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Central Role of Vasotocin in the Neuroendocrine Regulation of Stress Responses and Food Intake in Chickens, Gallus gallus

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Central Role of Vasotocin in the Neuroendocrine Regulation of Stress Responses and Food Intake in Chickens, *Gallus gallus*.

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Abstract

It is well known that arginine vasotocin (AVT) in birds is involved in physiological homeostasis such as cardiovascular, osmotic regulation as well as reproductive functions. Pertinent to these physiological functions, AVT immunoreactive (-ir) neurons in the hypothalamus have been found associated with hemorrhage, dehydration, oviposition and other physiological regulation. Evidence, however, suggests that AVT also plays significant roles in modulating behavior, memory, stress, and food intake. This dissertation research addresses the latter two neuroendocrine functions of AVT in detail within the chicken brain. First, the functional role of AVT-ir neurons in conjunction with corticotropin releasing hormone (CRH)-ir neurons in the neuroendocrine regulation of stress is elucidated in meat type chickens. The results from research showed that a distinct type of AVT neurons (parvocellular - small sized) in the paraventricular nucleus (PVN) of the hypothalamus is activated in response to acute and chronic stress. Furthermore, in order to facilitate the neuroendocrine regulation of stress in chickens, expression of AVT in the PVN occurs in the latter phase of the stress period compared to earlier activity of CRH when birds are exposed to a stressor. Second, the neuromodulatory role of AVT to activate CRH neurons via vasotocin receptors (V1aR) within the avian brain is addressed. The results suggests that AVT could augment stress response by modulating CRH neuronal activity in a septal nucleus called the nucleus of the hippocampal commissure (NHpC). Third, the functional role of AVT via the V1aR in food intake regulation is characterized. Results show the association of AVT neurons (magnocellular – large sized), in the preoptic and hypothalamic brain structures, and CRH neurons, in the NHpC, following food intake in chickens. Hence, neuroendocrine regulations of AVT neurons in distinct brain structures in the stress response and food intake in chickens are characterized by this dissertation research.

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Second, I like to thank my committee members: Dr. Bottje, Dr. Kumar, Dr. Dridi and Dr. Kong who provide tremendous help and guided me through the course of this dissertation research. I thank Dr. Wideman, Dr. Erf, Dr. Rath, Dr. Thaxton, and Dr. Donoghue for their guidance, their encouragement and I believe that in some way they had contributed to my professional development. I thank some of them for providing me the opportunity to conduct experiments in their facility that was not part of my doctoral research. I thank Dr. Anthony for providing us enough birds to conduct preliminary experiments. I thank Dr. Alexander Jurkevich for teaching me the basic technique in neurobiology and for his guidance throughout my graduate research. I thank Seong Kang and Liz Greene for teaching me molecular biology techniques and guiding me through troubleshoots. I thank my department chair Dr. Michael Kidd who encouraged us to study the chick brain and helped us cultivate the importance of neuroendocrinology in poultry industry. I also thank him for providing me with an assistantship to continue my doctoral research.

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Dedication

This dissertation is dedicated to the scientific community.

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List of Abbreviations

3v - third ventricle
ABC - avidin biotin complex
aCSF - artificial cerebrospinal fluid
ACTH - adrenocorticotropic hormone
ADX - adrenalectomy
AVP - arginine vasopressin
AVT - arginine vasotocin or vasotocin
BDNF - brain derived neurotropic factor
BST - bed nucleus of stria terminalis
C - centigrade
CCD - charge coupled device
cDNA - complementary deoxyribonucleic acid
c-fos - cellular FOS
CORT - corticosterone
CRH - corticotropin releasing hormone
CSM - corticoseptomesencephalic tract
CVO - circumventricular organ
d - day(s)
DAB - diaminobenzidine
DEX - dexamethasone
FD - food deprivation
FITC - fluorescein isothiocyanate
FOS - Finkel-Biskis-Jinkins murine osteosarcoma virus oncogene
FSK - forskolin
g - gram(s)
GABA - gama aminobutyric acid
h/hr - hour(s)
hnRNA- heteronuclear RNA
HPA - hypothalamo-pituitary-adrenal
HRP - horseradish peroxidase
i.c.v. - intracerebroventricular injection
i.v. - intravenous
ir - immunoreactive
kg - kilogram(s)
µl - microliter(s)
µm - micrometer(s)
mg - milligram(s)
ml - milliliter(s)
mRNA - messenger RNA
MPO - medial preoptic nucleus

MTR - mesotocin receptor
NHpC - nucleus of the hippocampal commissure
Ni - nickle
NPY - neuropeptide Y
NST - nucleus of the solitary tract
OVLT - lamina terminalis of the organum vasculosum
OXY - oxytocin receptor
PB - phosphate buffer
PBS - phosphate buffer saline
PCR - polymerase chain reaction
PHN - periventricular hypothalamic nucleus
POMC - proopiomelanocortin
PPoN - periventricular preoptic nucleus
PVN - paraventricular nucleus
RNA - ribonucleic acid
SCM - standard culture medium
SCN - suprachiasmatic nucleus
SFO - subfornical organ
SO - supraoptic nucleus
SSO - subseptal organ
V (R) - vasotocin receptors

List of Published Papers

1. Nagarajan, G., Tessaro, B.A., Kang, S.W., Kuenzel, W.J., 2014. Identification of arginine vasotocin (AVT) neurons activated by acute and chronic restraint stress in the avian septum and anterior diencephalon. Gen. Comp. Endocrinol. 202, 59-68. (Chapter 2)

2. Nagarajan, G., Kang, S. W., & Kuenzel, W. J. (2017) Functional evidence that the nucleus of the hippocampal commissure shows an earlier activation from a stressor than the paraventricular nucleus: Implication of an additional structural component of the avian hypothalamo-pituitary-adrenal axis. Neurosci. Lett. 642, 14–19. (Chapter 3)

3. Nagarajan, G., Jurkevich, A., Kang, S. W., & Kuenzel, W. J. (2016). Diencephalic and septal structures containing the avian vasotocin receptor (V1aR) involved in the regulation of food intake in chickens, *Gallus gallus*. Physiol. Behav. 164, 268-276. (Chapter 5)

4. Nagarajan, G., Jurkevich, A., Kang, S. W., & Kuenzel, W. J. (2017) Anatomical and functional implications of CRH neurons in the nucleus of the hippocampal commissure of the avian brain: An emphasis on glia-neuron interaction via the V1a receptors. J. Neuroendocrinol. (to be submitted, Chapter 4)

Introduction

Chickens are the most widely domesticated birds in the modern world where the challenges for raising birds for meat consumption and egg production are large. Lots of progress have been made in different fields of poultry in the last 6 decades to improve growth, health, feed efficiency, managements, etc., however, ongoing research at the cellular and molecular level is yet to bridge the knowledge gap between physiology and behavior of the birds. External factors, such as predatory threats, heat, cold environment, etc., and internal factors, such as thirst, hunger, pain, infections etc., are perceived by birds and corresponding changes occur in the internal milieu to facilitate physiological and behavioral responses. Changes in the internal milieu of an organism are brought by biochemical substances released from distinct types of tissues in response to external or internal stimuli. Particularly, the nervous system and neurochemical substances play a major role in maintaining the internal milieu. For instance, the classical 'fight or flight' stress response involves the sympathetic nervous system where norepinephrine acts as a major neurotransmitter, however, at the same time passive necessary processes such as food intake and reproduction are suppressed. Hence, the complexity of neural connections between different brain structures performing various vital functions are large and there is a paucity of information on how neural substrates modulate physiology and behavior.

This dissertation research focuses on one of the most studied neurohormones, arginine vasotocin (AVT) and its functions in the neuroendocrine regulation of stress and food intake. In recent decades AVT has gained particular attention because of the following reasons: 1) AVT has diverse physiological and behavioral functions, 2) Detailed localization and projections of AVT synthesizing neurons in the avian brain, 3) Distinct functions of AVT are associated with particular sets of neural substrates containing AVT neurons, 4) feasibility of laboratory

techniques to study activity of individual types of AVT neurons following a particular stimuli and 5) sequence availability of vasotocin receptors and neuroanatomical localization of vasotocin receptors in different brain sites. All these available features makes AVT an interesting neurohormone whose neuroendocrine regulation is least understood in the stress pathway and food intake regulation within the chicken brain and hence the focus of this research.

Chapter 1

Vasotocinergic system in the avian brain: A literature review.

1. Arginine vasotocin (vasotocin/AVT)

Since the discovery of presser effects of pituitary (hypophysis) extracts in mammals (Oliver and Schäfer, 1895), studies were pursued to identify pituitary extracts from several non-mammalian vertebrates to show similar pressor response (Heller and Pickering, 1961). A surge in research to identify the properties of the compounds in the pituitary extract resulted in the discovery of vasopressin, oxytocin, vasotocin, lysipressin in several vertebrates (Katsoyannis and du Vigneaud, 1958; Sawyer et al., 1960; Archer, 1960; Munsick et al, 1960) including chickens. These structurally similar nine peptide (nonapeptide) hormones are secreted from the neurohypophysis or posterior pituitary (vasopressin, oxytocin, vasotocin, lysipressin, etc.) and were found to be associated with physiological functions such as to maintain blood pressure, anti-diuresis/osmotic regulation and reproduction (Heller 1941; Sawyer, 1960; Heller and Pickering, 1961). The hormone acts on the collecting duct of nephrons to increase the permeability of water to get reabsorbed and help in the retention of water (Grantham and Burg, 1966), thus giving its classical name, anti-diuretic hormone. In birds, arginine vasotocin (AVT), a homolog of arginine vasopressin (AVP) in mammals, has been historically recognized as physiological regulator of water balance (Munscik et al., 1960). Similar to mammals, AVT is primarily released from neurosecretory cells into the neurohypophysis (posterior lobe of the pituitary), and executes it function in the kidney to reabsorb water at times of water loss in the body (Skadhauge and Schmidt-Nielsen, 1967).

From the pioneering work of Dr. Ernst Scharrer and others, the hormones that exert pressor and anti-diuretic function were found to be released from neurosecretory cells of the hypothalamus (Bargmann and Scharrer, 1951). These neurosecretory cells or magnocellular neurons are primarily found in two subpopulation on either side of the hypothalamus (bilateral) and its organization is more or less phylogenetically conserved throughout vertebrate evolution. Anteriorly, a population of neurons is present at the base of the brain near the preoptic region, supraoptic nucleus (SO). The other can be found posteriorly (caudally) near third ventricle, the paraventricular nucleus of the hypothalamus (PVN) (Bargmann and Scharrer, 1951; Yasuda, 1954; Farner and Oksche, 1962; Swanson and Sawchenko, 1983). Magnocellular neurosecretory neurons in these nuclei project their axons into the neurohypophysis (posterior pituitary) via the internal zone of the median eminence and this system is called as the hypothalamo-neurohypophyseal nerve tract (Oksche et al., 1963; Mikami et al,. 1978). Hence, the secretory neurohormone released from the posterior pituitary for fine tuning of homeostatic regulation of pressor activity or electrolyte balance or reproductive function, was found to be synthesized in neurons of the SO, PVN and preoptic regions.

The advent of immunohistochemical methods facilitated the identification of AVP immunoreactive (-ir) neurons in the rodent brain (Vandesande and Dierickx, 1975). A few years later, AVT-ir neurons were also identified using a specific antibody targeting AVT, in several avian species (Goossens et al., 1977). This sophisticated technique identified different types of neurons stained for AVT. First, using immunohistochemical and tract tracing methods, large or magnocellular AVT-ir neurons were identified as the sources of neurohormone release into the peripheral system (Mikami, et al., 1978, 1986). Second, another type of AVP/AVT neurons was found present in the hypothalamus that was smaller in size and has been called parvocellular

neurons. These parvocellular neurons have two sets of projections. Some project their axons into the median eminence where they release their peptides into the portal system to modulate physiological responses (by acting on pituitary cells), while others project to brain stem and spinal cord to modulate autonomic functions. In this review, AVT neurons in these systems will be discussed and followed by the current knowledge on the neuroendocrine regulation of AVT on stress and its possible role in the regulation of food intake. Since a plethora of information is available in mammals, rodent studies are used whenever there is a lack of data in avian studies.

1.1. Distribution of AVT neurons

Although, AVP (mammals) and AVT (birds and reptile) were separated during the course of vertebrate evolution (Hoyle, 1998), they differ only by one amino acid at position 3 where phenylalanine in AVP is mutated to isoleucine in AVT. Nonetheless, major populations of AVP/AVT neurons are conserved across classes of vertebrates (Moore and Lowry, 1998). Distribution of AVT-ir neurons has been studied in several avian species (including domestic pigeons, starlings, canaries, doves, quail, finches, mallards, juncos, blue tits, budgerigars and chickens) using the immunocytochemical procedure incorporating antibodies specific to the AVT peptide (Goossens et al., 1977; Bons, 1980; Berk et al., 1982; Tennyson et al., 1985; Panzica 1985; Mikami, 1986; Kiss et al., 1987; Panzica et al., 1999; Fabris et al., 2004; Montagnese et al., 2015). These studies clearly showed that AVT-ir neurons are found predominantly in three regions of the avian brain: the preoptic region, anterior hypothalamus and mid-hypothalamic structures (Fig. 1). Specifically, AVT immunoreactivity was found in neurosecretory magnocellular neurons of the SO and PVN described above (Mikami 1986). In the preoptic region, AVT is clearly present in two subnuclei of the SO (external and ventral subdivisions), preoptic recess, and periventricular preoptic nucleus (PPoN). Caudally, PPoN

AVT-ir neurons also have some small perikarya (cell body). In the anterior hypothalamic region, AVT-ir neurons can be identified in the periventricular hypothalamic nucleus (PHN), rostral PVN, the lateral group of neurons and the dorsolateral anterior nucleus.

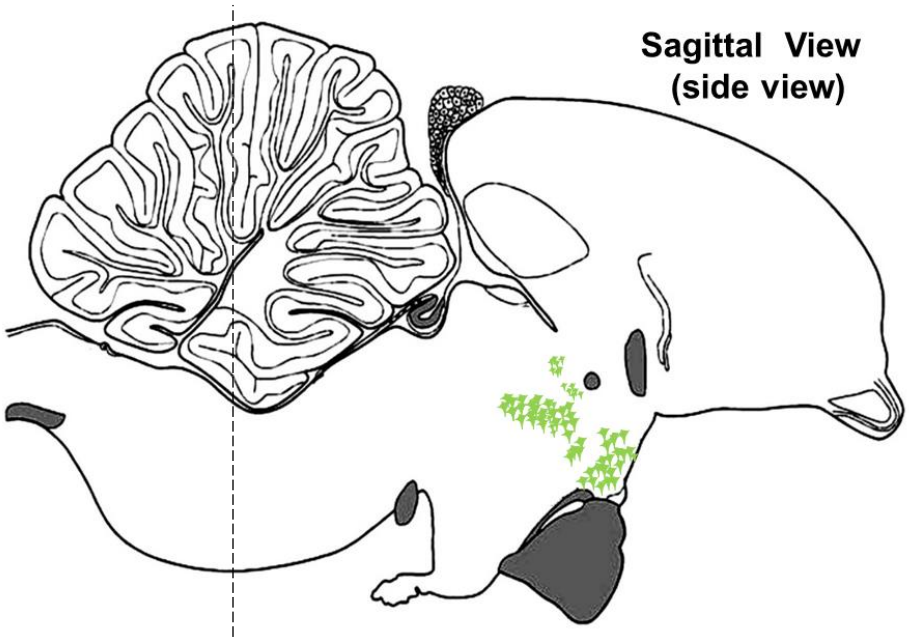

Fig. 1 (a-h). Schematic representation of side view of a chick brain containing vasotocin neurons. Coronal (cross section) reference atlas plates (Kuenzel and Mason, 1988) and digital images of brain sections (boxed in region) containing vasotocin neurons (brown) in different nuclei of the chicken diencephalon are shown in Fig. 1a through 1h. Different nuclei or brain structures in the reference atlas plates can be distinguished by different color codes. The numbers in the atlas plates refers to the location anterior (A, in mm) to the zero reference point (dashed line) in the side view of the chick brain. Atlas plates in Fig. 1a-h were obtained from http://avianbrain.org/nomen/Chicken_Atlas.html and were modified. Digital images in Fig. 1a-Fig. 1h were taken by GN.

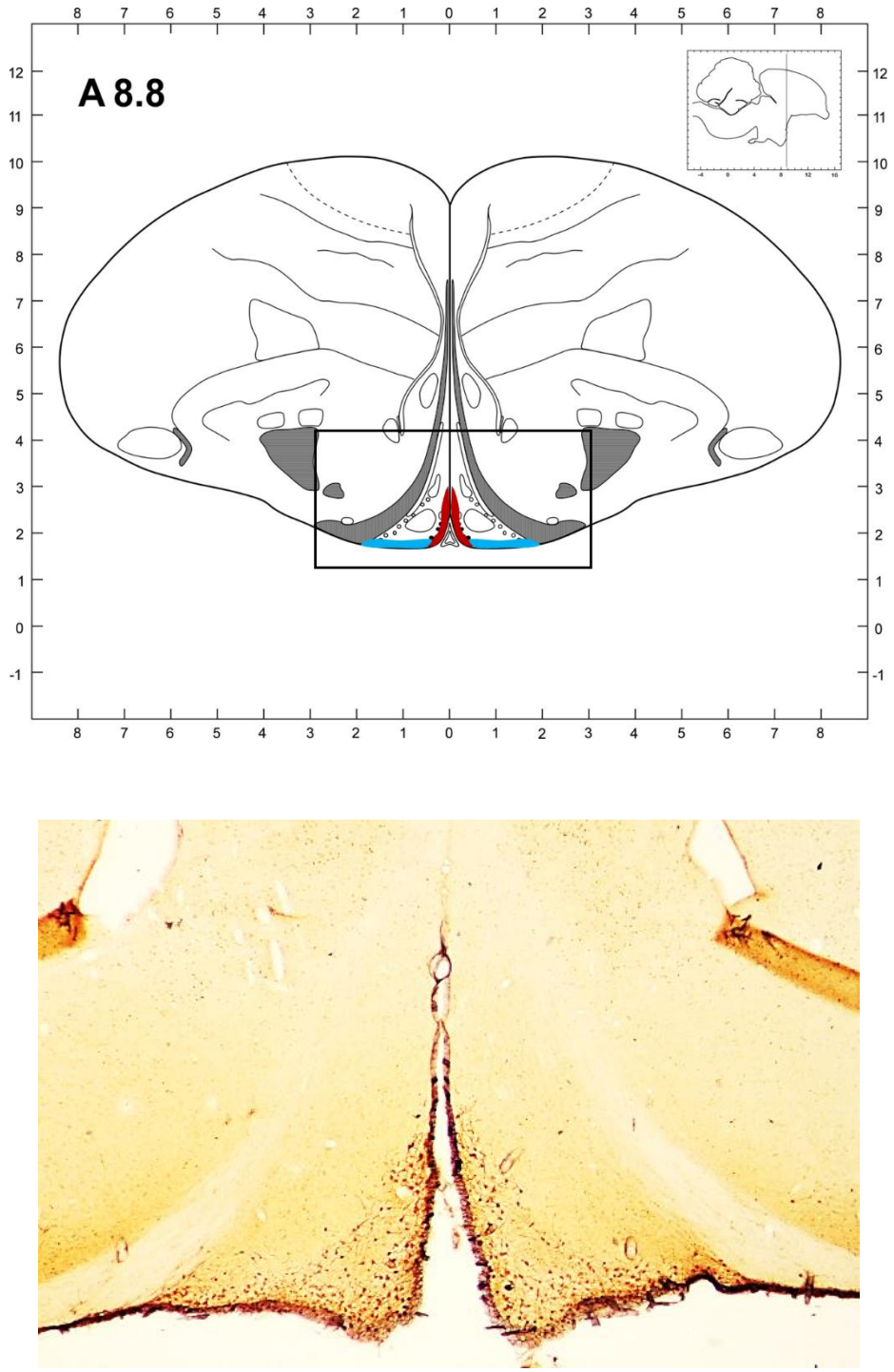

Fig. 1a. Vasotocin neurons in the anterior part of the chicken diencephalon. Vasotocinergic neurons in the periventricular preoptic nucleus (PPoN) are shown in brown and vasotocinergic neurons in the supraoptic nucleus (SO) are shown in blue.

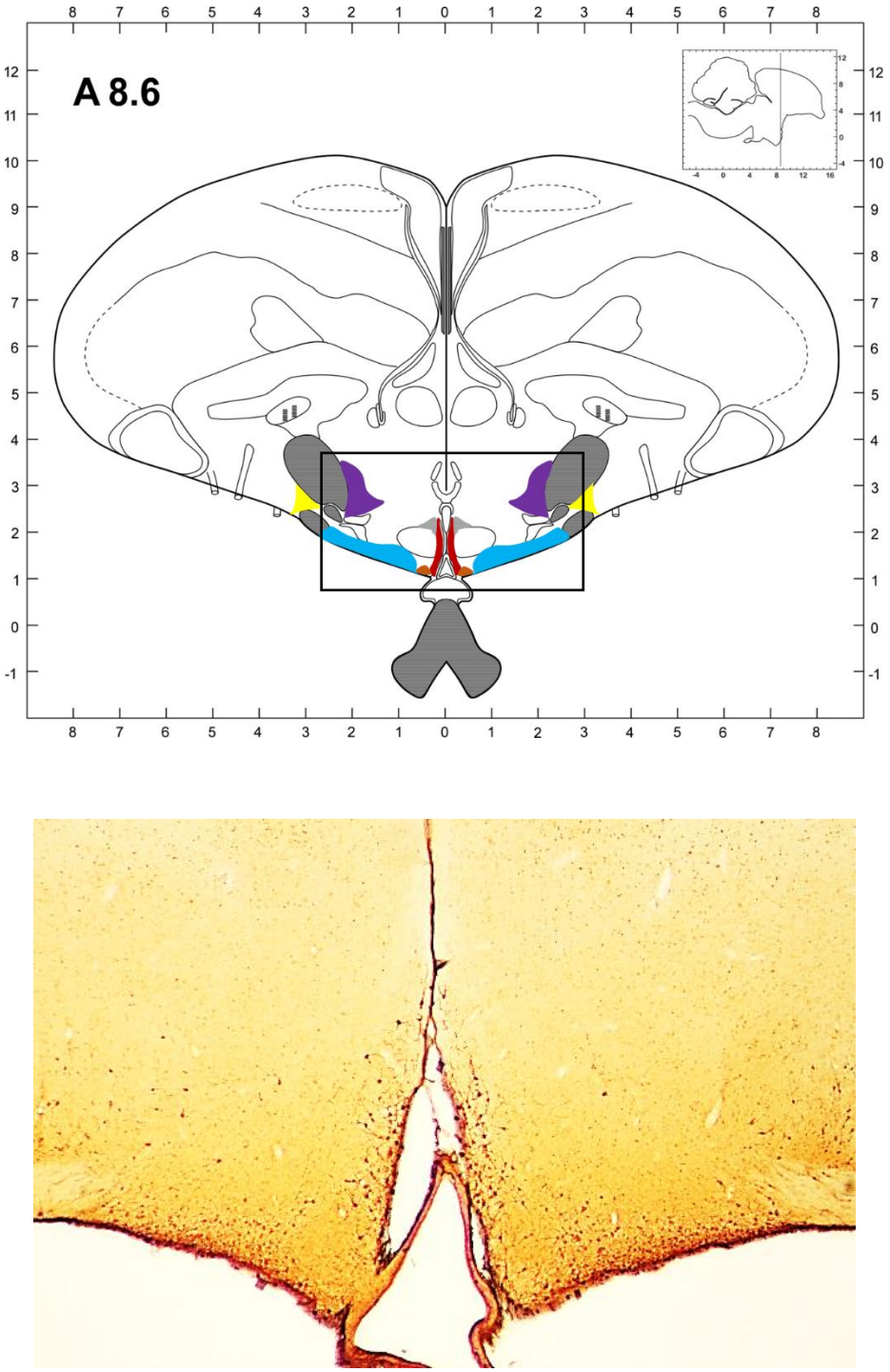

Fig. 1b. Separation of vasotocinergic neurons in the periventricular preoptic nucleus (PPoN brown) and supraoptic ventral subnucleus (SO, blue) is observed at A8.6. At the same level the lateral group of neurons (shown in purple) begin to appear. The external supraoptic subnucleus is shown in yellow.

