arginine vasotocin (AVT) and corticotropin releasing-hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus (Ganong, 1963). The production of the CRH from the hypothalamus stimulates the anterior pituitary gland to produce and secrete adrenocorticotropin (ACTH) into the bloodstream. In turn, adrenocorticotropin stimulates the release of steroid hormones, particularly corticosterone in birds (Nagra et al., 1963; Holmes and Philips 1976, Siegel et al., 1980) and rodents (de Roos, 1960) or cortisol (in humans and other mammalian species) (Heftmann and Mosettig, 1970) from the adrenal cortex in mammals. In birds, the interrenal tissue, arranged in cords (each cord composed of a double row of adrenocortical cells) releases the stress hormone. Corticosterone, synthesized from cholesterol in the steroidogenic pathway, is the major glucocorticoid in birds and in rodents (Hanukoglu, 1992). It is high in stressed birds' plasma subjected to different types of physical stressors such as osmotic stressors (Ludwig et al., 1994; Fitts et al., 2004) and psychological stressors including immobilization or restraint (Koolhaas et al., 1999; Kuenzel and Jurkevich, 2010; Selvam et al., 2013). The hypothalamo-pituitary-adrenal (HPA) axis is responsible for the adaptation mechanism of animals or birds to various stressors described by Selye as the adaptation syndrome (Selye, 1963). In non-mammalian vertebrates, arginine vasotocin, homologous to mammalian arginine vasopressin and

CRH are major secretagogues of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Castro et al., 1986; Cornett et al., 2013). Previous studies have shown that AVT potentiates the action of CRH on ACTH, and therefore, AVT and CRH are synergistic in in their effect on plasma corticosterone that may involve the potentiation of the signal transduction pathway of CRH (Kuenzel and Jurkevich, 2010; Cornett et al., 2013). The effects of AVP/AVT and CRH are mediated by their interactions with seven transmembrane G-protein-coupled receptors (GPCR) (Birnhaumer, 2000; Gimpl and Fahrenholz, 2001; Mikhailova et al., 2007; Cornett et al., 2013).

In mammals, AVP, a nona-peptide, has been shown to have three receptors subtypes, namely, V1aR, V1bR, and V2R (Lolait et al., 1992; Morel et al., 1992; Sugimoto et al., 1994). This neuropeptide essentially exerts a vasoconstrictive action on vascular smooth muscle cells (Morel et al., 1992), stimulates the HPA axis (Ostrowski et al., 1994; Sugimoto et al., 1994), and has an antidiuretic effect in the kidneys (Lolait et al., 1992). In addition, the mammalian V1aR is expressed in gonadotropes of the anterior pituitary affects release of gonadotropins. The V1aR is also found in vascular smooth muscle cells, in liver, and throughout the brain including three circumventricular organs, specifically the pineal gland, choroid plexus, and area postrema (Morel et al., 1992; Orcel et al., 2002). In contrast, the V1bR is predominantly found in corticotrophs of the anterior pituitary and mediates the action of AVP on ACTH release (Antoni, 1993; Sugimoto et al., 1994). The vasopressin V1bR is also expressed in the brain, including the hippocampus, hypothalamus, and amygdala (Young et al., 2006). The vasopressin receptor subtype 2 (V2R) is located in the kidney, where it regulates water reabsorption (Birnkaumer et al., 1992; Lolait et al., 1992). Furthermore, arginine vasopressin acts through the V1a and V1b receptors and is mediated by the phospholipase C/protein kinase C Ca^{2+} signaling pathway, while its action through the V2R is mediated through the adenylate cyclase / Protein kinase A cAMP signaling pathway (Liu and Wess, 1996).

In contrast, at least four vasotocin receptor subtypes have been identified in non-mammalian vertebrates (Ocampo et al., 2012; Yamaguchi et al., 2012). The avian AVT receptors are of major interest in the present study. They were originally named according to their time sequence of discovery: vasotocin receptor one (VT1R), vasotocin receptor two (VT2R), vasotocin receptor three (VT3R), and vasotocin

receptor four (VT4R). Their gene and their amino acid sequence data have strongly suggested which vasotocin receptors are homologous to the appropriate receptor in the vertebrate vasotocin/vasopressin receptor family (Table 1). Recently, the V2R in fish has been subdivided into two subtypes: V2aR (homologous to the conventional mammalian V2R) but uniquely stimulates the calcium signaling pathway rather than the cAMP pathway, and the V2bR (homologous to avian VT1R) maintaining the more ancestral calcium pathway (Tan et al., 2000; Ocampo et al., 2012; Yamaguchi et al., 2012).

Table 1: Avian AVT receptors, their proposed homologous fish and mammalian receptors and their receptor functions.

Avian AVT receptor subtypes	**Teleost fish AVT/IT receptor subtypes**	**Mammalian AVP/OT Subtype receptor homolog**	**AVT functions in birds**	**AVP functions in mammals**	**AVT functions in teleost fish**
VT1R (14)	V2bR (15)	-	Oviposition	-	Osmoregulation
-	V2aR (11,15)	V2R (9)	-	Water balance	Osmoregulation
VT2R (2,3,5)	V1a-2R (6,7)	V1bR (8)	ACTH release	ACTH release	Reproduction behavior
VT3R (2,4)	OTR (9)	OTR (1)	Egg laying	Milk ejection, parturition	-
VT4R (13)	V1a-1R (6)	V1aR (10,12)	ACTH release	Blood pressure regulation, glycogenolysis, reproduction,	Reproductive behavior, Vision, Olfaction

Adan et al., 1995 (1); Baeyens and Cornett, 2006 (2); Cornett et al., 2003 (3); Gubrij et al., 2005 (4); Jurkevich et al., 2005 (5); Kline et al., 2011 (6); Lema et al., 2010 (7); Lolait et al., 1995 (8); Maybauer et al., 2008 (9); Ostrowski et al., 1992 (10); Ocampo et al., 2012 (11) ; Orcel et al., 2002 (12); Selvam et al., 2013 (13); Tan et al., 2000 (14); Yamaguchi et al., 2012 (15).

Recent studies have shown that vasotocin receptor two (VT2R/V1bR) and vasotocin receptor four (VT4R/V1aR) are expressed in the corticotrophs of the anterior pituitary and mediated the release of ACTH in response to acute immobilization stress in chicken (Jurkevich et al., 2005,. 2008; Cornett et al., 2013; Kuenzel et al., 2013; Selvam et al., 2013). Like the mammalian V1aR, Selvam et al., (2014). These studies have discovered that the VT4R is highly expressed throughout the brain including all ten

circumventricular organs in the avian brain. Two of the avian CVOs, the organum vasculosum of the lamina terminalis (OVLT) and the subseptal organ (SSO)/ the subfornical organ in mammals (SFO), have shown high levels of VT4R/V1aR immunoreactivity suggesting that the chicken VT4R may be associated with osmoregulation. The typical vertebrate CVO usually displays specialized ependymal cells, has an incomplete blood-brain barrier, contains cerebrospinal fluid (CSF)-contacting neurons, and is located adjacent to the ventricles of the brain (Vigh, 1973). Mammals have less than ten, usually eight CVOs, three of which are regarded as sensory: the subfornical organ, homologous of avian subseptal organ, organum vasculosum of the lamina terminalis, and area postrema (APa). Several studies have confirmed that the SFO and the OVLT are involved in drinking behavior and osmoregulation through the action of angiotensin II on AVP/AVT release in mammals (McKinley et al., 1992; McKinley et al, 1998) and in birds (Gerstberger et al., 1987; Simon-Oppermann et al., 1988; Simon et al., 1992). The recent VT4R has been identified in chickens; however**,** very little data about its function is documented except for its involvement in psychogenic stress. Therefore, the objective of the study was to test the possible function of the VT4R within the OVLT and the SSO with regard to osmoregulation in the chicken. Experiments were, therefore, designed to test whether or not a physical stressor, hyperosmotic saline, would affect the immunohistochemistry or gene expression of VT4R located in the OVLT and/or the SSO.

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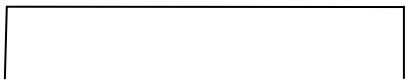
CHAPTER I - Review of Literature

1. Arginine vasopressin (AVP)/ Arginine vasotocin (AVT)

1.1. Structure and synthesis of AVP/AVT

Arginine vasotocin (AVT) in non-mammalian vertebrates is homologous to mammalian arginine vasopressin (AVP). Arginine vasotocin is a highly conserved nona-peptide (9 amino acids). It contains a ring-like structure created by disulfide bonds between cysteine residues at positions 1 and 6. Arginine vasotocin differs from AVP by only one amino acid at the position 3 where isoleucine (Ile) in AVT is substituted for Phe in AVP (Acher and Chauvet, 1995; Goldstein, 2006) (Fig 1).



	1	2	**3**	4	5	6	7	8	9
Vasotocin	Cys	Tyr	**Ile**	Gln	Asn	Cys	Pro	Arg	Gly
Vasopressin	Cys	Tyr	**Phe**	Gln	Asn	Cys	Pro	Arg	Gly

Fig. 1. Amino acid sequences of the avian and mammalian AVT/AVP (Goldstein, 2006).

Arginine vasotocin or arginine vasopressin is synthesized in the magnocellular neurons of the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), and parvocellular neurons in the PVN. In mammals, AVP is derived from a precursor preproarginine vasopressin consisting of the following structural sequence: signal peptide - vasopressin - neurophysin II - glycopeptide or copeptin (Acher et al., 1955; Brownstein, 1980; Acher and Chauvet, 1995; De Bree et al., 2003), while the non- mammalian AVT precursor, preproarginine vasotocin, has two linked stuctures, AVT and neurophysin II, without any glycopeptide (Acher and Chauvet, 1995)**.** Arginine vasotocin/arginine vasopressin precursors are packaged in secretory granules of both hypothalamic PVN and SON neurons. Therefore, it is cleaved to generate AVT/AVP during its transport from the PVN and the SON in the posterior pituitary (neurohypophysis) where it is released into the bloodstream to regulate the salt and the fluid balance (Rinaman et al., 1995). The arginine vasotocin/ arginine vasopressin synthesis and release are significantly induced by a physical stress such as an osmotic stimulation (an increased or a decreased

plasma hyperosmolarity or a hypovolemia) (Falke, 1991). The cleavage of the AVT/AVP precursor is mediated by the ordered actions of these enzymes: dibasic endopeptidase, carboxypeptidase, peptidylglycine monooxygenase, and alpha-amidating lyase. The arginine vasopressin/ arginine vasotocin is carried into the caudal region of the posterior pituitary gland where it is released into the bloodstream. A second source of the arginine vasopressin in mammals (Gillies et al., 1982) or the arginine vasotocin in birds (Mikhailova et al., 2007; Cornett et al., 2013; Kuenzel et al., 2013) originates from parvocellular neurons in the PVN where it can potentiate the corticotrophin releasing hormone (CRH) effect. Both parvocellular AVT neurons and CRH neurons terminate in the median eminence and bind to receptors on anterior pituitary corticotrophs, and, thereby, activating the hypothalamic-pituitary-adrenal axis (HPA) (Carsia et al., 1986; Familari et al., 1989).

1.2. Functions of AVP/AVT

1.2.1. Role of AVP/AVT in the central nervous system

In mammals, arginine vasopressin, located in the central nervous system or the brain, plays a variety of physiological functions, including drinking behavior (de Arruda Camargo et al., 2003), learning and memory process (de Wied, 1997; Everts and Koolhaas, 1999) , social recognition (Everts and Koolhaas, 1999; Bielsky and Young, 2004), sexual behavior (Gash and Boer, 1987), aggression (Goodson and Bass, 2001), anxiety (Everts and Koolhaas, 1999), depression, and stress response (Familari et al., 1989). In birds, AVT is known to modulate several behaviors, including vocalization or singing (Maney et al., 1997; Goodson, 1998), sexual behavior (Kihlstrom and Danninge, 1972; Jurkevich and Grossmann, 2003), aggression (Goodson, 1998), and stress response (Carsia et al., 1986; Kuenzel and Jurkevich, 2010; Kuenzel et al., 2013; Kang and Kuenzel, 2014).

1.2. 2. Peripheral effects of AVP/AVT

The circulating AVP/AVT mainly regulates water homeostasis, maintains body blood pressure, and activates ACTH release induced by the stress in mammals (Grantham and Burg, 1966; Lolait et al., 1992; Morel et al., 1992; Sugimoto et al., 1994) and in birds (Gerstberger et al., 1984; Simon-Oppermann, 1984; Hughes, 2003) respectively. Indeed, AVP/AVT is primarily known as the anti-diuretic hormone (ADH)

causing the water absorption in collecting ducts of the kidney to create the hyperosmotic urine in mammals (Grantham and Burg, 1966). However, it is involved a complex osmoregulation system which includes the kidney, the gastrointestinal system, and the salt glands in birds (Gerstberger et al., 1984; Hughes, 2003). The increase in plasma osmolarity such as water deprivation (Koike et al., 1977; Árnason et al., 1986) or hypertonic saline infusion (Koike et al., 1979) appears to be the principal stimulus for AVT release from the posterior pituitary gland. For example, an increase of the AVT synthesis has been correlated with an increased gene expression of the AVT mRNA in the hypothalamic paraventricular nucleus and the supraoptic nucleus in birds (Chaturvedi et al., 1997). In other words, any osmotic stress results in the activation of the AVT magnocellular neurons in the PVN and the SON synthesizing the AVT, which is releasing from the posterior pituitary gland directly into the bloodstream to restore water balance in birds (Chaturvedi et al., 1997).

2. Arginine vasopressin (AVP)/Arginine vasotocin (AVT) Receptors

Different subtypes of AVP/AVT and oxytocin (OT)/Mesotocin (MT) receptors have been identified among species. The new classification of these subtypes is based on phylogenetic analyses, and their respective functions of all mammalian and avian AVP/AVT subtype receptors are summarized in the table 1.

2.1. Structure of VT4R/V1aR or recent adopted acronym AVPR1A

The mammalian vasopressin subtype 1a receptor (V1aR or AVPR1A) belongs to the guanine protein-coupled receptor (GCPR) family. This class of receptor consists of 7 hydrophobic transmembrane alpha helices joined by three extracellular and three intracellular loops, an extracellular N-terminal region, and an intracytoplasmic C-terminal region (Barberis et al., 1998). The binding sites for the ligand are significantly expressed in the conserved extracellular loops 1 and 2 (Sharif and Hanley, 1992). The vasopressin subtype 1a receptor is located on the chromosome 12 and contains 394 amino acids. This receptor is characterized by two major features: 3 N-glycosylation on the asparagine (Asn) are present on the extracellular domains (Asn 14 and Asn 27 on N-terminal; Asn 198 in second extracellular loop) and 3 disulfide bonds (one between cysteine (Cys) residues in the second and third extracellular loop; two on Cys residues within C-terminal domain (Thibonnier et al., 1994; Oychinnikov et al., 1998; Robert and Clauser, 2005). The N-glycosylated sites on the V1aR modulate its receptor expression level at the extracellular domain region, and, therefore, are required for normal receptor expression as well as for the

efficient protein folding. In contrast, this N-glycosylation is not involved in the ligand (AVP/AVT) recognition for the intracellular pathway activation (Hawtin et al., 2001). Moreover, the disulfide bonds are required for the correct folding of the V1aR. All major features on the mammalian V1aR suggest that the structure of this receptor has been well-established among different mammalian species in a number of studies. However, the structure of the chicken VT4R is 419 amino acids in length, shares 69% similarity with the V1aR of mammals and not well studied (Baeyens and Cornett, 2006; Jayanthi et al., 2013). Jayanthi et al. (2014) have recently discovered binding site similarities with the well-established V1aR using three-dimensional molecular modeling studies of the VT4R. **2.2. Functions of V1aR/AVPR1A**

In mammals, specifically, the V1aR is abundantly found in a variety of specific regions of the brain and in diverse peripheral organs suggesting that it may possess multiple functions. Several mammalian studies have established both central and peripheral functions of the V1aR. It plays a major role in the cardiovascular system and influences the arterial blood pressure. Importantly, this receptor mediates the vasocontrictive effects of AVP, resulting in an increased blood pressure in response to any systemic decrease in blood pressure (Johnston, 1985; Knepper, 2000; Lange, 2007). More specifically, the binding of the AVP to the V1aR induces the activation of the phospholipase C (PLC) pathway leading to the release of Ca^{2+} into the vascular smooth muscle cells. Thus, the pathway provides Ca^{2+} required for the contraction of blood vessels. The V1aR also mediates the effects of the AVP in the liver (glycogenolysis) and the platelets (aggregation). In addition, the V1aR mRNA, highly expressed in brain including three circumventricular organs (choroid plexus, area postrema, and subfornical organ), is consistent with the possible role of the arginine vasopressin V1aR in the central control of the osmoregulation (Schultz et al., 1977; Katusic et al., 1984; Ostrowski et al., 1994). Additionally, the V1aR mediates diverse behavioral effects in mammals, including social recognition (Bielsky and Young 2005), avoidance behavior (Kovacs et al., 1979), social memory and learning process (Dantzer et al., 1988; de Wied et al., 1993), anxiety (Caldwell et al., 2008), fear (Viviani and Stoop, 2008), locomotion (Tsunematsu et al., 2008), aggressive and maternal behavior (Nephew and Bridges, 2008), and pair bonding (Walum et al., 2008). The vasopressin V1a receptor was found in gonadotropes of the anterior pituitary gland, thereby could modulate the reproductive function (Orcel et al., 2002). The mammalian V1aR was not located on corticoptrophs. In contrast, the chicken VT4R/V1aR is highly expressed on corticotrophs and shown to be involved in psychological stress (Kuenzel, 2013; Selvam et al., 2013; Kang and Kuenzel, 2014). In the

mammalian pituitary gland, vasopressin through the V1bR is known to be involved in the neuroendocrine hypothalamo-pituitary adrenal axis in response to stress (Antoni, 1993; Sugimoto et al., 1994). The V1aR controls major behavioral functions and may shut off or decrease the gonadal function with a continued stress. Since the V1aR is associated with several physiological roles in mammals, it makes sense to explore other possible effects of the avian VT4R rather than a restraint stress, and an obvious one is the osmoregulation due to the presence of the VT4R/V1aR in CVOs associated with water balance.

