Differential responses of the vasotocin 1a receptor (V1aR) and osmoreceptors to immobilization and osmotic stress in sensory circumventricular organs of the chicken (*Gallus gallus*) brain

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ABSTRACT

Past studies have shown that the avian vasotocin 1a receptor (V1aR) is involved in immobilization stress. It is not known whether the receptor functions in osmotic stress, and if sensory circumventricular organs may be involved. An experiment was designed with four treatment groups including a 1h immobilization acute stress (AS) group, an unstressed acute control (AC), a third given an intraperitoneal (ip) hypertonic saline injection (HS) and isotonic saline controls (IC) administered ip. One set of chick brains was perfused for immunohistochemistry while a second was sampled for quantitative RT-PCR. Plasma corticosterone (CORT) and arginine vasotocin (AVT) concentrations were significantly increased in the immobilized and hypertonic saline groups ($p < 0.01$) compared to controls. Intense staining of the V1aR occurred throughout the organum vasculosum of the lamina terminalis (OVLT) and subseptal organ (SSO)/subfornical organ (SFO). The immunostaining allowed the boundaries of the two circumventricular organs (CVOs) to be described for the first time in avian species. Both treatment groups showed marked morphological changes in glia within the OVLT and SSO/SFO. The avian V1aR, angiotensin II type 1 receptor (AT1R), and transient receptor potential vanilloid receptor 1 (TRPV1) mRNA levels were increased in the SSO/SFO in hypertonic saline treated birds compared to isotonic controls. In contrast, the latter two genes (*AT1R* and *TRPV1*) were significantly decreased in the OVLT of birds subjected to hyperosmotic stress, while all three genes were significantly up-regulated after immobilization. Taken together, results show a possible differential function for the same receptors in two anatomically adjacent CVOs.

Key Words: avian AT1R, TRPV1, subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT).

1. Introduction

In non-mammalian vertebrates, arginine vasotocin (AVT), and corticotropin-releasing hormone (CRH) are major secretagogues stimulating release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Castro et al., 1986) and shown to be effective in the release of corticosterone (CORT) from the adrenal gland of male and female birds (Madison et al., 2008). Two vasotocin receptors, type two (VT2R; Jurkevich et al., 2005, 2008; Kuenzel and Jurkevich, 2010; Cornett et al., 2013) and type four (VT4R; Selvam et al., 2013; Kuenzel et al., 2013; Kang and Kuenzel, 2014) have been shown to be involved in the neuroendocrine hypothalamo-pituitary-adrenal (HPA) axis of chickens. Due to the genome sequence and anatomical and functional similarity of the two avian receptors to the mammalian vasopressin V1b and V1a receptors, it has been proposed that the VT2R and VT4R be termed the avian V1bR (Cornett et al., 2013) and avian V1aR (Kuenzel et al., 2016), respectively. We have utilized an acute psychogenic stressor, immobilization, and showed that both the avian V1bR and V1aR as well as the CRH receptor 1 (CRH-R1) and CRH-R2 are involved in acute and chronic stress along with the release of CORT (Kuenzel et al., 2013; Selvam et al., 2013; Kang and Kuenzel, 2014).

Studies performed in birds to date have shown that the avian V1bR has not been found in the chicken brain (Jurkevich et al., 2005), nor in the brain of the white-throated sparrow or zebra finch (Leung et al., 2011). In contrast the avian V1aR has been reported in both the white-throated sparrow and zebra finch brains (Leung et al., 2011) and throughout the chicken brain (Selvam et al., 2015). Curiously, the V1aR was found in glia in all ten circumventricular organs (CVOs) of the chick brain (Selvam et al., 2015). A typical vertebrate CVO usually displays specialized ependymal cells, an incomplete blood-brain barrier, cerebrospinal fluid (CSF)-contacting neurons, and is located adjacent to the ventricles of the brain (Vigh and Vigh-

Teichmann, 1973). Using an antibody to the avian V1aR, immunoreactivity (V1aR-ir) was documented in glia located in two sensory CVOs, the organum vasculosum of the lamina terminalis (OVLT) and subseptal organ (SSO), homologous to the mammalian subfornical organ (SFO). The finding prompted us to perform a detailed anatomical study of the distribution of the V1aR in the two CVOs as it was reported earlier using classical stains that the OVLT of birds was extensive and appeared to span from the base of the preoptic region to the anterior commissure (Dellmann, 1964). This observation was later confirmed by immunocytochemistry showing terminal fields of gonadotropin releasing hormone type one (GnRH-1) along the entire lamina terminalis (Kuenzel and Golden, 2006). However, what was not clear was where the OVLT ended and the SSO/SFO began as data from the duck suggested that the SFO likewise began at the dorsal region of the anterior commissure (Schmidt, 1995). In the chick brain, it was not documented where the SSO/SFO first appeared in brain sections, and more importantly, where the lateral extent of the two CVOs occurred in this or other avian species.

In addition to clarifying anatomical data, the location and expression of the V1aR in glia of the two CVOs may determine whether both the OVLT and SSO/SFO play major roles in osmoregulation as demonstrated in mammals, rather than the SFO being the primary CVO as reported in the duck brain (Simon et al., 1992). Simpson and Routtenberg (1973) demonstrated in mammals that the SFO was the site where circulating angiotensin II (ANG II) mediated the onset of drinking behavior. Subsequent studies showed that the rodent SFO sends major projections to the OVLT and nucleus preopticus medianus (MnPO; Miselis et al., 1979, 1987). All three structures (OVLT, SFO, MnPO) are also involved in osmoregulation through the action of angiotensin II on vasopressin release in mammals (McKinley et al., 1998, 1999). High affinity binding sites for ANG II were identified in the mammalian brain (Mendelsohn et al., 1984) and subsequently characterized as AT₁ and AT₂ receptors (Wright and Harding, 1994; Aguilera et al., 1995). In birds, it likewise has been shown that avian species drank water after

administration of ANG II (Wada et al., 1975; Evered and Fitzsimons, 1976; Takei, 1977a) and an anatomical site, the SFO, in Japanese quail showed sensitivity to ANG II (Takei, 1977b). Autoradiographic localization of ANG II receptors was found in the SFO and anteroventral third ventricle (AV3V) region, an area where the avian OVLT was also located (Gerstberger et al., 1987, 1992; Simon et al., 1992).