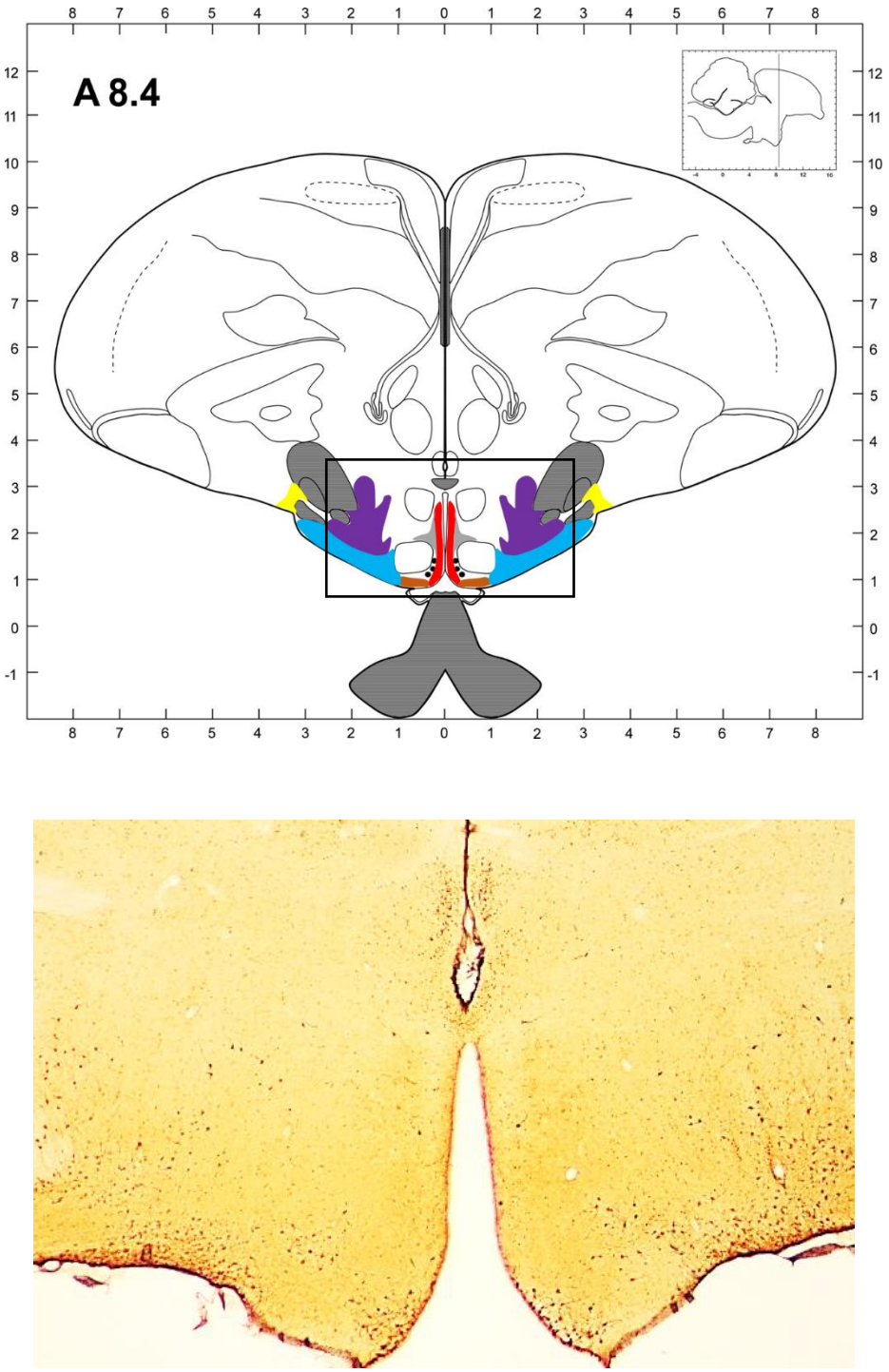

Fig. 1c. Vasotocinergic neurons in the supraoptic nucleus (SO, blue) and the lateral group (purple) are shown at the level A8.4. Vasotocinergic neurons present in the suprachiasmatic nucleus (SCN, light brown) are located at the ventromedial base of the 3rd ventricle.

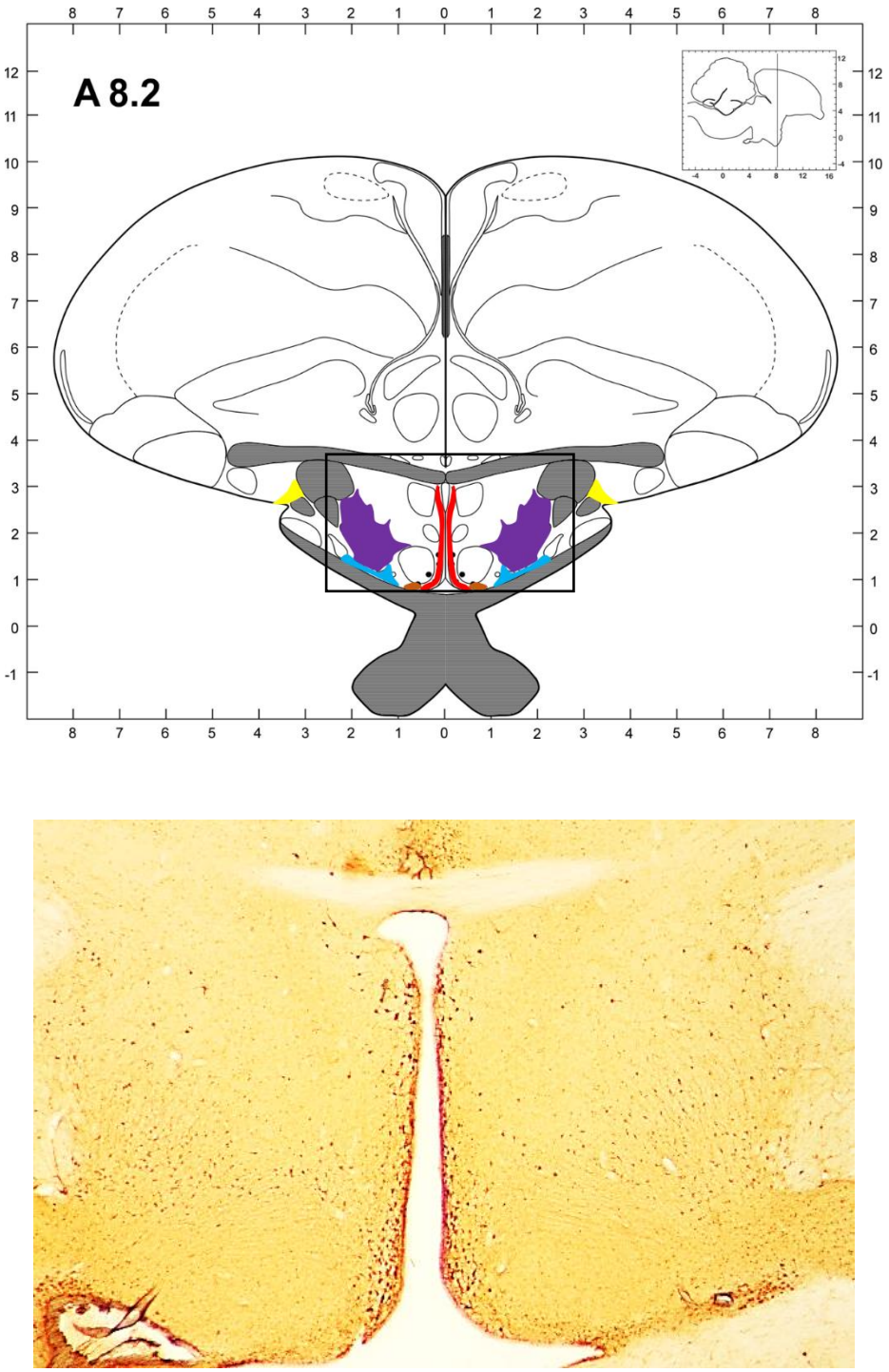

Fig. 1d. Vasotocinergic neurons are prominently observed near the wall of the 3rd ventricle in the periventricular preoptic nucleus (PPoN, red). The lateral group (purple) expands in area at this level and the supraoptic nucleus (SO, blue) vasotocinergic neurons disappears.

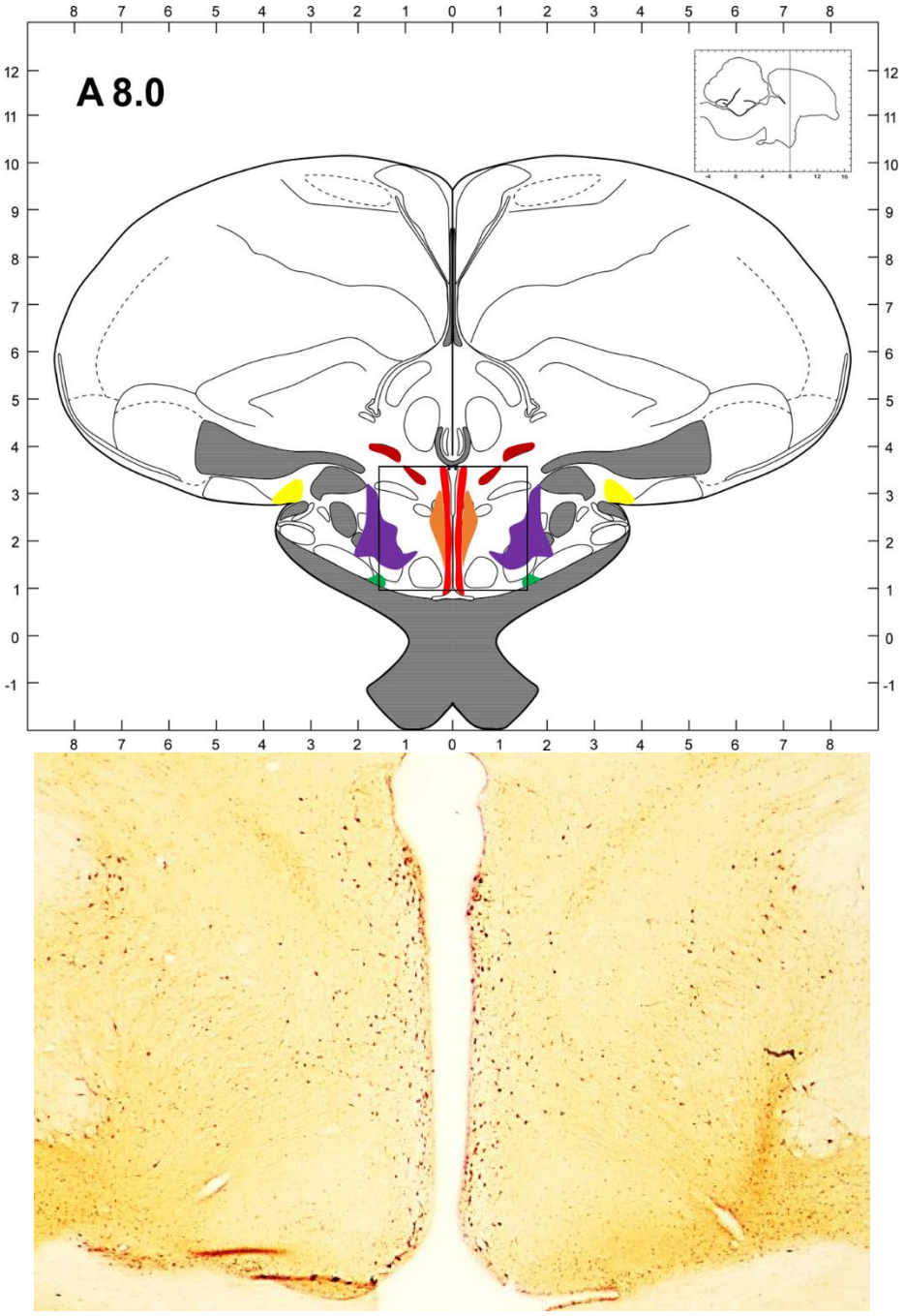

Fig. 1e. At A8.0, vasotocinergic neurons in the paraventricular nucleus (PVN, orange) are prominently observed in medial portion of the hypothalamus. A group of parvocellular neurons in the bed nucleus of stria terminalis (BST) is shown in dark brown color. Vasotocin neurons in the periventricular preoptic nucleus (Fig. 1d - A8.2, red) continues into the hypothalamic region called the periventricular hypothalamic nucleus (PHN, A8.0, red).

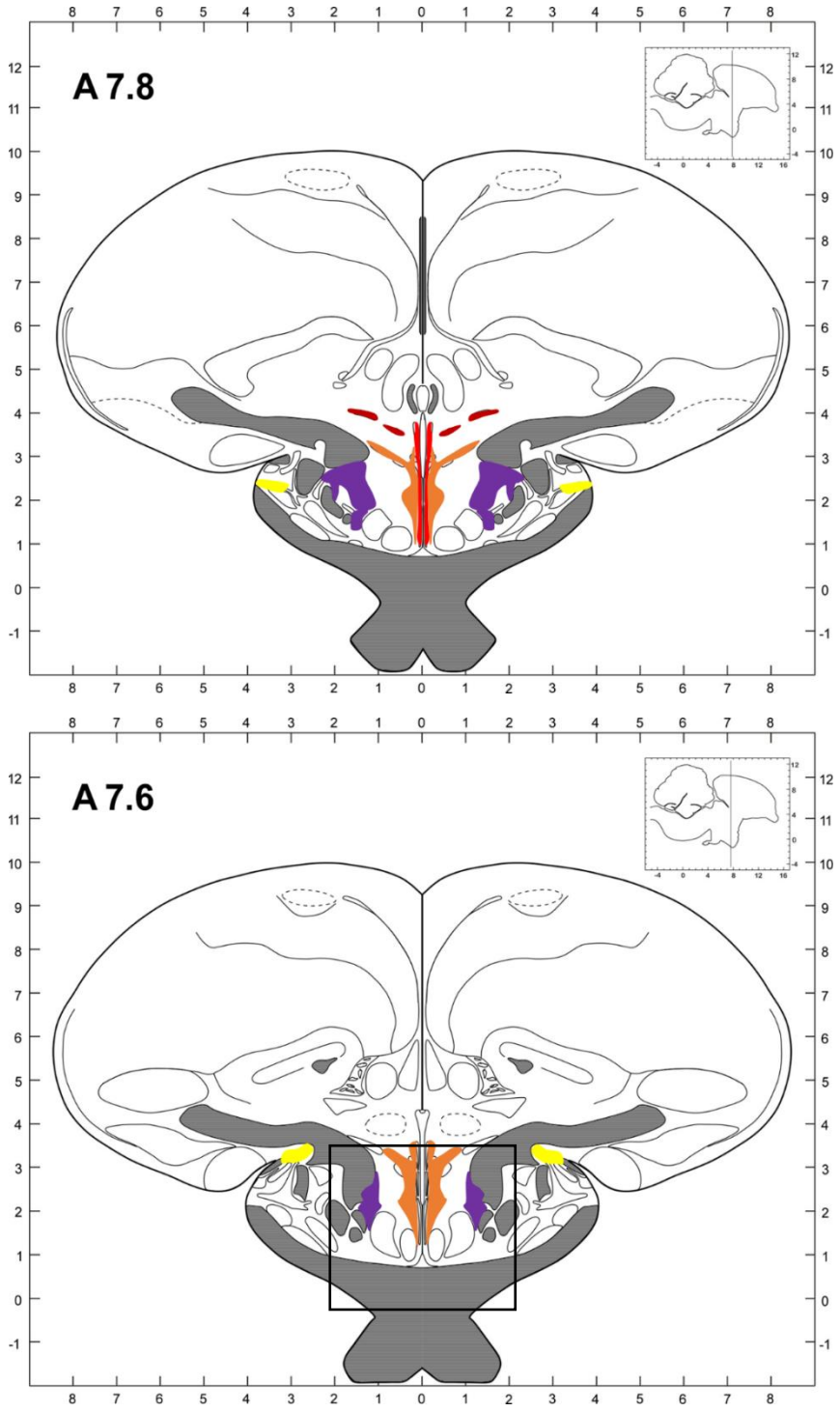

Fig. 1f. At A7.8 and A7.6 vasotocinergic neurons in the paraventricular nucleus (PVN, orange) expands laterally from the medial region. On the lateral side, the PVN vasotocinergic neurons are continuous with the lateral group (purple), where the lateral group begins to disappear.

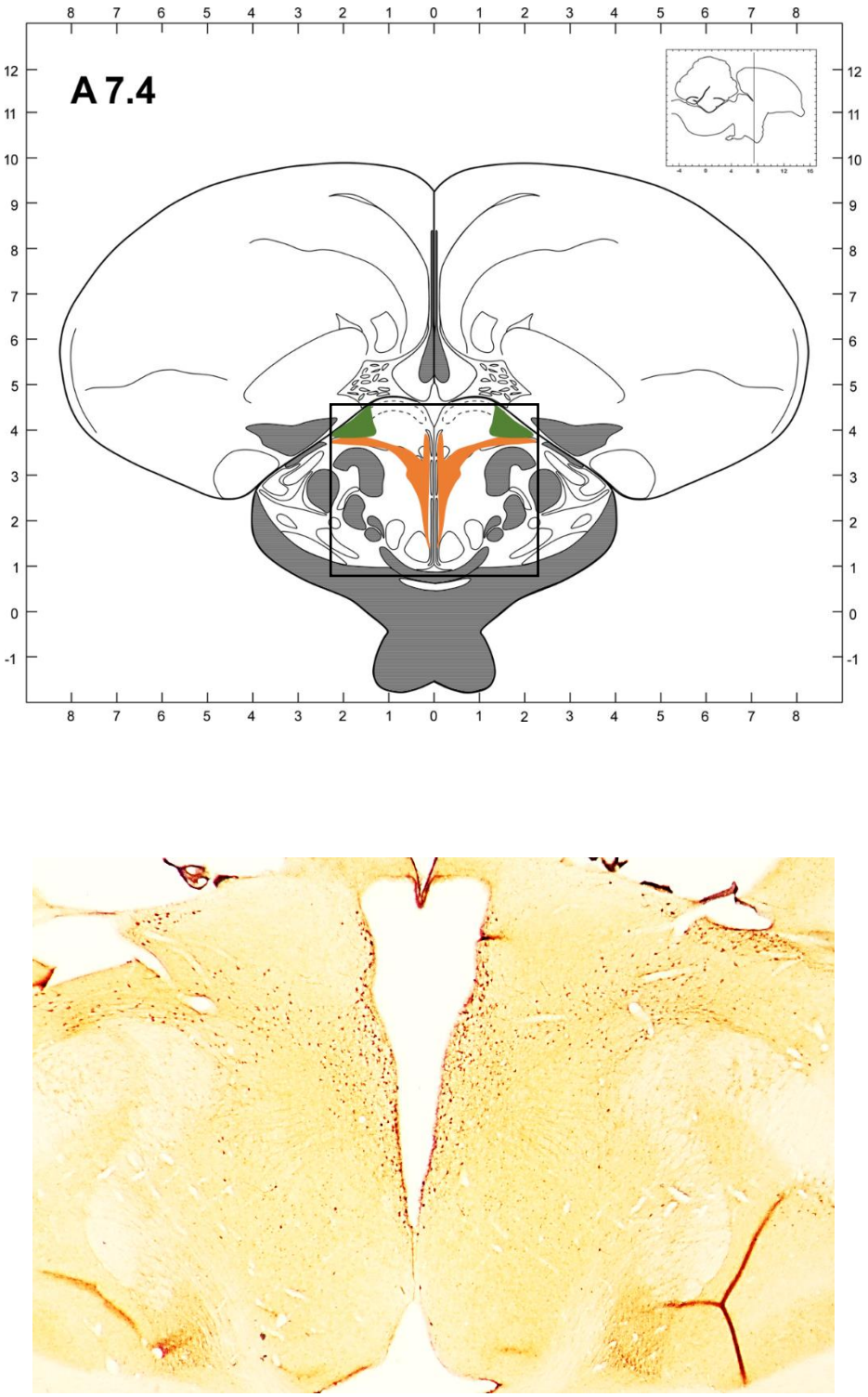

Fig. 1g. At A7.4, where septum separates from the hypothalamus, the paraventricular nucleus (PVN) vasotocinergic neurons extends laterally to form a wing shaped pattern. At this level dorsal thalamic area contains a population of vasotocin neurons (green).

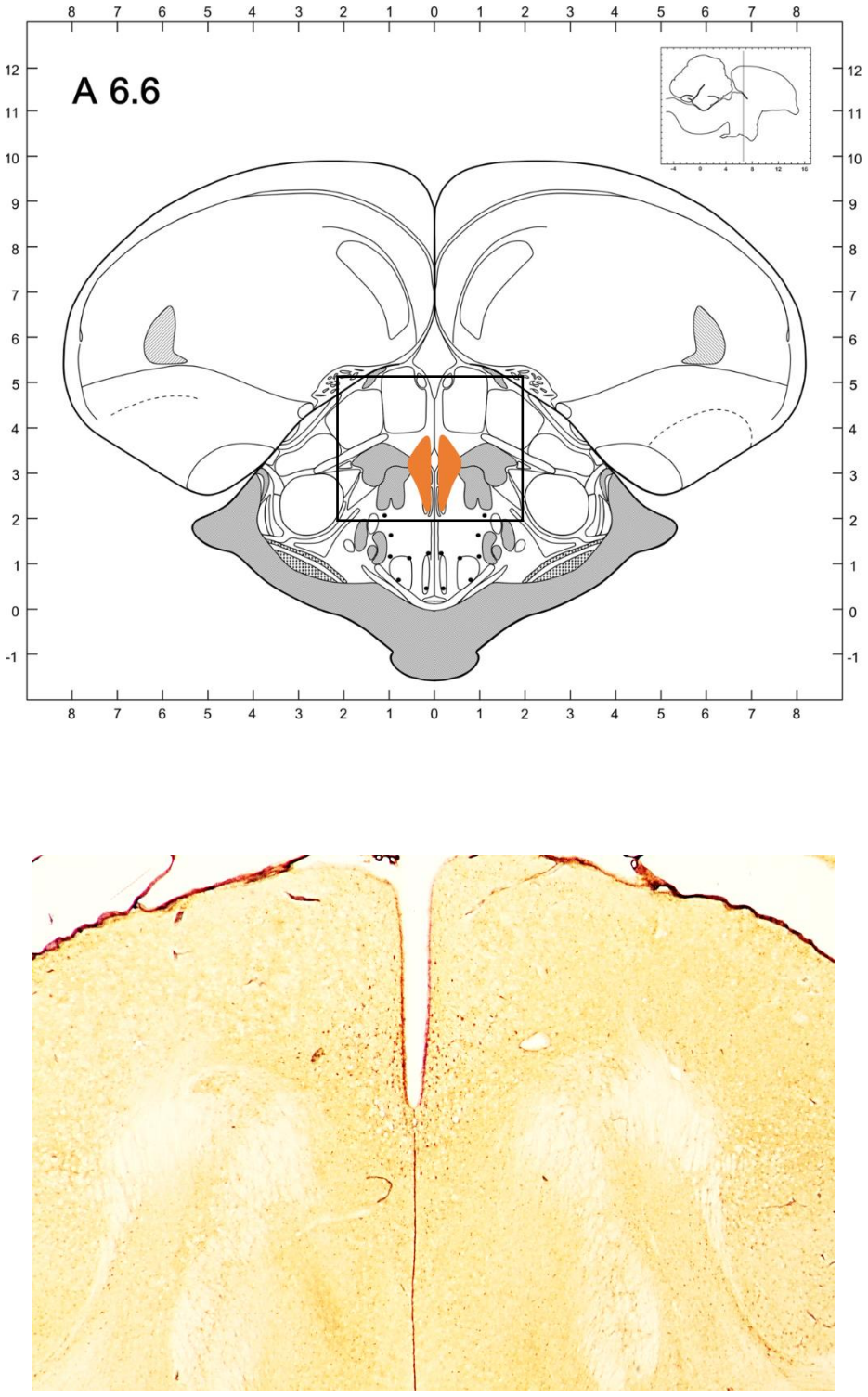

Fig. 1h. In the mid-hypothalamic region, the paraventricular nucleus (PVN) vasotocinergic neurons are present in dorsomedial portion and they disappears posteriorly.