2.3. Antagonists of VT4R/V1aR

The AVP in mammals regulates many essential functions through the V1aR centrally and peripherally. The functions of V1aR are summarized in the table 1 and 2.2 of the literature review. Because of the important and extensive roles of the vasopressin system in humans and other mammals, different antagonists of the V1aR have been developed for therapeutic purposes. The V1aR antagonists have been subdivided into two major classes based upon their specificity: (1) selective V1aR antagonists including SR49059 (Serradeil et al., 1993), OPC-21268 (Yamamura et al., 1991) and (2) relatively non-selective antagonists of the V1aR, known as mixed antagonists such as Manning compound (V1aR/OTR) [Manning et al., 2008], YM087 (V1aR/V2R) [Gieldon et al., 2001] , and JTV-605 (V1aR/V2) [Serradeil et al., 2001]. Because of their high stability and specificity, SR49059 and OPC-21268 are considered as the most efficacious antagonists. Of relevance, several mammalian V1aR antagonists such SR49059, OPC-21268, and YM-087 show a high binding affinity for the chicken VT4R (Jayanthi et al., 2014). Moreover, Jayanthi et al. (2014) have recently identified a number of VT4R antagonists that reduce POMC hnRNA expression in the primary avian pituitary cell cultures following a stimulation of a cocktail of CRH/AVT neuropeptides. Note that POMC is a useful indicator of the ACTH activation. Each of the four following blockers (SR-49059, H-6722, OPC-21268, and H-5350) reduced the expression of the POMC hrRNA by 55%, 44%, 39%, 35% when applied, respectively, to the culture system. To date, SR49059 is the most potent selective antagonist of the avian V1aR due to its high binding affinity and its significant attenuation of the poultry pituitary POMC expression.

3. Signal transduction pathways (Second messenger systems)

Arginine vasopressin exerts its physiological action by binding to a specific extracellular domain in a transmembrane region of a distinct AVP subtype receptor, and, thus, leading to a change in a particular signal transduction pathway for its stimulation or its inhibition. The mammalian arginine vasopressin subtype 2 receptor (V2R), highly expressed in kidney, activates a G_s subunit of the G protein, which stimulates the adenylate cyclase causing the production of the cAMP/protein kinase A (Orloff and Handler, 1967; Liu and Wess, 1996) that is required as second messengers to mediate antidiuretic effects of AVP (Figure 2).

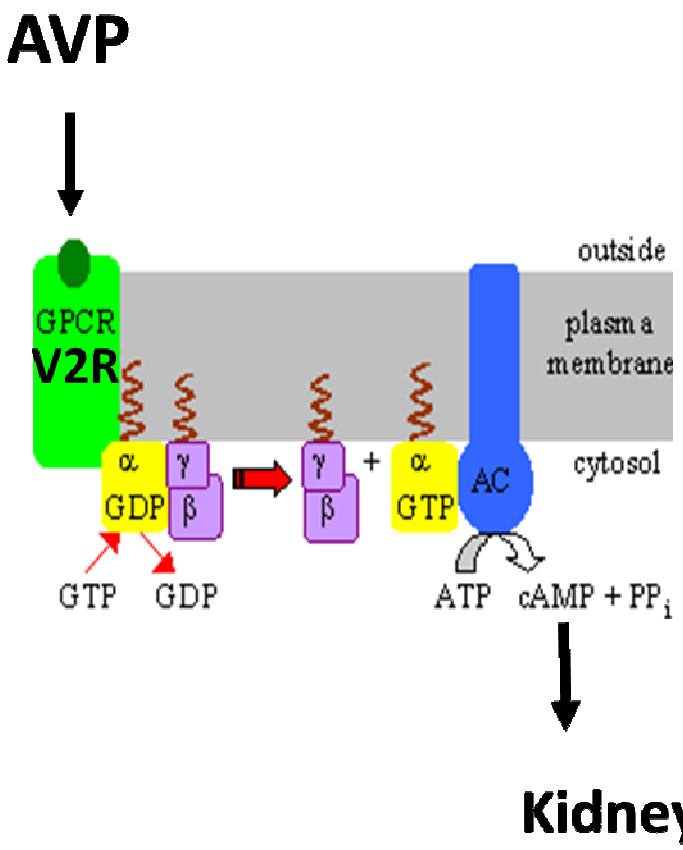


Fig. 2: Diagram depicting the signal transduction adenylate cyclase/cAMP pathway upon the binding of the AVP to mammalian the V2R (homologous to chicken VT1R) monitoring the antidiuretic effects of the AVP in the kidney. AC: adenylate cyclase, GPCR: Guanine-protein-coupled receptor, G_α (α): G-protein

subunit alpha; G□□: G-protein subunits gamma beta, AVP: arginine vasopressin, V2R: arginine vasopressin subtype 2 receptor (https://www.rpi.edu).

In addition, the arginine vasopressin subtype 1b receptor (V1bR) expressed in the anterior pituitary and the subtype 1a receptor (V1aR) located on smooth blood vessels mediate AVP actions through phospholipase C pathways (Fig. 3) (Morel et al., 1992). Similar to the mammalian V1aR, the avian vasotocin subtype 4 receptor (VT4R) mediates AVT effects through the calcium pathway (Ocampo et al., 2012; Yamaguchi et al., 2012). Subsequently, the binding of the arginine vasopressin with the V1aR activates $G_{q,}$ subunit of the G protein, and, then, stimulates the phospholipase C (PLC). The phospholipase C hydrolyses phosphotidyl inositol 4, 5 bi-phosphate (PIP_2) into inositol 1, 4, 5-triphosphate (IP_3) and diaglycerol (DAG) (Thibonnier et al., 1996). Second messengers (IP_3 and DAG) cause the Ca^{2+} increase in the cytosol. The inositol 1,4,5-triphosphate binds to the inositol 1,4,5-triphosphate receptor (IP_3-R) located on the endoplasmic reticulum (ER) to release the Ca^{2+} leading to activation of the calcium-calmodulin complex, while the DAG affects the protein kinase C to produce cytosolic Ca^{2+} through voltage-gated Ca^{2+} channels. High concentrations of the Ca^{2+} results in the vasoconstriction of the vascular smooth muscle cell (Lange et al., 2008). However, a striking new finding among non-mammalian AVT receptors involves the classification of the avian VT1R among the vertebrate family of vasotocin receptors. Molecular phylogenetic and functional analyses have recently categorized the mammalian V2R into V2aR and V2bR (Table 1). The V2aR, the conventional mammalian V2R, is primarily associated with the adenylate cyclase/cAMP pathway. In contrast, V2bR, present in all other classes of vertebrates and homologous to chicken VT1R, switches its signal mechanism to mediate the PLC/Ca^{2+} pathway rather than the cAMP pathway (Ocampo et al., 2012; Yamaguchi et al., 2012). The finding suggests that the cAMP signal transduction pathway for this particular receptor subtype may be unique to mammals.

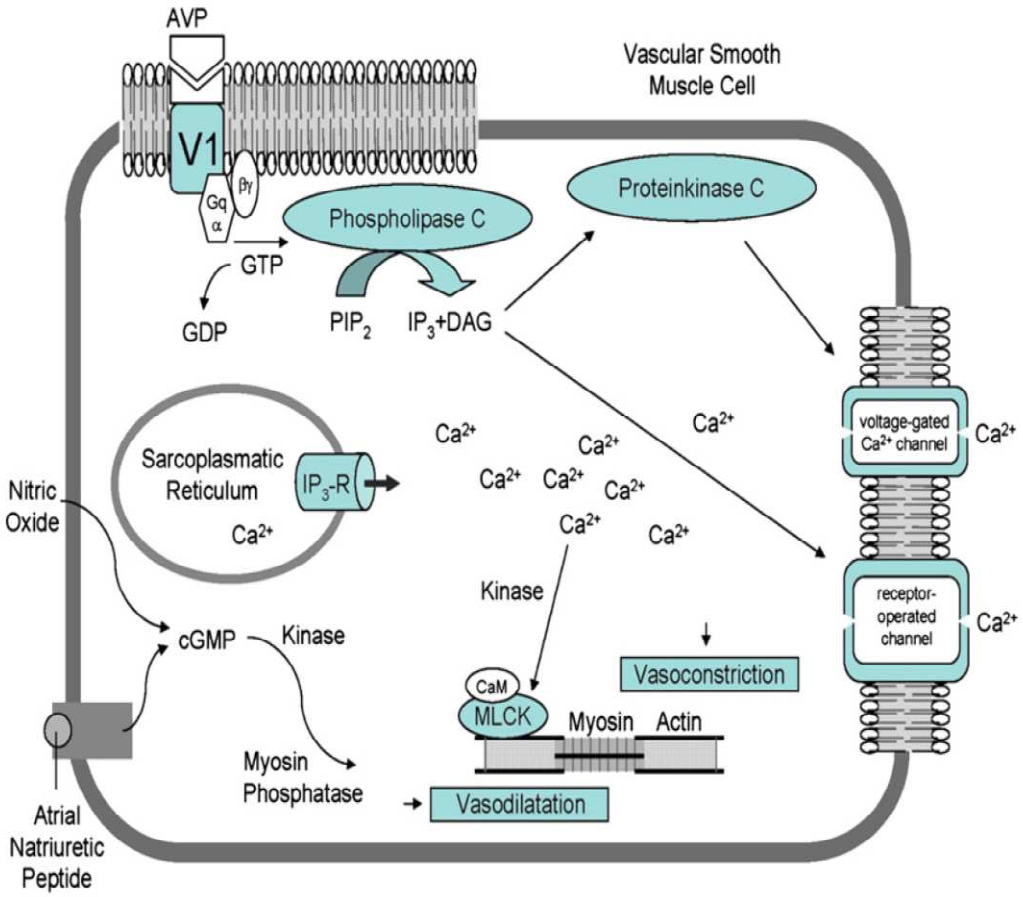


Fig. 3: Diagram showing the signal transduction PLC/Ca^{2+} pathway of the mammalian V1aR (homologous to chicken VT4R) mediating the major AVP functions (Lange et al., 2008).

4. Regulation of Stress

4.1. Physical and psychological stressors and their mechanisms

Magnocellular neurons in the brain are stimulated by physical stressors such as osmotic stimuli, while psychological stressors are thought to stimulate parvocellular neurons in both mammals and avian species (Bourque, 2008; Ulrich-Lai and Hermann, 2009).

Additionally, magnocellular AVP/AVT-neurons of the PVN and the SON are thought to be uniquely responsive to physical stressors, particularly those affecting osmolality changes (dehydration, hemorrhage, hyperosmotic saline, food deprivation). These changes cause the activation of the hypothalamo-posterior pituitary system (figure 4) to release the AVP into the bloodstream in mammals

(Ludwig et al., 1994; Fitts et al., 2004) or the AVT in birds (Ruch et al., 1975; Goto et al., 1986; Stallone and Braun, 1986; Simon-Oppermann et al., 1988). The circulating AVP/AVT primarily exerts: (1) its vasoconstrictive effects on smooth muscle cells through the mammalian V1aR; (2) its antidiuretic actions on the kidney via the V2R in mammals or possibly the VT1R in chickens. Furthermore, the parvocellular AVP/AVT-neurons of the PVN primarily react to other types of stressors called psychological stressors or emotional stressors including immobilization or restraint in mammals (Antoni, 1993; Sugimoto et al., 1994) and birds (Hermann, 1993; Kuenzel and Jurkevich, 2010). Upon the activation of the parvocellular neurons of the PVN by the neural input generated by psychological stressors, the classic hypothalamo-pituitary adrenal axis (Fig. 4) is activated in the following sequence: (1) release of AVP/AVT and CRH from hypothalamic neurons into the median eminence and their transport via the portal capillary system to the anterior pituitary gland, (2) binding of the neuropeptides to their respective receptors located on corticotrophs with the subsequent release of the ACTH from the anterior pituitary gland into the bloodstream, and (3) binding of the ACTH to receptors on cells of the adrenal cortex (mammals) or the interrenal tissue (birds) that stimulate the production of the cortisol in some mammals (Wiegand and Price, 1980; Familari et al., 1989; Kempainen et al., 1993) and the corticosterone in birds and rodents (Carsia et al., 1986; Romero et al., 1998; Kuenzel and Jurkevich, 2010), known as the primary stress hormone. Data have shown that the AVP/AVT can potentiate the neuroendocrine effect of CRH in mammals (Swanson and Kuypers, 1980; Gillies et al., 1982) and birds (Mikhailova et al., 2007; Kuenzel and Jurkevich, 2010; Cornett al., 2013). In other words, the corticotropin releasing factor (CRH) (Figure 4) stimulates the ACTH release from the anterior pituitary gland through the CRH-R1in birds or the CHR-R1and the CRH-R2 in mammals. In addition, the AVP/AVT (Fig. 4) also affects the release of the ACTH from the avian anterior pituitary gland via VT2R/V1bR and VT4R/V1aR (Jurkevich et al., 2005,. 2008; Kuenzel et al., 2013; Kang and Kuenzel, 2014). The adrenocorticotropic hormone is carried via the peripheral circulation to increase the glucocorticoid release from the adrenals into the bloodstream. As a result, glucocorticoids affect physiological functions of diverse organs, including the reproductive system, the digestive system, the cardiovascular system, the immune system, and the brain. Although the high plasma corticosterone levels are controlled by negative feedback mechanisms on the HPA axis to

maintain corticosterone/cortisol plasma levels within the physiological range of mammals (Dallman et al., 1992; Barden et al., 1995) and birds (Carsia et al., 1986).

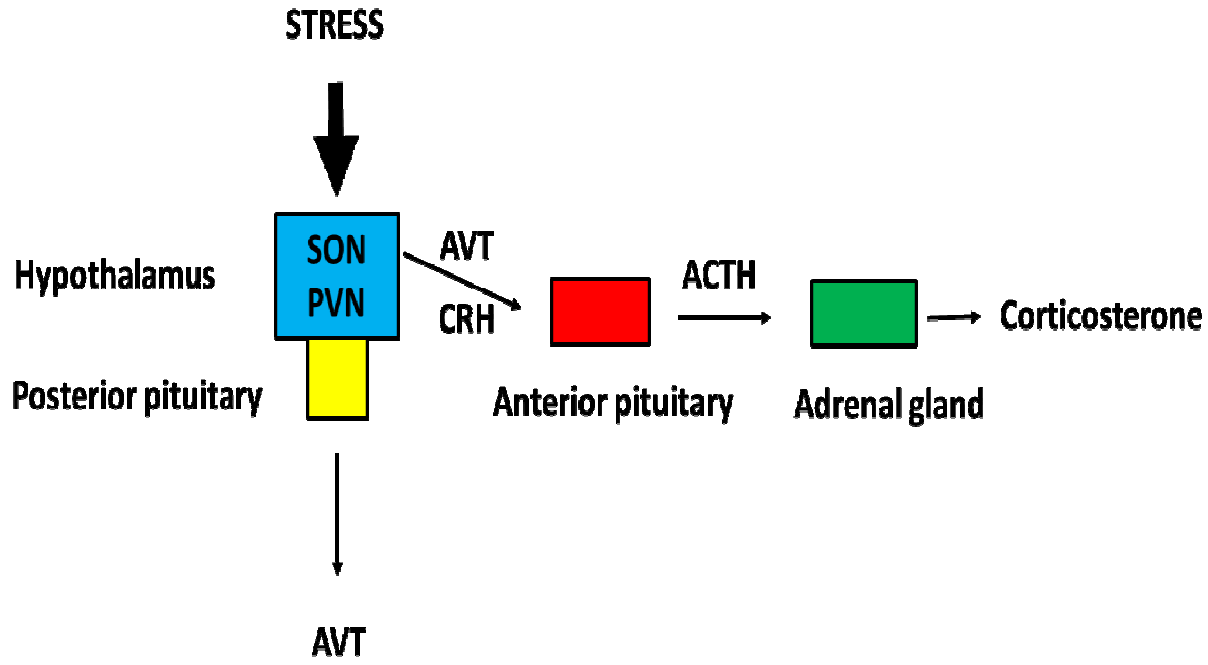


Fig. 4: Two major response stress pathways: hypothalamo-anterior pituitary-adrenal gland axis and hypothalamo-posterior pituitary system. AVT (arginine vasotocin), ACTH (adrenocorticotropic hormone), CRH (corticotropin-releasing hormone), SON (supraoptic nucleus), PVN (paraventricular nucleus). (Adapted from Cornett et al., 2013)

4.2. Implication of CVOs in water intake regulation

Several studies in mammals and birds have established that particular cells having binding sites or receptors for angiotensin II located in the SFO, a CVO, regulate water intake (Gerstberger et al., 1987; Vivas et al., 1990). Both avian and mammalian studies have also reported that stressful inputs can affect specific CVOs that, in turn, have established to send connections to the hypothalamic PVN and SON (Iovino and Steardo, 1984; Philips, 1987). The end result is the activation of the neurohypophyseal system (neural axis, NHS), and/or the neuroendocrine hypothalamo-pituitary-adrenal (HPA) axis. A recent discovery in our laboratory showed immunoreactive VT4R/V1aR within the same CVOs, OVLT and SSO,

associated with the regulation of the drinking behavior (Selvam et al., 2014). The finding was unique for the VT4R/V1aR suggesting, perhaps, a functional role of the VT4R/ V1aR in the osmotic balance.