The SFO and OVLT in mammals have been proposed to not only affect water intake and conserve body water but also function in osmotic and body fluid homeostasis (Sunn et al., 2003). In particular, a family of transient receptor potential vanilloid (TRPV) receptors have been discovered and proposed as candidates for vertebrate osmoreceptors in the SFO and OVLT (Liedtke et al., 2000). In the duck brain, the rostral extension of the third ventricle where the lamina terminalis occurs appeared to be the comparable site for stimulating drinking and antidiuresis (Gerstberger et al., 1987; Simon-Oppermann et al., 1988). To the best of our knowledge, no study has been reported in birds where a particular member of the TRPV family, nor a particular member of the ANG receptor family, has been shown to play a role in osmoregulation within the avian SFO or OVLT. Our discovery that the avian V1aR occurs in glia located in those two CVOS suggests that perhaps the V1aR may also be involved in osmoregulation in birds. We therefore subjected a group of chicks to a physical stressor (intraperitoneal administration of hypertonic saline) and another treatment group subjected to a psychological stressor (immobilization) along with their respective control groups. Our hypotheses were 1) both the SFO and OVLT would be associated with sensing osmotic stress, and 2) the V1aR and one of the receptors in the TRPV and/or AT family may play a role in either one or both avian CVOs.

2. Results

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

The avian V1aR antibody was effective in identifying the location of circumventricular organs (CVOs) as glia are major components of CVOs and all 10 avian CVOs have glia containing the V1aR (Selvam et al., 2015). In particular, the avian V1aR has been shown to be abundant in both the OVLT and SSO and therefore the receptor has been an effective marker in defining the boundaries of the two CVOs in the chick brain. As can be seen in the following set of coronal images, the OVLT begins as a small triangular shape at the base of the preoptic area (Plate A8.8, Fig. 1, Fig. 2A, 2B) and lines the anterior most wall of the third ventricle (3V) called the lamina terminalis. Caudally, the OVLT assumes a more dorsal position as it approaches the anterior commissure (CA, Fig. 2C-2F), a prominent fiber tract that marks the dorsal region of the anterior hypothalamus. The OVLT passes in front of the CA and ends at its final location just dorsal to the CA (Fig. 2F). At approximately the mid-region of the CA and just dorsal to it, the SSO begins (Fig. 2G) and thereafter occupies the ventral part of the nucleus of the hippocampal commissure (NHpC), previously called the bed nucleus the pallial commissure. Glial cells within the SSO show ir for the V1aR along the roof of the 3V and V1aRs are also seen surrounding blood vessels located along the midline of the SSO (Fig. 2H-2J). Posteriorly, the SSO occupies a greater proportion of the NHpC (Fig. 2I-2K). At the caudal end of the SSO, a transition occurs and the space previously occupied by blood vessels enlarges and becomes filled with cerebrospinal fluid (Fig. 2K-2L). Immediately posterior, the enlarged, dorsal space at midline joins the 3V below it and the two ventral arms of the lateral ventricles join the 3V to form the interventricular foramina where the choroid plexus resides. A most characteristic part of the SSO is its caudal-most structure, a ventral projection into the 3V showing dense V1aR-ir (Fig. 2K-2L).

One objective of the study was to determine if any anatomical changes occurred to the cells immunoreactive for the V1aR in the OVLT or SSO following the two distinct stressors (immobilization or administration of hypertonic saline) compared to their respective control

groups. The OVLT, had one site that displayed consistent anatomical changes in both stress treatment groups. It was located at the ventral region of the OVLT, just below the attachment of the OVLT to the brain parenchyma on each side of the base of the preoptic area (Fig. 2D). Note the triangular-shaped ring comprising the major structure of the OVLT and its two lower attachments to the brain. A narrow, vertical space (the cistern (CIS)) is evident just dorsal to each of the two attachment sites (Fig. 2D). The CIS most likely is filled with cerebrospinal fluid similar to the 3V. The glial cells immunoreactive for the V1aR just lateral to the major ring structure of the OVLT, suggest that they too should be included as part of the OVLT structure, due to their clear and restricted immunostaining. Just ventral to the thin space (the CIS) on the right and left sides of the brain are where changes in glial structures were noted among the four treatment groups. Images of the isotonic saline controls (IS, Fig. 3A) and acute immobilization controls (AC, Fig. 3E) and their respective boxed-in images at higher magnification (Fig. 3C, 3G), respectively, show immunoreactive glial processes oriented in consistent, parallel, horizontal rows. In contrast, the boxed-in areas of the ventral portion of the OVLT in the HS (Fig. 3B, 3D) and AS (3F, 3H) stressed groups show parallel glial processes that curve ventromedially toward the 3V at midline. An attempt was made to quantify the intensity of immunostaining in this particular region among the four treatment groups. No significant treatment differences were obtained (data not shown).

A morphological difference was also noted by immunoreactive glial cells in the SSO. Thin, glial processes in both control groups (IS and AC birds), show a dorsal-ventral orientation and appear to connect blood vessels located dorsally at midline to the 3V positioned ventrally (Fig. 4A, 4C). When glial processes in the same region are examined in the two groups subjected to stress (HS and AS birds), note the lightly stained areas that form a horizontal row or rows between midline blood vessels and 3V. The overall effect suggests that the vertically oriented processes in both stress groups form discontinuous sets of parallel glial processes (Fig. 4B,

4D). Perhaps glial connections between blood vessels and the 3V are modulated, particularly for the HS group that showed fewer glial processes contacting the 3V (Fig. 4B).