Other regions in the anterior hypothalamus containing AVT-ir neurons are the two subdivisions of the SCN (Panzica 1985; Cantwell and Cassone, 2006 a and b), where the medial SCN neurons are present close to the preoptic recess and the third ventricle and contain more AVT-ir parvocellular neurons than the lateral SCN. The lateral SCN neurons are present in the lateral part of the hypothalamus and ventral to the lateral group of AVT-ir neurons (Cantwell and Cassone, 2006b). In the mid-hypothalamic region, the PVN and the lateral group of neurons appear to be continuous from the anterior hypothalamic region. Interestingly, the avian PVN contains heterogeneous population of neurons including magnocellular and parvocellular neurons (Panzica and Viglietti-Panzica, 1981; Panzica and Viglietti-Panzica 1983; Korf, 1984), as shown in Fig. 2.

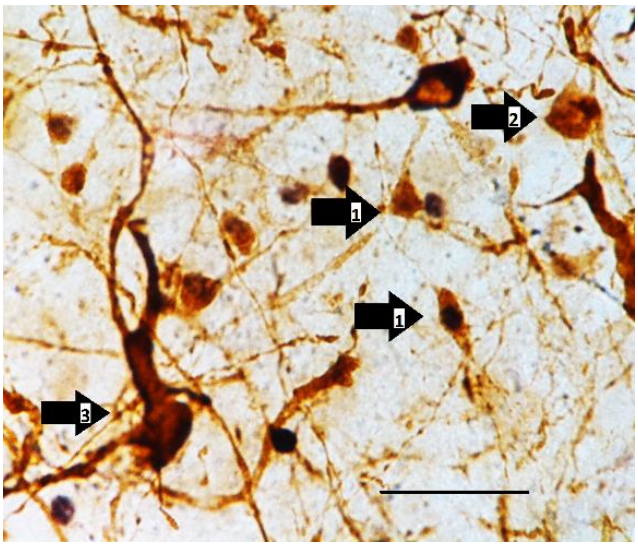

Fig. 2. Heterogeneous population of AVT-ir neurons in the paraventricular nucleus. 1) Parvocellular neurons, 2) medium sized neurons and 3) magnocellular neurons. Scale bar – 50 µm. (Digital image was taken by GN).

Although the distribution of AVT-ir neurons in these major nuclei are conserved across avian species, subtle differences can be observed in some nuclei of aves (Panzica, 1985; Moore and Lowry, 1998). For example, differences in the distribution pattern of AVT-ir neurons were

observed among birds in the SO and PVN (Goossens et al., 1977,) and posterior extent of AVT-ir neurons in the SCN are more densely packed in quail than in chickens (Panzica, 1985). Nonetheless, the SO and PVN gained particular attention because of dense population of neurosecretory magnocellular neurons stained with AVT (Goossens et al., 1977; Mikami 1986) and their functions are conserved in most vertebrates.

AVT neurons in different brain structures described above are located in the hypothalamus, however, some extra-hypothalamic structures also contain AVP/AVT neurons in both mammals and birds. Other brain structures that contains AVT-ir neurons, are the bed nucleus of the stria terminalis (BST), dorsolateral to the nucleus rotundus and the dorsolateral thalamus (Tennyson et al., 1985; Panzica, 1985; Aste et al., 1996). The BST contains parvocellular type AVT-ir neurons and are sexually dimorphic in birds (Jurkevich et al., 1996; Jurkevich et al., 1999; Aste et al., 1997; Aste et al., 2016), while the latter two nuclei contains medium sized AVT-ir neurons. Similarly, in mammals use of colchicine (axon transport blocker) revealed the presence of vasopressin neurons in the BST, dorsomedial hypothalamus, medial amygdala, and locus coeruleus (Caffe and Van Leeuwen. 1983; Van Leeuwen and Caffe, 1983). However, the latter three nuclei have not yet been found to contain AVT-ir neurons in birds and therefore possibly involved in differences observed among classes of vertebrates as reflected among the distribution of vasotocinergic/vasopressinergic systems in the brain (Lowrey and Moore, 1998). Nonetheless, both hypothalamic and extrahypothalamic distributions of AVT-ir neurons are presumed to play distinct roles in the physiology and behavior of birds.

1.2. Efferent projections of AVP/AVT neurons

Several distinct brain regions were found to contain AVT-ir fibers. Major projections of AVT-ir magnocellular neurons (the largest neurons in the central nervous system) run through a common course (neurohypophyseal tract) and have been implicated in classical neurohypophyseal functions. While other nuclei have distinct characteristic projections within the avian brain (Robinzon et al., 1988, Panzica et al., 1988, Sugita, 1994), and are likely engaged in various behavioral and physiological functions (described in section 1.5). Since, it has been estimated that a single neuron in the hypothalamic and extra-hypothalamic structures could have about 600 and 3000-4000 terminals, respectively (Palkovits, 1991), the permutation and magnitude of functions associated with a particular population of neurons are large.

Neurons at the basal part of the corticoseptomesencephalic tract (CSM), the SO, and the periventricular midhypothalamic structures, the PVN and PHN, have processes that are part of the neurohypophyseal tract (Mikami et al., 1986). Furthermore, projections of neurons in the preoptic recess (PPoN) were also found to course through the neurohypophyseal tract (Berk et al, 1982), while some of its terminals were found to project to the 3rd ventricle. A closer look at neurons of the preoptic recess was found to have three sets of projections: 1) dorsal set of neurons contact the lumen of the 3rd ventricle, 2) middle set contact the lamina terminalis of the organum vasculosum and 3) the ventral set project towards the dorsal border of the CSM and its axons runs along with the SO neurons in the caudal region. Like neurons in the preoptic recess, some dorsal set of the PHN AVT-ir neurons contact the 3rd ventricular surface (Tennyson et al., 1985). However, a large number of the dorsal set and the ventral set of PHN magnocellular neurons course through the lateral group of neurons and join the neurohypophyseal tract that eventually end in the neurohypophysis.

Processes of the lateral group of AVT-ir neurons pass along the AVT-ir neurons in the SO, project towards the quantofrontal tract to the CSM and runs parallel to the optic tract and continues with the external division of the SO. However, the final destination of axons of these neurons has not been identified until now, leaving the functions of these neurons unidentified. While a majority of the PVN magnocellular neurons have processes that project through the lateral part of the hypothalamus and joins the neurohypophyseal tract, some of these neurons also project to the lumen of the 3rd ventricle. In addition to these classical finding, evidence in mammals also suggests that magnocellular neuron send axon collaterals within the brain (Mason et al., 1984; Hawthorn et al., 1985; Knobloch et al., 2012), particularly to the nucleus accumbens and central nucleus of the amygdala (Knobloch et al., 2012). Such distinct projections of magnocellular neurons are quite possible, however, have not been characterized within the avian brain.

AVT-ir fibers (terminals) are also present in the circumventricular organs (CVO) such as the organum vasculosum laminae terminalis, subfornical organ, subcommissural organ, median eminence and prosencephalic structures such as the medial and lateral geniculate nucleus, nucleus of the hippocampal commissure, ventral paleostriatum, subdivision of mammillary nucleus, mesencephalic structures such as the area ventralis of Tsai, substantia nigra, optic tectum, nucleus intercollicularis, ventral tegmental area, lateral reticular formation, pedunculopointine tegmental nucleus, periaqueductal gray, locus coeruleus and raphe nucleus in the brain stem region and nucleus of the trigeminal nerve (Panzica et al.,1988; Sugita, 1994).

A distinct set of brain regions are known to contain AVT terminals, however, the origin of these fibers is not yet clear in avian species. Nonetheless, rodent studies have provided a wealth of information such that mammalian data could be used to bridge the lack of information

in birds. Although, AVP/AVT magnocellular neurons contribute minor projections within the brain, AVP/AVT parvocellular neurons are known to provide extensive projections within the brain. The first set of parvocellular AVT-ir neurons was observed in the PVN whose axonal terminals were suggested to be present in the external zone of the median eminence, Fig. 3, (Goossens et al., 1977; Blasher, 1984), however, these finding are yet to be confirmed.

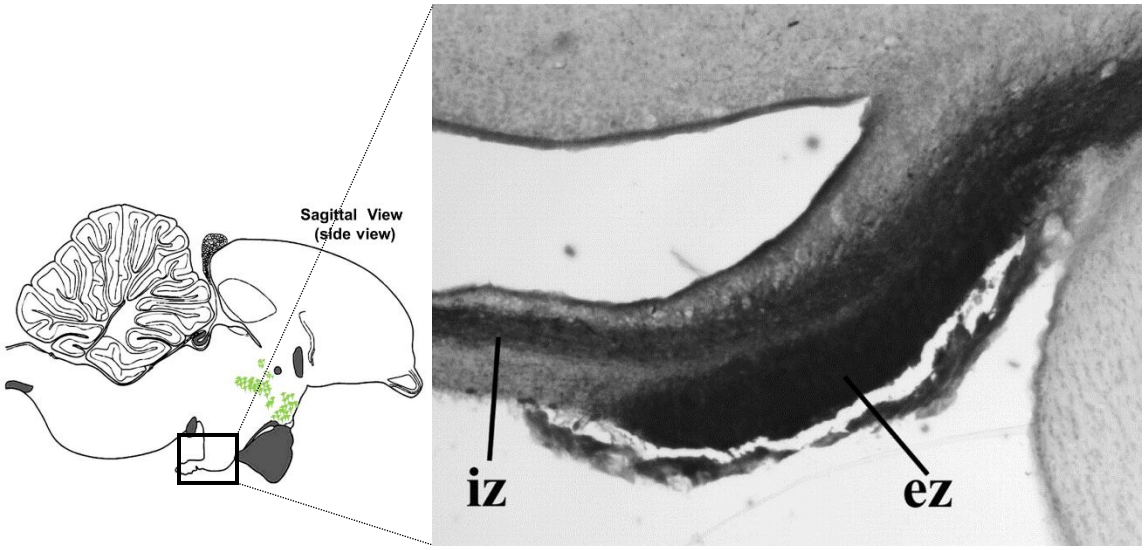

Fig. 3. External and internal zone of the median eminence, where axons of magnocellular neurons pass through the internal zone (iz) and parvocellular neurons terminate in the external zone (ez). (from Kuenzel and Jurkevich, 2010).

The PVN parvocellular neurons also project to midbrain and hindbrain regions including the parabrachial nucleus, lateral reticular nucleus, vagal complex, nucleus ambiguous (Buijs, 1978) and also to spinal cord regions such as the lateral funiculus, intermediolateral column, dorsal horn (Swanson, 1977; Swanson and McKellar, 1979). The second set of AVT-ir parvocellular neurons is present in the SCN of chickens (Panzica, 1985) and the SCN has several efferent connections within the brain, particularly to the hypothalamic, thalamic and tectal nuclei (Cantwell and Cassone, 2006a) and the medial and lateral SCN have reciprocal connections between them (Cantwell and Cassone, 2006 a and b). The highly studied third group of AVT-ir

parvocellular neurons is present in the medial preoptic nucleus (MPO) and BST (Panzica et al., 2001). Similar to AVP fibers in mammals (De Vries and Buijs, 1983), BST has projection to the lateral septum and MPO (Panzica et al., 2002; Absil et al., 2002; Jurkevich et al., 2003) which was confirmed by the tract tracing method (Montagnese et al., 2008). In addition, it was also identified that the BST AVP-ir neurons have processes to the lateral habenular nucleus, midbrain central gray, dorsal raphe nucleus, pontine peripeduncular nucleus and locus coeruleus in mammals (De Vries and Buijs, 1983; Caffe et al., 1987).

Thus, a majority of AVT-ir magnocellular neurons present in the SO, PVN, PHN, and PPoN projects to the neurohypophysis (posterior pituitary) via the internal zone of the median eminence and AVT-ir parvocellular neurons project extensively within the brain as well as the external zone of the median eminence.

1.3. Afferent connections to AVP/AVT neurons

For a particular set of AVP/AVT neurons to get activated and perform a specific function, these neurons must receive appropriate afferent (input) signals. Delineation of afferent connections requires careful retrograde tracing as well as combination of immunocytochemical procedure with particular neuropeptides/neurotransmitters of interest. Of the different types of AVP/AVT-ir neurons the most investigated are magnocellular neurons present in the SO and PVN, because their large perikarya size makes them easy targets for identifying afferent connections after tracer injections. On the other hand, only a few studies have shown projections towards parvocellular type neurons.

The neurosecretory neurons have been known to receive connections from two circumventricular organs namely, the SSO and OVLT (Miselis et al., 1979; Muller et al., 1994; Richard and Bourque, 1995; Gerstberger et al., 1987). Other forebrain regions that provide

afferents to the SO and PVN are the MPO, lateral septum, diagonal band of broca, basal nucleus of Meynert, entorhinal complex, ventral hippocampus. Additionally, magnocellular neurons are well known to receive information from the brain stem structures such as the nucleus of the solitary tract (NST), dorsal parabrachial nucleus, nucleus ambiguous (Tribollet et al., 1985; Mckinley et al., 1996) and intermediolateral column of spinal cord (Buijs, 1987). Neuropeptides and amines containing neurons in these brain regions modulate the activity of magnocellular neurons in response to a specific stimulus. For example, angiotensin II in the SSO have projections to the SO (Jhamandas et al, 1989; Gerstberger et al., 1987). Noradrenergic cell groups, specifically in ventrolateral medulla and NST, have afferent connections to AVP-ir neurons in the SO and the PVN (Sawchenko and Swanson, 1982; McKellar and Loewy, 1981; Sladek and Zimmerman, 1982; Day, 1989). Although only a few brain structures project to the core of the SO and PVN, a number of different brain structures, including structures in the limbic system, brain stem and the preoptic nucleus, projects to region surrounding (perinuclear region) the SO and PVN (Sawchenko and Swanson, 1982; Silverman and Oldfield, 1984; Oldfield et al., 1985; Tribollet et al., 1985; Oldfield and Silverman, 1985) and even contact dendrites of magnocellular neurons in axo-dendritic fashion (Silverman et al, 1985). With respect to midbrain regions, magnocellular neurons receive projections from serotoninergic neurons in the medial and dorsal raphe nucleus (Sawchenko et al., 1983); dopaminergic neurons in the substantia nigra, ventral tegmental area and anterior hypothalamic region (Cheung et al., 1998; van Vulpen et al., 1999); histaminergic input from tuberomammillary nucleus (Weiss et al., 1989). The PVN and SO neurons also receive connections from the anterior and dorsomedial hypothalamus and the BST, which provide glutamatergic and GABAergic (gamma aminobutyric

acid) afferents (Theodosis et al., 1986; Boudaba et al., 1996; Boudaba et al.,1997), likely involved in modulating/gating neuronal activity (Israel et al., 2010).

Parvocellular AVP/AVT-ir neurons receive afferents from a number of brain regions. AVP neurons in the mBST appear to receive projections from the olfactory system and to certain extent from the medial amygdalar nucleus (Weller and Smith, 1982, Dong et al., 2001). The lateral or visual SCN neurons are the recipients of tectal afferents. Both medial and lateral SCN receive afferents from several other brain structures including limbic, preoptic, hypothalamic, thalamic and midbrain regions (Cantwell and Cassone, 2006b). Interestingly, the SCN glutamatergic and GABAergic neurons were shown to project to the SO in mammals (Cui et al., 1997b). Thus, different brain structures modulate the activity of AVP/AVT neurons found in distinct brain structures.

1.4. Vasotocin receptors

Knowledge on functional characteristics of receptors in birds have gained particular attention in the past decades because of their sequence availability. Since, neurohormones (ligands) bind to their specific receptors and initiate a response, it is important to understand the receptor mechanism as well as the expression of hormones or ligands. Receptors of AVP/AVT are distributed throughout the body, performing vital functions. Different functions of vasopressin receptors have been identified including those in the visceral system and central nervous system to facilitate physiological as well as behavioral functions (De Weid et al., 1984; Koshimizu et al., 2012). The vasotocin receptors belong to the G-protein coupled receptors family and are present in the membrane of a variety of cells including the cardiovascular system, kidney, brain, pituitary and platelets. Over the course of the past decade, four different types of vasotocin receptors has been identified in birds, namely V1a, V1b, V2 and V3. (Note: the

nomenclature of each receptor has been changed since the receptors were named with respect to the time and order in which they were discovered. V2 = V1, V1b = V2, V1a = V4, V4 = V3/MTR/OXYR). Since, they belong to the G-protien 7 transmembrane receptor family, these receptors are homologous to the 4 types of mammalian receptors and appear to be phylogenetically conserved structurally and functionally throughout evolution (Ocampo Daza et al., 2012; Yamaguchi et al., 2012). Although, the AVP/AVT receptors are conserved, the second messenger system within the cells varies to a smaller extent depending upon the receptor subtype. In a variety of cells, the V1a, V1b and V3 receptors were found to have signal transduction pathway associated with phosphatidylinositol breakdown leading to calcium signaling (Woods et al., 1986; Hatton et al., 1992; Dayanithi et al., 1996; Cornett et al., 2003), while V2 receptors involved activating adenylate cyclase by which cyclic adenosine monophosphate (cAMP) acts as the second messenger.

The first type of vasotocin receptor identified in birds was the V2 receptor and was identified in the egg shell gland and brain of chickens (Tan et al., 2000). Although, V2 receptors have been identified in the kidney of mammals (Bankir, 2001), and associated with the regulation of ionic balance, its function in the avian kidney is unknown. The second receptor type identified was the V1b receptor and was found in the anterior pituitary of chickens (Cornett et al., 2003; Jurkevich et al., 2005), particularly the corticotropes of the anterior pituitary gland. The V1a receptors, on the other hand, have been found in both the brain and pituitary of chickens (Selvam et al., 2013 and 2015) as well as in the brain of song birds (Leung et al., 2011). The last of the four different types, the V3 receptors homologous to the oxytocin receptors in mammals, has been identified in shell glands of birds (Gubrij et al., 2005). Unlike, V1a and V2 receptor subtypes, V1b receptors (Jurkevich et al., 2005) and V3 receptors have not been identified in the

chick brain. Although V3 receptors have not been studied in the chicken brain, evidence using in situ hybridization show that V3 receptors are expressed in several brain regions of white-throated sparrow (*Zonotrichia albicollis*) and zebra finch (*Taeniopygia guttata*) (Leung et al., 2011).

Within the brain, subtypes of vasotocin receptors were found in pallial, subpallial, hypothalamic, thalamic and brainstem regions (Leung et al., 2011; Selvam et al., 2015), however, only some brain structures show localization of vasotocin receptors matching with vasotocin fibers (Voorhuis et al.,1988; Leung et al., 2009). The phenomenon associated with this anatomical mismatch is discussed in section 1.5.4.

1.5. Anatomical to functional implications of AVP/AVT neurons

Specific brain structures respond to a particular stimulus in a multimodal fashion, such that integration of different afferents determine the activity of a particular set of a neurohormone containing neurons. The afferent signals could contain several types of endogenous factors. Vasopressin, and probably AVT, neuronal activities are modulated by neurotransmitters and neuromodulators including acetylcholine, dopamine, noradrenaline, histamine, serotonin, GABA, glutamate, opioids, cholecystokinin, prolactin, kisspeptin, angiotensin II, neuropeptide Y, α-melanocyte stimulating hormone, somatostatin, galanin, atrial natriuretic peptide, inhibin, substance P (Renaud and Bourquet, 1991; Brown et al., 2013). Moreover, AVP neurons were also shown to be regulated by AVP released from their dendrites (Ludwig, 1998; Ludwig and Leng, 2006) and likely act via their own receptors (Hurbin et al., 1998; Hirasawa et al., 2003). Thus, a multitude of neurohormones and neurotransmitters modulate activity of AVP/AVT neurons through paracrine and autocrine regulatory mechanism.

1.5.1. Osmoregulation

Classically, AVP/AVT is released in the systemic circulation in response to changes in plasma osmolality. Changes in osmolality is detected by osmosensitive neurons present in the circumventricular organs within the brain, the SFO and OVLT and activate magnocellular neurons via angiotensin II (Jewell and Verney, 1957; McKinley et al., 1996; Richard and Bourque, 1995; Gerstberger et al., 1987; Muller et al., 1994; Anderson et al., 2000; Ciura et al., 2006). Activation of CVO's is particularly noted in hypertonic saline stressors that stimulate magnocellular neurons (Oldfield et al., 1994; Shi et al., 2008). Activation of magnocellular neurons in the SO and PVN (Muhlbauer et al., 1992; Jaccoby et al., 1997) leads to the release of AVT in the blood (Nouwen et al., 1984; Arad et al., 1986), to facilitate retention of water in the kidney by water reabsorption (Skadhauge and Schmidt-Nielsen, 1967).

1.5.2. Baroregulation

Both high and low blood pressure modulate activity of magnocellular AVP neurons. Such baroreflex responses are mediated by baroreceptors present in the carotid sinus and aortic nerve, providing two types of information to the brain. In hypotensive condition, information from baroreceptors are relayed through the brainstem structures including the locus coeruleus, periaqueductal grey, ventrolateral medulla, parabrachial nucleus to activate AVP neurons in the SO and PVN, probably via catecholaminergic neurons (Sladek and Zimmerman, 1982; Bank and Harris, 1984; Day and Renaud, 1984; Raby and Renauld, 1989; Dampney and Horiuchi, 2003). Thereby secretion of AVP/AVT into the blood facilitates its classical pressor function. On the other hand, in hypertensive condition, information from baroreceptors are relayed through the brainstem structures (the nucleus of solitary tract, area posterma, ventrolateral medulla, parabrachial nucleus) to GABAergic neurons in the diagonal band of broca thereby inhibiting

AVP/AVT neurosecretory neurons (Blessing and Reis, 1982; Dampney and Horiuchi, 2003) and leading to the cessation of the activity of AVP/AVT magnocellular neurons for high blood pressure. Hence, AVP/AVT neuronal activity is modulated by both excitatory and inhibitory neural circuits to regulate cardiovascular homeostasis.

Another factor that can influence baroreceptors is loss of blood (hemorrhage). Hemorrhage is also known to trigger the release of vasopressin (Ginsburg and Brown, 1956) and vasotocin in birds (Bottje et al., 1989; Jaccoby et al., 1997), where the latter species requires a large drop in blood volume to activate release of AVT and work in conjunction with the renin-angiotensin system (Thrasher, 1994).

1.5.3. Other visceral sensory regulation

Similar to osmoregulation and baroregulation (blood pressure), other peripheral sensory stimuli are also known to regulate the activity of AVP/AVT neurons via brain stem structures described above. Similar to mammals where maternal activities including parturition, lactation and feeding pups involves activation of brain stem regions and magnocellular neurons (Bealer and Crowley, 1998; Meddle et al., 2000; Lipschitz et al., 2004), oviposition in hens leads to release of AVT in the blood (Arad and Skadhauge 1983; Shimada et al., 1987) for the contraction of endometrial lining, myometrium (Sawyer, 1977; Koike et al., 1988), probably mediated through V2 and V4 receptors (Tan et al., 2000; Gubrij et al., 2005).

The nucleus of the solitary tract serves as the mediator of the signals from viscera including those involved with feeding. Visceral signals via cholecystokinin are transmitted through the nucleus of the solitary tract adrenergic cells and to the magnocellular neurons (Renaud et al., 1983; Verbalis et al., 1986; Onaka et al., 1995), however, centrally orexigenic signals from NPY neurons also project to the PVN magnocellular neurons (Sawchenko et al.,

1985; Jhanwar-Uniyal et al., 1993; Baker and Herkenham et al., 1995) and leading to release of AVP (Willoughby and Blessing, 1987; Leibowitz et al., 1988). Thus, both central and peripheral signals tend to regulate the activity of magnocellular neurons in the SO and PVN.