5. Circumventricular Organs (CVOs)

5.1. Characteristic features of CVOs

In mammals, eight or less CVOs have been identified in the brain. They are differentiated into secretory structures, including neural and intermediate lobes of the pituitary gland, median eminence (ME), subcommissural organ (SCO), and pineal gland (PIN). Sensory CVOs include organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and area postrema (AP_a). There is also a specialized secretory CVO known as the choroid plexus (PC) located within the lateral, third, and fourth ventricles. The choroid plexus is responsible for the production of the cerebrospinal fluid (Petrov et al., 1994; Duvernoy and Risold, 2007).

Unlike mammals, at least ten avian CVOs have been identified, including OVLT, SSO (homologous of SFO), AP_a, ME, PIN, PC, SCO, lateral septal organ (LSO), paraventricular organ (PVO), and subtrochlear organ (STO) (Fig. 5) (Kuenzel and van Tienhoven, 1982).

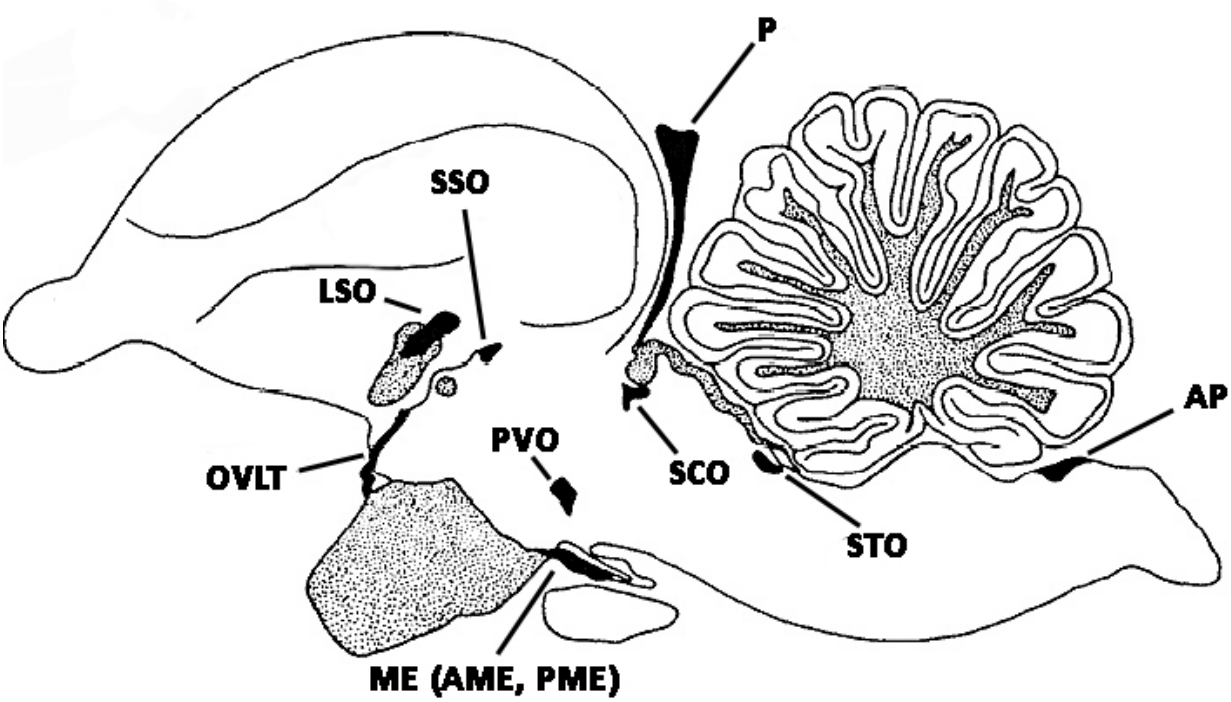


Fig. 5: Sagittal view: Circumventricular organs in the avian brain. ME: Median eminence, SCO: subcommissural organ, P: pineal gland, OVLT: organum vasculosum of lamina terminalis, SFO: subfornical organ, AP_a: area postrema, LSO: lateral septal organ, PVO: paraventricular organ, STO: subtrochlear organ, PC: choroid plexus (not shown as it is lateral to this midline view of the chick brain). (Kuenzel and van Tienhoven, 1982).

Most CVOs share the following general criteria:

- contain specialized ependymal cells;

- are rich in a vascular network of fenestrated capillaries that have an incomplete blood-brain barrier (BBB) (Leonhardt, 1980);

- may have cerebrospinal fluid-contacting neurons

- and are found adjacent to the ventricles within the brain (Vigh, 1971).

A general function of CVOs is to provide communication between the peripheral organs and the brain through the blood and the cerebrospinal fluid (CSF) respectively. In fact, blood borne substances consisting of ions (sodium, potassium, calcium, chloride) and/or hormones (AVP/AVT, calcitonin, atrial natriuretic factor, angiotensin II) can access the brain from the blood by transport to the CSF by specialized neurons (Quirion et al., 1984; Rouleau et al., 1984; Patel et al., 1986; McKinley et al., 1990). Consequently, ions, hormones, and other molecules in the CSF can be monitored by osmoreceptors present in sensory CVOs. The best known example is the renin angiotensin system where circulating ANG II once it binds to the ANG II, AT1 receptor in the SFO mediates the water intake (Nishimura and Bailey, 1982; Nishimura et al., 1984). Thereafter, a secondary effect is the release of antidiuretic hormone (ADH/AVP), which is responsible for the water reabsorption at the level of the kidneys (Palkovitis, 1987; Johnson et al., 1992; Johnson and Gross, 1993). The osmosensitivity of osmoreceptors within sensory CVOs is critical to maintain water and salt balance homeostasis. Interestingly, any hyperosmotic stressor (hypertonic saline solution or injection) can be sensed by the OVLT and mediated through of the transient receptor potential vanilloid 1 (TRPV1) gene expressed in the OVLT. The transient receptor potential vanilloid 1 gene (TRPV1) detects an osmotic change at the molecular level (Ciura et al., 2011). Subsequently, the change in Trpv1 gene expression induces a physiological modification in cation channels located in OVLT neurons. Consequently, the hyperosmolarity exerts a mechanical effect on the cell shrinking that is detected (Ciura et al., 2011). The transient receptor potential vanilloid 1 gene appears to be a non-selective cation channel that is activated during hypertonicity-evoked shrinking of osmosensory neurons (Sharif-Naeini et al., 2006; Prager-Khoutorsky et al., 2014). As a result, the shrinkage of the neuronal cells within the OVLT compresses the microtubule system, making the microtubules push against TRPV1, and, therefore, directly opening the calcium channel (Andres and Göpfert, 2014; Prager-Khoutorsky et al., 2014).

5.2. Organum Vasculosum of the Lamina Terminalis (OVLT)

5.2.1. Anatomy of OVLT

The organum vasculosum of the lamina terminalis is a highly variable structure among mammalian and avian species based upon capillary networks and its dorsal or posterior extension dependent upon a particular animal species (Duvernoy and Risold, 2007).

In mammals, the OVLT, part of the anteroventral third ventricle (AV3V) region, is reduced to a superficial capillary network located at the base of the third ventricle in small rodents, while the OVLT is more developed in the rabbit. Moreover, the anteroventral third ventricle region is a unique structure located in periventricular tissue between the anterior commissure (CA) and optic chiasma (OC) consisting of OVLT, preoptic periventricular area, and median preoptic nucleus (MnPO). The median preoptic nucleus is also known as the nucleus medianus of the medial preoptic area (NM; Brody and Johnson, 1980). Morphologically, the OVLT appears as a triangular-like structure located at midline within the AV3V structure dorsal to optic chiasma and ventral to median preoptic nucleus (Figure 6). In other words, the OVLT, located at the anterior edge of the optic chiasma at the base of the brain, extends dorsally toward the anterior commissure; however, its dorsal direction is restricted to the suprachiasmatic region. Thus, the organum vasculosum of the lamina terminalis does not continue to the anterior commissure. The dorsal end of OVLT is strikingly unique in mammals and ends abruptly (Miselis, 1981; Thrasher and Keil, 1987). The median preoptic nucleus occurs dorsal to the OVLT. The median preoptic nucleus splits into two wings, and, thereby, making the dorsal boundary of the OVLT unclear anatomically in the rat and other mammals. To distinguish better the dorsal end of the OVLT from the beginning of the MnPO in mammals, immunohistochemistry using anti-calretinin has been used. The calretinin positive immunoreactivity helps to determine the dorsal boundary since it shows where the blood brain barrier is lacking (main feature of CVOs), and, thus, validating the presence of the OVLT. In contrast, the calretinin negative immunoreactivity above the dorsal limit suggests that the blood brain barrier is intact indicating the beginning of the MnPO (McKinley et al., 1997; McKinley et al., 1998). In addition, neural connections exist between the SFO and the AV3V area which strongly enables the identification of the AV3V (Fig. 6).

The median preoptic nucleus is involved in the water and the salt balance in mammals (Miselis et al., 1979; Miselis, 1981; Saper and Levisohn, 1983; Johnson, 1985).

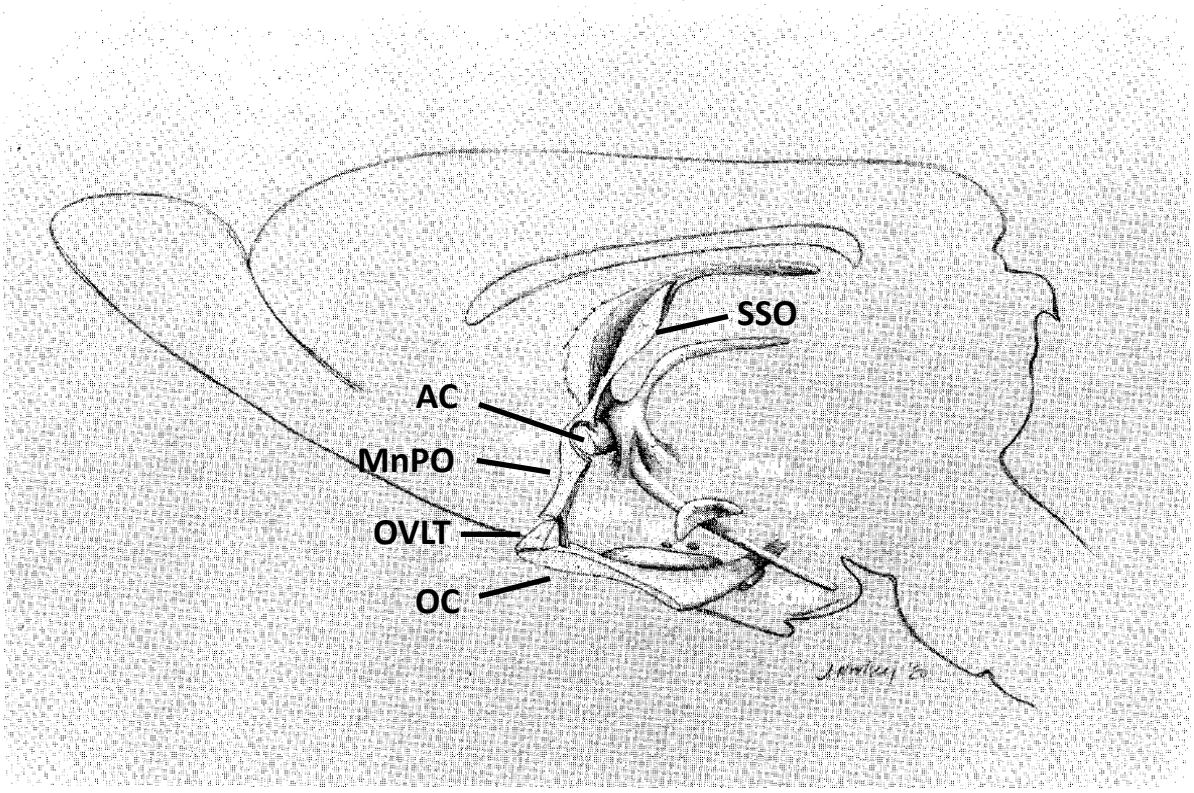


Fig. 6: Three dimensional sagittal view of the rat brain. The subfornical organ/subseptal organ lies in the anterior dorsal region of the third ventricle and contacts the dorsal part of MnPO. The median preoptic nucleus begins above the anterior commissure, moves in front of it and continues ventrally along the anterior border of the third ventricle just above the OVLT where it divides into two wings passing laterally and ventrally on either side of the OVLT. The anteroventral third ventricle (AV3V) includes the OVLT, MnPO, and periventricular nucleus (PeV) (not shown on this diagram) (Miselis, 1981).

In birds, the fenestrated capillary network accompanying the OVLT has a more extensive development. The organum vasculosum of the lamina terminalis, first appearing as triangular-like shape, is primarily located at the anterior edge of the optic chiasma at the base of the brain, ventromedial to the periventricular preoptic nucleus. It proceeds dorsally along the third ventricle, passes in front of the

anterior commissure, and ends at the base of the nucleus of the hippocampal commissure (NHpC) where it ends directly dorsal to the anterior commissure (Kuenzel and Golden, 2006). Objectively, in mammals, two distinctive regions have been defined based on the rostral region of the third ventricle: (1) the anterior dorsal wall of the third ventricular region including the SFO and the anterior-ventral region for third ventricle (AV3V). The autoradiographic techniques using ANG II have identified ANG II binding sites in AV3V region, including OVLT, MnPO, and PeV in mammal (Fig. 6). Mammalian AV3V could be similar to avian AV3V. In contrast, several authors might mention neither ANG II binding sites in MnPO nor the possible presence of MnPO in ducks (Fig. 7) (Gerstberger et al., 1987; Simon et al., 1992, Natke et al., 1996). As a result, the avian OVLT could be either different from the AV3V region or be part of the AV3V region. Surprisingly, Dellmann (1964) has reported the different portions of the OVLT on a sagittal view as follow: prechiasmatic section, thin and thick middle portion, subcommissural section, precommissural section, and supracommissural section. Furthermore, Korf (1984) has described that afferent connections to the avian PVN derived from the OVLT and the SSO. Subsequently, autoradiographic ANG II binding studies have identified the OVLT and the AV3V region. Both anatomical (Korf, 1984; Dellmann, 1964) and autoradiographic studies (Fig. 7) (Shigematsu et al., 1986; Gerstberger et al., 1987; Simon et al., 1992; Natke et al., 1996) have probably not mentioned the homolog to the MnPO in avian species. However, further studies should be done in avians to investigate whether or not they contain an equivalence of the mammalian MnPO.

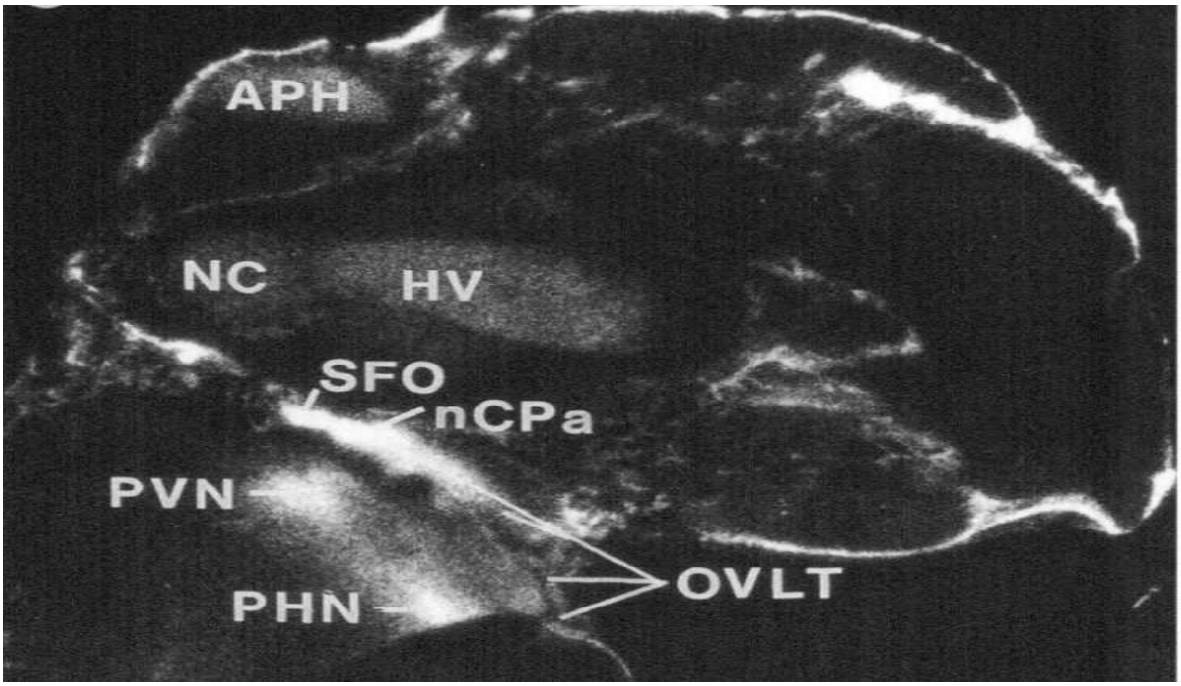


Fig. 7: Sagittal section of the chicken brain. ANGII labelling shows OVLT, SFO, and other brain structures. There is no structure showing the equivalent of the mammalian MnPO. The anteroventral third ventricle (AV3V) seems to have the OVLT and the PeV (not shown) (Natke et al., 1996)

5.2.2. Function

The organum vasculosum of the lamina terminalis sends major projections to the magnocellular neurons of the PVN and the SON. Therefore, the pathway suggests one function of the OVLT, which mediates the release of the AVP/AVT into the bloodstream from the hypothalamo-posterior pituitary system to conserve body water as a result of physical stressors in mammals (Philips, 1987; Honda et al., 1990; Armstrong, 1996) and in birds (Korf, 1984; Koike et al., 1979; Sharp et al., 1995). The organum vasculosum of the lamina terminalis also shows projections to the parvocellular neurons of the PVN causing the stimulation of the AVP/AVT and the CRH secretion which activate the hypothalamo-pituitary-adrenal (HPA) axis resulting from various psychogenic stress threats in mammals (Saper and Levisohn, 1983;). Additionally, immunohistochemical and autoradiographic studies have reported that the OVLT is

an osmoreceptive area containing mostly V1R, particularly V1aR (Jurzak et al., 1995), as well as ANG II receptor subtype T1A involved in the control of any sodium imbalance and osmolality change to maintain bodily ion osmotic homeostasis (Gerstberger et al., 1987; Richard and Bourque, 1995; Lenkei et al., 1997).