2.2. Functional studies involving the OVLT and SSO

Both the immobilization (psychogenic stressor) as well as the hypertonic saline (physical stressor) resulted in a significant elevation of plasma corticosterone (CORT). Plasma CORT in acute immobilization birds (1.22 ± 0.26 ng/ml) showed a 5-fold increase compared to their controls (0.25 ± 0.041 ng/ml). A 9-fold increase of plasma corticosterone concentration occurred in the HS treated birds (2.46 ± 0.56 ng/ml) compared to their respective controls administered isotonic saline (0.27 ± 0.05 ng/ml; Fig. 5A; $p \leq 0.01$). Additionally, plasma arginine vasotocin (AVT) was determined and it was shown significantly elevated in both the AS and HS treatment groups compared to controls. Plasma AVT in the immobilized group (7.97 ± 0.95 pg/ml) was nearly 1 fold higher compared to the acute control group (4.19 ± 0.59 pg/ml; Fig. 5B). Hypertonic saline treated birds showed a 1.9-fold higher level of plasma AVT concentration (17.06 ± 4.78 pg/ml) compared to 5.89 ± 0.32 pg/ml shown by isotonic controls (Fig. 5B; $p \leq 0.01$).

Since the SFO in mammals and SSO in birds had previously been determined to function in water intake, selected genes associated with water intake and osmoregulation as well as the avian V1aR were quantified after the two stressors were imposed to determine if the three selected genes were differentially regulated depending upon the type of stressor imposed. The first gene examined was the avian V1aR, due to its major involvement in the stress response of birds. Two additional genes examined were the angiotensin II type 1 receptor (*AT1R*) and the transient receptor potential cation channel vanilloid type 1 (*TRPV1*). The *AT1R* and *TRPV1* were both shown to be involved in water intake (Bottari et al., 1993) and body fluid

osmoregulation, particularly hypertonicity in mammals (Liedtke et al., 2000; Sharif Naeini et al., 2006; Ciura and Bourque, 2006).

The first brain tissue samples examined were dissections that included the SSO. Note that birds subjected to immobilization stress for 1h (AS) when compared to controls (AC), showed no significant differences either in the AT1R nor the TRPV1 following the acute stressor (Fig. 6C, 6E). Nonetheless when the avian V1aR was determined, the AS groups showed a significant increase in the V1aR compared to the AC birds (Fig. 6A). In contrast, both the AT1R and TRPV1 were significantly elevated following hypertonic saline (HS) administration (Fig. 6C, 6E) compared to controls (IC). Similar to the AS birds, the HS treatment effected a significant elevation in the avian V1aR (Fig. 6A) compared to the IS controls.

Curiously, the dissected samples containing the OVLT responded differently. The AT1R and TRPV1 showed a significant increase in gene expression following immobilization stress (AS, Fig. 6D, 6F) while both genes were significantly down regulated due to the physical stressor, hypertonic saline (HS, Fig. 6D, 6F). Both stressors, applied to the AS or HS treatment groups, displayed significant elevations of the avian V1aR (Fig. 6B), similar to the results for the SSO.

The paraventricular nucleus (PVN) in birds (Berk et al., 1982 Tennyson et al., 1985; Richard et al., 2004), like mammals (Swanson and Kuypers, 1980; Cummings et al., 1983), contains major groups of AVT and CRH neurons. Contradictory results have been reported in the response of the major CRH receptor, CRH-R1, in the PVN following hypertonic saline administration. Imaki et al. (2001a, 2001b) have recorded increased expression while Jessop et al. (1990) and Larsen et al. (1993) have shown decreased CRH gene expression following osmotic stimulation. Since subsequent data have shown that there exist a direct, positive relationship between CRH mRNA and CRH-R1mRNA in the PVN (Rivest et al., 1995), one

would expect decreased CRH-R1 gene expression in the studies of Jessop et al. (1990) and Larsen et al. (1993) if measured. Therefore, we examined the response of avian CRH-R1 in the PVN following each of our two stress models. Brain samples were dissected containing the PVN and mRNA was determined for CRH-R1. A differential expression of the CRH-R1 was obtained in the PVN dependent upon the stressor. Immobilization stress (AS group) resulted in a significant increase in CRH-R1 gene expression while hypertonic saline administration (HS group) showed a significant down regulation compared to controls (Fig. 7).

3. Discussion

3.1. Anatomy of the OVLT and SSO/SFO

Early studies showed that the avian OVLT occurs for a considerable distance along the anterior wall of the 3V, beginning at the base of the preoptic region and extending up to the anterior commissure (CA; Dellmann, 1964; Mikami, 1976; Weindl and Sofroniew, 1982). The mammalian OVLT occupied a similar location, immediately dorsal to the anterior edge of the optic chiasma, however, its dorsal extent was normally about half the distance to the CA (Mergner, 1959; Weindl, 1973; McKinley et al., 1983). The current study using an antibody to the avian V1aR has clearly shown for the first time the anterior/posterior and lateral extent of the avian OVLT. The set of images provided in the Results section show that the glial component of the OVLT is a major characteristic. Throughout the OVLT a high density of V1aRs occurred. Importantly the OVLT extends the length of the lamina terminalis from where it first appears anteriorly at the base of the preoptic area just prior to the optic chiasma and continues along the entire anterior wall of the 3V until the OVLT passes in front of and dorsal to the CA. A similar anterior-posterior extent of this CVO was likewise documented by gonadotropin releasing hormone fibers occurring throughout the chick OVLT (Kuenzel and Golden, 2006). For the first time, the lateral extent of the OVLT was clearly defined by V1aR immunoreactive glia. Note the

two contact points at the base of the preoptic region located in the preoptic region supporting the central, triangular portion of the OVLT (Fig. 2D). Hence cells showing V1aR-ir line the anterior region of the lamina terminalis and basal preoptic, parenchyma forming a cistern (Fig. 2C, 2D; Fig. 8), specifically forming the pericisternal zone (PCZ, Fig. 8). At this level, the PCZ encloses the periventricular zone (PVZ), the central part of the OVLT. Both the PCZ and PVZ are components of this CVO. Cerebrospinal fluid appears to occur not only within the PVZ but also within the PCZ. Posteriorly, the two zones completely fuse (Fig. 2E).