Hypothalamic structures including the preoptic nucleus integrate thermosensory information from the periphery via the spinal cord and brain stem regions (Van Tienhoven et al., 1979; Boulant, 2000). AVP/AVT is known to play a role in thermoregulation such that intrahypothalamic and peripheral presence of the hormone is beneficial in hypothermic (Hassinen et al., 1994) as well as hyperthermic conditions (Wang et al., 1989) in birds, similar to the effects of AVP observed in mammals (Cooper et al., 1970). Some evidence suggests that magnocellular neurons that project into the ventricle could also have an effect in physiological functions of animals (see Buijs, 1987). Such release into ventricles could bind to the receptors (V1a) present in glial cells lining the circumventricular organs (Selvam et al., 2015), possibly associated with osmotic homeostasis, thirst and even cardiovascular functions. Hence, both the PVN and SON play a diverse physiological roles in birds and mammals.

Not only neurotransmitters/neuromodulators containing neurons regulate the activity and release of AVP/AVT neurons but also glial cells facilitate magnocellular neuronal activity. Pituicytes are special types of glial cells in the neurohypophysis that control the release of AVP from neuron terminals into the blood circulation (Hatton, 1988). Electron microscopic studies have shown that physiological stimuli such as dehydration-osmotic imbalance-lactation-parturition in rodents lead to retraction of pituicytes from magnocellular terminal fields thereby mediating the release of the neurohormone into the circulation. On the other hand, removal of stimuli lead to encapsulation of pituicytes back onto the terminal fields of magnocellular neurons resulting in the arrest of neurohormone release. Likewise, glial cells ensheathing magnocellular

neurons in the supraoptic nucleus (Montagnese et al., 1988, Oliet et al., 2008) and GnRH nerve terminals (Ojeda et al., 2008) undergo similar mechanistic changes. Plasticity of pituicytes have been extensively studied and is probably mediated through cytoskeletal proteins and cell adhesion molecules in the pituicytes (Miyata et al., 2001). These structural changes within pituicytes and intercellular structural proteins are accompanied by changes in calcium binding proteins and calcium associated proteins in the magnocellular axonal terminals for hormone release. Thus, integrated activity of afferent connections and glial cells regulate neurosecretion of the nonapeptide into the blood to maintain physiological homeostasis.

1.5.4. Neuromodulation

Based upon anatomical information it has been generally emphasized that the AVP/AVT not only controls physiological homeostasis through the release from the neurohypophysis, but also modulates behavioral functions by acting as a neuromodulator within the brain. The neuromodulator characteristics of AVP is supported by the presence of AVP terminals found in several brain regions. For example, AVP neurons in the SCN have dense projections to the PVN, (Buijs et al., 1998) and are probably involved in a circadian pattern of release of hormones (Kalsbeek, et al., 1996). Although, such projections and facilitation of SCN AVT neurons are yet to be found in the birds (Cantell and Cassone. 2006), diurnal release of hormones has been demonstrated in birds (Kovacs et al., 1983; Lauber et al., 1987).

Furthermore, the nonapeptide is not only released from magnocellular neurons axon terminals in the neurohypophysis but is also found to be released from magnocellular neurons within the brain (Ludwig and Leng, 2006). This concept is further emphasized by 1) the presence of some magnocellular axons collaterals in the lateral hypothalamus (Mason et al., 1984) and central nucleus of amygdala (Knobloch et al., 2012), which influence both physiology

and behavior (Epstein et al., 1990; Huber et al., 2005). 2) Nonapeptides are not only synthesized in the perikarya and transported to the terminals, but also evidence indicate sources of hormone synthesizing machinery occur in axon terminals. This concept is evident from the presence of AVP mRNA in AVP axons in the neurohypophysis (Trembleau et al., 1995; Jung et al., 2012) and presence of neurosecretory granules in both axons and dendrites (Silverman et al., 1983). 3) Somatodentritic release of peptides has been demonstrated (Pow and Morris, 1989; de Kock et al., 2003). 4) Autoregulation of magnocellular neurons occurs in the SO and PVN (Ludwig and Leng, 1997; Hirasawa et al., 2003). Furthermore, AVP/AVT neurons from a few brain structures have projections within the entire brain (section 1.2; Buijs, 1987; Viglietti-Panzica, 1986; Panzica et al., 1988). Hence, release of AVP/AVT within the brain seems to have a specific function or functions based upon the sites of release.

Importantly, localizations of the V1a receptors (Leung et al., 2011; Selvam et al., 2015) does not appear to completely match the sites of AVT-ir terminals or fibers within the avian brain (Voorhuis et al., 1988; 1991; Sugita, 1994; Leung et al., 2009). Such anatomical mismatch has been well documented in mammals as well. This led to the hypothesis of volume transmission (Agnati et al., 1995; van den Pol, 2012), suggesting that long distance diffusion of this abundant nonapeptide is possible within the brain (Ludwig and Leng, 2006). Moreover, the rate of central degradation of the hormone is slower than that of the peripheral release and hence the effect of the hormone in the brain is much longer (Burbach et al., 1983; 1984). Thus, different stimuli could have both spatial and temporal effects in distinct brain regions (Buijs 1987; Ludwig and Leng., 2006) modulating behavior of an organism at different levels.

1.5.4.1. Regulation of reproductive behavior

Unlike neurosecretory magnocellular neurons in the hypothalamic structures, parvocellular AVT neurons in the BST and medial preoptic nucleus exhibit dimorphism between males and females (Van Leeuwen et al., 1985; Jurkevich et al., 1996; Panzica et al., 2002). Expression of these neurons are under tight regulation of gonadal steroid hormones (testosterone) in both mammals and birds (Aste et al., 1997; De Vries and Panzica 2006), providing a unique characteristic feature with respect to reproductive behavior. Thus, AVT neurons in the BST and MPO (Panzica et al., 2002; Absil et al., 2002; Jurkevich and Grossmann, 2003), could play a role in copulation in birds (Wild and Balthazart, 2013). Similar to neurons in the BST, AVP/AVT-ir terminals in the lateral septum also exhibit sexual dimorphism with males having higher AVT-ir terminals than females (De Vries et al., 1983; Panzica et al., 2002) and is completely eliminated in castrated animals (De Vries and Panzica, 2006). Concomitantly, such steroid dependent change in the AVT terminals correlates with changes in V1a receptors expression in the lateral septum (Grozhik et al., 2014) and are associated with aggressive behavior in birds (Goodson et al., 1998).

Much of the physiological and behavioral implications of AVP/AVT is presumed to be associated with groups of neurons present in specific nuclei. Nonetheless, the distinction between central and peripheral functions of the nonapeptide is clear and separate. However, functions of AVT neurons in some brain regions remain unknown. More importantly, the avian PVN contains a mix of both parvocellular and magnocellular AVT-ir neurosecretory neurons that receive afferents from several brain regions (Korf, 1984). Although functions of AVT-ir magnocellular neurons have been elucidated in the past, the role of the avian PVN AVT parvocellular neurons in the stress pathway (hypothalamo-pituitary-adrenal axis) has not been

elucidated and it has been described in the next section in comparison to the mammalian stress pathway.

2. Hypothalamo-pituitary-adrenal (HPA) axis: AVP/AVT and CRH

Stress is perceived by a complex set of neuronal circuits (ranging from psychogenic neural circuits in forebrain to visceral sensory neural circuits in brainstem) resulting in the activation of stress hormone (Pacak and Palkovits, 2001; Brunton and Russell, 2008). The cascade involves activation of two arms of the stress pathway: sympathoadrenomedullary axis and hypothalamo-pituitary-adrenal (HPA) axis. The neuroendocrine regulation of AVP/AVT involves the latter axis and this review addresses the HPA axis. Following stress, unidirectional activation of specific cell groups, in the hypothalamus, anterior pituitary and adrenal glands results in release of the stress hormone, corticosterone (CORT) in rodents, birds, and reptiles. This cascade of events is called the HPA axis in vertebrates. Thus, physiological or environmental threats to an organism result in the perception of the signals via limbic forebrain and brainstem structures in the brain leading to the activation of the HPA axis.

Sensitivity of the HPA activity depends on the type, degree or intensity of stressors (Pacak and Palkovits, 2001) and predetermined by exposure to CORT (Buckingam, 2006). Initial studies on the activation of the HPA axis via the release of ACTH from the pituitary cells were associated with hypothalamic factors (Harris, 1952). It was discovered that in both *in vivo* and *in vitro* systems AVP/AVT facilitate the release of ACTH from the anterior pituitary cells. However, the efficacy of AVP on the release of ACTH was found to be lower than the other hypothalamic or pituitary stalk extracts (Gillies et al., 1978; 1982), and doses that larger than physiological levels such as pharmacological doses of AVP were required for the release of

ACTH. The discovery of a 41 amino acid peptide hormone in the early 1980's (Vale et al., 1981) confirmed that the increased efficacy of the hypothalamic extract in the release of ACTH was indeed provided by corticotropin releasing hormone (CRH). Eventually, it was found that both CRH and AVP/AVT work in a synergistic fashion that results in augmented release of ACTH (Gillies et al., 1978; Castro et al., 1986). This synergistic action of AVP/AVT and CRH on the release of ACTH likewise concurs with immunohistochemical evidence showing AVP and CRH colocalization within parvocellular neurons in the PVN (Sawchenko et al., 1984) as well as in axon terminals present in the external zone of the median eminence (Antoni, 1993) and has been reported in birds (Kuenzel and Jurkevich, 2010).

In mammals, AVP and CRH parvocellular neurons target the median eminence where both CRH and AVP are released and transported to the anterior pituitary via the portal vasculature to trigger the release of ACTH from the anterior pituitary (Gillies et al., 1982; Antoni, 1993). Both CRH and AVP neurons are present in a homogeneous population in the medial parvocellular division of PVN (Sawchenko et al., 1992) and about 50% of the cells contains both CRH and AVP within the perikarya (Whitnall, 1993). These neurons are controlled by distinct cell groups in the limbic system, brainstem and other perinuclear hypothalamic regions (Sawchenko and Swanson, 1982; 1983; Cunningham and Sawchenko, 1988; Sawchenko et al., 1996). Instead of homogeneous population of neurons, however, the avian PVN contains a heterogeneous population of both parvocellular and magnocellular neurons. Much like the mammalian PVN the avian counterpart receives projections from a variety of brain structures (Korf, 1984) including limbic system and brainstem (Berk 1987), described previously. While a wealth of information is available regarding the regulation of

AVT magnocellular neurons following physical stressors, there is a dearth of information in the precise localization and regulation of CRH and AVT parvocellular neurons in the avian PVN.

In the anterior pituitary, both CRH and AVP/AVT act on a specific pituitary cell type called corticotropes. Binding of CRH and AVT to their respective receptors (CRH – CRHR1/CRHR2 and AVT – V1a/V1b) on corticotropes stimulates the synthesis and release of ACTH into the systemic circulation. Interestingly, the synergistic release of both CRH/AVT coincides with receptors activation. Upon binding of CRH and AVP/AVT to their cognate receptors, dimerization of CRHR1 and V1b receptors occur on corticotropes of mammals (Young et al., 2007) and birds (Mikhailova et al., 2007). Activation of these receptors lead to the synthesis of ACTH from its precursor, proopiomelanocortin, via a cAMP dependent pathway. The end product of the HPA axis, CORT, is then released from the adrenal glands when ACTH acts on the zona fasciculata (mammals) or adrenocortical cell (birds) (Carsia, 2015). Thus, AVP/AVT system was also found to be associated with activation of the stress pathway in conjunction with CRH.

Hence, the vasotocinergic system in the hypothalamus of the vertebrate brain is involved in physiological homeostasis via the hypothalamic-neurohypophyseal system (HNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Thus, it is necessary to identify functional characteristics of neurons associated with the neuroendocrine stress mechanism. Such characterization would be beneficial to understand the role AVT in poultry stress research. Since expression of vasotocin neurons at the time of hatching is similar to that of mature chickens (Arnold-Aldea and Sterritt, 1996; Tennyson et al., 1986), it is speculated that AVT is involved the neuroendocrine regulation of stress from hatch until senescence.

2.1. Plasticity of neurons in response to stress

In mammals, parvocellular neurons have the ability to synthesize different types of neuropeptides (Sawchenko et al., 1983). Different neurohormones are expressed in particular sets of neurons where their expressions differ temporally following stressors (Bartanusz et al, 1993; Ma et al., 1997). For example, a more potent stressor (ether inhalation stress) triggers expression of hnCRH primary transcripts (hn-heteronuclear) as early as 5 min, whereas expression of hnAVP primary transcript occurs at 1 h(Kovacs and Sawchenko, 1996; Ma et al., 1997). Likewise, temporal expression of CRH and AVP mRNA following stressors were also noted in a number of studies (Lightman and Young, 1988; Bartanusz et al., 1993). This difference in the expression pattern of CRH and AVT suggests that different regulatory mechanisms are involved in the same neuronal type. Several regulatory factors such as CREB (cAMP response element binding protein) and immediate early genes such as c-fos, NGFI-B (nerve growth factor inducing factor- B) (Chan et al., 1993; Kovacs, 1998) have binding sites in the promoter regions of CRH and AVP (Chan et al., 1993) and have been associated with the differences in their expression patterns.

Further, it was proposed in the 1990's that AVP has a distinct connection to a phenomenon in stress called 'stress memories' and has the ability to be hyper responsive to further homotypic stressors (stressors of same type) (Aguilera, 1994), with the ability to be co-released with CRH in response to the subsequent homotypic stressor (de Goeij et al., 1992). Since the avian PVN contains a mixed population of magnocellular and parvocellular neurons in the medial regions of the PVN, effects of distinct types of stressor are largely unknown, except for magnocellular neurons descried previously (section 1.5.1). Furthermore, whether both CRH

and AVT neurons in the avian PVN respond to stressors similar to the mammalian PVN remains unknown.

2.2. Effects of glucocorticoids on AVP/AVT neurons

Steroid hormones have a profound effect on the expression of AVP/AVT neurons in regulating physiology and behavior. One of the effects was described in section 1.5.4.1, regarding effects of testosterone in the expression of AVP/AVT neurons in the BST. Likewise, glucocorticoids (CORT), the principle output of the HPA axis, modulate the expression of AVP containing neurons in the mammalian PVN via glucocorticoid receptors. In the HPA axis, increased CORT concentration have inhibitory effects at the level of pituitary (ACTH) and PVN (CRH and AVP). Such manifestations are critical to maintain normal physiological limits of stress hormone, which would otherwise be detrimental to the animal. Glucocorticoids affect expression of CRH and AVP through a negative feedback mechanism (Kovacs et al., 1986; Swanson and Simmonds, 1989). Consistent with this phenomenon, absence of CORT, such as in adrenalectomy, increases expression of CRH and AVP in the PVN (Sawchenko, 1987; Shibata et al., 2007). Furthermore, a forward shift and elevation in the expression of AVP hnRNA is also observed in the absence of CORT (ADX) (Herman and Cullinan, 1997). Thus, CRH and AVP play a role in the stress axis and are involved in feedback glucocorticoid inhibition. Although much is not known about the central effect of CORT in the regulation of CRH and AVT in the avian PVN, the feedback effect of CORT has been shown in the pituitary of birds as early as embryonic stage E11 by exogenous application of the CORT (Jenkins and Porter, 2005). Thus, the functional role of CORT in birds appears to be similar to mammals, however, such a possibility has not been tested extensively in the avian hypothalamus.

3. Metabolic control of AVP/AVT neurons

Several avian brain regions are involved in feeding control (Kuenzel et al., 1994; 1999) and a few have most of the orexigenic and anorexigenic peptidergic/neurotransmitter containing neurons (Kuenzel 1994; Boswell, 2005; Richard and Proszkowiec, 2007; Tachibana and Tsutsui, 2016) in order to maintain energy homeostasis. Since food intake neural circuits are complex and involves more than 30 different neurohormones in the regulation of food intake (Tachibana and Tsutsui, 2016), the role of AVP/AVT neurons constitutes part of a complex neural network in energy metabolism. In mammals and birds AVP/AVT acts as an anorexic agent (Meyer et al., 1989, Tachibana et al., 2004), however, little evidence is available regarding direct central role of AVP/AVT neurons in food intake (Pei et al., 2014). Although, the nonapeptide's main function is to maintain water balance, there appears to be three distinct mechanisms through which AVP/AVT is associated with food intake regulation.

First, a suppressive effect on food intake was evident from both peripheral (Langhans et al., 1991) and central administration of AVP (Ikemura et al., 2004) and AVT (Tachibana et al., 2004). Ikemura and colleagues showed that intraperitoneal administration of AVP in rats suppressed both food intake and water intake, however, when injected intracerebroventricularly it only decreased food intake and not water intake. Thus, AVP/AVT acts within the brain to regulate food intake in mammals and birds. Evidence shows that one of the receptors of AVP/AVT is involved in the modulation of food intake. In V1a-/- mutant mice, neuropeptide Y augmented food intake in V1a-/- mice when compared to wild type mice (Aoyagi et al., 2007). Furthermore, administration of an antagonist to V1a showed an increase in food intake (Mechaly et al., 1999). A similar attempt in chickens also showed that the avian V1a receptor is involved

in food intake regulation (Kuenzel et al., 2016). Thus, in both mammalian and avian brains, AVP/AVT appear to have a role in the regulation of food intake via V1a receptors.

Second, with respect to changes in energy levels, some recent studies showed that magnocellular neurons in the PVN and SON were able to detect glucose levels (Mitra et al., 2010; Song et al., 2014). Consistent with this finding, following a restricted feeding regime, a significant increase in FOS was observed in magnocellular neurons of fed animals (Johnstone et al., 2006; Poulin and Timofeeva, 2008) compared to non-fed animals. Further evidence shows that hypothalamic magnocellular neuronal activation occurs at 30 min, peaked around 60 min and the activity was reduced at around 3 h after food was presented (Johnstone et al., 2006). The same results were observed in magnocellular neurons of the hypothalamus, when glucose levels were artificially increased (Nagai et al., 1995; Briski and Brandt, 2000) or when food intake was artificially induced by central injection of neuropeptide Y (Li et al., 1994). Thus, activation of AVP magnocellular neurons immediately after a fed state suggest these neurons respond to nutritional status, either associated with mobilization of glucose (Nagai et al., 1995; Briski and Brandt, 2000) or an anticipatory event (Mandelblat-Cerf et al., 2017). At least one phenomenon appears to exist in birds. One of the functions of AVT was its association with energy mobilization (John and George, 1973), since an increase in AVT levels was noticed in pigeons after flight that was neither associated with a change in osmolality (George et al., 1992) nor stress (Viswanathan et al., 1987). However, whether activity of AVT magnocellular neurons in energy metabolism is just a correlation or causation remains unknown.

Finally, the SCN was found to participate in food entrainment (Angeles-Castellanos et al., 2011) and the SCN contains AVP/AVT neurons. In mammals, neuronal connectivity between the arcuate nucleus and ventromedial SCN (Yi et al., 2008) and between the

intergeniculate nucleus and SCN (Saderi et al., 2013) are known to have metabolic controls. Further, SCN lesions could result in blocking vagal nerve activity in controlling glucose and insulin rhythms (Strubbe et al., 1987). Thus, SCN containing AVP neurons not only control circadian rhythms but also appear to entrain food intake in mammals, however such possibilities remain to be tested in birds.

Overall, answers to whether AVP/AVT neurons have a direct inhibitory role on food intake or activated as a consequence to food consumption or an anticipatory event has begun to emerge using modern techniques (Watts, 2017).

4. Goals of this dissertation research

Understanding neuroanatomical structures and their functions in *Gallus gallus* (domestic chickens) have a great importance in poultry. An extensive collection of literature is available on physiological, reproductive and social behavior function of AVT in birds, yet little is known about the neuroanatomical and functional role of AVT in stress and food intake regulation. The goals of this research are to understand the role of AVT neurons in specific brain structures associated with stress and food intake using basic neuroscience and molecular techniques. The dissertation research is focused on these two aspects in poultry for the following reasons.

1) Stress is an important aspect in the life of birds and helps prepare them from underlying threats in their surrounding environment. Nonetheless, stress could be detrimental if an underlying threat persists and could not be compensated by homeostasis mechanisms (Virden and Kidd, 2009). Depending on the intensity and duration of stressors, adverse effects of stress could affect both the physiology and behavior of the birds at different levels ranging from adrenal enlargement to anxiety/fear and beyond. Hence, stress is

considered a major welfare concern in farm animals (Forbes 2007). Basic understanding of the neuroanatomy and neurohormones involved in stress pathways is thus critical in evaluating welfare conditions of poultry.

2) The current direction of the poultry industry to improve meat production lead to the identification of factors involved in energy metabolism, food intake and feed efficiency. Several factors are involved in the regulation of food intake and energy metabolism. Vasotocin is one of the factors associated with food intake, however, little is known about the functional regulation of AVT neurons in poultry. Complexity in the neural network involved in food intake and energy metabolism drives the need for better understanding of the neuroanatomy and neurohormones associated with feed consumption in poultry.

Thus, the dissertation research aims to provide the central role of vasotocin neurons (both parvocellular and magnocellular) in the regulation of stress and food intake in chicken and a possible central role of vasotocin receptors in control of food intake. The research also aims to provide a clear map of avian brain structures associated with stress and food intake, which have not been defined to date. The data from these research studies will help further improve the basic understanding of the stress mechanism and food intake in chickens.

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Chapter 2

Identification of AVT neurons activated by acute and chronic restraint stress.

Excerpts from "Nagarajan, G., Tessaro, B.A., Kang, S.W., Kuenzel, W.J., 2014. Identification of arginine vasotocin (AVT) neurons activated by acute and chronic restraint stress in the avian septum and anterior diencephalon. Gen. Comp. Endocrinol. 202, 59-68."

Abstract

Effects of acute and chronic psychogenic stress in the brain of domestic avian species have not been extensively studied. In this study, acute and chronic restraint stress were applied to chickens in order to identify activated arginine vasotocin (AVT) neurons (parvocellular or magnocellular), in the paraventricular nucleus (PVN), supraoptic nucleus (SO), lateral groups of neurons (L), periventricular nucleus of the preoptic region and hypothalamus, using immunocytochemistry. The core subnucleus of the PVN (PVNc) was known to contain distinct groups of AVT-ir neurons, parvocellular (small), medium sized and magnocellular (large). Using FOS, a neuronal activation marker, activated AVT neurons were identified following restraint stress. Of all the structures containing AVT-ir neurons, only AVT-ir parvocellular neurons in the PVNc were found activated in both acute and chronic stress. In addition to AVT, an attempt was made to visualize corticotropin releasing hormone (CRH) -ir neurons using colchicine. Although AVT-ir parvocellular neurons coexist with CRH-ir neurons in the PVNc, only few neurons were shown co-localized with AVT and CRH in the PVN after acute restraint stress. Interestingly, the nucleus of the hippocampal commissure (NHpC) was shown to contain a dense population of CRH-ir neurons and the structure displayed significant increase and decrease in the number of FOS-ir cells following acute and repeated restraint stress, respectively.

Results of this study indicate that AVT-ir parvocellular neurons in the avian PVNc and most likely CRH-ir neurons in the NHpC are associated with psychogenic stress.
Key Words: corticosterone, hypothalamus, paraventricular nucleus, parvocellular neurons, co-localization.

1. Introduction

Two major neurohormones involved in the stress response of birds are corticotropin releasing hormone (CRH) and arginine vasotocin (AVT), the latter, a homolog of mammalian arginine vasopressin (AVP). Both CRH and AVT comprise the neuroendocrine components of the classical hypothalamic-pituitary-adrenal (HPA) axis. In rodents, neurons containing CRH and AVP (Burlet et al., 1974; Bloom et al., 1982; Cummings et al., 1983; Swanson et al., 1983; Sawchenko et al., 1984) have been extensively studied and their anatomical regions in the brain have been well characterized following different types of stressors such as immobilization, water deprivation and social interaction (Swanson et al., 1986; de Goeij et al., 1992, Aguilera and Kiss, 1993; Bartanusz et al., 1993; Ebner et al., 1999; Ma et al., 1999; Ho et al., 2010). The distribution of AVT immunoreactive (-ir) neurons in the avian hypothalamus has also been well documented (pigeon - Berk et al., 1982; chicken - Tennyson et al., 1985, Jurkevich et al., 1997; quail - Viglietti Panzica 1986, Panzica et al., 1988; canary - Kiss et al., 1987; zebra finch - Voorhuis and De Kloet, 1992; junco - Panzica et al., 1999; budgerigar - Fabris et al., 2004) and has provided possible anatomical brain regions involved in physical and social stress in birds (Muhlbauer et al., 1992; Jurkevich et al., 1996; Jaccoby et al., 1997; Xie et al., 2010; Goodson et al., 2012).