5.3. Subseptal Organ (SSO)/ Subfornical Organ (SFO)

5.3.1. Anatomy of subseptal Organ (SSO)/ subfornical Organ (SFO)

The avian subseptal organ is homologous to the mammalian subfornical organ. The anterior border of the SFO/SSO varies based upon the avian species (Schmid, 1995).

In mammals, for example, the anterior extension of the rat SFO has a defined anatomical border. The rat SFO is located at the meeting point of the horn of the lateral ventricle with the third ventricle (Duvernoy and Risold, 2007). The point of the horn is located dorsal to anterior commissure and ventral to the fornix in mammalian species (Dellmann and Simpson, 1979). The subseptal organ moves posteriorly and protrudes slightly into the third ventricle resulting in a finger-like structure (Song K, 1992). The subfornical organ, similar to the OVLT, sends projections into the PVN and the SON (Miselis, 1981; Fitts et al., 2004).

Unlike the mammalian SFO, the avian subseptal organ lacks the fornix. In duck and other avian species, the SSO does not have a clear anatomical anterior or lateral border, while it is in continuity with the nucleus of the hippocampal commissure and the anteroventral third ventricle region. Schmid (1994) has used functional studies with Evans blue, which stained the SSO devoid of blood brain barrier (BBB) to clarify the SSO boundaries in duck and its extension (broadwell and Sofroniew, 1993). He has found that Evans blue primarily stained around the large central blood vessel and its perivascular space which originates from the anterior commissure and passes posteriorly through the entire duck SSO. In addition, the stained Evans blue was seen dorsally to the roof of the third ventricle at the rostral or anterior end of the anterior commissure and dorsally and laterally from the central blood vessel. No Evans blue staining was observed neither in nucleus of the hippocampal commissure and the posterior part of anterior

commissure nor the nucleus of the hippocampal commissure and the end of the SSO. As a result, the absence of Evans blue staining in these regions, represented the anterior and lateral border of the SSO, suggested the leakiness of BBB in the SSO from the anterior commissure to the caudal end of SSO where SSO emerges with the plexus choroid (Schmid, 1994). Moreover, the chicken SSO has been described by the VT4R and the GnRH-1 terminal field immunoreactivities (Kuenzel and Golden, 2006; Selvam et al., 2014). On cross-sections, posteriorly, the OVLT changes position and continues at the base of the lateral septal region where it is located just below the nucleus of the hippocampal commissure. The organum vasculosum of the lamina terminalis location below the NHpC is known as the transition region between the OVLT and the SSO. The beginning of the SSO is medial and ventral to NHpC. As the SSO moves posteriorly, it shows intense dense VT4R immunoreactive glial cells around an increasing central chamber at the SSO dorsal region. At more posterior, the SSO has a finger-like projection from the roof of third ventricle into the third ventricle space containing the cerebrospinal fluid at the ventral SSO region. (Kuenzel and van Tienhoven, 1982; Kuenzel and Golden, 2006; Selvam et al., 2014)

5.3.2. Function of subseptal Organ (SSO)/ subfornical Organ (SFO)

The subfornical organ plays a role in regulating water intake in mammals and birds. The subseptal Organ contains ANG II subtype 1 receptors (AT1R) which mediate changes in the behavior and the physiology of birds (Murphy et al., 1993; Kempf et al., 1996; Sch□fer et al., 1996; Kempf et al., 1999) and mammals (Hohle et al., 1995; Lenkei et al., 1995; lenkei et al., 1997) to regulate the osmotic balance. Moreover, in rats, few V1aR labelled cells were found in the SFO, while high levels of the V1aR were expressed in the pineal gland, the choroid plexus, and the area postrema. The V1aR within the SFO mediates the effects of the AVP (Ostrowski et al., 1994). Similarly, novel avian VT4R/V1aR immunoreactivity has been shown present in the chicken SSO. Knowing that the SFO/SSO is involved in the AVP/AVT release from the hypothalamo-posterior pituitary system to regulate the water balance in both mammals and avian species (Iovino and Steardo, 1984; Jonhson et al., 1992), the avian VT4R/V1aR immunoreactivity within SSO is an additional evidence to examine this avian CVO to ascertain whether or not the evidence can be obtained to support its role in osmotic regulation.

6. Arginine vasopressin/AVT control of osmoregulation

Mammalian AVP and avian AVT are well known by their other name, antidiuretic hormone (ADH), because they regulate water and salt balance in the body (Goldstein, 2006). In fact, any increase in plasma osmolality results in a drinking behavior, and the kidneys respond by activating their antidiuretic function to restore the osmolality (Bourque, 2008). Water deprivation or hyperosmotic stimulus induces the activation of the renin-angiotensin system which triggers the release of ANG II (Nishimura et al., 1982; Nishimura et al., 1984). The angiotensin II, in turn, activates osmoreceptors such as ANG II type1 receptor in the OVLT and the SSO neurons in mammals (Honda et al., 1987; Vivas et al., 1990; Richard and Bourque, 1995) and in birds (Kempf et al., 1996; Sch□fer et al., 1996). Subsequently, the two CVOs send output signals to magnocellular neurons of the hypothalamic PVN and SON which synthesize the AVP/AVT. They are released from the posterior pituitary gland into the bloodstream (Stallone and Braun, 1986; Ludwig et al., 1994; Fitts et al., 2004) to normalize plasma osmolality through the aquaporin 2 channel located at collecting ducts of the kidney (Fitts et al., 2004; Yang et al., 2004; Starbuck and Fitts, 1998). In mammals, the AVP exerts its action on the collecting ducts in the kidney to favor water reabsorption. This results in hyperosmotic urine in response to hypertonic osmolality (Grantham and Burg, 1966). However, the osmoregulation in avian species appears more complex due to roles played not only by the kidney, but also the gastrointestinal tract and the salt glands (Hughes, 2003).

7. Hypothesis

What is unknown is whether the VT4R present in the avian OVLT and SSO plays a role in water balance. It is hypothesized that the avian VT4R located in the OVLT and the SSO responds to physical stressors that affect osmotic homeostasis.

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CHAPTER II - Structural and Functional Analyses of the CVOs in Chicken Brain

1. Introduction

Recent studies have shown that vasotocin receptor two (VT2R/V1bR) and vasotocin receptor four (VT4R/V1aR) are expressed in corticotrophs of the anterior pituitary and mediated the release of ACTH in response to acute immobilization stress in chicken (Jurkevich et al., 2005., 2008; Cornett et al., 2013 ; Kuenzel et al., 2013 ; Selvam et al., 2013). Like the mammalian V1aR, Selvam et al (2014) have discovered that the VT4R is highly expressed throughout the brain including all ten circumventricular organs in the avian brain. Since two of the avian CVOs, the organum vasculosum of the lamina terminalis (OVLT) and the subseptal organ (SSO)/ the subfornical organ in mammals (SFO), have shown high levels of VT4R/V1aR immunoreactivity suggesting that the chicken VT4R may be associated with osmoregulation (Selvam et al., 2014). Several studies have confirmed that the SFO and OVLT are involved in drinking behavior and osmoregulation in mammals (McKinley et al, 1998) and in birds (Gerstberger et al., 1987). The recent VT4R has been identified in chickens; however, very little data about its function is documented except for its involvement in psychogenic stress. To evaluate the possible function of the VT4R within the OVLT and SSO with regard to osmoregulation in chicken, experiments were, therefore, designed to test whether or not a physical stressor, hyperosmotic saline, would affect the immunocytochemistry and/or gene expression of VT4R located in the OVLT and/or SSO.

2. Materials and Methods

2.1. Animals

Three-week old male chickens were housed in individual cages under a 16:8 hours light/dark cycle with lights on at 6:00 am. Birds were provided food and water *ad libitum*. Birds were randomly selected into 2 sets / four treatment groups (n=8/treatment). The set 1 included acute controls (not handled) and acute immobilization stressed birds prevented from standing or moving their wings. They had water accessibility during 1 hour. The set 2 was composed of hypertonic saline (3.0 M NaCl) and isotonic saline (0.15 M NaCl). The birds of set 2 were injected intra-peritoneally (i.p) with either 3 M NaCl or .15 M NaCl at a dosage of 5 ml/kg. The birds were brought back to their cages for 1 hour without any water access. All animals used in this study were treated in accordance with protocols approved by the university of Arkansas Institutional Animal Care and Use Committee.

2.2. Radioimmunoassay of corticosterone

The blood samples were taken from the brachial vein in 3-week old male chickens subjected to 1 h of acute immobilization stress (n=8), and 1 h of hyperosmotic stress (n=8). Blood samples were centrifuged at 3000 rpm for 20 mn at 4℃, and the p lasma samples were stored at - 20℃ until assayed. Plasma corticosterone (CORT) concentrations were determined by radioimmunoassay (Madison et al., 2008; Poudman and Opel, 1988). Briefly, plasma samples (200 µl) were first extracted with 2 ml of ethyl ether in borosilicate glass tubes (12 x 75 mm). All tubes were vortexed for 30 min at room temperature and the water-soluble fraction was separated in a methanol/dry ice bath. The liquid fraction of each sample was transferred to a new tube and dried at 37℃ in an evaporator. Dried extracts were reconstituted with 400 µl of assay buffer (0.1 M PBSG, pH 7.0), vortexed for 5 min, and equilibrated overnight at 4℃. In duplicate assay procedure, 100 µl of anti-corticosterone purchased from Fitzgerald Comp (Concord, MA, USA) and 100 µl of ^{125}I corticosterone tracer purchased from MP Biomedicals Inc. (Orangeburg, NY, USA) were added to each sample and standard tube and incubated for at least 24 hours at 4℃ . The bound anti-corticosterone was se parated from the unbound by precipitation using sheep anti-rabbit antibody purchased from MP Biomedicals Inc. (Orangeburg, NY, USA) and 6% of

polyethylene glycol. The supernatant of each sample and standard was discarded, dried, and read using Cobra Quantum gamma-counter.

2.3. Radioimmunoassay of AVT

Plasma arginine vasotocin (AVT) concentrations were estimated using a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Inc Burlingame, CA, USA). Briefly, plasma AVT samples were extracted using C-18 SEP- COLUMNS. Each C-18 SEP-COLUMN was placed on 15 ml centrifuge tube and equilibrated by buffer B (1ml) followed by buffer A (3 ml, 3 times). Plasma samples (1 ml) were acidified with 1 ml of buffer A. The acidified plasma solution was loaded to equilibrated C-18 SEP- COLUMN. The acidified plasma passed through the column over variable time (30 mn to 4 hours depending on the presence or not of bubbles).The column was washed slowly with buffer A (3ml, twice) and discarded. The buffer B (3ml) was used to elute AVT from column and the eluted solution was dried in SpeedVac concentrator. The dried extract was reconstituted in 250 µl of RIA buffer and 100 µl x 2 of reconstituted samples and standards were assayed. In duplicate assay procedure, 100 µl of rabbit anti-AVP antibody (the RIA for AVT in birds was developed using the high cross-reactivity of AVT with AVP raised in rabbits, Möhring et al., 1980) was added to samples and standards tubes and incubated for 16-24 hours at 4℃. Then, the ^{125}I AVP tracer (100 µl) was added to tubes and incubated for 16-24 hours at 4℃. The free and bound AVT were separated using th e second antibody, goat anti-rabbit (100 µl), followed by normal rabbit serum (100 µl) and RIA buffer (500 µl). After the tubes were centrifuged at 3000 rpm for 20 min at 4℃, the supernatant was removed by aspiration, and the precipitate was counted using gamma-counter.

2.4. Immunohistochemistry

2.4.1. Tissue preparation

Birds were anesthetized with an I.V injection of sodium pentobarbital (30 mg ml/kg). Each anesthetized bird was perfused through left heart ventricle and carotid arteries with 150 ml of ice cold heparinized PBS containing 0.1 M PBS with 0.1% sodium nitrite, pH 7.4 immediately followed by 250 ml

of freshly prepared, ice cold Zamboni's fixative solution, pH 7.4 containing 4% paraformaldehyde with 15% picric acid in 0.1 M PBS buffer at pH 7.4. The brains were blocked in a stereotaxic instrument (Kopf Instrument, Tujunga, CA) and post-fixed in the same fixative overnight at 4℃. Blocked brains were cryoprotected in 30 % sucrose in 0.1 M PB at 4℃ un til they sank, frozen in dry ice, wrapped in parafilm and aluminium foil, and stored at - 20℃ until sect ioned. Blocked brains were embedded in Jung OCT medium (freezing media, Leica Microsystems, Wetzlar, Germany) and sectioned at 40 µm in a coronal plane on a cryostat (Leica CM 3050S,Leica Microsystems, Austin, TX, USA) between anterior and posterior planes of atlas plates A 8.8, to A 7.6 using an atlas of the chick brain (Kuenzel and Masson, 1988). The A 8.8 stands for 8.8 mm anterior to the zero coordinate which was the centered ear bars of the stereotaxic instrument. Brain sections were collected on 24-well plates containing cryoprotective solution, sealed using parafilm, and kept at - 20℃ .

2.4.2. Bright field immunohistochemistry

Free-floating sections were rinsed several changes (6 times x 10 min) of PBS at room temperature to remove cryoprotectant solution, incubated for 30 min (2X15min) in PBS containing 0.6% of hydrogen peroxidase (2 ml of 30% H2O2 in 98 ml of 0.02 M PBS) to suppress endogenous peroxidase activity and then permeabilized with 0.4% Triton X-100 in 0.02 M PBS for 15 min. Subsequently, sections were placed into 5% normal goat serum (NGS) in PBS with 0.1% sodium azide and 0.2% Triton X-100 for 30 min to block non specific binding sites. The sections were then incubated with rabbit antibody against chicken VT4R diluted 1:2500 in 0.02 M PBS containing 1%NGS, 0.4% Triton X-100 , and 0.1% sodium azide for overnight at 4℃ on a belly dancer. Follo wing incubation, sections were rinsed in 6 times at 10 min each and incubated with goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS with 0.2% Triton X-100 for 90 min at room temperature (RT). After 4 timed rinses at 10 min each, the sections were incubated with Vectastain Elite ABC peroxidase complex (Vector Laboratories) diluted 1:5 in PBS containing 0.1% crystallized bovine serum albumin (BSA), and 0.2% Triton X-100 for 90 min. The sections were rinsed 4 times at 10 min each in PBS, followed by 15 min-rinse in 0.175 M sodium acetate buffer, pH 6.0 and immediately immunostained through glucose oxidase-diamino benzidine-nickel method (Shu et al., 1988). After 5-7 min (up to 20 min), this reaction was

stopped by a short rinsing in 0.175 M sodium acetate buffer. Sections were immersed in PBS and distilled water, mounted on gelatin-coated glass slides, air-dried overnight and coverslipped with histomount (National Diagnostic, Atlanta, GA).

2.5 Treatment Groups of Birds and Sampling Procedure for Gene Expression Involving two Circumventricular Organs

2.5.1 Birds, treatments, sampling procedures

The birds used for the gene expression experiment were subjected to the same treatments as described in 2.1 of material and methods with n=8 birds per treatment. Birds were killed by cervical dislocation, and their brains were removed from the skull, put into the 2-methylbutane, frozen in dry ice, and stored at - 80℃ until sectioned procedure.