The SFO in birds has been examined at the ultrastructural (Mikami and Asari, 1978; Takei et al., 1978; Tsuneki et al., 1978) and light-microscopical levels (Weindl and Sofroniew, 1982; Schmid, 1995). A detailed anatomical study of the duck SSO compared to the SFO of the rat showed that the duck SSO extended from the CA posteriorly until it met the choroid plexus medially (Schmid, 1995). The use of Evans blue showed that a large, fenestrated blood vessel resided in the center of the avian SFO along its entire anterior to posterior extent. Anatomically, a diffuse labeling of Evans blue occurred laterally from the central blood vessel indicating that the mid- and caudal regions of the CVO lacked a blood-brain barrier (Schmid, 1995). The chicken SSO shows a comparable, anatomical structure beginning at the CA and ending where the 3V joins the lateral ventricles forming the interventricular foramina occupied by the choroid plexus.

3.2. Anatomical changes following stress

Anatomical changes were noted in the OVLT and SSO/SFO following both stress treatments. At the ventral most region of the lateral cisterna (Fig. 2C, 2D) where the central, triangular component of the OVLT attaches to the brain parenchyma, both types of stressors, HS and AS, resulted in curved, oriented glial processes (Fig. 3B, 3F) compared to controls that exhibited more horizontally oriented ones (Fig. 3A, 3E). The anatomical changes observed in

270 the V1aR immunoreactive glia following the two stress types nicely reflects changes observed in
271 V1aR gene expression (described below), however, the functional significance of this
272 anatomical finding remains unknown. A more striking difference was observed within the
273 SSO/SFO. Specifically, the long, delicate, vertically oriented glial processes that appeared to
274 connect blood vessels within the SSO/SFO to cerebrospinal fluid within the 3V showed
275 discontinuities compared to controls. In the hypertonic stress group, decreases in glial endings
276 contacting the 3V were consistently observed compared to controls. Additionally, in both the HS
277 and AS groups, anatomical areas of discontinuity were seen, marked by horizontal, 'open' areas
278 with less V1aR-ir. The ends of glia not clearly contacting the 3V (Fig. 4B) and 'open' areas (Fig.
279 4B, 4D), suggest movements and retractions of glial processes containing V1aR, perhaps
280 resulting in the physical inhibition of ligand binding to available receptor sites or ephaptic
281 coupling of neuronal fibers or components as documented in the following studies. At the
282 ultrastructural level retraction of fine glial processes were reported in the supraoptic nucleus
283 following dehydration and rehydration in rat neurosecretory neurons (Tweedle and Hatton,
284 1977). In birds a terminal field of gonadotropin-releasing hormone (GnRH) axons in the median
285 eminence (ME) were shown completely surrounded by specialized glia whose processes
286 retracted when birds were transferred to a long photoperiod or triiodothyronine was applied to
287 that brain region (Yamamura et al., 2006). In addition to the reported dynamic movements of
288 glial processes at the nano- and micro-domain levels, a cellular domain level composed of
289 columnar or spherical organization of glia and neurons has also been described having a
290 specific functional organization (Parpura et al., 2012). We propose such a cellular domain
291 exists within the SSO responsible for what appears to be a dynamic retraction of glial processes
292 from the 3V as well as other sites between blood vessels at midline and the 3V, particularly
293 following the hypertonic treatment. The mechanism of action is unknown, hence our
294 interpretations of the histological data are speculative. Considerable data are needed to resolve

the unusual changes in patterns of V1aR-ir shown by the SSO and OVLT among treatments. Importantly a quantitative, proteomics analysis of the V1aR is needed to determine whether changes in the protein reflect the significant elevation of gene expression of the *V1aR* displayed by both the acute stress and hypertonic saline treatment groups.

3.3. The avian SSO/SFO and OVLT show differential gene expression for genes associated with osmotic balance and psychological stress

The mammalian SFO was first shown to be the site where circulating ANG II stimulated water intake (Simpson and Routtenberg, 1973). Thereafter, it was demonstrated that avian species drank water after administration of ANG II (Wada et al., 1975; Evered and Fitzsimons, 1976; Takei, 1977a) and an anatomical site, the SSO/SFO, in Japanese quail showed sensitivity to ANG II (Takei, 1977b). Ablation of the CVO reduced water intake stimulated by Ang II infusions in mammals (Johnson et al., 1992; Phillips, 1987) and birds (Volmert and Firman, 1992). Extensive anatomical and electrophysiological studies were conducted in the Pekin duck. A large number of SSO/SFO –intrinsic neurons responded to ANG II with markedly enhanced electrophysiological discharge rates (Matsumura and Simon, 1990; Schmid and Simon, 1992) and ANG II likewise inhibited salt gland secretion in ducks (Gerstberger and Gray, 1993; Simon et al., 1992). Utilizing autoradiography, highest density of Ang II receptors were found in the SSO/SFO, followed by the supraoptic nucleus, anterior ventral third ventricle (AV3V) region and PVN (Gerstberger et al., 1987). In mammals, two ANG II receptors were initially characterized, angiotensin 1 (AT₁) and 2 (AT₂) and the former, located in the brain, was shown to be associated with thirst and sodium appetite and blocked by the AT₁ receptor antagonist losartan (Beresford and Fitzsimons, 1992; Bottari et al., 1993). In birds however, the receptors found for ANG II appeared to be distinct from mammalian receptors (Kocsis et al., 1994; Le Noble et al., 1993; Murphy et al., 1993; Nishimura et al., 1994). To further