Past studies have shown that AVT neurons present in the supraoptic nucleus (SO) and the paraventricular nucleus (PVN) showed a significant increase in the number of AVT neurons (Chaturvedi et al, 1994) and up regulation of AVT gene expression (Jaccoby et al., 1997) following dehydration or hyperosmotic stimuli. In birds, AVT is also known to influence social behavior. Peripheral administration of an analog to AVT in canary (song bird) affected seasonal singing behavior (de Kloet et al., 1993) and central administration of AVT in zebra finches affected aggression (Goodson et al., 2004). Arginine vasotocin neurons in the medial bed nucleus of the stria terminalis (BSTM) were shown to be involved in appetitive sexual behavior (Xie et al., 2011) and silencing of AVT neurons, using a knockdown procedure, in the BSTM resulted in a lack of preference for large social group sizes in gregarious birds (for review see Goodson et al., 2012). Thus, AVT-ir parvocellular neurons in the BSTM were associated with social stress (Panzica et al., 2002; Xie et al., 2011; Goodson et al., 2012).

Past studies cited above show by immunocytochemical and in situ hybridization results that AVT-ir magnocellular neurons in the SO and PVN respond to physiological challenges and AVT-ir parvocellular neurons in the BSTM are activated by social challenges in chickens, however it is not clear whether that separation of function, based upon the sizes of neurons (magnocellular or parvocellular) holds following psychogenic stress. Thus, dual immunocytochemistry was utilized to identify activated AVT-ir neurons (using FOS, a neuronal activation marker) and to assess the structural relationship between AVT-ir and CRH-ir neurons in the PVN and adjacent regions.

2. Materials and methods

2.1. Facilities and animals

Day old male broiler chicks were obtained from a commercial chicken hatchery. Chicks were raised in battery brooder cages for their first two weeks beginning at 32 °C and dropping the temperature 3 °C per week until 23 °C where it was maintained until the end of experiments. At two weeks of age birds were randomly distributed to cages (two per cage). Birds were fed a standard broiler diet ad libitum and maintained with a photoperiod of 16 h of light and 8 h of darkness each day. All procedures and experimental protocols for use in chickens were approved by the University of Arkansas Institutional Animal Care and Use Committee (#16043).

2.2. Experimental groups and sampling procedure

Birds were randomly selected and distributed to the following four experimental groups at 5 weeks of age: acute control, acute stress, chronic control and chronic stress (n=5/group). Acute stress birds were subjected to one h restraint stress for one day. Chronic stress birds experienced one h restraint stress for ten consecutive days. Birds were taken out of their cages, placed in a harness secured with Velcro straps for one hr. Birds could not stand nor flap their wings and had access to water during each stress period. Birds in the acute and chronic control groups were taken out of their cages carried to the corridor of the room and put back to their home cage. The experiment was designed so that all birds in the four treatment groups were at the same age when sampled. At the end of each treatment a blood sample was taken from each bird. Birds were then anesthetized with sodium pentobarbital solution (27mg/kg, *i.v.*) and perfused via the carotid arteries with 150 ml of heparinized phosphate buffer (0.1 M PB with 0.05% sodium nitrite) followed by 200 ml of Zamboni's fixative (0.1 M PB containing 4% paraformaldehyde and 15% saturated picric acid, pH 7.4). The brain sample of each bird was

post fixed with the same fixative overnight at 4 °C. They were then blocked in a stereotaxic instrument (Kopf, Tujunga, CA) and cryoprotected in 30 % sucrose (in 0.1M PB) until each brain sank to the bottom of the vial. Thereafter, brain samples were stored at -80 °C until they were sectioned at 40 µm thickness using a cryostat (Leica CM3050 S, Leica Microsystems, Frisco, TX). Brain sections were then kept in cryoprotective solution (-20° C) until assayed using immunocytochemistry (ICC).

2.3. Radioimmunoassay

All blood samples were centrifuged, plasma removed and stored at - 20° C until assayed for corticosterone (CORT). Plasma CORT concentration was measured using a radioimmunoassay described previously (Proudman and Opel, 1989; Madison et al., 2008). Intra-assay coefficients of variance was 11.5%. All samples were assayed in duplicate.

2.4. Dual immunocytochemistry

Brain sections that matched chick atlas plates A 9.0 to A 6.8 (Kuenzel and Masson, 1988) were chosen for an immunocytochemical procedure. Sections were incubated in 5% normal goat serum (NGS) for 30 min to minimize non-specific binding and were incubated in the primary polyclonal anti-FOS antibody (host – rabbit; sc-253, LOT#C2112, Santa Cruz Biotechnology; 1:3000 dilution), for at least 24 h at 4 ºC. Sections were then incubated for 90 minutes in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA; 1: 500 dilution), followed by incubation for 90 minutes in Avidin Biotinylated Horseradish Peroxidase Complex (ABC-HRP complex, Vector Laboratories; 1:5 dilution). Nickle-Diaminobenzidine (DAB) was used as a chromogen (black color) to visualize the immunoreactivity. Sections were rinsed with buffer solutions between incubations. To visualize arginine vasotocin neurons in the same sections, a primary antibody raised in rabbit (kindly

provided by Dr. Gray) was used at a dilution of 1:10,000. Immunoreactivity of AVT containing neurons was visualized using solely diaminobenzidine giving a brown color.

To assess AVT/CRH co-localization in the PVN, dual fluorescence ICC was performed. In a separate study, birds were anesthetized and their heads positioned in the stereotaxic instrument to implant a guide cannula (Plastic One, Roanoke, VA) at the following coordinates (-1.0 mm anterior to the lambda suture and + 0.8 mm lateral to midline) to target the dorsal region of the lateral ventricle. The depth of the guide cannula was 3.0 mm. Birds were allowed to recover post-surgery for at least two days prior to being tested for cannula position using the reliable angiotensin II drinking response (Maney and Wingfield, 1998). Colchicine (16 µg/bird) was injected centrally and 24 h later birds were either restrained (stress) for 1 h or were unrestrained (controls). Colchicine blocks the axonal transport of peptides from the perikarya of neurons and it has been used to visualize CRH-ir neurons (Bloom et al., 1982; Jozsa et al., 1984). Twenty four h after colchicine injection, brain sections were obtained as described earlier. Sections were washed with 0.02M PBS and treated with 0.4% triton X-100. Sections were incubated in 5% normal donkey serum followed by incubation in a mixture of primary antiserum containing CRH (host - guinea pig; T-5007, Bachem; 1:2000) and AVT (host - rabbit; 1:10,000) for 40 h at 4 °C. Following incubation, sections were washed and incubated in a mixture of secondary antibodies (CRH - red, Cy3 and AVT - green, FITC). Sections were then mounted and coverslipped using Vectashield. Brightfield and fluorescence images were taken using a Zeiss Imager M2 microscope and digital camera (Hamamatsu, Orca-ER) and processed using Image Pro Plus (Media Cybernetics, Inc.).

2.5. Quantification

Sections were quantified for AVT-ir and AVT/FOS-ir in specific brain nuclei (Fig 2). Regions of interest were quantified at either 200X or 400 X depending on the density of neurons. Four to five brain sections per bird were chosen for quantification of each specific nucleus. Based upon the diameter of perikarya, three distinct types of AVT-ir neurons were distinguished - parvocellular neurons, 8-10 µm; medium sized neurons, 12-20 µm and magnocellular neurons, >20 µm in the PVNc (Viglietti-Panzica and Panzica, 1981; Panzica and Viglietti-Panzica, 1983; Tennyson et al., 1985). Since the PVN, in particular, was found to have heterogeneous populations of AVT-ir neurons in different subnuclei, quantification was carried out for the three types of neurons in the PVN and the periventricular hypothalamic nucleus (PHN).

2.6. Statistical analysis

Data in the results section represent the total number of AVT-ir neurons and AVT-ir neurons containing a FOS-ir nucleus quantified in a defined area within the region of interest. One way ANOVA was used to determine a significance level and if significant, a Tukey HSD test was used to do a pairwise comparison among treatment groups. All data are presented as mean ± SEM and significance level utilized was $p < 0.05$.

3. Results

3.1. Restraint stress induced corticosterone concentration

Plasma CORT concentrations measured by RIA were significantly higher in both acute (3.2 ± 0.6 ng/ml) and chronic stress groups (1.5 ± 0.3 ng/ml) compared to their respective controls, 0.44 ± 0.2 ng/ml and 0.42 ± 0.1 ng/ml ($p < 0.01$), Fig. 1.

3.2. Quantification of FOS positive AVT neurons

Dual immunostaining in brain structures (AVT and FOS) that showed significant differences among treatment groups are given in Table 1. Note that the number of AVT-ir neurons did not vary in each subnucleus among the treatment groups. The ventral periventricular hypothalamic nucleus (PHNv), the core paraventricular nucleus (PVNc) and the dorsolateral paraventricular subnucleus (PVNdl) contained an abundant number of activated AVT-ir medium sized neurons. However, the differences between the treatment groups and their respective controls were not significant. The thalamolateral paraventricular subnucleus (PVNtl) had a high number of activated AVT-ir magnocellular neurons and showed significantly decreased activation only in the acute stress. The parvocellular neurons in the PVNc are the only neurons that showed significant activation following restraint stress ($p < 0.05$).

3.3.1. The core paraventricular nucleus (PVNc)

Parvocellular neurons were the only AVT-ir neurons that showed significant activation in the PVNc. The acute and chronic stressed groups showed significantly higher FOS positive AVT-ir neurons compared to their respective control groups (Fig. 3). Curiously, the chronic control group had a very low number of activated AVT –ir neurons.

3.4. AVT-ir and CRH-ir neurons in PVNc

Visualization of CRH-ir perikarya was made possible with the central administration of colchicine (Jozsa et al., 1984). Corticotropin releasing hormone immunoreactive neurons were abundant in the PVNc. Although, AVT-ir and CRH-ir neurons coexist in the PVNc no co-localization was observed in controls. Sparse non-significant co-localization was, however, observed in the rostro-caudal extension of the PVNc after one h restraint stress (Fig. 4).

3.5. CRH-ir neurons in the NHpC

Interestingly, the use of colchicine also revealed that the NHpC contained a high density of CRH-ir neurons (Fig 5c). Note that a significant elevation of FOS-ir neurons was detected in the dorsal region of the NHpC following acute stress (Fig 5a) compared to controls (Fig 5b).

4. Discussion

4.1. Stress hormone levels

In mammals and birds it is known that both CRH and AVT are secretagogues of ACTH (Rivier and Vale, 1983; Westerhof et al, 1992). Application of restraint stress has been shown to elicit stress levels in both rodents (Gibbs, 1984) and birds (Beuving and Vonder, 1978). In our study using chickens, plasma CORT concentration was significantly higher following one h restraint stress as well as following repeated restraint stress. Chronic (repeated) restraint stress, however, showed a one fold decrease in CORT concentration compared to acute stressed birds. These data are consistent with mammalian studies showing that repeated exposure to the same stressor results in decreased stress levels compared to the imposed initial exposure (Pitman et al., 1988; Chen and Herbert, 1995; Ma and Lightman, 1998), perhaps representing an adaptation of birds to repeated stressor.

4.2. AVT-ir neurons and restraint stress

An abundant number of AVT-ir neurons were quantified in the SO, PVN and a lateral group of neurons (L, as designated by Tennyson et al., 1985), but significant differences in the activation of AVT-ir neurons were found only in the PVN. The PVNc appears to play an important role for two reasons. A higher number of FOS-ir cells were found in the PVNc of the acute and chronic stress groups. Secondly, the PVN has long been known to contain

heterogeneous populations of neurons - magnocellular, medium sized and parvocellular neurons in rats (Swanson and Kuypers., 1980) and chickens (Viglietti-Panzica and Panzica, 1981; Panzica and Viglietti-Panzica, 1983). In our current study, only the AVT-ir parvocellular neurons in the PVNc were activated in both acute and chronic stress. An increase in AVP mRNA levels and the number of AVP-ir parvocellular neurons has been shown in the PVN of rats following repeated immobilization stress (Bartanusz et al., 1993). Although, our current study in chickens relies solely on immunocytochemical data the number of AVT-ir parvocellular neurons in the PVNc did not change following repeated stress (Table. 1). The absence of the increase in the AVT-ir neurons following repeated stress could be due to species differences. This difference in species has also been reported in sheep (Rivalland et al., 2007). In a pilot study, we were able to determine that these AVT-ir parvocellular neurons were not co-localized with CRH neurons in the PVNc and after one h of restraint stress only a few AVT-ir parvocellular neurons were co-localized with CRH neurons (Fig 4). This result is consistent with studies in rodents, however, AVT immunoreactivity was found significantly increased in the CRH-ir parvocellular neurons in the PVN following chronic stress (de Goeij et al., 1992; Bartanusz et al., 1993) and following adrenalectomy (Sawchenko et al., 1984). In chickens, the pattern of AVT immunoreactivity in CRH-ir parvocellular neurons following chronic stress is yet to be determined.

Arginine vasotocin magnocellular neurons present in the SO and the PVNc are involved in osmotic balance (Muhlbauer et al., 1992; Jaccoby et al., 1997). In our current study magnocellular neurons in the SO and the PVNc did not show increased activation following psychogenic stress and this result is similar to rodent studies (Goeij et al., 1992; Bartanusz et al., 1993). Interestingly, AVT-ir magnocellular neurons in the thalamolateral subnucleus of the PVN

(PVNtl) showed a decreased activation in acute stress (Table.1). It is possible that magnocellular neurons in the PVNtl were sensitive to the negative feedback mechanism by CORT. A main outcome of our current study is that AVT-ir parvocellular neurons in the PVNc of chickens are involved in psychogenic stress.

An interesting observation was found in the NHpC. Utilizing colchicine administration, that enhances the visualization of CRH-ir perikarya, we were able to observe CRH-ir neurons in the NHpC (Fig 5c), particularly in its dorsal region. Since we were able to observe a significant increase in FOS-ir neurons in the dorsal NHpC of birds subjected to acute stress and a decrease in their number after chronic stress, data suggest the structure is involved in the avian stress response. Since plasma CORT showed a significant elevation following acute stress and declined significantly with chronic stress, it is possible that CRH-ir neurons within the NHpC may best reflect changes in plasma CORT concentrations in chickens due to habituation.

In conclusion, chickens subjected to either acute or chronic restraint stress showed a similar pattern in plasma CORT to that of rodent studies. The PVNc, a primary AVT-ir structure adjacent to the third ventricle, showed a significant activation of AVT-ir parvocellular neurons to the psychogenic stressor. Thus, AVT-ir parvocellular neurons in the PVNc play a role in both acute and chronic psychogenic stress (Fig 6). Based upon other studies, AVT-ir magnocellular neurons in the PVNc have been shown to be active in osmotic and dehydration stress, however these neurons were not active following restraint stress used in this study. This implies that a particular type of AVT-ir neurons is activated differentially due to different kinds of stressors.

Table 1. Average number of AVT neurons and FOS+AVT neurons quantified following restraint stress in the chick brain.

AVT-ir Region	Area Quantified	Type of neurons	Acute Control (n=5)		Acute Stress (n=5)		Chronic Control (n=5)		Chronic Stress (n=5)	
			AVT	AVT+FOS	AVT	AVT+FOS	AVT	AVT+FOS	AVT	AVT+FOS
PHNv	0.1 mm^2	Parvocellular	3±1	1	4±1	1	3±1	1	5±1	2±1
		Medium Sized	23±2	8±1	23±1	10±2	25±1	11±2	27±2	11±2
		Magnocellular	2±1	1	1	1	1	1	1	-
PVNc	0.4 mm^2	Parvocellular	48±11	17±2	49±10	33±7*	54±8	1	50±10	28±6*
		Medium Sized	77±7	24±8	66±5	17±5	73±9	32±9	65±5	20±6
		Magnocellular	7±3	5±1	7±4	3±1	9±2	5±2	8±1	3±1
PVNdm	0.03 mm^2	Parvocellular	-	-	-	-	-	-	-	-
		Medium Sized	8±2	3±1	8±1	3±1	9±1	4±1	10±3	4±4
		Magnocellular	-	-	-	-	-	-	-	-
PVNdl	0.1 mm^2	Parvocellular	2±1	-	2±1	-	2±1	-	2±1	-
		Medium Sized	30±2	7±3	27±3	3±1	29±4	13±4	23±2	6±2
		Magnocellular	-	-	-	-	-	-	-	-
PVNtl	0.1 mm^2	Parvocellular	-		-		-		-	
		Medium Sized	-		-		-		-	
		Magnocellular	24±5	11±3	19±6	6±2*	22±6	14±2	25±8	11±3
SOv	0.02 mm^2	Parvocellular	-	-	-	-	-	-	-	-
		Medium Sized	-	-	-	-	-	-	-	-
		Magnocellular	23±1	11±3	25±1	9±2	26±1	16±1	23±1	11±3

Table 1. Average number of AVT neurons and FOS+AVT neurons quantified following restraint stress in the chick brain. (Cont.)

AVT-ir Region	Area Quantified	Type of neurons	Acute Control (n=5)		Acute Stress (n=5)		Chronic Control (n=5)		Chronic Stress (n=5)	
			AVT	AVT+FOS	AVT	AVT+FOS	AVT	AVT+FOS	AVT	AVT+FOS
PPoN	0.15 mm^2	Parvocellular	-	-	-	-	-	-	-	-
		Medium Sized	30±4	13±4	27±3	8±2	33±4	25±5	27±4	13±3
		Magnocellular	-	-	-	-	-	-	-	-
L	0.36 mm^2	Parvocellular	-	-	-	-	-	-	-	-
		Medium Sized	44±7	13±6	53±8	11±5	39±1	14±1	41±3	14±4
		Magnocellular	-	-	-	-	-	-	-	-

PHNv – ventral periventricular hypothalamic nucleus; PVNc – core paraventricular nucleus ; PVNdm – dorso medial paraventricular nucleus; PVNdl– dorsolateral paraventricular nucleus; PVNtl – thalamolateral paraventricular nucleus. SOv– ventral supraoptic nucleus; PPoN – periventricular preoptic nucleus; L – Lateral group of neurons (designated by Tennyson et al., 1985). *represents values significantly different ($P<0.01$) when compared to their respective controls. ¯ indicates the absence of the specific type of neurons. Data are represented as Mean ± SEM.

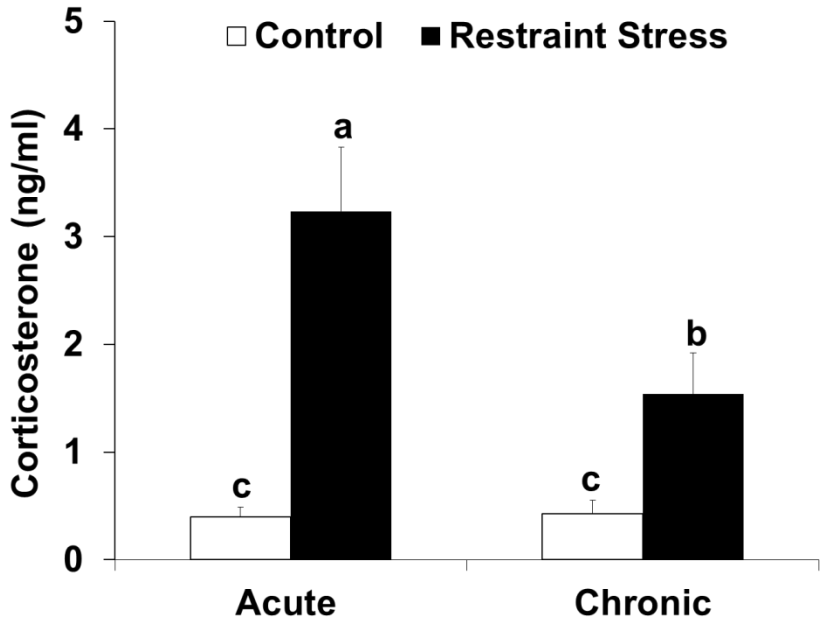
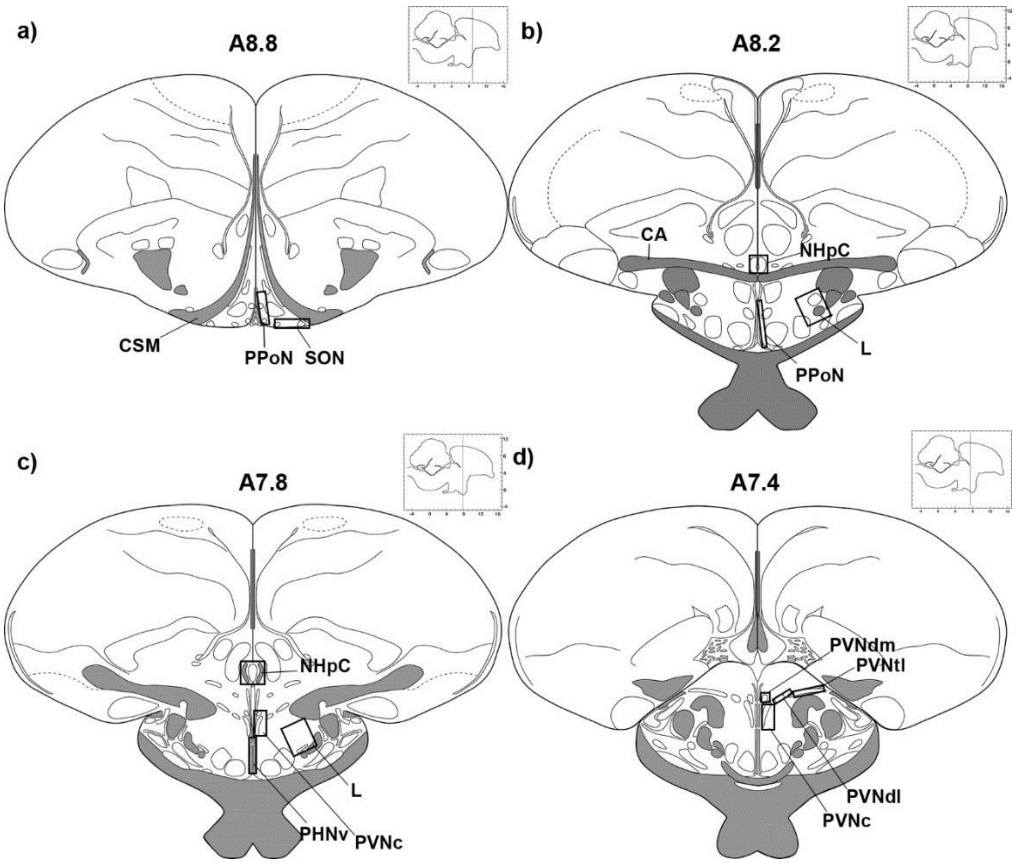


Fig. 1. Plasma corticosterone (CORT) concentrations following acute (1 hr; n=5) and repeated (1 h for 10 consecutive days; n=5) restraint stress. Error bars in each histogram indicate the standard error of the mean.



F**ig. 2.** Schematic diagrams from a chick brain atlas (Kuenzel and Masson, 1988), representing different planes of brain cross-sections (Plates A8.8 – A7.4) containing nuclei that were quantified for AVT-ir neurons and FOS-ir cells. A-D represents cross sections from chicken brain (plate A8.8 – A7.4). The boxed region in each schematic diagram represents the quantified area for AVT-ir or FOS-ir and AVT-ir following restraint stress. Bilateral quantification of activated AVT-ir neurons were performed in SOe - external supraoptic nucleus; SOv - ventral supraoptic nucleus; PPoN – periventricular preoptic nucleus; L - lateral group of AVT-ir neurons (following nomenclature from Tennyson et al., 1985); PHNv – ventral periventricular hypothalamic nucleus; PVNc - core paraventricular nucleus; PVNdm - dorsomedial paraventricular subnucleus; PVNdl - dorsolateral paraventricular subnucleus; PVNtl - thalamolateral paraventricular subnucleus. NHpC - nucleus of the hippocampal commissure.