2.5.2 Sectioning the OVLT and SSO for Gene Expression of Selected Genes

Frozen chick brains were embedded in Jung OCT medium (freezing media, Leica Microsystems, Wetzlar, Germany) and sectioned at 200 µm in horizontal planes on a cryostat (Leica CM 3050S, Leica Microsystems, Austin, TX, USA) between anterior and posterior plans corresponding respectively A 8.8 and A 8.2 for OVLT (Fig.1), A8.0 and A 7.6 for SSO and PVN (Fig.1) of chicken brain atlas (Kuenzel and Masson, 1988). Each brain section was collected in a 1.5 ml tube containing 100 ml of Trizol (Qiagen, Valencia, CA, USA) and kept at - 80℃. Each brain s ection had average size as follow: OVLT (Length (L) x width (w) x thickness (th); 2 mm x 1.5 mm x 0.2 mm respectively); SSO (1.5 mm x 1.5 mm x 0.2mm); PVN (2 mm x 1.5 mm x 0.2 mm).

Anterior

Posterior

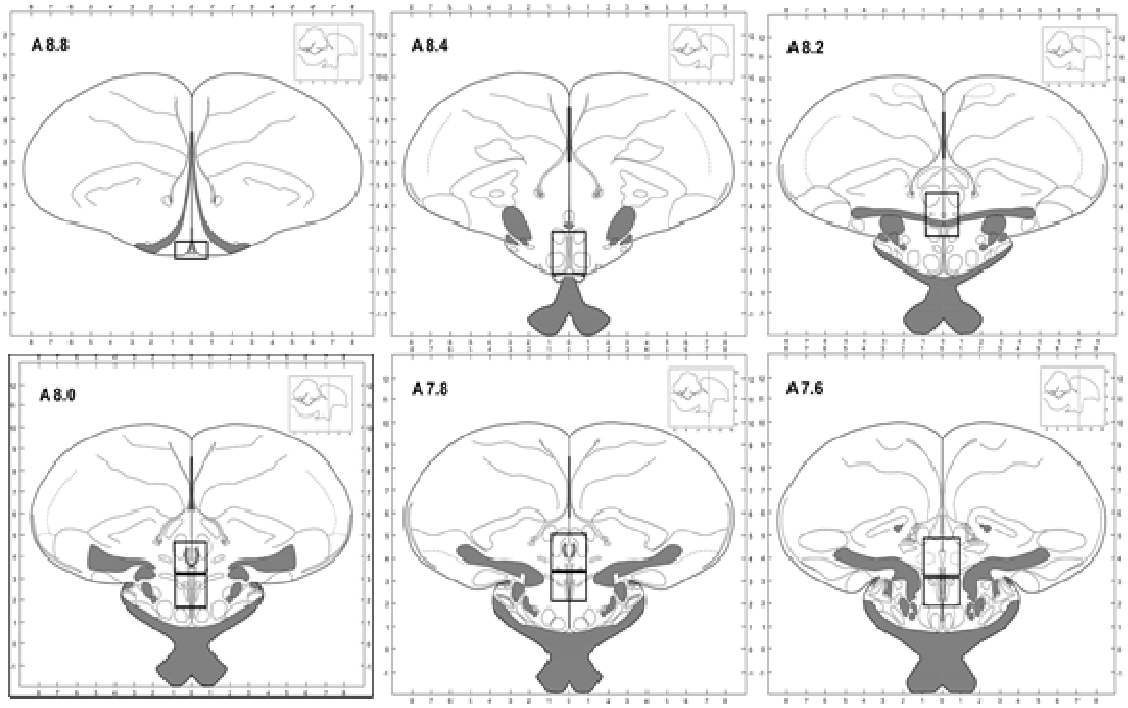

Fig. 1: Schematic plates A8.8, A 8.4, and A 8.2 (Anterior region of the hypothalamus) of the chick brain show the organum vasculosum of lamina terminalis (OVLT). The boxed-in areas depict the dissected regions for the OVLT. Schematic plates A8.0, A 7.8, and A 7.6 show more posterior regions of the chick hypothalamus. Just dorsal to the hypothalamus are the three upper boxed-in areas showing the tissue dissected that included the subseptal organ (SSO). The lower three boxed-in regions show the areas dissected that included the paraventricular nucleus (PVN) (Modified from Kuenzel and Masson, 1988).

2.5.3 RNA isolation and two-step real-time quantitative RT-PCR

Total RNA was extracted from each sectioned brain using Trizol® reagent (Life Technologies, Palo Alto, CA, USA) following the DNase I treatment (Invitrogen, Carlsbad, CA, USA) and purification by RNeasy Micro Kit (Qiagen, Valencia, CA, USA). The RNA quality and quantity were determined using agarose gel electrophoresis and Gen.5 Synergy HT (BioTek, Winooski, VT, USA). Single-stranded cDNA was synthesized from 2 µg total RNA using oligo d(T)16 primer and superscript III (Invitrogen, Carlsbad, CA, USA), as previously described (Kang et al, 2007; Kang et al., 2010; Selvam et al, 2013). The PCR primer pairs for VT4R and CRH-R1 were previously reported (Kang and Kuenzel, 2014, Selvam et al., 2013). The specific oligonucleotide primers for AT1 and TRPV1 were designed by the PRIMERS3 program (http://frodo.wi.mit.edu).The best primer pairs were selected from several pairs based on PCR product quality and lengths after electrophoresis on a 3 % agarose gel. A portion of 4 ul of cDNA was subjected to a quantitative real time RT- PCR using 7500 Real Time PCR system (Applied Biosystems LLC, Foster, CA, USA) with Power SYBR Green PCR Master Mix (Invitrogen Grand Island, NY, USA). Conditions of the real time qRT-PCR were 1 cycle at holding stage (50℃ for 2 min, 95℃ for 10 min), 4 0 cycles; denaturation (at 95℃) for 60 s, annealing (at 58-60℃) for 30 s for AT1, TRPV1, and β-actin: 58℃, for VT4R, CRH-R1, and GAPDH; 60℃, extending (at 72℃) for 3 min; holding stage (72℃ for 10 min). The NCBI accession numbers, PCR product size and primer sequences used in the present study are: VT4R [NM001110438, 137 bp (VT4R-F: 5-GGT TGC AGT GTT TTC AGA GTC G-3; VT4R-R: 5-CAA GAT CCG CAC CGT CAA G-3)], CRH-R1 [NM_204321, 141 bp (CRH-R1-F: 5-CCCTGCCCCGAGTATTTCTA-3; CRH-R1-R: 5- CTTGCTCCTCTTCTCCTCACTG-3)], AT1 [NC_006096.3 ; 138 bp (AT1-F: 5-CTGTTTCAGGAAGGCACAGT-3; AT1-R: 5-TGCTGGCCACTGTTTTTAAT-3)], TRPV1[NC_006106.3 ; 131 bp (TRPV1-F: 5-AAAGGCTGCCTGTTCATCAT-3; TRPV1-R: 5-TTGTCAGCTGTTTCCAGTGC-3)] GAPDH [NM204305, 128 bp (GAPDH-F:5- CTTTGGCATTGTGGAGGGTC-3; GAPDH-R: 5-ACGCTGGGATGATGTTCTGG-3)], β- actin [L08165, 158 bp (Actin-F: 5-CACAATGTACCCTGGCATTG-3; Actin-R: 5-ACATCTGCTGGAAGGTGGAC-3)]. The chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

and β-actin gene were as internal controls. The fold change values for the stressed groups compared with controls were determined by the ΔΔCT method.

2.6. Statistical analysis

Statistical analyses were performed using software JMP® Pro 11.0 (SAS Institute Inc. Cary, NC). Differences among the four treatment groups (same sample size) were analyzed using one way analysis of variance (ANOVA). Pairwise comparisons between the groups were analyzed with Fisher's least significant difference (LSD) test. Data from each group are expressed as Mean ± SEM. A probability level of $p < 0.05$ or $p<0.01$ was considered statistically significant.

3. Results

In our laboratory, we showed that the vasotocin receptor, VT4R/V1aR, was found in glial cells in the CVOs of the avian brain and were particularly dense in the OVLT and the SSO/SFO. Since vasopressin is also known as antidiuretic hormone, and AVT is homologous to vasopressin, we wished to test the hypothesis that the avian VT4R/V1aR within the OVLT and/or SSO functions in water balance or specifically osmotic balance. Our past studies have focused on examining psychological stress using immobilization as a model. Results have shown that the stress hormone, corticosterone, is significantly elevated following acute or chronic restraint stress, and four receptors associated with corticotrophs in the anterior pituitary are involved strongly suggesting that the classical hypothalamo-pituitary-adrenal axis was activated. In our laboratory, we have previously not examined a physical stressor. Since osmoregulation and water balance primarily involve a physical stressor, we needed to select a means of disrupting the normal ionic balance in a bird, and first determine whether it was truly stressful. If it was shown stressful, based upon elevated corticosterone levels, we would then utilize that procedure to examine whether it affected the anatomy and gene expression of the two CVOs. As controls, birds were either subjected to a psychological stressor, immobilization, treated with an isotonic solution that did not change the osmotic balance of their bodies or were not handled.

3.1. Plasma Corticosterone and Arginine Vasotocin Concentrations

To determine the overall systemic effect of each stressor, blood samples were collected after 1hour of acute immobilization stress or 1h following 3 M sodium chloride IP injections and assayed for corticosterone (CORT) and arginine vasotocin (AVT). Results are shown in Fig. 2 and 3.

3.1.1. Plasma corticosterone concentrations

Plasma levels of corticosterone were significantly different in the treatment group given hypertonic saline IP ($p<0.05$) and the group subjected to immobilization ($p<0.01$) compared to their respective controls. Plasma levels of corticosterone in acute immobilization birds (1.22 ± 0.26 ng/ml; mean ± SEM) showed a 5-fold increase compared to their controls (0.25 ± 0.041 ng/ml). Similarly, subjecting birds to a physical stressor, hypertonic saline administration resulted in a significant increase in their stress hormone levels. Specifically, a 9-fold increase of plasma corticosterone concentration was obtained in hypertonic birds (2.46 ± 0.56 ng/ml) compared to their respective controls, administered isotonic saline (0.27 ± 0.05 pg/ml; Fig. 2). Taken together, the hypertonic group displayed more than a 2-fold increase in CORT than the other treatment group subjected to immobilization (Fig. 2).

3.1.2. Plasma arginine vasotocin concentrations

Figure 3 shows the plasma concentration of arginine vasotocin following 1hour of hypertonic saline administration or acute immobilization stress. Plasma AVT in the immobilized group (7.97 ± 0.95 pg/ml) was significantly different ($p < 0.01$) from the acute control group (4.19 ± 0.59 pg/ml). The injection of 3.0 M NaCl of hypertonic saline to birds was a 2.9-fold higher level of plasma AVT concentration (17.06 ± 4.78 pg/ml) compared to its isotonic controls (5.89 ± 0.32 pg/ml).

As a result, the brains of chicken having high corticosterone and arginine vasopressin were selected for determining the impact of hypertonic saline on the VT4R within SSO/OVLT using anti-VT4R for immunohistochemistry and then investigating gene expressions of interest expressed with real time RT-PCR to test our hypothesis.

3.2. Anatomy of the OVLT/SSO in the Avian Brain

The distribution of the VT4R in the organum vasculosum of lamina terminalis and the subseptal organ was examined in coronal sections of the chick brain.

3.2.1. Organum Vasculosum of the Lamina Terminalis

The organum vasculosum of the lamina terminalis begins at the anterior edge of the optic chiasma located at the base of the brain forming a triangular-like shape (Fig. 4A). The structure continues dorsally along the third ventricle (Fig. 4B-4E), moves in front of the anterior commissure (Fig. 4F), and ends at the base of the nucleus of the hippocampal commissure where it resides directly dorsal to the anterior commissure. The organum vasculosum of the lamina terminalis having glia containing the avian V1aR contacting the cerebrospinal fluid in the rostral portion of the third ventricle suggest strongly that this CVO can bind available arginine vasotocin within the cerebrospinal fluid and ,perhaps, respond to any changes in its concentration (Fig. 4D).

3.2.2. Subseptal Organ

The subseptal organ appears to begin immediately after the anterior commissure (Fig. 5A), continues posteriorly and dorsally (Fig. 5B-5E), and ends as a fingerlike form projecting into the third ventricle (Fig. 5E, 5F). In other words, in figure 6 (5B-5D), the VT4R immunoreactive glial fibers in the anterior region of the SSO occurs around chambers containing CSF which increasingly enlarge as SSO continues posteriorly. The glial cells containing the avian VT4R have the capability of binding the circulating AVT in the CSF. As it continues posteriorly, the SSO shows increased VT4R immunoreactivity particularly at the base of the NHpC where the head of the glia contact the chamber within the posterior SSO that contains the cerebrospinal fluid and their terminal processes form the fingerlike projection (Fig 5E, 5F) that enters the ventral portion of the third ventricle. Therefore, this structure has the components capable of binding the AVT within the CSF, and, perhaps, respond to changes in AVT levels within the third ventricle.

3.3. Structural Analyses of the OVLT/SSO

To test our hypothesis that the VT4R located in OVLT/SSO is associated with osmoregulation, we examined morphological changes of VT4R immunoreactive glial cells within OVLT/SSO following 1h of 3 M sodium chloride compared to its isotonic control as well as a group subjected to immobilization and its respective control.

3.3.1. Organum Vasculosum of Lamina Terminalis

The VT4R immunoreactive ependymal cells were present within the OVLT of hypertonic saline and acute immobilization stress groups as well as their respective controls. We are focused on the ventral region of the main triangular-like part of the OVLT attached to the brain parenchyma (Fig. 4D; boxed area on the brain in Fig. 6A, 6B, 8B). In the other words, we examined morphological changes that occurred under the chamber (not involved in our analysis) caused by the split between the main portion of the OVLT and the brain parenchyma. The organum vasculosum of lamina terminalis of both hypertonic saline (Fig. 6B, 6D) and immobilization group (Fig. 8B, 8D) distinctly displayed curved immunostained VT4R glial processes. The head of curved immunoreactive VT4R glial processes originated either at the brain parenchyma or at the split between the proper OVLT and the brain parenchyma, moved down as curving manner in the third ventricle contacting the CSF compared to the controls (Fig. 6A, 8A). In control birds, the curving processes were not clearly observed; however, the head of immunoreactive VT4R glials also derived from the brain parenchyma and moved horizontally to the third ventricle. In addition, the marked change in orientation and the increased number of VT4R/V1aR immunoreactive glial processes in the hypertonic saline group ($p<0.01$) compared to their isotonic controls were observed (Fig. 7B). No significant change was observed in the acute immobilization group (Fig. 7A).

3.3.2. Subseptal Organ

The VT4R immunoreactive ependymal cells were present within the SSO of the hypertonic saline and acute immobilization stress groups as well as in their controls, respectively. Examining coronal sections revealed no significant morphological changes in the dorsal portion of the SSO where glial cells

appeared to surround chambers containing the CSF. However, significant changes were observed in the orientation of VT4R immunoreactive glial fibers contacting CSF in the mid-and ventral regions of the SSO. The head of glial processes originated from the third ventricle in the ventral region moved discontinuously to dorsal region of the SSO in association with horizontal compact bands of fibers in the hypertonic saline (Fig. 9B) and immobilization group (Fig. 9D) compared with their respective controls. The controls showed vertical and parallel orientation of VT4R immunoreactive glial fibers connecting ventral and dorsal regions of SSO without any visible horizontal fiber bands (Fig.12A, 12C). The organization of glial cells in compact bands in both experimental groups (Fig. 9B, 9D) rendered their quantification variable and inconsistent. Therefore, the quantification of immunoreactive glial processes or thicker bands was not completed in this CVO.

3.4. Functional Analyses of SSO/OVLT

In order to confirm the results from the immunohistochemical changes of VT4R immunoreactive within the OVLT/SSO in the experimental groups, particularly following the hyperosmotic, physical stressor, we isolated total mRNA from the OVLT/SSO in 3-week old male chicken preoptic regions for quantitative RT-PCR to determine the VT4R gene expression and other relevant genes. We first checked the change of angiotensin II subtype 1A receptor mRNA (AT1AR mRNA) gene expression. It is known to mediate water regulation within the OVLT/SSO. Second, we tested the TRPV1 mRNA gene expression. It senses the hypertonicity caused by the shrinking of cells from a hyperosmotic, physical stressor such as the one used in our study.

3.4.1 Angiotensin II Subtype 1A Receptor (AT1AR mRNA) mRNA Gene Expression in the SSO and OVLT

Expression levels of AT1AR mRNA (ANG II subtype 1A receptor mRNA) were measured in the subseptal organ and the organum vasculosum of lamina terminalis of the 1h acute immobilization stressed birds and following 1h of administration of hypertonic saline, designated as the hypertonic stressed group. The two treatment groups were compared to their respective controls (acute control; isotonic saline control). A significant increase of the AT1AR mRNA gene expression in the hypertonic

saline birds (95%, p=0.05) occurred compared to the isotonic saline controls in the SSO (Fig. 10A). No change in the AT1AR occurred; however, in the acute immobilization stressed birds compared to their controls (Fig. 10A). In contrast, within the OVLT, there was a significant increase of the AT1AR mRNA in the immobilized treatment group compared to its control while the hypertonic saline group displayed a significant decrease in gene expression compared to its isotonic saline controls. In other words, in the OVLT, the AT1AR mRNA was increased (upregulated, 1.48-fold) in immobilized birds, while decreased (slightly downregulated) in hypertonic saline birds (Fig. 10B).