characterize the avian ANGII receptors and possibly resolve the issue of receptor differences, both electrophysiological and pharmacological receptor analytical methods were undertaken to examine the duck SFO (Schäfer et al., 1996). Indeed, the ANG II-specific binding sites of the duck SFO/SFO revealed lower affinity for losartan (mammalian AT₁ specific antagonist) compared to CGP-42112A (mammalian AT₂ specific antagonist). Nonetheless, both central as well as peripheral ANG II receptor types in ducks were sensitive to sulfhydryl reducing agents as shown for the mammalian AT₁- but not the AT₂-receptor subtype (Simon et al., 1992; Tsutsumi and Saavedra, 1991). The authors suggested that the avian-specific ANG II receptor in the brain shared more properties with the mammalian AT₁-receptor subtype than with the AT₂- receptor subtype.

We therefore examined whether the AT₁- receptor subtype in the avian brain might play a significant role, using a chicken physical stress model, intraperitoneal injections of hypertonic saline, compared to a common psychogenic stress model, immobilization. Importantly, the role of the OVLT in fluid and electrolyte homeostasis has not been addressed in avian species. The availability of two tools, the discovery of the V1aR, residing in all CVOs of the chick brain (Selvam et al., 2015) and the discovery in mammals of a class of receptors that detected osmotic stimuli, the vanilloid receptor-related osmotically activated channels (Liedtke et al., 2000), stimulated interest in that receptor family. Early studies suggested that the TRPV2 (Muraki et al., 2003) or TRPV4 (Liedtke and Friedman, 2003) were viable candidates. More recent data, using knock-out (*TRPV1*^{-/-}) mice, showed a lack of an electrophysiological response to systemic hypertonicity as well as attenuated water intake compared to wildtype controls where the TRPV1 receptor occurred within the OVLT (Ciura and Bourque, 2006). A full-length TRPV1 was also identified in the mammalian supraoptic nucleus and found to be located in vasopressin, not oxytocin neurons (Moriya et al., 2015). Hence we examined gene expression

of the avian *V1aR*, *AT1R* and *TRPV1* in the SSO/SFO and OVLT following the use of a physical stress model, osmotic imbalance, compared to a psychogenic stressor, immobilization.

Results showed that in examining the SSO/SFO following osmotic imbalance, a significant increase in gene expression for the *AT1R* and the *TRPV1* occurred while no changes were detected in the same two genes after the psychogenic stressor. In contrast, the OVLT showed significantly decreased gene expression for the *AT1R* and the *TRPV1* following hypertonic saline administration while both genes were significantly upregulated after the psychological stressor. The results are intriguing in that they suggest both the avian SSO/SFO and OVLT are involved in osmoregulation while the OVLT serves an additional function related with psychological stress. The significant elevation of plasma AVT in both stress treatments as well as the significant rise in *V1aR* in the SSO/SFO and OVLT following hypertonic administration as well as immobilization, reinforces that view. The CRH-R1 mRNA transcript data from dissections of the PVN likewise matched gene expression data obtained for the OVLT. Specifically, CRH-R1 mRNA was upregulated following immobilization while significantly downregulated after hypertonic saline administration.

Gene expression data suggest a separation of function, with the SSO/SFO and OVLT involved in osmoregulation, while the OVLT has an additional function associated with the regulation of psychological stress. Nonetheless, we cannot explain the unexpected significant decrease in gene expression of the *AT1R* and *TRPV1* genes in the OVLT of the HS treatment group. In particular, data in mammals demonstrate that the primary location for osmosensors in the mammalian brain is the OVLT (Johnson and Gross, 1993; McKinley et al., 1998). Importantly, recent electrophysiological data in mice showed intrinsic osmoreception in the OVLT. Enhanced extracellular recordings were recorded after systemic hyperosmolality while current-clamp data showed osmotically stimulated intracellular recordings. In contrast, no

electrophysiological changes were obtained in knockout mice lacking the *TRPV1* gene (Ciura and Bourque, 2006). We therefore expected a significant increase in the avian *TRPV1* in the HS birds. Perhaps a species difference is a major reason for the data obtained for the avian OVLT. Nonetheless, a future, time series of sampling appears necessary in birds to determine the dynamic interactions between the avian SSO/SFO and OVLT regarding their responses to hypernatremia. Our tentative interpretation of the opposite responses of the *AT1R* and *TRPV1* genes in the SSO/SFO compared to the OVLT after 1h of a hyperosmotic challenge is that the OVLT is serving as a negative feedback. In this manner, the OVLT attenuates the large water shifts caused by the sudden increased plasma tonicity. A more regulated return to the set-point of the body's systemic osmotic pressure thereby occurs.

A second unresolved issue is what appears to be an inconsistent finding of significantly increased V1aR mRNA and less dense peptide ir in parts of the OVLT and SSO/SFO in the two stressed groups. In the stressed groups, the morphological observation of decreases in glial endings contacting the 3V compared to controls and discontinuous areas or rows shown in the SSO/SFO due to less V1aR-ir does not necessarily indicate a significant decrease of protein expression. It is not uncommon to have an inconsistency between protein expression and gene expression (Greenbaum et al., 2003; Ko et al., 2000). The inconsistency between V1aR immunoreactive protein and mRNA expression level can be explained not only by technical limitation, but also biological reasons. Regarding the former, may be the limitation of immunohistochemistry sensitivity for quantification. Biologically, there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well-defined to be able to estimate protein concentrations from mRNA expression levels. In fact, considering the importance of post-transcriptional regulatory mechanisms, mRNA expression level could be very distant from the protein one (Tiseo et al., 2013). Role of post-transcriptional mechanisms in the modulation of V1aR expression was suggested in mammals,

indicating that mRNA stability of V1aR may play a role in protein expression during the myogenic differentiation process (Alvisi et al., 2008). The results of current study indicate that *in vivo* half-lives of the avian V1aR proteins in brain may be substantially short to give a significant immunoreactive change following stressors.