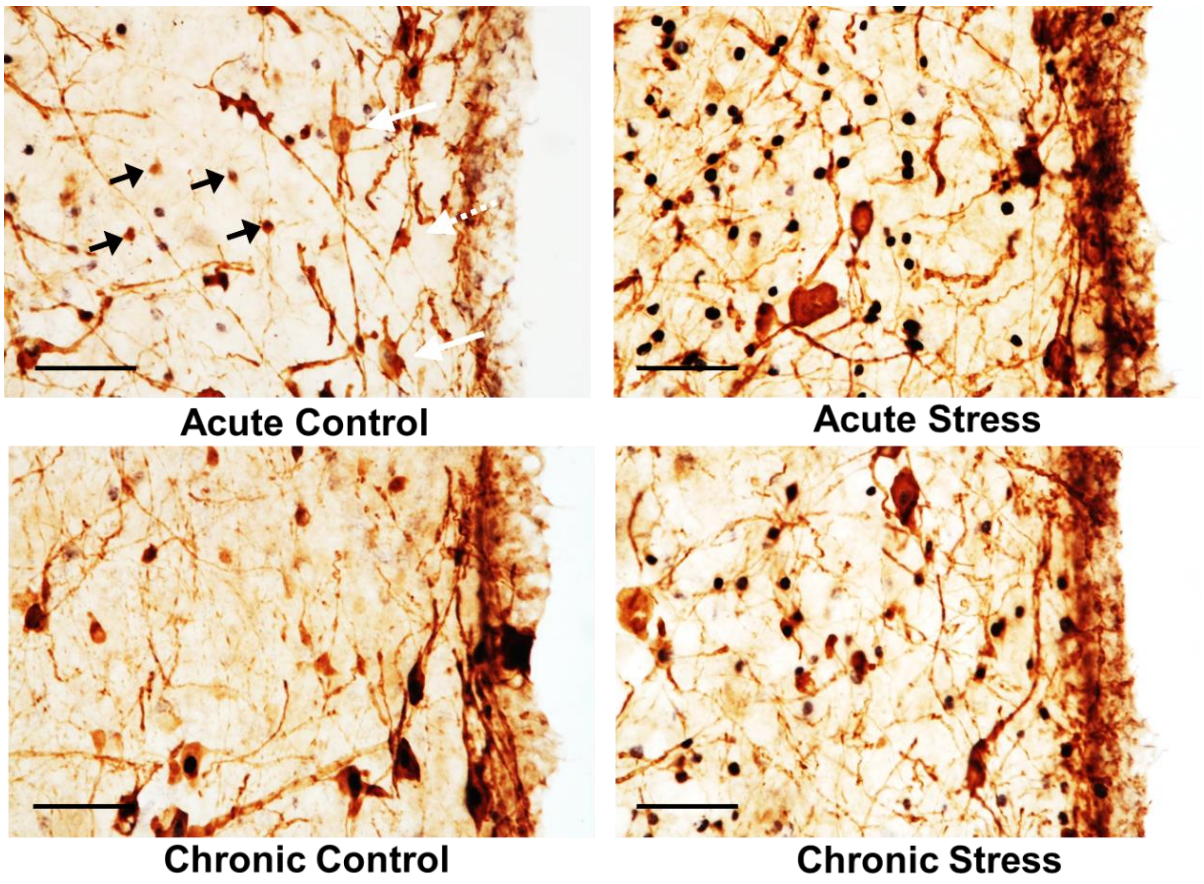


Fig. 3. Digital images show dual immunostaining of FOS and AVT in the PVNc of the acute and the chronic groups compared to their respective controls. In the PVNc parvocellular neurons - solid black arrows, medium sized neurons – dotted white arrow and magnocellular neurons – solid white arrows. Scale bar - 100 µm.

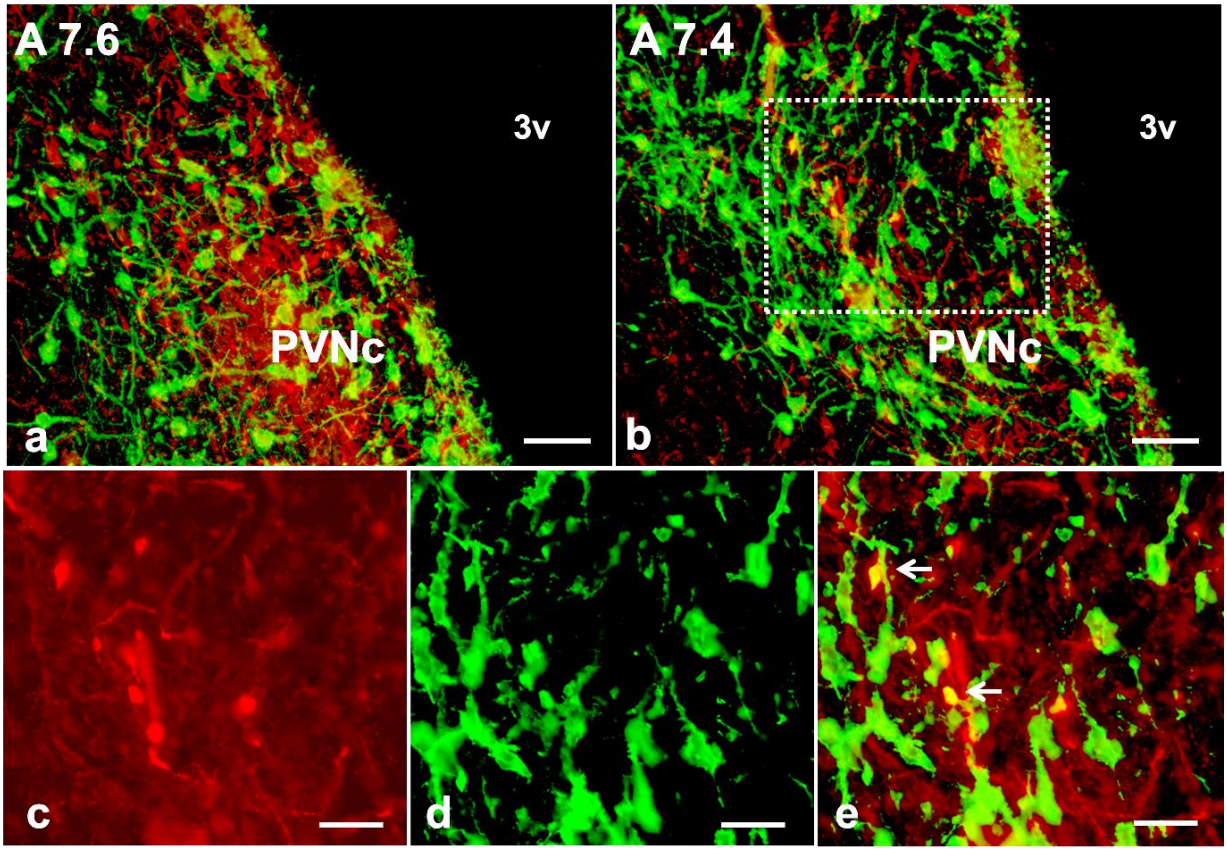


Fig. 4. Co-localization of arginine vasotocin (AVT - green) and corticotropin releasing hormone (CRH - red) immunoreactive neurons in the core region of the paraventricular nucleus (PVNc) of the chicken hypothalamus. The PVNc at the brain atlas plate A 7.6 (a) has a greater number of CRH and AVT neurons compared to the PVNc at plate A 7.4 (b) where few CRH neurons were observed. b) Scarce co-localization of CRH with AVT was documented in parvocellular neurons following one h restraint stress. c), d) and e) represent the boxed-in region in b) where rare co-localization of AVT and CRH was seen (arrows in e). The third ventricle is represented as 3v. Scale bars in a and b: 100µm; c, d and e: 50 µm.

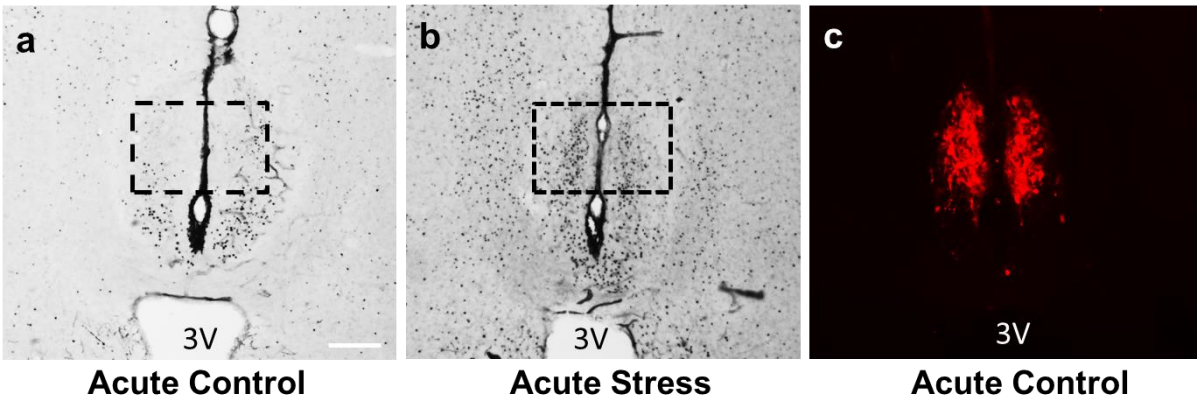


Fig. 5. Restraint stress induced FOS activation in the nucleus of the hippocampal commissure (NHpC) containing CRH-ir neurons. a) FOS-ir in a brain section from an acute control bird and b) FOS-ir in a brain section from a one h restraint stressed bird. Boxed-in region in a) and b) represent the region containing CRH-ir neurons in c). The third ventricle in a, b and c represented as 3v. Scale bars in a, b and c: 200 µm.

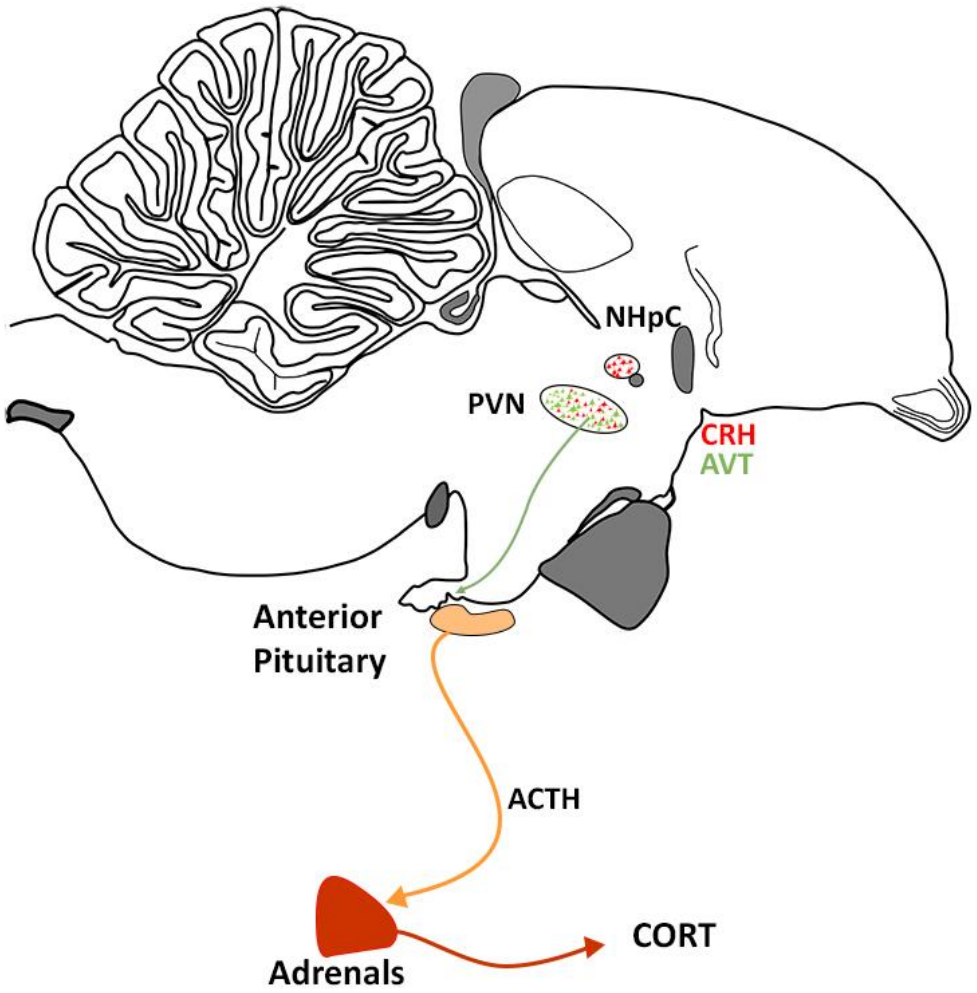


Fig. 6. Schematic representation of the hypothalamo-pituitary-adrenal (HPA) axis in birds. Arginine vasotocin (AVT) containing parvocellular neurons are activated in response to acute and chronic stress. These parvocellular synthesize and release AVT in the median eminence. In conjunction with corticotropin releasing hormone (CRH), AVT activates anterior pituitary corticotropes resulting in cleaving of a preprohormone (proopiomelanocortin – POMC) and release of adrenocorticotropic hormone (ACTH) in the blood. Adrenocortical cells in the adrenal gland are activated by ACTH to synthesize and release corticosterone (CORT), the final end product of the HPA axis. Of particular interest, CRH neurons in the NHpC also appear to respond to the stress response in birds whose details are described in Chapter 3.

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Chapter 3

Functional evidence that AVT is involved in the later phase of the stress response.

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Abstract

Despite extensive data addressing the regulation of the hypothalamo-pituitary-adrenal (HPA) axis in vertebrates the neuroendocrine regulation of stress in birds remains incomplete. The paraventricular nucleus (PVN) contains the key neuropeptides, corticotropin releasing hormone (CRH) and arginine vasotocin (AVT), containing neurons. However, another population of CRH neurons was recently identified in a septal nucleus called the nucleus of the hippocampal commissure (NHpC). Therefore, the current study investigated changes in gene expression of CRH and AVT in the PVN and CRH in the NHpC, as well as changes in plasma corticosterone concentrations following a stressor, food deprivation. In the NHpC, a rapid increase in CRH mRNA levels was observed as early as 2 hr, while relative CRH mRNA expression in the PVN increased thereafter from 4 to 12 h of food deprivation. On the other hand, relative mRNA levels of AVT in the PVN were not significantly different until 12 h and peaked at 24 h following food deprivation. Furthermore, at the level of the anterior pituitary, relative expression of proopiomelanocortin transcripts followed gene expression patterns of CRH and AVT in the

brain. In the absence of food, the pattern of plasma CORT showed an initial rise at 2 h and a fourfold increase was measured at 4 h that was sustained through 24 hr. Taken together, results from this study suggest that 1) CRH neurons in the NHpC are first to respond and appear to be very responsive to stress stimuli compared to those in the PVN and 2) CRH is predominant in the early phase of stress while AVT is involved in the later phase of the stress period.

Keywords: corticotropin releasing hormone, vasotocin, septum, hypothalamus, food deprivation, corticosterone.

1. Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is the fundamental stress pathway documented in vertebrates. Corticotropin releasing hormone (CRH) and arginine vasotocin (AVT) are major regulatory neurohormones of the HPA axis in birds. These neurohormones, synthesized by neurons in the hypothalamus, are involved in the activation and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Carsia et al., 1986; Mikami, 1986). Once in the systemic circulation ACTH increases corticosterone (CORT) concentrations (Castro et al., 1986; Romero et al., 1998), the chief output glucocorticoid of the HPA axis. Several adverse conditions are known to activate the HPA axis and lack of availability of food is one of the stressful factors that increases CORT (Freeman et al., 1980).

Classically, both CRH and AVT are synthesized and released from heterogeneous populations of neurons present in the paraventricular nucleus (PVN) of the avian hypothalamus (Blasher, 1984; Tennyson et al, 1985; Jozsa et al., 1984; Richard et al., 2004). These neurons respond to a multitude of stressors and activate the HPA axis (Sharp et al., 1995; Jaccoby et al., 1997; Nagarajan et al., 2014; Cramer et al., 2015). Interestingly, another population of CRH

neurons was recently identified in a septal region of the avian brain called the nucleus of the hippocampal commissure (NHpC) (Nagarajan et al., 2014), previously termed the nucleus of the pallial commissure. Using an immediate early gene (FOS - neuronal activation marker), immunocytochemical studies have shown that the NHpC also responds to a variety of stressors (Sharp et al., 1995; Xie et al., 2010; Nagarajan et al., 2014). Thus, neurons in these two nuclei, the PVN and NHpC, seem to be involved in the regulation of the stress response. Nonetheless, there is a paucity of direct evidence addressing the neuroendocrine activation of the HPA axis by CRH and AVT.

Although extensive data are available on behavioral and physiological effects of CRH or AVT (Romero et al., 1998; Tachibana et al., 2004; Madison et al., 2008), the neuroendocrine mechanism through which the HPA axis becomes activated is still unclear. If the neuroendocrine stress hormones are potent mediators of systemic CORT concentration, then a systematic activation of neurohormones would be expected to occur in order to increase as well as maintain high levels of CORT, particularly if the stressor persists. Therefore, we designed a short term food deprivation study for 24 h to document the dynamics of CRH and AVT synthesis during the neuroendocrine regulation of the avian stress response.

Since the NHpC was found to contain CRH neurons, we hypothesized that the regulation of CRH neurons in the NHpC and PVN would be synchronized in an avian species for activation of the stress response. The present study investigated gene expression in the NHpC and PVN following time dependent food deprivation. Neuroendocrine gene expressions were then compared with expression of proopiomelanocortin (POMC) in the anterior pituitary and plasma CORT concentrations over time to determine whether the NHpC, along with the PVN, is involved in the temporal regulation of the HPA axis.

2. Materials and methods

2.1 Animals and sample collection

Day old male Cobb 500 chicks were obtained from a commercial hatchery and reared in a controlled heated environment at 32°C. A weekly reduction in temperature (2.5°C) was applied until 22°C was reached. Birds were provided with water and a standard starter diet (22% protein and metabolizable energy of 3100 kcal/kg) ad libitum. At 2 weeks of age, birds were weighed and birds with comparable body weights at that age were randomly distributed to cages. Birds were maintained in a constant photoperiod of 16L:8D with lights on at 6 AM. The food deprivation experiment was conducted when birds were 5 weeks of age (with unlimited access to water). Chicks were subjected to one of the following food deprivation treatments, beginning at 8 AM, along with their respective controls: 2 hr, 4 hr, 8 hr, 12 hr, and 24 h (n=7-8 birds/group). Blood samples were collected from the brachial vein of each bird for each sampling time period. Birds were then cervically dislocated, brain and anterior pituitary immediately dissected. Brain samples were frozen by immersion in two-methyl butane at -30°C followed by dry ice to maintain structural morphology of the brain for cryo-sectioning. Brain and anterior pituitary samples were stored at -80°C until processed. All of the experimental procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (#16043).

Plasma was obtained from each blood sample by centrifugation at 1200g for 20 min at 4°C and stored at -20 °C until processed. Cross sections of brain samples were cut at 100 µm thickness using a cryostat (Leica CM3050 S, Leica Microsystems, Frisco, TX) and target regions were either punched (brain punch, Palkovits, 1973) using a glass pipette (NHpC – 1.4 mm diameter including atlas plates A8.2-A7.6) or knife cut (PVN – 2x2 mm covering atlas plates A8.2-A6.4) (Kuenzel and Mason, 1988). All brain sections were dissected inside the cryostat

chamber maintained at -12°C. The anterior commissure was used as a landmark for dissections (Kuenzel and Mason, 1988). To determine the accuracy of the boundaries of punched or dissected structures, immediately after all punches or knife cuts, and the most anterior and posterior sections that were not dissected were Nissl stained and examined. Tissues samples were collected in Trizol and stored at -20°C until processed for RNA extraction.

2.2. Immunocytochemistry

In a separate experiment dual immunofluorescent staining was performed to display two phenotypic neurons: CRH and AVT. In order to visualize CRH-ir cell bodies in the NHpC, colchicine was administered intracerebroventricularly (16µg/bird, n=3) by targeting the dorsal horn of the lateral ventricle using the following coordinates (-1.0 mm anterior to the lambda suture, +0.8 mm lateral to midline and -3.0 mm ventral from cranium) as described previously (Chapter 2, Material and Methods). Brains were sampled and sectioned at 40 µm thickness. Brain sections covering the chick brain atlas plates A8.2-A7.6 (Kuenzel and Mason, 1988) were used for the assay. Dual immunostaining was performed as follows. Sections were rinsed in 0.02M phosphate buffer saline (PBS, pH 7.4) several times followed by treatment with 0.4% Triton X-100 in PBS for 15 min. The sections were then incubated in 5% normal donkey serum (0.1% sodium azide, 0.2% Triton X-100 in 0.02M PBS) for 30 min. After incubation, sections were transferred to a primary antibody solution (1% normal donkey serum, 0.2% Triton X-100 in 0.02M PBS) containing a cocktail of primary antibodies (CRH, host – guinea pig; T-5007, Bachem; 1:2000 and AVT, host – rabbit; a gift from Dr. Gray, 1:10,000) and incubated for at least 48 h at 4° C. Later sections were washed several times in 0.02M PBS and incubated in a cocktail secondary antibody solution (0.2% Triton X-100 in 0.02M PBS) for 90 min. Secondary antibodies used were anti-guinea pig IgG conjugated with Cy3 and donkey anti-rabbit IgG

conjugated with FITC (1:500, Jackson Immuno Research, West Grove, PA). Sections were then washed in 0.02 M PBS followed by a wash in distilled water and mounted on clean glass slides and cover slipped using Vectashield. Dual fluorescent digital images were acquired using a Zeiss Imager M2 microscope (Carl Zeiss Microscopy, LLC. Thornwood, NY) with attached CCD camera (Hamamatsu, Orca ER, Bridgewater, NJ) and Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD). Adobe Photoshop CS6 was used to create a montage, adjust brightness and contrast of digital images.

2.3. RNA isolation and gene expression assay

Trizol-chloroform (Life Technologies) based RNA extraction procedure was used to extract total RNA from micro dissected brain and anterior pituitary tissues (n=4-5/group). Total RNA was purified using RNeasy mini kit (Qiagen) and RNA quality of each sample was determined using Synergy HT multi-mode microplate reader (Biotek). Total RNA (200ng) was converted into cDNA using SuperScript III reverse transcriptase (Invitrogen) as per the manufacturer's protocol. The best primer set from a set of primer pairs (retrieved using the PRIMER3 program) for each gene was selected based on the quality of PCR products obtained after electrophoresis (3% agarose) analysis. Primer sets used in the assays are provided in Table 1. Product of each sample was mixed with Power SYBR green PCR Master Mix and were amplified using real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system). The assay was performed using the following conditions: 1 cycle at 95°C for 10 m and amplified for 40 cycles at 95°C for 15 s, 60°C for 1 m. The chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. At the end of amplification melting curve was utilized to eliminate nonspecific PCR product contamination. Relative gene

expression levels of each specific gene were determined by the $2^{-\Delta\Delta ct}$ method (Schmittgen and Livak, 2008).

2.4. Plasma corticosterone and osmolality

Plasma CORT concentration was measured using a radioimmunoassay described previously (Madison et al., 2008). A rabbit polyclonal primary antibody against corticosterone (Fitzgerald Inc., Concord, MA, USA) and secondary antibody (sheep anti rabbit) was used in the assay. The corticosterone isotope, ^{125}I, (MP Biomedicals Inc.) was used for the competitive binding assay. All samples were assayed in duplicate. Intra-assay coefficients of variance was 10.7%. Plasma osmolality was measured using the freezing point depression method (Model 3250 Osmometer, Advanced Instruments, Inc). Plasma osmolality was measured to check whether increased AVT mRNA levels in the PVN is not a result of osmotic stimuli following food deprivation.