3.4.2. Receptor Potential Vanilloid Type 1 mRNA (TRPV1mRNA) Gene Expression in the SSO/ OVLT

To determine whether TRPV1 mRNA is associated with hypertonicity sensing in the chicken, we performed the RT-PCR from extracted SSO/OVLT tissues to examine TRPV1 mRNA change from either acute hypertonic saline injection or acute immobilization stress. The 1h hypertonic saline administration significantly increased TRPV1 mRNA levels (189 %, p=0.05) in the SSO (Fig. 11A) and significantly decreased TRPV1 mRNA expression levels (37%, $p< 0.05$) in the OVLT (Fig. 11B) compared to their respective controls. The Acute immobilization had no significant effect on TRPV1 mRNA change (p=0.6437) within the SSO compared to its controls (Fig. 11A). In contrast, acute immobilization stress significantly increased TRPV1 mRNA levels (116%, $p<0.05$) in the OVLT (Fig. 11B).

3.4.3. Vasotocin subtype 4 receptor mRNA (VT4R mRNA) gene expression in SSO and OVLT

Within the SSO, there was a significant increase of VT4R mRNA in the hypertonic saline group and acute immobilization stress group (19%, p=0.05; 39%, p=0.05 respectively) compared to their respective controls (Fig.12A). Within the OVLT, a significant increase of VT4R mRNA levels were observed in both injected hypertonic saline and immobilization birds (60%, $p<0.05$; 20%, p=0.05) compared to their respective controls (Fig. 12B). Both experimental groups showed upregulated VT4R mRNA levels; however, VT4R mRNA was more highly upregulated in the hypertonic saline birds compared to immobilized birds within the OVLT (Fig. 12B).

3.4.4. Corticotropin releasing hormone subtype 1 receptor mRNA (CRH-R1 mRNA) expression in paraventricular nucleus (PVN)

The paraventricular nucleus contains CRH-R1 and has connections with both SSO/OVLT, so the study of CRH-R1 mRNA gene expression would assess the activation of the hypothalamo-neurohypophyseal system or of the hypothalamo-pituitary adrenal axis following hypertonic stress. The CRH-R1 mRNA levels were significantly increased and upregulated (64%, $p<0.01$) in immobilized birds compared to their controls, while CRH-R1 gene expression was significantly decreased (36%, $p<0.01$) in the hypertonic saline administered birds compared to their controls in the paraventricular nucleus (Fig.12C).

4 Discussion

4.1 Administration of hypertonic NaCl is an effective, physical stressor in broilers

Results of our study (Fig. 2 and 3) provide evidence that 3-week male chickens are more responsive to physical stress such as hypertonic saline than psychogenic stress, immobilization. In fact, hypertonic stimuli released more corticosterone (2-fold increase) and arginine vasotocin than acute immobilization stress. These results corroborate those observed by other studies (Nouwen et al., 1984; Harbuz and Lightman, 1989; Shibasaki et al., 1993). Data suggest that hypertonic saline is a highly effective and potent stimulator of plasma corticosterone secretion, and, therefore, the physical stressor likely affects the SSO/OVLT, the focused areas of our study. However, both stressors appear to have significant and specific effects on anatomical as well as gene expression changes in the two CVOs examined in this study.

4.2 The structure and extent of the SSO and OVLT within the avian brain can be identified using an antibody to the VT4R/V1aR.

Examining coronal sections revealed VT4R-immunoreactive glial cells throughout the extent of the OVLT, one of the sensory brain CVOs. In the past, it has attracted attention because of its unusual characteristic position, in front of the beginning of the third ventricle and moving dorsally toward the anterior commissure and above it. This unique OVLT structure in chicken was first described by Dellmann

(1964). Additional anatomical characteristics for this CVO in the chicken (Kuenzel and van Tienhoven, 1982; Kuenzel and Masson, 1988; Kuenzel and Golden 2006; Selvam et al, 2014) and duck (Matsumura and Simon, 1990; Natke et al., 1996) brain have been provided. The avian VT4R antibody, however, is most useful in defining the extent of the OVLT in the avian brain. Additionally, the VT4R immunoreactive glial cells found within the SSO, homologous to mammalian SFO, distinctly described the SSO morphology in the chicken. Our findings corroborate previous data in the chicken (Kuenzel and Golden, 2006; Selvam et al, 2014), pigeon (Weindl and Sofroniew, 1982) and duck (Schmid, 1994). As a result, the immunohistochemistry using anti VT4R was positive and revealed the presence of immunostained-VT4R glia throughout the SSO and the OVLT, particularly around blood vessels and lining part of the third ventricle where the two CVOs resided, critical for the regulation of water balance in chicken in response to physical stressors of interest.

4.3 Anatomical changes in specific regions of the OVLT and SSO occurred following physical and psychological stressors

The present study shows significant immunohistochemical changes of the chicken VT4R immunostained glial fibers around the third ventricle within the SSO/OVLT following a physical stressor such as hypertonic saline and psychogenic stressor, including acute immobilization stress compared to their respective controls. The presence of chicken VT4R immunoreactivity changes noted around the third ventricle within the SSO/OVLT may suggest that the chicken VT4R could bind AVT, and, thereby, sensing any change in CSF concentrations resulting from plasma osmolarity changes imposed by a physical stressor. The V1aR results herein provide new data to support the functional role of angiotensin II regarding osmotic regulation based upon autoradiographic studies in mammals (Mendelsohn et al., 1984; Simon-Oppermann and al., 1988; Oldfield et al., 1994) and birds (Gerstberger et al. 1987; Natke et al., 1996). Indeed, the circulating angiotensin II bind to the ANG II subtype 1 receptor within the SSO/OVLT to regulate water balance in response to any osmolarity change. The immunohistochemical changes of VT4R glial cells within the SSO/OVLT seem to have the same location pattern as the ANG II and could mediate AVT actions affecting osmoregulation in the chicken. In addition, in our study, the high plasma AVT levels in 3-week male chickens subjected to physical stress in association with marked

changes in glial VT4R immunoreactivity glial changes provide strong evidence that the VT4R within the OVLT and the SSO could be associated with osmotic stress. Similarly, the psychological stressor, immobilization, affected a VT4R immunoreactive glial change in the SSO/OVLT. The chicken OVLT and SSO could also be associated with the classical HPA stress response involving the neuroendocrine system. In other words, anatomical changes within the SSO/OVLT, particularly in chicks given hyperosmotic saline have shown that the VT4R might be involved in avian osmoregulation. The functional study of *VT4R* gene and other genes related to water balance have provided additional evidence supporting our hypothesis that the OVLT and SSO appear to be involved in osmotic regulation.

4.4 Real-time RT-PCR data support the functional role of the SSO in osmotic stress based upon significantly increased expression of the following genes: *AT1aR*, *Trpv1* and the *VT4R/V1aR* after an imposed hyperosmotic challenge

In order to completely validate our hypothesis: determine whether or not a physical stressor, hyperosmotic saline, would affect the gene expression of VT4R located in the SSO, we performed RT-PCR of genes involved in osmoregulation. Our results show that TRPV1 mRNA levels are highly increased in the SSO (Fig. 11A). This result is consistent with mammalian studies which have confirmed that the SSO is considered to be an osmosensor and has the receptors to initiate water intake (Johnson and Gross., 1993; Sladek and Johnson, 2013). Transient receptor potential vanilloid type 1 mRNA, an osmosensory transducer located within the both CVOs, might participate in hypertonicity sensing. In mammals, it has been shown to be responsive to hypertonic saline stimulation and to release AVP from magnocellular neurons in mammals (Sharif- Naeini et al, 2006; Liedtke, 2007; Cuiri et al, 2011; Sladek and Johnson, 2013). Thus, the up-regulated *TRPV1* gene in our study shows that the gene is present in the SSO and responds following a physical stressor. Moreover, in our study, acute hypertonic saline i.p injection and the acute immobilization stress have opposite effects on chicken CRH-R1 mRNA expression in the hypothalamic paraventricular nucleus (PVN) (Figure 12C). Our results are similar to previous reports utilizing mammals (Makino et al, 1999; Steckeler and Hosboert, 1999). The CRH-R1 receptors are found in the anterior pituitary gland of mammals and birds (Potter et al., 1994; De Groef et al., 2004; Kuenzel et al., 2013) and widely expressed in the central nervous system, including, amygdala,

hippocampus, lateral septal nucleus, the vasopressin/vasotocin-containing neurons in the supraoptic nucleus and the paraventricular nucleus (Potter et al., 1994; Imaki et al., 2001; De Groef et al., 2004) in mammals and birds. Moreover, several studies have identified neural connections between SSO and PVN, PVN and pituitary in mammals and birds (Miselis, 1981; Natke et al, 1996). Thus, the circulating ANG II act via the AT1AR located within the SSO (Figure 10A). The subseptal organ thereafter projects to PVN. Subsequently, parvocellular CRH neurons of PVN (Fig. 12C, upregulated PVN CRH-R1 mRNA by acute immobilization stress) activate the classical HPA axis, while magnocellular AVT neurons (Fig. 12C, downregulated PVN CRH-R1 mRNA by acute hypertonic saline injection) stimulate the HNS system for AVT release. Hence, the change of chicken CRH-R1 mRNA gene expression in an opposite direction in the PVN may suggest that CRH-R1 is involved in differential roles regarding osmoregulation as well as the classical stress pathway.

Finally, our findings have shown that VT4R mRNA levels were increased in the SSO following hypertonic saline administration (Figure 12A). Previous studies have shown that the VT4R was present in corticotrophs of the anterior pituitary and downregulated in the cephalic lobe following psychogenic stress (immobilization) suggesting the VT4R is associated with the neuroendocrine HPA pathway (Selvam et al, 2013). In contrast, its homologous, the mammalian V1aR has not been shown to be associated with the HPA pathway (Orcel et al., 2002). Therefore, the following data about effects of physical stress in our study: (1) anatomical change in VTR4 immunoreactivity within the SSO; (2) functional change of the AT1AR mRNA and TRPV1 mRNA within the SSO; (3) CRH-R1 mRNA changes in PVN; and (4) the unique functional change in VT4R mRNA expression in SSO strongly support the view that the avian VT4R/V1aR is involved in osmoregulation. The data strongly suggest that the VT4R/V1aR plays a major role in avian water balance.

4.5 Real-time RT- PCR data support the functional role of the OVLT in psychological stress based upon significantly increased expression of the following genes: *AT1aR*, *Trpv1* and the *VT4R/V1aR* after an imposed immobilization stress

Besides the stated hypothesis in our study, we examined the effect of psychological stress, immobilization stress on VT4R gene expression located in the OVLT. In our study, TRPV1 mRNA levels were highly upregulated in the OVLT (Fig. 11B) by the psychological stressor. Our data support the role of the OVLT in psychogenic stress based upon the response of the three genes analyzed. The Data suggest that psychogenic stress activates an osmosensor detector gene within the OVLT. Further studies will be necessary to verify whether or not the TRPV1 functions as an osmosensor located in the OVLT and also plays a role in responding to acute immobilization, a psychological stressor. Additionally, the VT4R mRNA gene expression was shown to be moderately increased in the OVLT following acute immobilization stress (Fig. 12B). Selvam et al (2013) have provided anatomical and gene expression evidence of the VT4R operating within the classical HPA axis following immobilization stress. Thus, morphological change of VT4R immunoreactive glials cells within the OVLT, gene expression change of VT4R mRNA in OVLT, and the contrasting direction of CRH-R1mRNA in PVN provide evidence that the psychological stressor affects the avian VT4R located in the OVLT. Consequently, the chicken VT4R located in the OVLT appears to be associated with the neuroendocrine stress pathway (HPA) in response to immobilization stress.

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6. Tables and figures

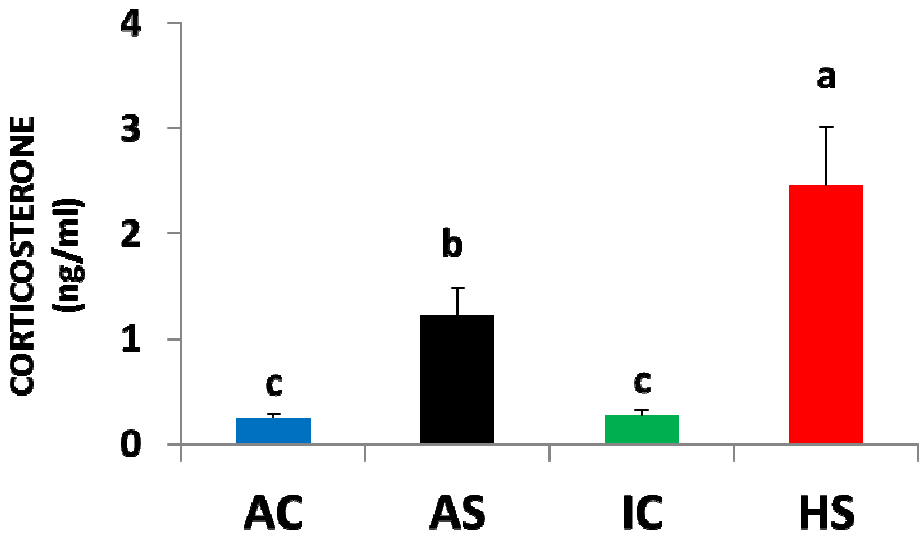

Fig. 2: Plasma corticosterone levels in response to 1h of acute immobilization stress and acute hypertonic saline administration compared to their respective controls. Different lower case letters show group means that are significantly different ($p<0.05$ or $p<0.01$). AC, acute immobilization control; AS, acute immobilization stress; IC, isotonic saline control; HS, acute hypertonic saline. (n=6 birds/group); Error bar: SEM (standard error of mean).

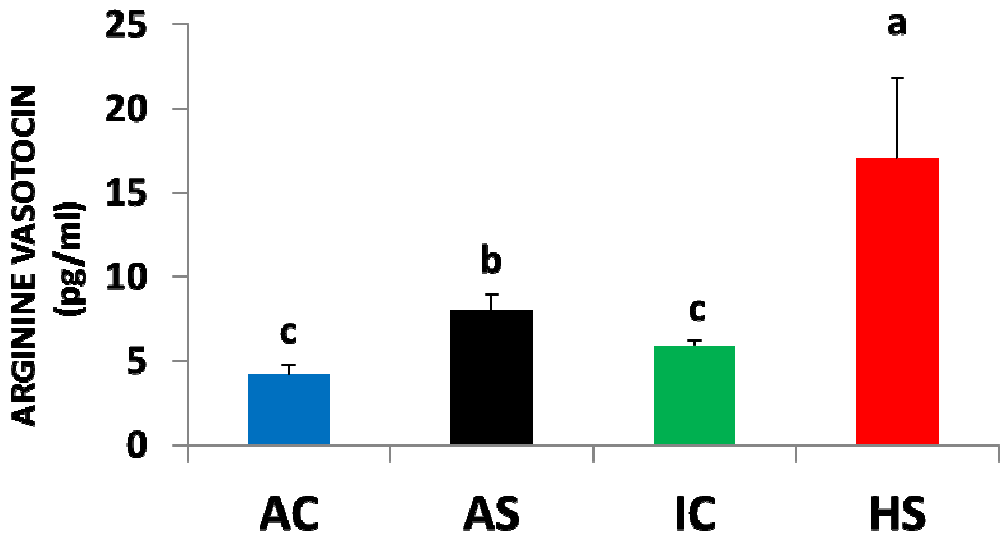

Fig. 3: Plasma arginine vasotocin levels in response to 1h of acute immobilization stress and acute hypertonic saline administration compared to their respective controls. Different lowercase letters show group means that are significantly different ($p<0.01$). AC, acute immobilization control; AS, acute immobilization stress; IC, isotonic saline control; HS, acute hypertonic saline. (n=5 birds/group); Error bar: SEM (standard error of mean).