In conclusion, a major source of the protein product of the V1aR mRNA transcripts was shown to be located in glial cells, revealed by dense V1aR-ir in two sensory circumventricular organs (CVOs), the OVLT and SSO/SFO, following immunohistochemistry. A psychogenic stressor, immobilization, as well as a physical stressor, hypertonic saline, resulted in significant elevations of plasma corticosterone and arginine vasotocin and significant increases in gene expression of the avian V1aR compared to controls. The avian OVLT and SSO/SFO function in osmoregulation in birds, similar to mammals. Likewise, based upon gene expression data, both CVOs house osmoreceptors. Significant changes in mRNA transcripts for the AT1R following hypertonic saline challenge suggest that in addition to the SSO/SFO, the avian OVLT plays a role in the regulation of water intake. The OVLT also appears to be associated with psychogenic stress.

4. Experimental Procedure

4.1. Animal housing and diets

Male broiler Cobb 500 chicks were obtained from a commercial hatchery at day of hatch and raised in brooder cages for the first three weeks. They were exposed to continuous light for the first three days so that the chicks could locate both the feed and water. Thereafter birds were provided with a photoperiod of 16h of light, 8h of dark with lights on at 6:00AM. Birds were fed a standard commercial chick starter diet containing 22% crude protein and a metabolizable energy value of 3100 kcal/kg. Food and water were provided *ad libitum*.

4.2. Treatment groups

At 3-weeks of age broilers were transferred to cages and housed individually. Birds were randomly selected into four treatment groups (n=8/treatment): (1) Acute stress (AS), birds were secured in a harness where they could not move their wings nor stand, however, did have access to water during a one hour (1h) restraint period. The remaining 3 treatments were: Acute controls (AC), birds remained in their cages until sampled; Hypertonic saline (HS), birds administered *ip* hypertonic saline (HS) at a concentration of 3.0 M NaCl in a volume of 5ml/kg (Jaccoby et al., 1997) and, Isotonic saline controls (IC), birds given 0.15 M NaCl *ip*. Birds in the last two groups after *ip* injections, were brought back to their cages for 1h and did not have access to water nor feed during that time interval. All procedures that were imposed on birds in the study followed protocols that were approved by the University of Arkansas Institutional Animal Care and Use Committee.

4.3. Radioimmunoassay (RIA) for plasma corticosterone

Blood samples were taken from the brachial vein of 3-week old male chicks subjected to 1 h of acute immobilization stress (n=8), and 1 h of hyperosmotic stress (n=8), including their respective controls. Blood samples were centrifuged at 3000 rpm for 20 min at 4°C, plasma samples collected and stored at - 20°C until assayed. Plasma corticosterone (CORT) concentrations were determined by RIA (Madison et al., 2008). 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 fresh tube and dried at 37°C in an evaporator. Dried extracts were reconstituted with 400 µl of assay buffer (0.1 M phosphate buffered saline containing 1% gelatin PBSG, pH 7.0), vortexed for 5 min, and equilibrated overnight at 4°C. Using a duplicate assay procedure, 100 µl of the primary

antibody (polyclonal rabbit anti-CORT), purchased from Fitzgerald Inc.(Concord, MA, USA) and 100 µl of ¹²⁵I corticosterone tracer purchased from MP Biomedicals Inc. (Orangeburg, NY, USA) were added to each sample and incubated for at least 24 hours at 4°C . Sheep anti-rabbit antibody was used as secondary antibody (MP Biomedicals Inc.,Orangeburg, NY, USA) and 6% of polyethylene glycol was used to precipitate bound tracers. The supernatant of each sample and standard was discarded, the remaining liquid dried, and counts/tube were determined.

4.4. RIA for plasma arginine vasotocin

Plasma arginine vasotocin (AVT) concentrations were assayed using a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Inc Burlingame, CA, USA) and the assay was performed according to the manufacturers recommendation. Briefly, plasma AVT samples were extracted using C-18 SEP- COLUMNS. Each C-18 SEP-COLUMN was fit onto a 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 onto an equilibrated C-18 SEP- COLUMN. The acidified plasma passed through the column over a variable time period (30 min to 4 hours depending upon the presence or absence of bubbles). The column was washed slowly with buffer A (3ml, twice) and discarded. The buffer B (3ml) was used to elute AVT from the column and the eluted solution was dried using a 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 a 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, (Möhrling et al., 1980) was added to samples and standard tubes and incubated for 16-24 hours at 4°C. Then, the ¹²⁵I AVP tracer (100 µl) was added to tubes and incubated for 16-24 hours at 4°C. The free and bound AVT were separated using the 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°C, the supernatant was removed by aspiration, and the precipitate was counted using gamma-counter.

4.5. Immunohistochemistry

4.5.1. Animal and Tissue preparation. Birds were anesthetized with an intravenous (*iv*) injection of sodium pentobarbital (27mg/kg). Each anesthetized bird was perfused through the left heart ventricle (150 ml) followed by 50 ml via the carotid arteries using ice cold heparinized 0.1M phosphate buffer saline (PBS) containing 0.1% sodium nitrite, pH 7.4. Immediately thereafter birds were perfused with 250 ml of freshly prepared, ice cold Zamboni's fixative solution, containing 4% paraformaldehyde with 15% picric acid in 0.1 M PBS buffer at pH 7.4. Brains were blocked in a stereotaxic instrument (Kopf Instrument, Tujunga, CA) and post-fixed in the same fixative overnight at 4°C. Blocked brains were cryoprotected using 30 % sucrose in 0.1 M PB at 4°C until they sank. Brains were then frozen in dry ice, wrapped in parafilm and aluminium foil, and stored at - 20°C until sectioned. Blocked brains were embedded in Jung OCT medium (freezing media, Leica Microsystems, Wetzlar, Germany) and cross sectioned at 40 µm using a cryostat (Leica CM 3050S, Leica Microsystems, Austin, TX, USA) between anterior and posterior planes of atlas plates A 8.8 (preoptic region) to A 7.6 (anterior hypothalamus; Kuenzel and Masson, 1988). Sections were then stored at -20°C in an ethylene glycol based cryoprotectant solution until processed for immunohistochemistry.