2.5. Organotypic culture

In order to understand the regulation of CRH and AVT gene expression, in the hypothalamus and septal regions, an organotypic slice culture was performed. The Brain slice culture experiment was performed using brains obtained from 3-5 d old chicks. Birds were cervically dislocated, brains were dissected out of the cranium and placed in ice cold artificial cerebrospinal fluid (aCSF: 130mM NaCl, 3mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2.H_2O$, 26 mM $NaHCO_3$, 1.25mM $NaH_2PO_4.H_2O$ and 10mM D-glucose) saturated with carbogen gas (95% O2 and 5% CO2). Brains were then glued (Loctite superglue) to a vibratome stage and immediately covered with ice cold aCSF. The vibratome chamber was maintained in an ice cold condition at all times. Brains were sectioned at 350 µm thickness and horizontal sections were dissected to contain the NHpC and PVN (see Fig 5 for dissected area). Tissues (n=2 birds) were then placed

on each cell culture insert (Millipore PICM03050) saturated with 1 ml standard culture media – (SCM) [50% Eagle basal medium, 25% Hanks Blank salt solution, 25% heat inactivated horse serum, 25U/ml penicillin, 0.5% glucose and 0.5 mM L-glutamine] and maintained at 36° C and 5% CO_2. The procedure was performed in less than one h. Slices were maintained in the culture environment for 9-10 d and culture media was replaced every 2 or 3 d. One day prior to the treatments, SCM was replaced with serum free media (SFM) [100% Eagle basal medium, 25U/ml penicillin, 0.5% glucose and 0.5 mM L-glutamine]. Compounds and doses used in this study were as follows: forskolin (10 µg, F6886 - Sigma) and dexamethasone (500 ng, D-1756 - Sigma). Media containing DMSO was used as vehicle. After 24 h tissues were frozen, collected in Trizol solution and stored at -80° C.

2.6. Statistical analysis

Analysis of both gene expression data and hormone assay data were performed using JMP pro 11.0 (SAS Institute Inc). One way analysis of variance was used to find significant treatment effects among all treatment groups and student's t-tests were used for mean separation between controls and treatment groups. For gene expression analysis, Kruskal-Wallis tests were used to find overall significant difference and Wilcoxon test was used for mean separation between controls and treatment groups. Significance level used in all analyses was $p < 0.05$. Data in the results section are shown as mean ± SEM.

3. Results

3.1 Localization of CRH and AVT neurons

Dual immunostaining for CRH and AVT was performed in brain sections containing the NHpC and PVN (Fig. 1). The dorsal part of the NHpC contains a population of CRH-ir neurons

while the core region of the PVN contains heterogeneous populations of both CRH-ir and AVT-ir neurons.

3.2. Rapid activation of CRH mRNA in the NHpC following food deprivation

In the NHpC, relative CRH mRNA levels showed an overall significant difference among treatment groups ($p < 0.0009$). Gene expression of CRH significantly peaked at 2 h of food deprivation compared to controls (Fig. 2a, $p < 0.05$). Significant down regulation of CRH gene expression was observed at 4 h in food deprived birds, however CRH gene expression levels were higher than the control birds ($p < 0.05$). Gene expression levels at 8 h and 12 h food deprivation showed no differences when compared to controls. A 24 h absence of food significantly down regulated CRH below control values ($p < 0.05$).

3.3. Temporal regulation of CRH and AVT mRNA in the PVN

Overall, transcription levels of both CRH and AVT were found significantly different ($p < 0.0008$) and ($p < 0.0012$), respectively (Fig. 2b). While 2 h food deprivation showed no difference, CRH gene expression significantly increased at 4 h, 8 h and peaked at 12 h compared to controls ($p < 0.05$). Twenty four h food deprivation significantly downregulated CRH mRNA levels compared to the 12 h food deprivation period ($p <0.05$), while maintaining gene expression levels higher than control birds ($p < 0.05$). In contrast to CRH gene expression, AVT in the PVN did not show a significant increase until 12 h of food deprivation ($p < 0.05$) and its expression levels peaked at 24h following food deprivation ($p < 0.05$).

3.4. Food deprivation induced POMC expression in the anterior pituitary

Relative gene expression levels of both POMC hnRNA and mRNA in the anterior pituitary showed significant differences among the treatment groups ($p < 0.0006$) and ($p < 0.0006$), respectively (Fig. 2c). Two and four h food deprivation significantly increased POMC

hnRNA levels compared to controls ($p < 0.05$). Furthermore, 8 h, 12 h, and 24 h food deprivation showed significant increase in both hnRNA and mRNA compared to controls ($p < 0.05$).

3.5. Food deprivation induced corticosterone concentration

Corticosterone concentrations were measured from plasma samples of birds subjected to food deprivation for 2 h, 4 h, 8 h, 12 h and 24 h along with their respective controls. Food deprived birds showed an overall significance among the treatment groups (Fig. 3, $F_{10,81} = 13.06$, $p < 0.0001$). Although, a slight increase in CORT following food deprivation can be seen as early as 2 h, a significant fourfold increase was only observed at 4 h following withdrawal of food ($p < 0.0001$). This increase in CORT concentrations remained significantly higher at 8 to 12 h ($p < 0.05$) and appeared to plateau until the end of the 24h food deprivation time period.

3.6. Plasma osmolality following food deprivation

Plasma osmolality levels were measured in birds that were food deprived for 2 h, 4 h, 8 h, 12 h and 24 h. An overall significant change was observed among the treatment groups (Fig. 4, $F_{5,38} = 5.15$, $p < 0.0006$). Compared to birds on feed (0 h), plasma osmolality decreased immediately within 2 h following food deprivation ($p < 0.02$) and further significantly decreased until 24 h ($p < 0.05$).

3.7. Effects of forskolin and dexamethasone on AVT and CRH expression

Effect of forskolin and dexamethasone on gene expression of AVT or CRH was tested in slices containing the PVN or the NHpC for 24 h. Following administration of forskolin, slices containing the PVN showed a significant increase in AVT and CRH gene expression levels compared to controls given the vehicle ($p < 0.05$), Fig 6a and 6b, respectively. Forskolin induced increase in mRNA expression of both AVT and CRH was suppressed by dexamethasone

in slices containing the PVN ($p < 0.05$). The NHpC containing slices a showed similar pattern with forskolin treatment. A significant increase in CRH mRNA levels was observed compared to controls after 24 h forskolin treatment ($p < 0.05$, Fig 6c), while co-treatment of forskolin and dexamethasone showed a reduction in CRH gene expression compared to the forskolin treatment, however, it was not significantly different from the control group ($p > 0.05$).

4. Discussion

Based upon the evidence herein, the neuroendocrine regulation of the stress response following food deprivation in domestic birds involves CRH in both the NHpC and PVN followed by AVT in the PVN (Fig. 1 and 2). Utilizing gene expression of CRH and AVT the study examined the regulation of CORT in the absence of food for 24 h. Both gene expression and peripheral plasma CORT data suggest that a temporal regulation of CRH and AVT occurs for modulating the output of the HPA axis.

In mammals, activation of the classical HPA axis is well known to be potentiated by a combination of CRH and vasopressin (AVP), the primary secretagogues of POMC synthesis and ACTH release from the anterior pituitary. This sequence is followed by the release of the stress hormone CORT from the adrenal glands. Both *in vitro* and *in vivo* studies have shown that, when CRH and AVP were administered simultaneously, AVP augments CRH's normal action on the expected release of ACTH from corticotropes (Gillies et al., 1982; Rivier and Vale, 1983). Furthermore, *in vivo* studies have shown that following a particular stress stimulus, a characteristic pattern of CRH and AVP expression occurs such that gene expression of CRH precedes gene expression of AVP (Kovacs and Sawchenko, 1996). Similar to mammals, CRH and AVT act as secretagogues of ACTH in birds (Carsia et al., 1986; Castro et al., 1986).

However, the sequence of activation of neurohormones in the avian hypothalamus has remained unknown due to the following reasons: 1) a separate dense population of CRH neurons was identified in the NHpC, 2) CRH and AVT neurons are present, but scattered in heterogeneous populations within in the PVN and 3) lack of information regarding the temporal regulation of CRH and AVT following a particular stressor in birds.

For the first time we have found functional evidence that in birds a distinct group of CRH neurons located in the NHpC responds to stress stimuli. This is evident from the rapid induction of CRH mRNA in the NHpC followed by a significant rise in CRH mRNA in the PVN after subjecting birds to food deprivation. Previously, it was proposed that the NHpC may be part of the stress axis (Chapter 2). However, only indirect evidence showed that cells in the NHpC were activated following physiological challenges such as osmotic imbalance (Sharp et al., 1995), psychogenic stress (Chapter 2) and social stress such as territorial defense between roosters and consummatory reproductive behavior between roosters and hens (Xie et al., 2010). Hence, all of the studies just cited, including the current study, suggest that the NHpC is one of the first sites activated by a variety of acute stressors. Thereafter, CRH neurons in the PVN reinforce that early response resulting in the significant elevation of plasma CORT (Fig. 3). Later, maintenance of the higher levels of plasma CORT appears to occur due to the delayed but consistent elevation of AVT mRNA within the PVN (Fig. 2). Consistent with the stress studies in mammals (Kovacs and Sawchenko, 1996; Aguilera, 1998), the present study shows that gene expression levels of CRH and AVT varied temporally such that CRH gene expression occurred earlier than AVT. Furthermore, the increase in gene expression of AVT in the PVN following food deprivation was not associated with osmotic stimuli (Fig. 4). Thus, CRH and AVT

expression in birds shows a pattern of neuroendocrine activation of the HPA axis and exhibit a time dependent differential regulation to modulate the HPA axis.

Terminals of CRH and AVP in mammals (Bloom et al., 1982; Antoni, 1993) as well as CRH and AVT terminals in birds (Mikami, 1986; Kuenzel and Jurkevich, 2010) are found in the external zone of the median eminence, where both neurohormones are released and reach the anterior pituitary to act on corticotropes. Past *in vitro* studies have shown that CRH and AVT can induce corticotrope cell signaling. Specifically, dimerization between the mammalian V1b and CRH type 1 receptors (Young et al., 2007) and heterooligomerization between the avian V1b and CRH type 1 receptors (Mikhailova et al., 2007) have been demonstrated in cells transfected with the appropriate receptors. The latter avian study provided evidence that the apparent synergistic increase in plasma CORT following administration of CRH and AVT may be the result of enhanced release of the second messenger cAMP within the anterior pituitary cells and thereby altering the responsiveness of the HPA axis (Mikhailova et al., 2007). The present study concurs with this concept of the augmentation or synergistic effect of CRH and AVT. We examined gene expression of POMC in the pituitary, the preprohormone that is cleaved to release ACTH. The increase in CRH gene expression in both the NHpC and PVN corresponds to the early increase in POMC expression following food deprivation. However, the POMC expression further increased when there was an increase in AVT gene expression in the PVN (Fig. 2). Thus, CRH is sufficient to increase POMC expression and activate the HPA axis while the presence of AVT appears to be required for augmenting POMC expression in the anterior pituitary (Jayanthi et al., 2014) and thus maintaining the elevated plateau in peripheral CORT (Fig. 3).

The present study not only showed increased CRH gene expression in the NHpC and PVN in the early phase of the stress period, but also evaluated long term effects of CORT on gene expression of CRH because of the presence of glucocorticoid receptors (Kovács et al., 1989, Dickens et al., 2009, Dickens et al., 2011) in the NHpC and PVN. This prompted us to use forskolin and dexamethasone in organotypic slice cultures. Forskolin activates adenylate cyclase cell signaling and has been used for inducing CRH and AVP expression. While dexamethasone is a synthetic glucocorticoid and a more potent alternative for CORT. Use of forskolin increased CRH gene expression levels in both the NHpC and PVN (Fig 6), suggesting that CRH in these two structures could be activated by the adenylate cyclase dependent pathway. Likewise AVT in the PVN also appears to be influenced by the adenylate cyclase mechanism. On the other hand, glucocorticoids exert their inhibitory effect on the NHpC CRH expression as much as on CRH and AVT expression in the PVN (Bali et al., 2008). In food deprived birds, the NHpC CRH expression seems to be more sensitive to negative inhibition than CRH in the PVN (Fig 2). However, in *in vitro* experiment, inhibitory effect of glucocorticoids on gene expression of CRH is stronger in the PVN compared to the NHpC. This difference could be related to the following reasons: 1) the density of glucocorticoid receptors is higher in the PVN than the NHpC (Kovács et al., 1989, Dickens et al., 2009, Dickens et al., 2011) and 2) the number of replicates used for this experiment as well as the higher survival rate of CRH neurons in the PVN than the NHpC (data not shown). Nonetheless, downregulation of CRH observed could be a result of negative feedback effect of glucocorticoids following the stressor over longer period of time (Fig 2 and Fig 6).

In summary, significant elevation of the stress hormone, CORT, occurs within hours of food deprivation. The first significant contributor of that early induction was shown to be gene

expression of CRH mRNA in a septal structure called the NHpC followed by gene expression of CRH mRNA in the classical hypothalamic PVN. Thereafter, a delayed but consistent rise in AVT gene expression in the PVN appeared to sustain the elevated plateau of CORT, the avian stress hormone. Furthermore, glucocorticoids could have a direct inhibitory effect on CRH and AVT neurons in the PVN and CRH neurons in the NHpC if stress is prolonged for an extended time period. Data are also presented suggesting that a septal structure, the NHpC containing CRH neurons, is involved in the activation of neuroendocrine stress response and perhaps is part of the HPA or septo-hypothalamo-pituitary-adrenal axis in birds (Fig. 7).

Table 1. Primer set used for the gene expression analysis.

Gene	Genbank ID	Forward/Reverse primers	Product size
AVT mRNA	NM_205185	F- 5'CCTTCCCCGAACGCATAG3' R-5'GGGCAGTTCTGGATGTAGCAG3'	117bp
CRH mRNA	NM_001123031	F-5'GCCCACAGCAACAGGAAAC3' R-5' GTGATGGCTCTGGTGCTGAC3'	98bp
POMC mRNA	NM_001031098	F-5'GCCAGACCCCGCTGATG3' R-5'CTTGTAGGCGCTTTTGACGAT3'	56bp
POMC hnRNA	NM_001031098	F-5'ATTTTACGCTTCCATTTCGC3' R-5'AATGGCTCATCACGTACTTGC3'	141bp
GAPDH	NM204305	F-5'CTTTGGCATTGTGGAGGGTC3' R-5'ACGCTGGGATGATGTTCTGG3'	128bp

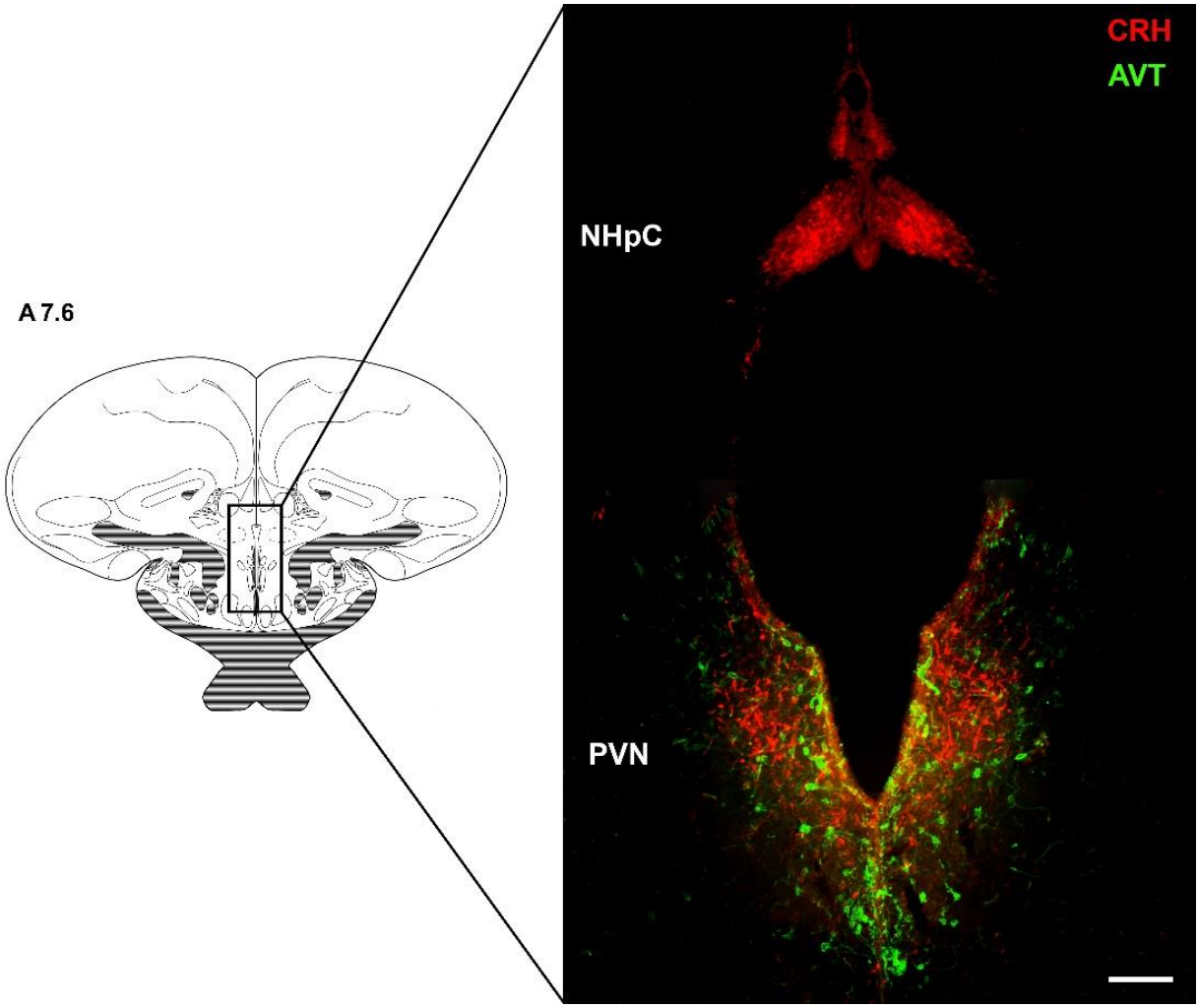

Fig. 1. Montage of a digital image containing dual immunofluorescent staining for corticoticortipin releasing hormone (CRH - red) and arginine vasotocin (AVT - green) immunoreactive neurons. The nucleus of the hippocampal commissure (NHpC) and the paraventricular nucleus (PVN) are shown with respect to chick brain atlas plate A7.6. Scale bar - 200 µm.

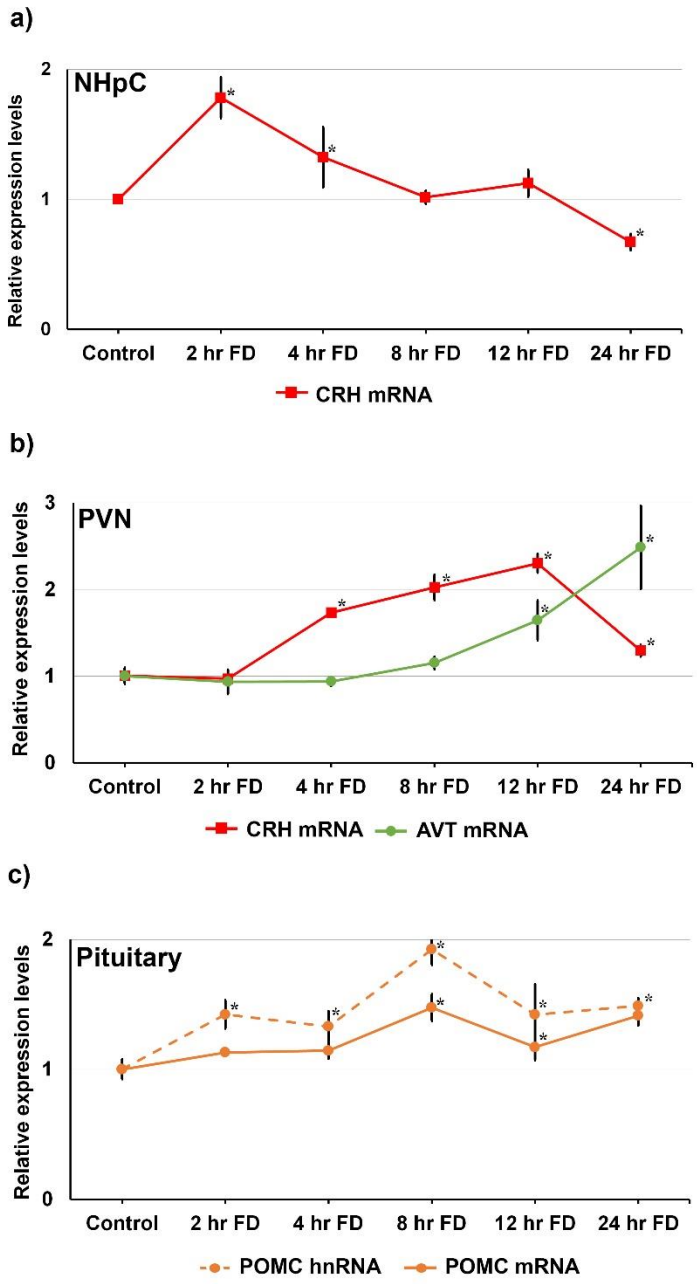

Fig. 2. Transcription levels of genes in the nucleus of the hippocampal commissure (NHpC), paraventricular nucleus (PVN) and the pituitary following food deprivation (FD) for 2, 4, 8, 12 and 24 h. Relative expression of CRH mRNA in the nucleus of the hippocampal commissure (NHpC) is shown in (a). Expression levels of CRH mRNA and AVT mRNA in the paraventricular nucleus (PVN) are shown in (b). Proopiomelanocortin (POMC) heteronuclear RNA (hnRNA) and mRNA expression levels in the pituitary are shown in (c). * represents the significant difference compared to controls ($p < 0.05$).

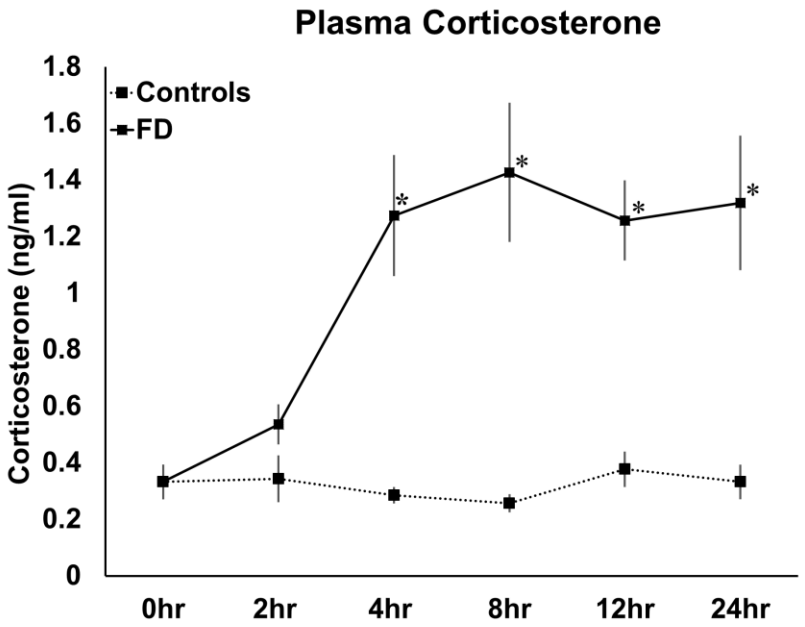

Fig. 3. Plasma corticosterone concentrations following food deprivation (FD). Birds were food deprived for 2, 4, 8, 12 and 24 h (solid lines) and are represented along with their respective controls where food and water were provided ad libitum (dotted lines). * represents significant differences between the controls and treatment groups ($p < 0.05$).

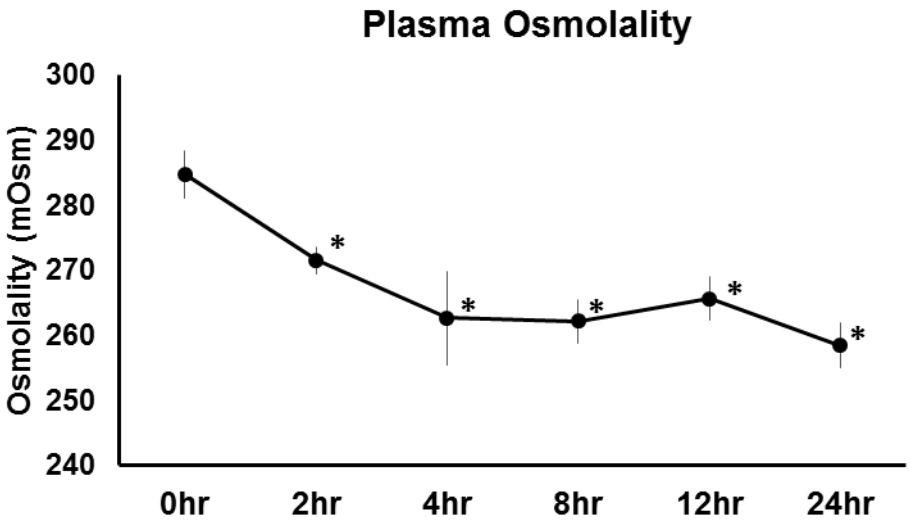

Fig. 4. Plasma osmolality following food deprivation for 2, 4, 8, 12 and 24 h. Zero h control birds had unlimited access to food and water. * represents significant differences between the controls and treatment groups ($p < 0.05$).