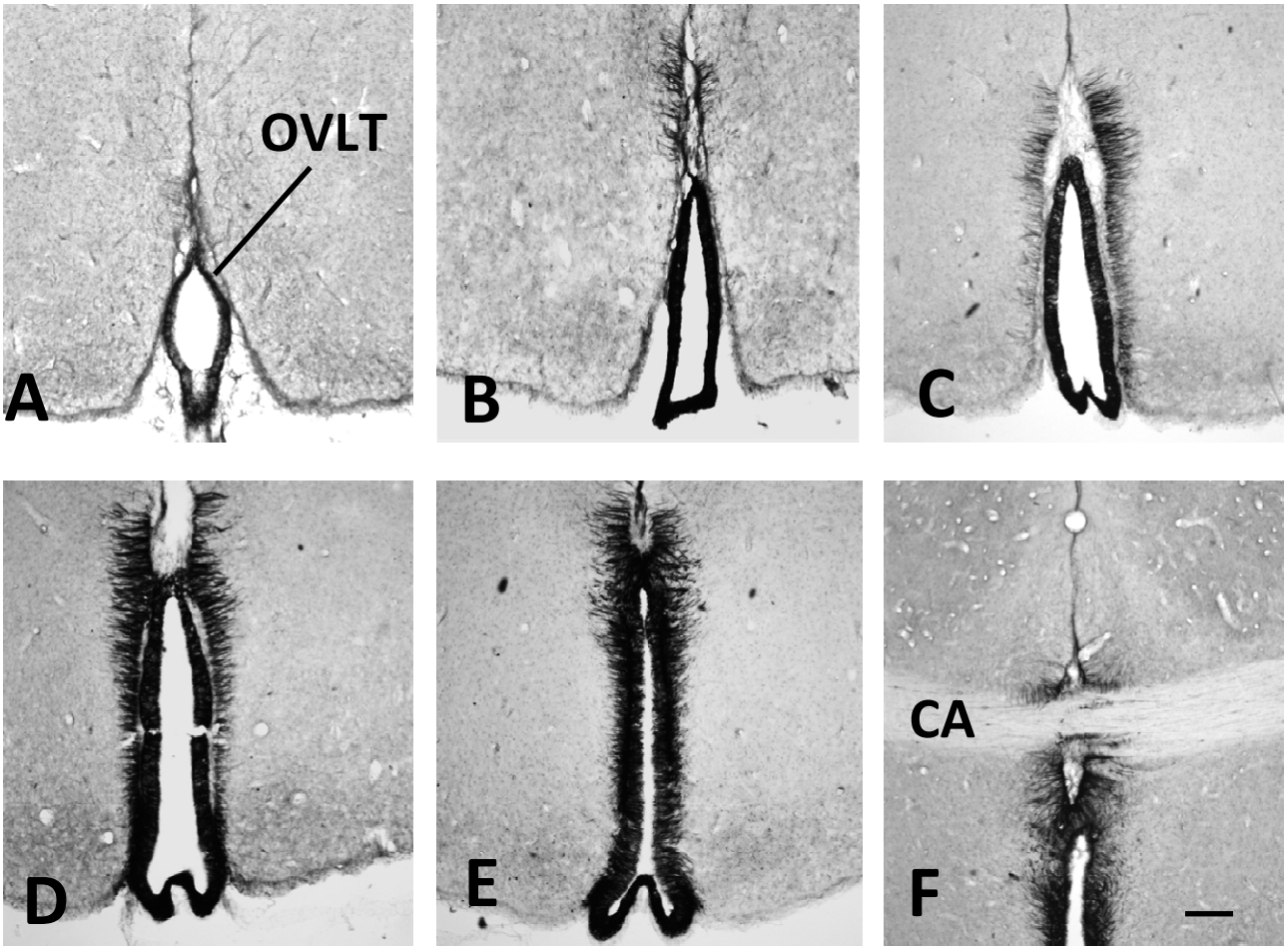

Fig. 4: Immunostaining of glia for arginine vasotocin subtype 4 receptor (VT4R), homologous to the mammalian vasopressin 1a receptor (V1aR). **A**. shows beginning of organum vasculosum of lamina terminalis (OVLT) at the base of the preoptic region. **B-E.** show progressive, dorsal movement of OVLT toward the anterior commissure (CA). **F.** the OVLT is shown in front of the CA and directly dorsal to the CA where it appears to end. Scale bar= 100 µm for A-F.

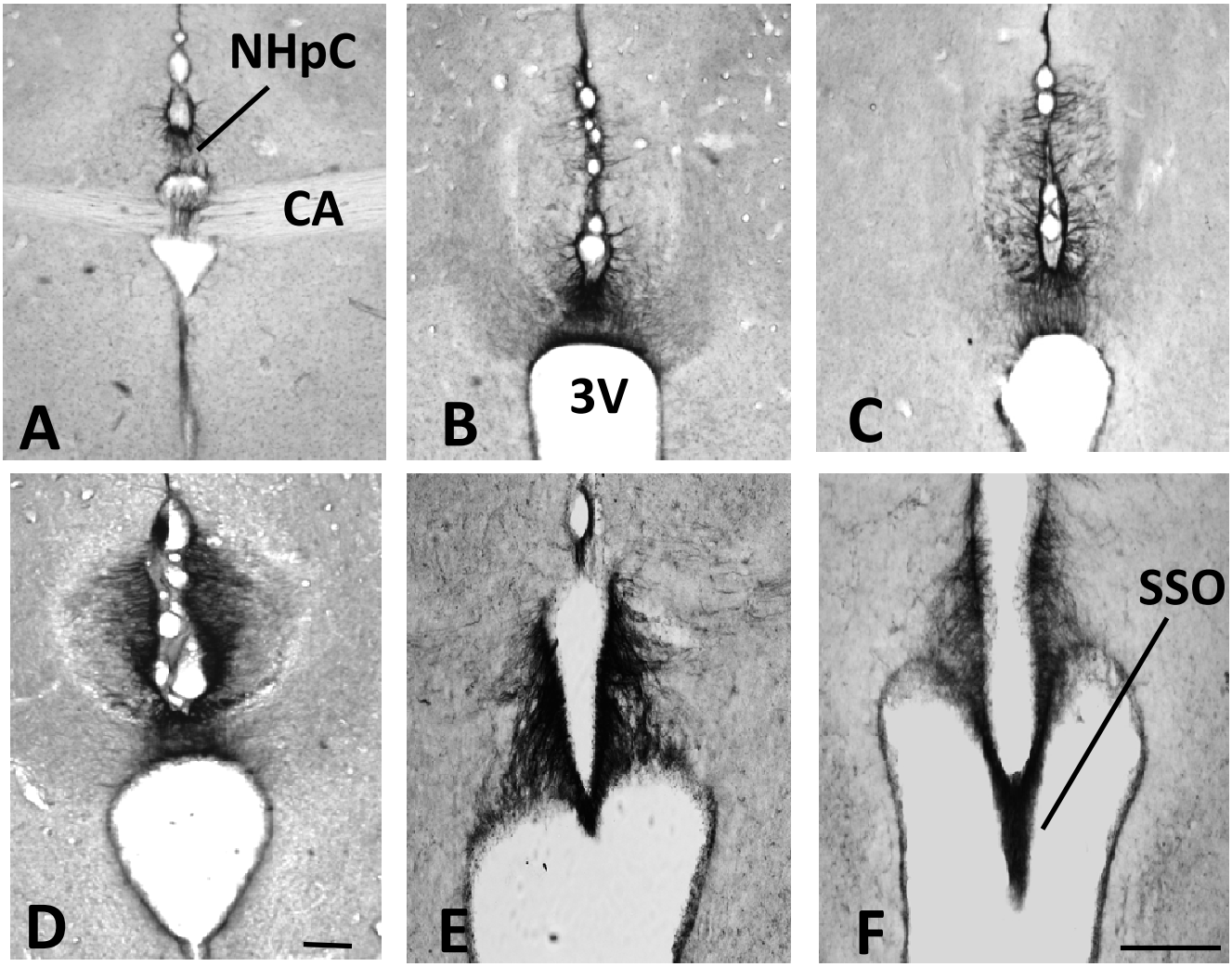

Fig. 5: Immunostaining of glia for arginine vasotocin subtype 4 receptor (VT4R) in the subseptal organ (SSO) homologous to the mammalian subfornical organ. **A**. Beginning of the SSO at the midline area adjacent to the nucleus of the hippocampal commissure (NHpC) and dorsal to the midline region of the anterior commissure (CA). **B-E**. Posteriorly immunostaining for VT4R in glia becomes particularly dense around the chamber dorsal to the 3V. **F.** at its most caudal level, the SSO sends a finger-like projection into the 3V. Scale bars for A-D=100 μm, E, F= 100 μm.

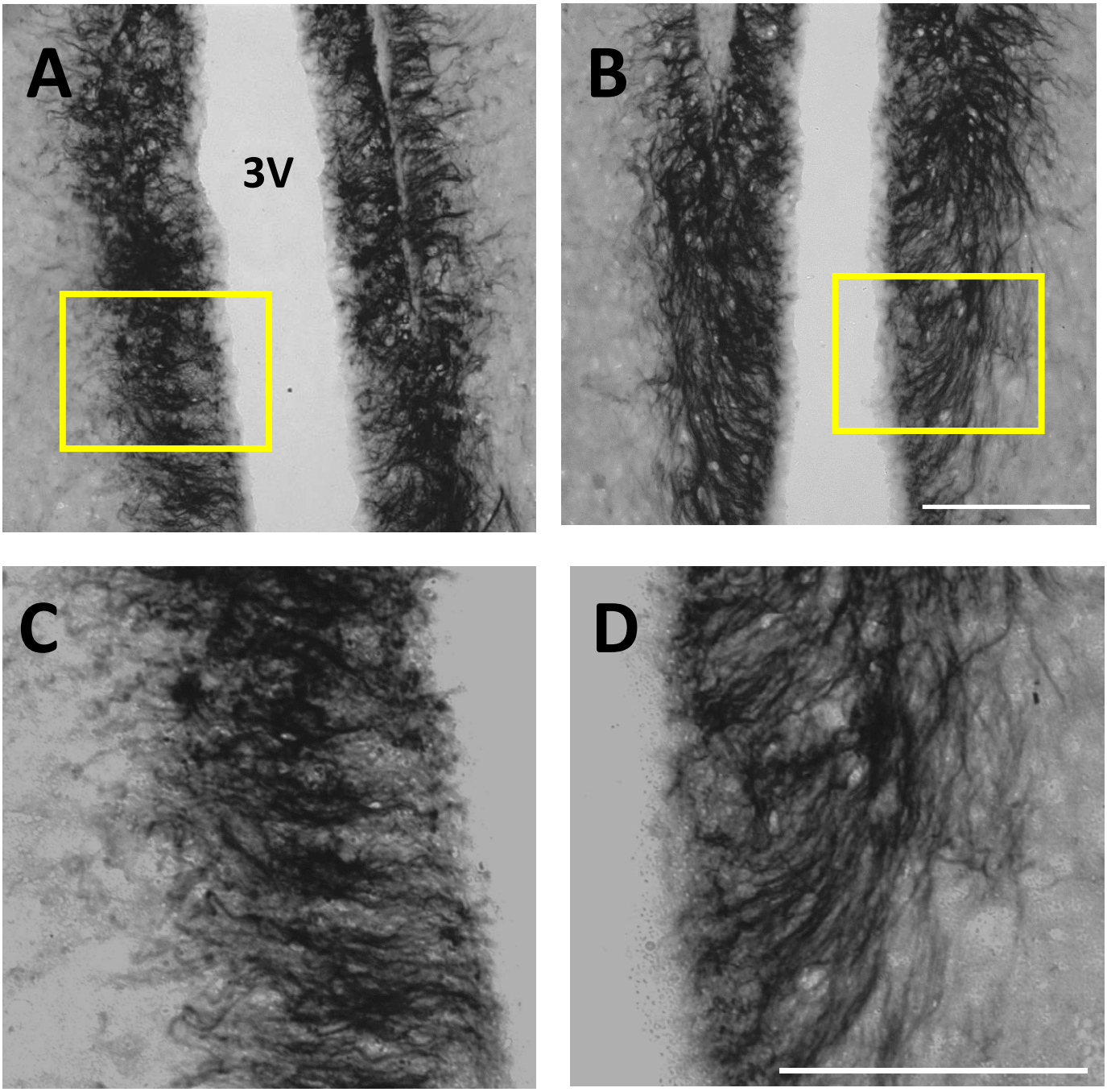

Fig.6: Immunohistochemical change within organum vasculosum of lamina terminalis (OVLT; box areas) following 1 h of hypertonic saline injection (**B**) compared to isotonic saline control (**A**). High magnification of OVLT in isotonic control (**C**) shows more horizontal orientation of bundles of glial fibers contrasting with parallel arrays of individual glial fibers in hypertonic saline (**D).** Glial fibers were quantified in 2 grids at 10x. Scale bar: A, B= 100 µm; C, D= 100 µm.

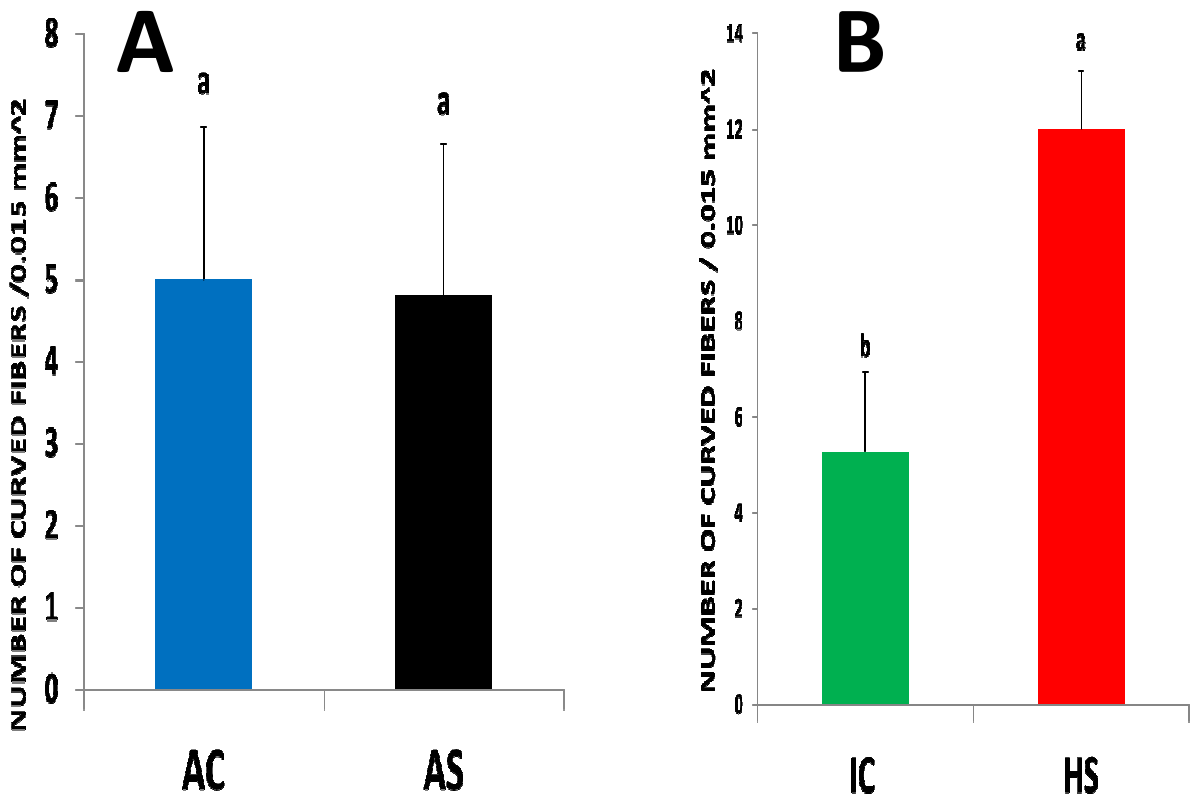

Fig. 7: Number of glial processes immunoreactive for VT4R/V1aR in the OVLT of the acute immobilization group (**A**) and in the hypertonic treatment group (**B**) compared to their respective controls. Different lowercase letters show group means that are significantly different ($p<0.05$). AC, acute immobilization control (n= 5 birds); AS, acute immobilization stress (n=4 birds); IC, isotonic saline control (n= 4 birds); HS, acute hypertonic saline (n= 4 birds). Error bar: SEM (standard error of mean).

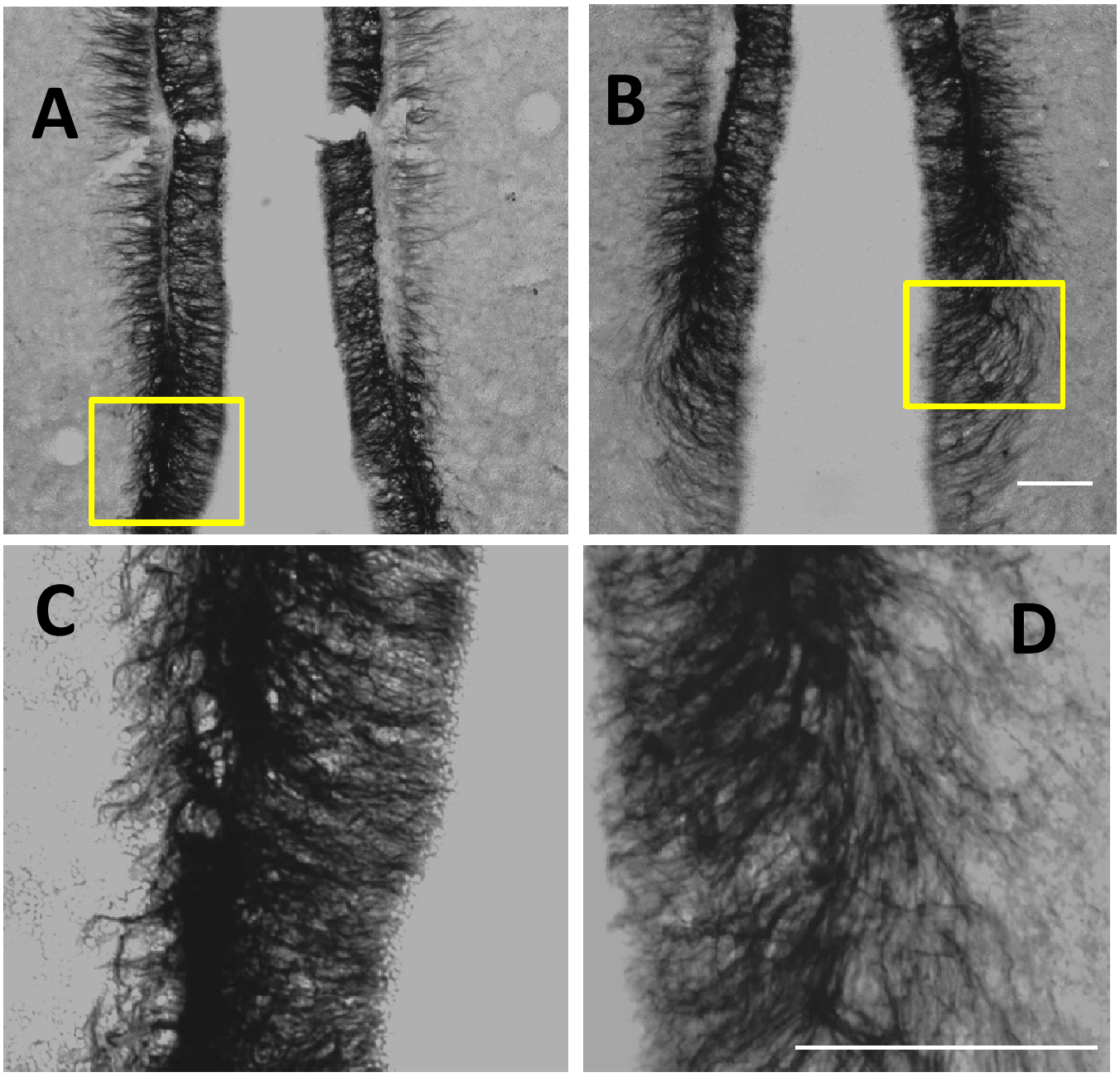

Fig. 8: Immunohistochemical change within OVLT following 1 h acute immobilization stress (**B**) compared to immobilization control (**A**). High magnification of OVLT in immobilization control (**C**) shows more horizontal orientation of bundles of glial fibers contrasting with parallel arrays of individual glial fibers in acute immobilization (**D**). Glial fibers were quantified in 2 grids at 10x. Scale bar: A, B=100 µm; C, D=100 µm.