4.5.2. Immunohistochemical procedure and imaging. Free-floating sections were rinsed in several changes (6 times x 10 min) of 0.02M PBS at room temperature to remove cryoprotectant solution, incubated for 30 min (2X15min) in PBS containing 0.6% of hydrogen peroxidase to suppress endogenous peroxidase activity and then permeabilized with 0.4% Triton X-100 in PBS for 15 min. Subsequently, sections were placed into 5% normal goat

serum (in PBS solution containing 0.1% sodium azide and 0.2% Triton X-100) for 30 min to block nonspecific binding sites. Sections were then incubated with rabbit antibody against chicken V1aR (Code: VT4-1, RRID: AB_2336062; Selvam et al., 2015). It was diluted 1:2500 in 0.02 M PBS containing 1%NGS, 0.4% Triton X-100 , and 0.1% sodium azide) for 48h at 4°C. Following incubation, sections were rinsed 6 times for 10 min each and incubated with goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS containing 0.2% Triton X-100 for 90 min at room temperature. 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. 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). Brain sections were then imaged using bright-field microscopy (Imager M2 microscope, Carl Zeiss Microscopy, LLC, Thornwood, N.Y.).

4.6. Treatment Groups and Sampling Procedure for the Gene Expression Study

4.6.1. Birds, treatments, sampling procedures. A second hatch of chicks was used for the gene expression experiment. Age of chicks, sex and experimental groups were subjected to the same treatments as described in 2.2 of material and methods section. Treatment groups were: (1) AS, (2) AC, (3) HS, and (4) IC with n=8 birds per treatment. Immediately after 1h of the acute stress treatments, birds were cervically dislocated, brains removed from skulls, placed into ice-

510 cold 2-methylbutane for initial freezing (20-25sec) followed by immersion in crushed dry ice,
511 wrapped in paraffin and aluminum foil, and stored at - 80°C until sectioned in a cryostat.

512 *4.6.2. Sectioning the OVLT, SSO and PVN for gene expression of selected genes.* Frozen
513 chick brains were embedded in Jung OCT medium (freezing media, Leica Microsystems,
514 Wetzlar, Germany), 200 µm cross-sections were made using a cryostat (Leica CM 3050S, Leica
515 Microsystems, Austin, TX, USA) between anterior and posterior planes corresponding to Plate A
516 8.8 through to Plate A 8.2 for OVLT, A8.0 through A 7.6 for SSO and PVN (Fig.1) of a chick
517 brain atlas (Kuenzel and Masson, 1988). Each brain section was collected in a 1.5 ml tube
518 containing 100 µl of Trizol (Life Technologies, Palo Alto, CA, USA) and stored at - 80°C. Each
519 brain section dissected had the following average size: OVLT (x-axis X y-axis X thickness):
520 1.5mm x 1.5mm x 0.2mm (section 1), 1.5mm x 2.0mm x 0.2mm respectively (sections 2 & 3);
521 SSO (1.5mm x 1.5mm x 0.2mm), 3 sections; PVN (1.5mm x 1.5mm x 0.2mm), 3 sections.

522 *4.6.3. RNA isolation and two-step real-time quantitative RT-PCR.* Total RNA was extracted
523 from each dissected brain structure using Trizol reagent following the DNase I treatment
524 (Invitrogen, Carlsbad, CA, USA) and purification by RNeasy Micro Kit (Qiagen, Valencia, CA,
525 USA). The RNA quality and quantity were determined using agarose gel electrophoresis and
526 Gen.5 Synergy HT (BioTek, Winooski, VT, USA). cDNA was synthesized from 2 µg total RNA
527 using oligo d(T)16 primer and superscript III (Invitrogen, Carlsbad, CA, USA), as previously
528 described (Kang et al., 2007). The PCR primer pairs for VT4R /V1aR and CRH-R1 were
529 previously reported (Kang and Kuenzel, 2014). The specific oligonucleotide primers for AT1R
530 and TRPV1 were designed by the PRIMERS3 program (<http://frodo.wi.mit.edu>). The best primer
531 pairs were selected from several pairs based on PCR product quality and lengths after
532 electrophoresis on a 3% agarose gel. A portion of 4 µl of cDNA was subjected to quantitative
533 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°C for 2 min, 95°C for 10 min), 40 cycles; denaturation (at 95°C) for 60s, annealing (at 58-60°C) for 30s for AT1R, TRPV1, and β -actin: 58°C, for V1aR, CRH-R1, and GAPDH; 60°C, extending (at 72°C) for 3 min; holding stage (72°C for 10 min). The NCBI accession numbers, PCR product size and primer sequences used in the present study are: V1aR [NM001110438, 137 bp (V1aR-F: 5-GGT TGC AGT GTT TTC AGA GTC G-3; VT1aR-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)], AT1R [NC_006096.3 ; 138 bp (AT1R-F: 5- CTGTTTCAGGAAGGCACAGT-3; AT1R-R: 5-TGCTGGCCACTGTTTTTAAT-3)], TRPV1[NC_006106.3 ; 131 bp (TRPV1-F: 5-AAAGGCTGCCTGTTCATCAT-3; TRPV1-R: 5-TTGTCACTGTTTCCAGTGC-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 $\Delta\Delta CT$ method.

4.7. Statistical analysis

Statistical analyses were performed using software JMP® Pro 11.0 (SAS Institute Inc. Cary, NC). Differences among the four treatment groups 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 $\pm$ SEM. A probability level of $p < 0.05$ was considered statistically significant.

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789 Figure legends

790 **Fig. 1.** Schematic plates **A8.8**, **A 8.4**, and **A 8.2** show the anterior region of the diencephalon
791 and septum of the chick brain depicting the beginning and end of the organum vasculosum of
792 lamina terminalis (OVLT). The boxed-in areas show the dissected regions for the OVLT.

793 Schematic plates **A8.0**, **A 7.8**, and **A 7.6** show mid-regions of the chick diencephalon and
794 septum. The ventral box on each of the 3 plates show the dissected region including the
795 paraventricular nucleus (PVN) while the dorsal box in all three plates depicts the region

dissected that included the cephalic (A8.0) and caudal (A7.6) areas of the subseptal organ (SSO). The SSO is considered homologous to the subfornical organ (SFO) of mammals. Abbreviation: CA = Anterior commissure (Modified from Kuenzel and Masson, 1988).

Fig. 2. Boundaries of the organum vasculosum of the lamina terminalis (OVLT) revealed by glia immunoreactive for the avian vasotocin 1a receptor (V1aR), homologous to the mammalian V1aR. **A.** Shows the beginning of the OVLT residing at the base of the preoptic region. **B-E.** Show the dorsal progression of the OVLT up 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; a portion of the OVLT in front of the CA was removed when sectioned. **G.** Beginning of the SSO/SFO at midline, directly dorsal to the CA and occupying the ventral region of the nucleus of the hippocampal commissure (NHpC). **H-J.** Glia immunoreactive for the avian V1aR are shown surrounding blood vessels at midline; densely ir glial processes contact the third ventricle (3V); a greater proportion of the NHpC is occupied by the SSO/SFO posteriorly. **K-L.** A transition occurs where a chamber appears containing cerebrospinal fluid dorsal to the 3V at the most caudal level of the SSO/SFO; a characteristic finger-like projection of the SFO projects into the 3V. Thereafter, the 3V joins the ventral horns of the lateral ventricles forming the interventricular foramina. Scale bars: A-J=100 μ m; K-L= 100 μ m.

Fig. 3. Changes in orientation of individual processes immunostained for the avian V1aR within the ventral region of the organum vasculosum of the lamina terminalis (OVLT). **A.** Isotonic saline (IS) control compared to **B.** Hypertonic saline (HS) treated bird. **C.** Higher magnification of boxed-in area of A for the control bird compared to **D.** Higher magnification of boxed-in area of B, the hypertonic saline treated bird. Note the curved processes of the HS bird compared to the linear ones displayed by the IS bird. **E.** Ventral region of the OVLT of a control bird not subjected to immobilization. **F.** Ventral OVLT region of a bird subjected to acute immobilization

820 stress (AS). Similar to the HS treated birds, the AS birds displayed curved processes. **G.**
821 Higher magnification of boxed-in area of E, control bird. **H.** Higher magnification of boxed-in
822 area F of a bird immobilized for 1h. Scale bar: A, B, E, F = 100µm; C,D,G,H = 50µm.

823 **Fig. 4.** Immunohistochemical changes within the subseptal organ/ subfornical organ
824 (SSO/SFO). **A.** Isotonic saline control. **B.** Bird subjected to hypertonic saline. **C.** A bird not
825 subjected to acute immobilization stress. **D.** Bird subjected to immobilization. See text for
826 description of changes. Scale bar, A-D = 100 µm.

827 **Fig. 5.** Plasma concentrations of **A.** Corticosterone, ng/ml, and **B.** Arginine vasotocin, pg/ml, in
828 response to 1h of acute immobilization stress and acute hypertonic saline administration
829 compared to controls, obtained by radioimmunoassay. Different lower case letters above
830 histograms show group means significantly different ($p \leq 0.05$). Abbreviations: AC = acute
831 immobilization control, AS = acute immobilization stress, IC = isotonic saline control, HS = acute
832 hypertonic saline stress. (n = 6 birds/group); Error bar: SEM (standard error of mean).

833 **Fig. 6.** Gene expression change of avian vasotocin 1aR (V1aR) mRNA in the **A.** Subseptal
834 organ (SSO/SFO) and **B.** Organum vasculosum of lamina terminalis (OVLT). **C.** Angiotensin II,
835 type 1a receptor (AT1aR) mRNA in the SSO/SFO and **D.** OVLT. **E.** Transient receptor potential
836 cation channel, vanilloid type 1 (TRPV1) mRNA in SSO and **F.** OVLT. Bird treatments included
837 acute controls (AC), birds subjected to 1 h of acute immobilization stress (AS), birds
838 administered isotonic saline, serving as controls (IC) or given hypertonic saline (HS). Different
839 lowercase letters show group means that are significantly different ($p < 0.05$) for each mRNA
840 transcript within each circumventricular organ. n = 6 birds/treatment, Error bar = SEM.

841 **Fig. 7.** Gene expression change of corticotropin releasing hormone-receptor 1 (CRH-R1) in the
842 paraventricular nucleus (PVN) following 1 h of acute immobilization stress or hypertonic saline

843 administration. Different lower case letters show group means that are significantly different
844 ($p < 0.01$). AC, acute immobilization control (n=5 birds); AS, acute immobilization stress (n=6
845 birds); IC, isotonic saline control (n=5 birds); HS, acute hypertonic saline stress (n= 6 birds).
846 Error bar: SEM.

847 **Fig. 8.** Schematic structure of the anterior region of the organum vasculosum of the lamina
848 terminalis (OVLT). Abbreviations: CIS – cistern, LT – lamina terminalis, PCZ – pericisternal
849 zone, PVZ – periventricular zone.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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Figure 7

Figure 8