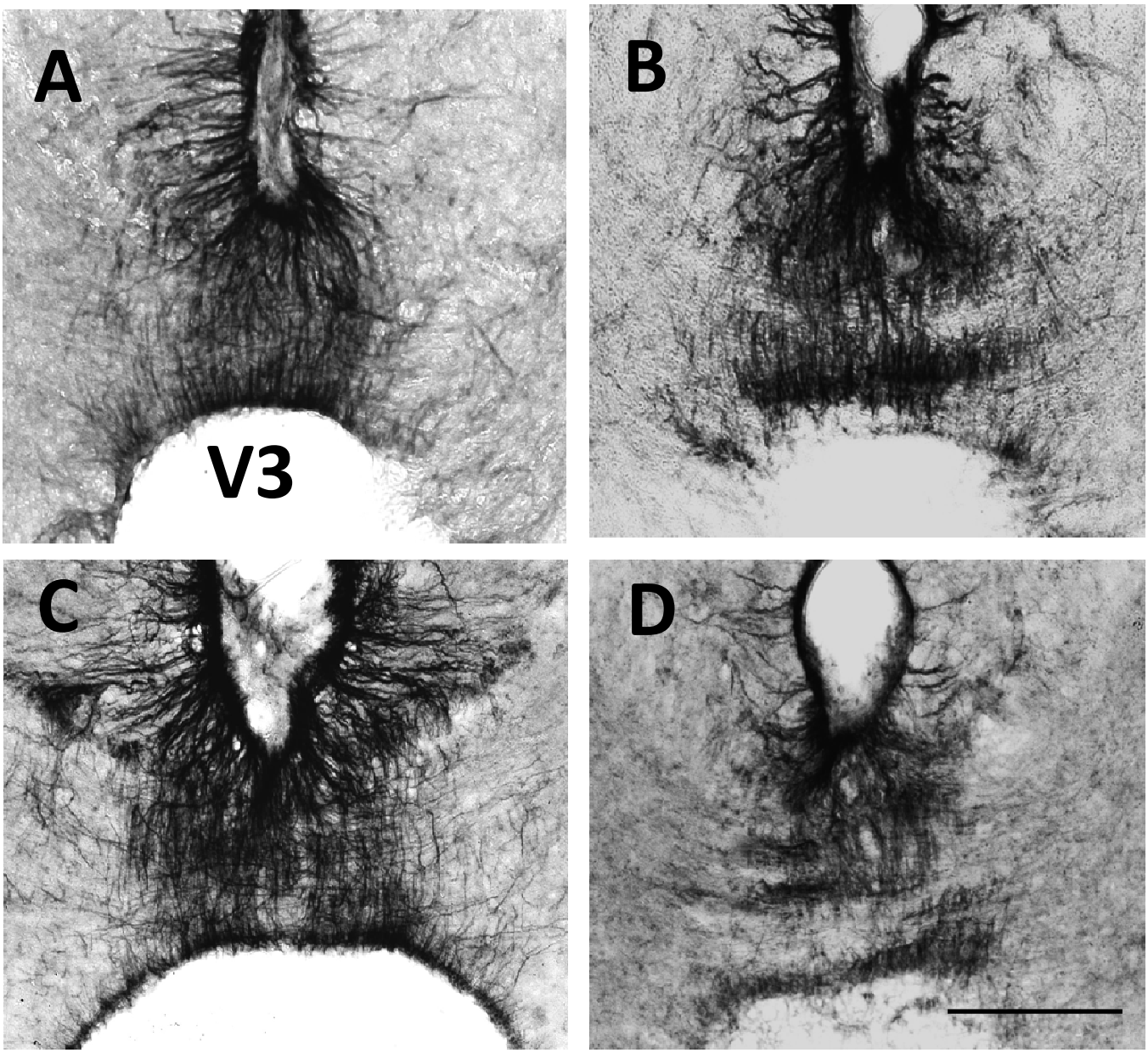

Fig. 9: Immunohistochemical changes within SSO following 1 h hypertonic saline injection **(B)** and 1 h acute immobilization stress **(D)** compared to their respective controls (isotonic saline, **A**; acute immobilization control, **C**)**.** Scale bar: 100 µm.

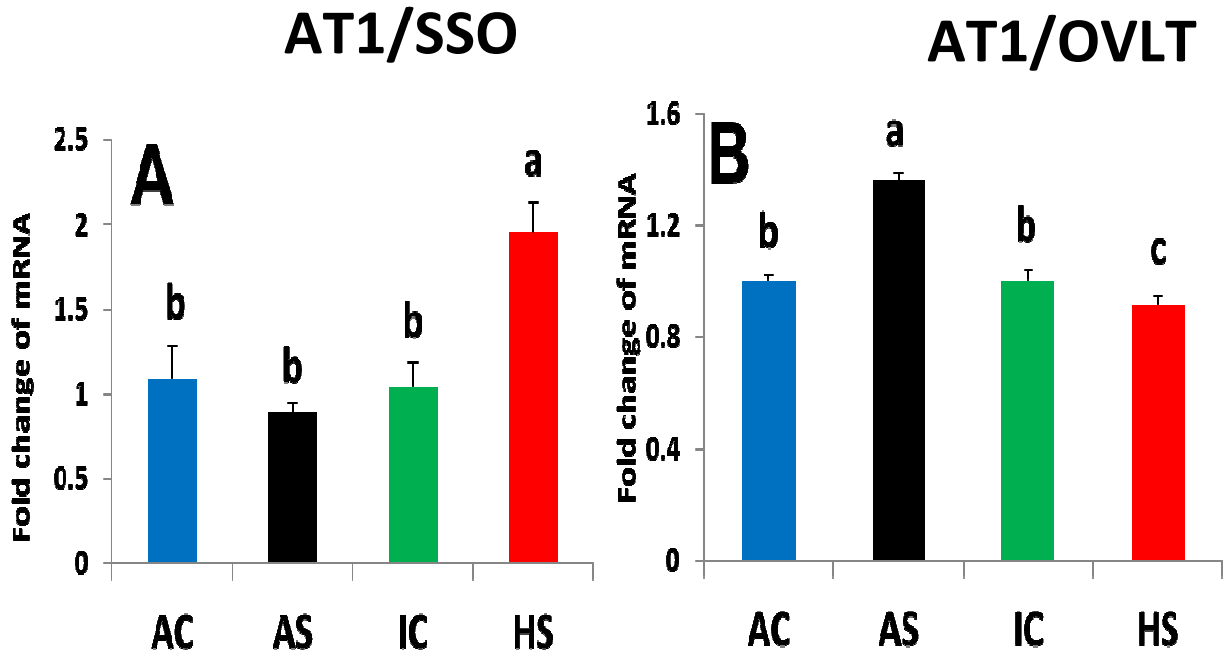

Fig. 10: Angiotensin 1 (AT1) receptor mRNA gene expression in the subseptal organ (SSO; **A**) and organum vasculosum of lamina terminalis (OVLT; **B**) following 1 h of acute immobilization stress and hypertonic saline injection. Different lowercase letters show group means that are significantly different ($p<0.01$). AC, acute immobilization control (n=5 birds); AS, acute immobilization stress (n=6 birds); IC, isotonic saline control (n=5 birds); HS, acute hypertonic saline (n= 6 birds). Error bar: SEM (standard error of mean).

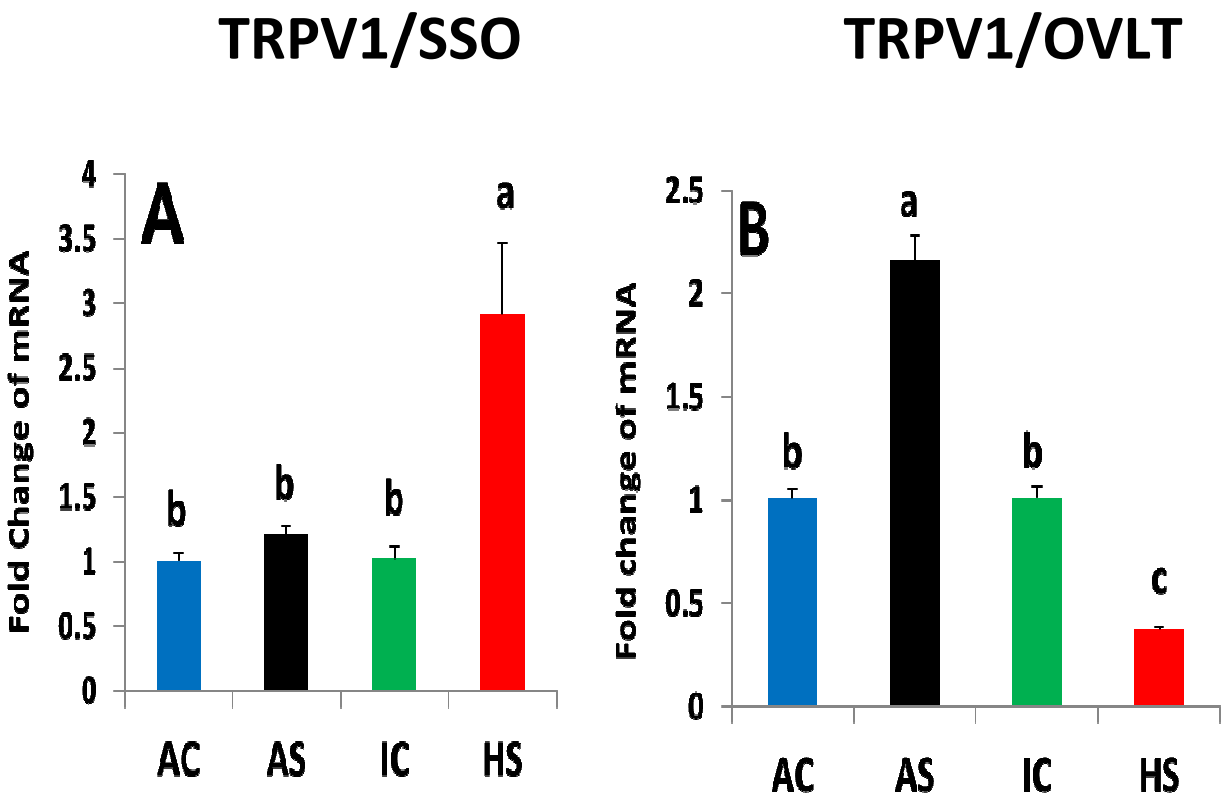

Fig. 11: Gene expression of TRPV1 mRNA in the subseptal organ (SSO; **A**) and organum vasculosum of lamina terminalis (OVLT; **B**) following 1 h of acute immobilization stress and hypertonic saline injection. Different lowercase letters show group means that are significantly different ($p<0.01$). AC, acute immobilization control (n=5 birds); AS, acute immobilization stress (n=6 birds); IC, isotonic saline control (n=5 birds); HS, acute hypertonic saline (n= 6 birds). Error bar: SEM (standard error of mean).

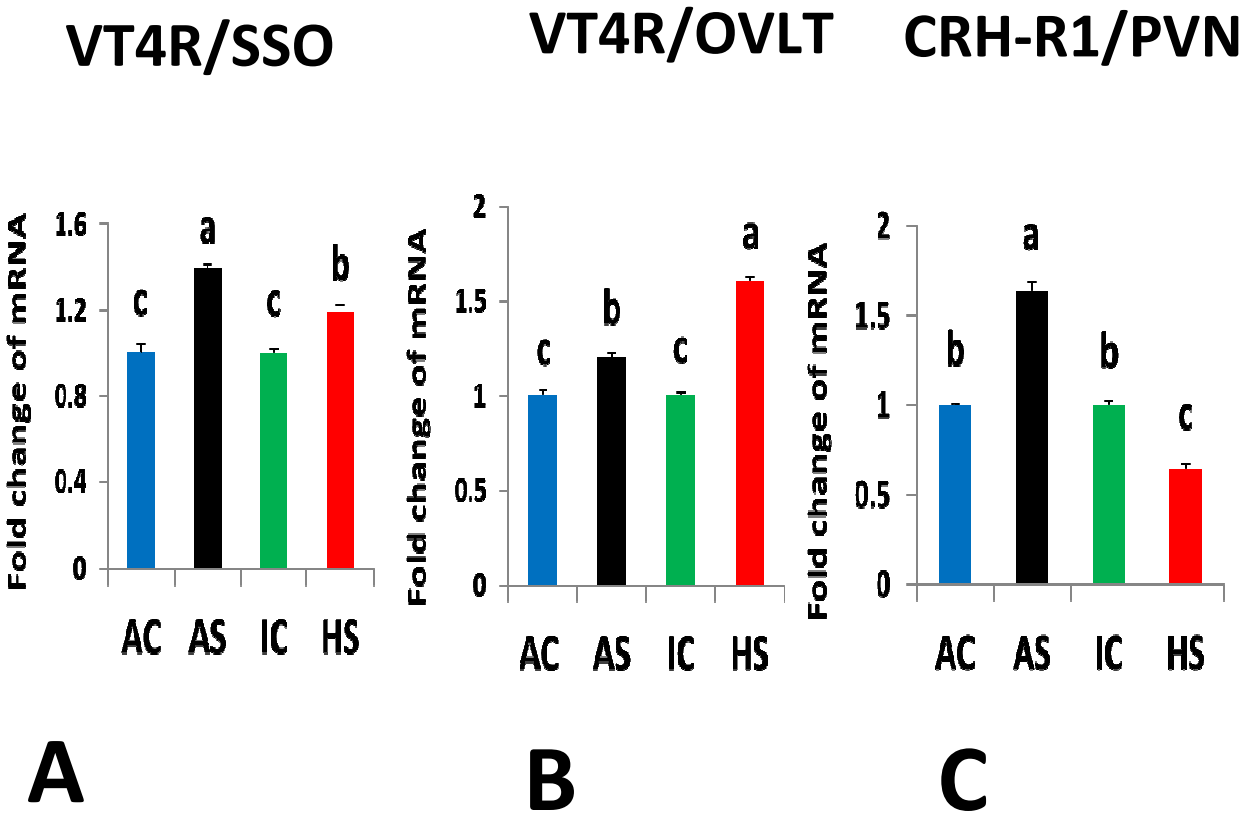

Fig. 12: Gene expression of the VT4R/V1aR in the subseptal organ (SSO; **A**) and organum vasculosum of lamina terminalis (OVLT; **B**) and, gene expression of corticotropin releasing hormone-receptor 1 (CRH-R1) in paraventricular nucleus (PVN; **C**) following 1 h of acute immobilization stress and hypertonic saline injection. Different lowercase letters show group means that are significantly different ($p<0.01$). AC, acute immobilization control (n=5 birds); AS, acute immobilization stress (n=6 birds); IC, isotonic saline control (n=5 birds); HS, acute hypertonic saline (n= 6 birds). Error bar: SE (standard error).

CONCLUSION

The levels of corticosterone and arginine vasotocin were more increased in the hypertonic saline groups showing that the physical stressor, hypertonic saline administration, was efficient, and, thus, probably would have desirable effects on VT4R immunoreactivity and on its gene expression within the SSO or OVLT. The physical and psychological stressors caused immunohistochemical changes of VT4R immunostained glial cells within SSO and OVLT. Those changes were more significant in the SSO and OVLT following physical stress. Gene expression of AT1AR, TRPV1, and VT4R were significantly increased in the SSO in response to physical stress. Significant changes of the gene expression were observed in the OVLT following psychological and physical stressors. The CRH-R1 mRNA gene expression in the PVN showed opposite directions: increased upon psychogenic stress, while decreased upon physical stress. These results strongly suggest that physical stress may affect the vasotocinergic system in SSO to regulate water balance through the VT4R, while psychogenic stress appears to affect the VT4R in the OVLT resulting in activation of the classical neuroendocrine HPA axis.