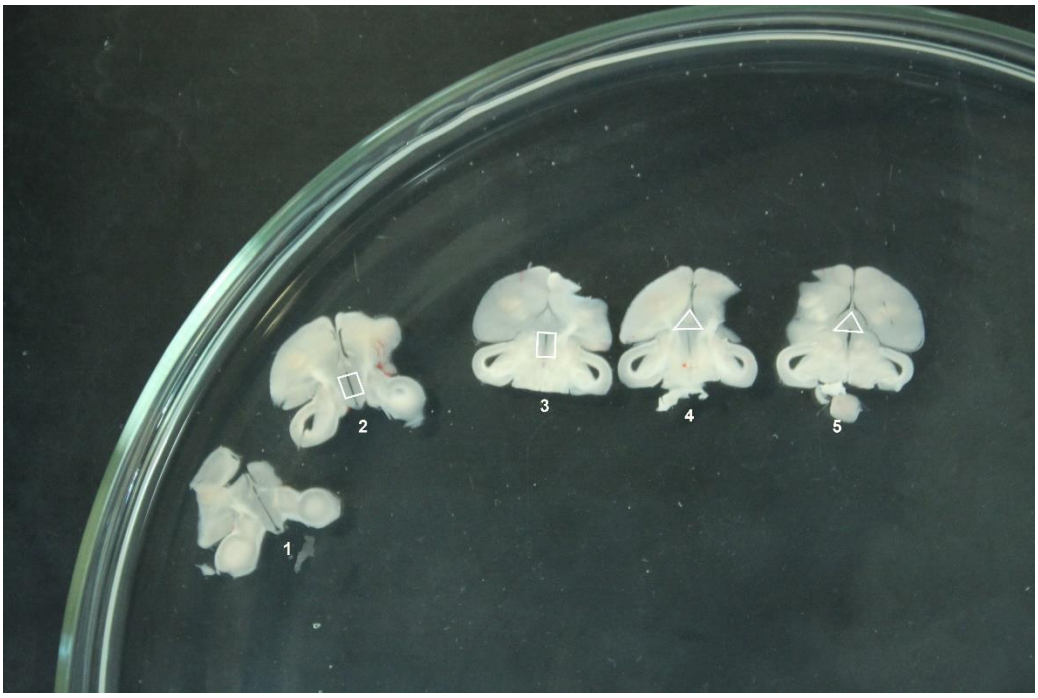

Fig. 5. Horizontal slices (350 µm) obtained from 3-5 d old chicks using a vibratome. Slices were trimmed at the marked regions to contain the paraventricular nucleus, PVN, (2 and 3) and nucleus of the hippocampal commissure, NHpC, (4 and 5) for slice culture experiments. The NHpC was dissected in a triangular shape to avoid obtaining CRH neurons from the bed nucleus of the stria terminalis.

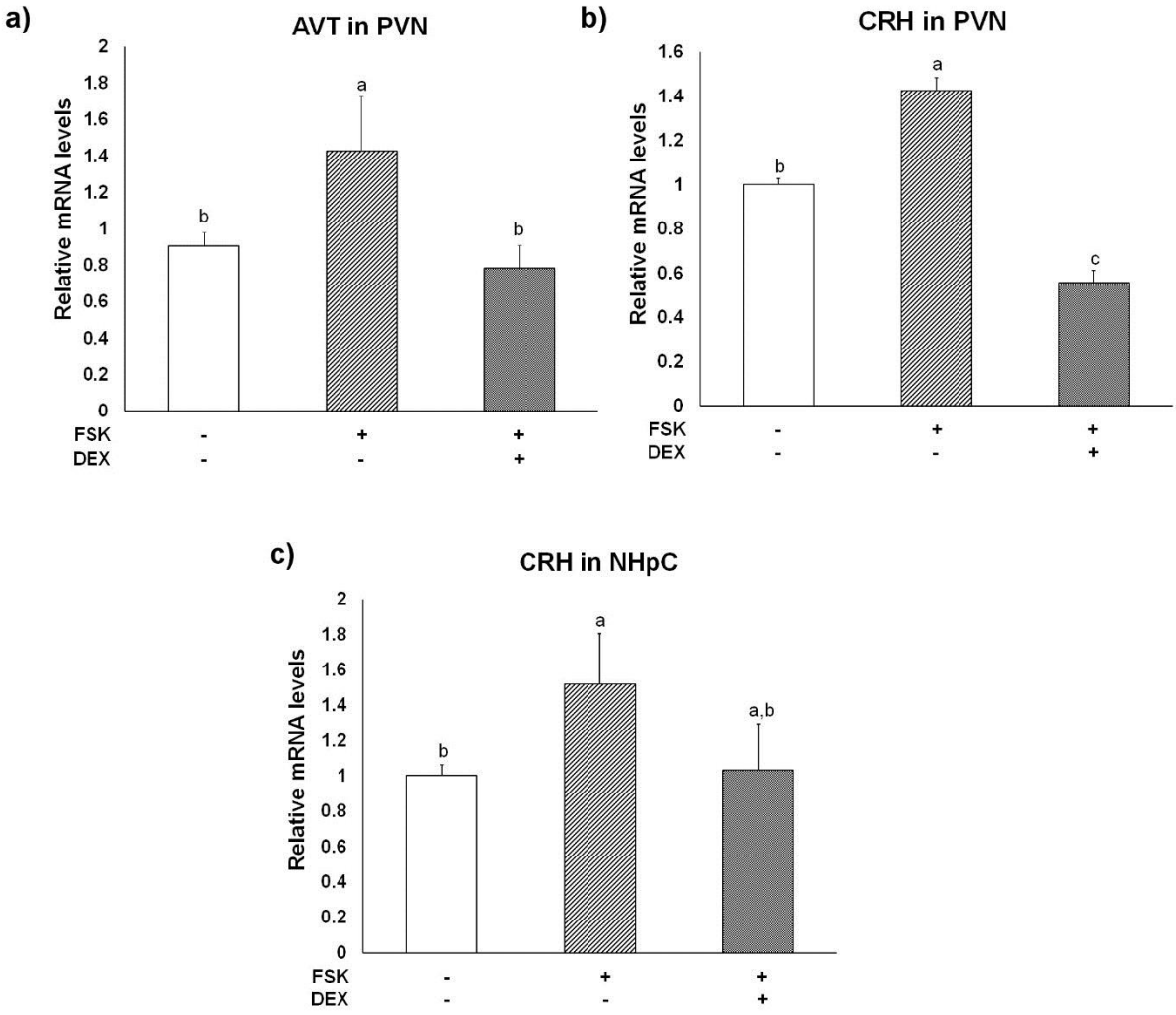

Fig. 6. Effect of forskolin (FSK) and dexamethasone (DEX) in cultured slices containing AVT and CRH neurons. a) and b) Relative expression of AVT and CRH mRNA levels in the paraventricular nucleus (PVN) are shown after FSK treatment or a combination of FSK and DEX treatment for 24 h. c) CRH mRNA levels in the septal slice culture containing the nucleus of the hippocampal commissure (NHpC) following FSK and/or DEX treatments. n=3. Different letters in each histogram set represent significant differences among the treatment groups.

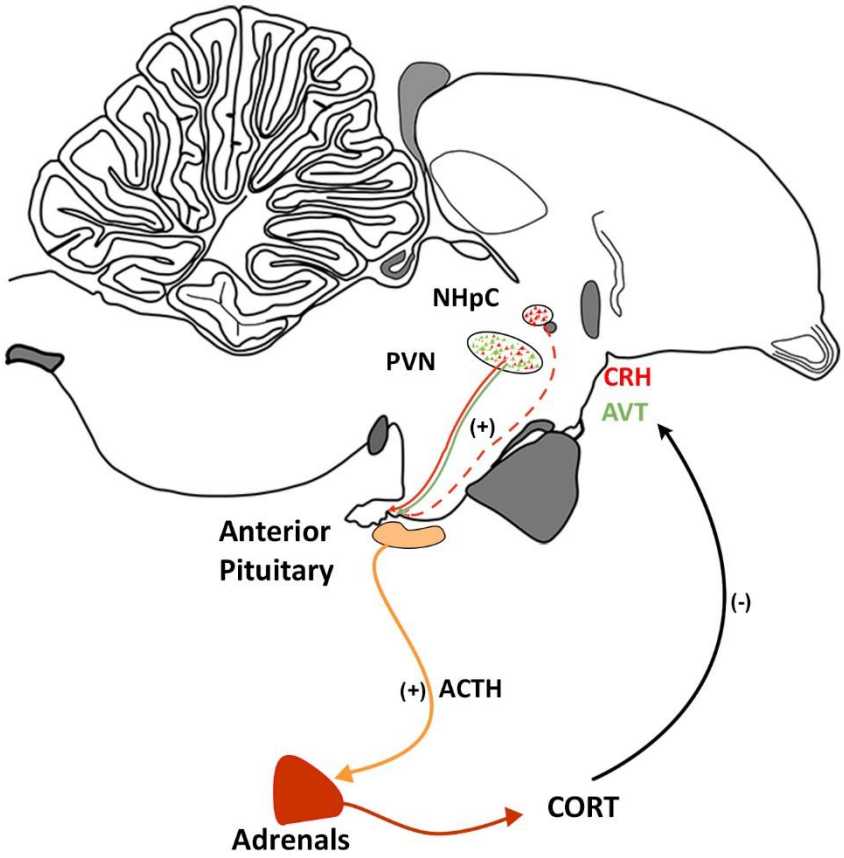

Fig. 7. Schematic representation of the septo-hypothalamo-pituitary-adrenal axis in birds. Two neural structures, the nucleus of the hippocampal commissure (NHpC) in the septum and the paraventricular nucleus (PVN) in the hypothalamus are activated in response to stress. The PVN contains corticotropin releasing hormone (CRH) and arginine vasotocin (AVT) neurons, while the NHpC contains CRH neurons. Temporal regulation of CRH and AVT results in the activation of anterior pituitary corticotropes resulting in cleaving of a preprohormone (proopiomelanocortin – POMC) and release of adrenocorticotropic releasing hormone (ACTH) in the plasma. Adrenocortical cells in the adrenal gland are activated by ACTH to synthesize and release corticosterone (CORT), the final end product of the septo-hypothalamo-pituitary-adrenal axis. Long term presence of CORT in the blood appears to influence synthesize of neurohormones (CRH and AVT) though negative feedback mechanism. + is stimulatory and – is inhibitory.

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Chapter 4

Neuromodulation of AVT on CRH neurons in the avian septum via V1a receptors in glia.

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Abstract

Previously, we showed that a septal structure containing corticotropic releasing hormone (CRH) immunoreactive neurons is associated with the stress mechanism within the avian brain. In the present study we characterized CRH-ir neurons in the septal region, called the nucleus of the hippocampal commissure (NHpC) and demonstrated functional regulation of AVT on CRH-ir neurons in the NHpC. Immunocytochemical and gene expression analysis were used to identify the anatomical and functional characteristics within the NHpC. A comparative analysis showed that CRH-ir neurons in the NHpC are larger (with two or more processes) than CRH-ir neurons in the paraventricular nucleus of the hypothalamus and lateral bed nucleus of the stria terminalis. Immunocytochemical evidence also showed that the NHpC CRH-ir neurons are surrounded by the vasotocinergic system, particularly with vasotocin (AVT) nerve terminals and V1a receptors within glia. Hence, it was hypothesized that AVT could modulate activity of CRH-ir neurons in the NHpC via V1aR in glia and was tested using organotypic slice cultures containing the NHpC. Gene expression analysis revealed that AVT treatment induced CRH mRNA levels, whereas a combination of AVT and V1aR antagonist treatment administered to cultured slices decreased

CRH mRNA expression. An attempt to identify the intercellular communication in a cascade involving AVT, V1aR in glia and CRH mRNA expression in the NHpC revealed that brain derived neurotropic factor (BDNF) and its receptors could be associated in the signaling mechanism. The results of the present study suggest that CRH-ir neurons in the NHpC are in part regulated via V1a-ir glia facilitated by AVT.

Keywords: glia, neurons, vasotocin, CRH, slice culture.

1. Introduction

In birds, major neurohormones involved in activation of the hypothalamo-pituitary-adrenal (HPA) axis are corticotropin releasing hormone (CRH) and arginine vasotocin (AVT). Parvocellular neurons that synthesize the neurohormones are found in the paraventricular nucleus (PVN) of the hypothalamus. Upon activation, CRH and AVT are synthesized in their respective neuronal phenotype and released into the external zone of the median eminence, and transported to the anterior pituitary via the portal system. In the anterior pituitary, they act on corticotropes to release their essential pituitary hormone (adrenocorticotropic hormone) that ultimately stimulates the adrenal glands within the HPA axis. Hypophysiotropic parvocellular neurons present in core region of the PVN respond to a variety of stressors, including hyperosmotic stress (Jaccoby et al., 1997; Sharp et al., 1995), heat stress (Cramer et al., 2015), social stress (Xie et al., 2010, Fokidis and Deviche, 2012) and psychogenic stress (Chapter 2), in order to initiate the stress response.

Recently, in addition to the PVN, an acute stress responsive neural structure was found in the septal region of the avian brain called the nucleus of the hippocampal commissure (NHpC). Previously, the NHpC was termed the nucleus of the pallial commissure or the nucleus of

commissural palli (Puelles et al., 2007). In situ hybridization and immunocytochemical studies using an immediate early gene, *c-fos* or its protein product (FOS), showed that the NHpC responded to acute stressors. Particularly, osmotic stress (Sharp et al., 1995), social stress (Xie et al., 2010), and restraint stress (Chapter 2) were shown to activate cells in the NHpC, in parallel with the classical PVN and thus suggesting an involvement of the two stress responsive neuronal structures in the avian brain (NHpC and PVN), discussed in Chapter 3. Interestingly, immunocytochemical evidence revealed CRH immunoreactive fibers in the NHpC in a variety of avian species, including galliformes (Wang and Millam, 1999; Richard et al., 2005; chapter 2), columbiformes (Péczely and Antoni, 1984) and passeriformes (Goodson et al., 2004). Furthermore, CRH mRNA expression rapidly increased in the NHpC compared to CRH mRNA expression in the PVN (Chapter 3). Hence, CRH in the PVN as well as in the NHpC, likely, contribute to the neuroendocrine stress pathway in birds. However, histochemical characteristics of the CRH cells in the NHpC have not been completely established. Thus, immunocytochemical assays were performed in this study to characterize the morphology of CRH-ir elements in the NHpC.

Studies have shown that the NHpC also contains the vasotocinergic system in chickens. Specifically, vasotocin terminal fields lay in the core structure of the NHpC (Jurkevich et al., 1996). Recently, presence of a specific vasotocin receptor subtype (V1aR) was identified in glia lining the walls of the midline (Selvam et al., 2015) that coexist with CRH-ir neurons (Nagarajan et al., 2016) in the NHpC. Moreover, intracerebroventricular administration of AVT in both roosters and hens increased plasma corticosterone concentrations (Madison et al., 2008), suggesting that AVT could act as a neuromodulator in the brain to augment stress response in birds. Hence, it was hypothesized that the NHpC containing CRH neurons are in part regulated

via V1aR-ir glia, where AVT acts as a neuromodulator. Thus, organotypic cultures were used to determine a plausible intercellular communication between V1aR-ir glia and CRH-ir neurons. To test this hypothesis, we used the natural agonist (AVT) and specific antagonist of V1aR (SR49059) in slice cultures containing the NHpC. Furthermore, the present study also attempted to find a possible neurotransmitter/neuromodulator involved in the V1aR glia – CRH neuron interaction.

2. Materials and methods

2.1. Animal Surgery

Male Cobb 500 birds were used in this study. Colchicine was administered intracerebroventricularly (i.c.v) to block axonal transport and obtain immunoreactive staining within the perikarya of CRH neurons. A problem with the visualization of CRH neurons is that rapid axonal transport of the peptide renders pale to negligible immunoreactivity of CRH perikarya. To overcome this known effect, colchicine has been used to block the transport of CRH from the cell body, allowing perikarya to be identified with CRH (Bloom et al., 1982; Józsa et al., 1984). At 24 d of age, birds were anesthetized with sodium pentobarbital solution (27 mg/kg, *i.v.*), and their heads secured in a stereotaxic instrument in order to implant a guide cannula (Plastic One, Roanoke, VA) at the following coordinates (1.0 mm anterior to the lambda suture and 0.8 mm lateral to midline). The dorsal horn of the lateral ventricle was targeted with a depth of the guide cannula at 3.0 mm from the top of the skull. Following cannulation surgery, birds were allowed to recover for at least 3 d. Cannula position was tested in each bird by recording each birds' dipsogenic response after injection of angiotensin II (80 ng/bird; Sigma Aldrich, St. Louis, MO). Days following the test, colchicine (40 µg/bird) in 8 µl sterile saline

solution, was injected i.c.v (n=3) and 24 h later birds were deeply anesthetized and brain of each bird was perfused with ice cold 0.1M phosphate buffer solution via the carotid arteries followed by perfusion with ice cold Zamboni fixative. Brain samples were then blocked, post fixed overnight, cryoprotected using 30% sucrose solution and stored at -80° C. Brain sections were cut at 40 µm using a cryostat. Sections were collected and the following immunocytochemical procedure was performed. All experimental protocols were approved by the University of Arkansas Institutional Animal Care and Use Committee (#16043).

2.2. Immunocytochemistry

Brain sections were rinsed in 0.02 M phosphate buffer saline (PBS) for several times before incubating them with 0.2% H_2O_2 for 30 min and followed by permeabilization treatment with 0.4% Triton X-100 for 30 min. Sections were incubated in 5% normal goat serum (containing 0.3% Triton X-100 in PBS) and immediately transferred to a primary antibody solution (0.02M PBS with 1% normal goat serum, 0.2% Triton X-100 and 0.05% sodium azide) for 48 h at 4° C. Primary antibodies used in this study are described in Table 1. After incubation sections were rinsed and incubated in secondary antibody solution containing biotinylated goat anti-guinea pig (Vector Laboratories, Burlingame, CA; 1:500 dilution) for 90min. This step was followed by incubation in Avidin biotinylated horseradish peroxidase complex (ABC-HP complex, Vector Laboratories) for another 90 min. Nickel diaminobenzidine (Ni-DAB) was used as a chomogen in a reaction (with 0.02% H_2O_2) resulting in deep blue color staining for CRH. In a different assay, CRH immunofluorescence was performed using a secondary antibody (donkey anti-guinea pig conjugated with DyLight 594; 1:400, Pierce-Thermo Scientific, Rockford, IL). Sections were then washed, mounted on clean glass slides and cover slipped.

In a separate experiment, a dual immunofluorescence assay was also performed for AVT and V1aR in the NHpC. Brains were collected as previously described. Sections containing the NHpC were incubated in 5% normal donkey serum followed by incubation in a cocktail of primary antibodies containing anti-AVP and anti-V1aR for 40 h at 4°C. After incubation, sections were washed and incubated in a cocktail of secondary antibodies containing donkey anti-guinea pig conjugated with DyLight 594 (1:400, Pierce-Thermo Scientific, Rockford, IL) and donkey anti-rabbit conjugated with FITC (1:500, Jackson Immuno Research, West Grove, PA). After several rinses in PBS and distilled water, sections were mounted on glass slides and cover slipped with Vectashield (Vector Laboratories, Burlingame, CA).

2.3. Organotypic culture

The Brain slice culture experiment was performed using brains obtained from 3-5 d old chicks. Birds were cervically dislocated, brains were dissected out of the cranium and placed in ice cold artificial cerebrospinal fluid (aCSF: 130mM NaCl, 3mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2.H_2O$, 26 mM $NaHCO_3$, 1.25mM $NaH_2PO_4.H_2O$ and 10mM D-glucose) saturated with carbogen gas (95% O_2 and 5% CO_2). Brains were then glued (Loctite superglue) to a vibratome stage and immediately covered with ice cold aCSF. The vibratome chamber was maintained in an ice cold condition at all times. Brains were sectioned at 350 µm and horizontal sections were dissected to contain the NHpC and PVN (see Fig 5 in Chapter 3). Tissues (n=2 birds) were then placed on each cell culture insert (Millipore PICM03050) saturated with 1 ml standard culture media (SCM) [50% Eagle basal medium, 25% Hanks Blank salt solution, 25% heat inactivated horse serum, 25U/ml penicillin, 0.5% glucose and 0.5 mM L-glutamine] and maintained at 36° C and 5% CO_2. The procedure was performed in less than one h. Slices were maintained in the culture environment for 9-10 d and culture media was replaced every 2 or 3 d. One d prior to the

treatments, SCM was replaced with serum free media [100% Eagle basal medium, 25U/ml penicillin, 0.5% glucose and 0.5 mM L-glutamine]. Compounds and doses used in this study were as follows: AVT (100 ng, Bachem) and SR49059 (50 ng, Sigma). After 24 h tissues were frozen, collected in Trizol solution and stored at -80° C.

Phenotype of neurons/glial cells and structural integrity of slices were tested using immunofluorescence. Cultured brain slices containing the NHpC were fixed in 4% paraformaldehyde for 2 h. The above described immunofluorescence procedure was performed to determine presence of CRH or V1aR immunoreactivity in slice cultures (see Fig 7).

2.4. RNA Isolation and gene expression assay

Trizol-chloroform (Life Technologies) based RNA extraction procedure was used to extract total RNA from microdissected brain tissues. Total RNA was purified using RNeasy mini kit (Qiagen) and RNA quality of each sample was determined using Synergy HT multi-mode microplate reader (Biotek). Total RNA (200 ng) from each sample was converted into cDNA using SuperScript III reverse transcriptase (Invitrogen). Reverse transcribed product of each sample was mixed with Power SYBR green PCR Master Mix and were amplified using real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with primers listed in Table 2. The best primer set from a set of primer pairs (retrieved using PRIMER3 program) for each gene was selected based on quality of PCR products obtained after electrophoresis (3% agarose) analysis. Real-time RT-qPCR was performed using the following conditions: 1 cycle at 95°C for 5 min and amplified for 40 cycles at 95°C for 30 sec, 56-60°C for 1 min. The chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. At the end of amplification, a melting curve was utilized to eliminate

nonspecific PCR product contamination. Relative gene expression levels of each specific gene were determined by the $2^{-\Delta\Delta ct}$ method (Schmittgen and Livak, 2008).

2.5. Imaging

Fluorescence digital images at 1µm distance were acquired using the Ziess Image M2 microscope (Carl Zeiss Microscopy, LLC., Thornwood, NY) with attached CCD camera (Hamamatsu, Orca ER, Bridgewater, NJ) and Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD). Digital images were then stacked using Image J. Bright field images were obtained using a digital camera (Canon EOS, Rebel T1i) attached to Olympus BX50 microscope (Olympus Optical Co, LTD, Japan). Morphometric analysis was performed using Image-Pro Plus software.

2.6. Statistical Analysis

Data in the results sections are presented as mean ± SEM. One-way analysis of variance was used to measure the overall significant difference in morphometric and gene expression analyses and student's t-test was used for pairwise comparison between treatments. Significance level used in the analysis was $p < 0.05$.

3. Results

3.1. CRH immunoreactive neurons

In the NHpC dense CRH-ir neurons were observed close to the midline and are present dorsal to the preoptic region, anterior hypothalamus and anterior commissure (Fig 1a) and mid-hypothalamic area (Fig 1b). Rostrocaudal extension of CRH-ir neurons in the NHpC began before the anterior commissure at the level A8.6 with few immunoreactive CRH neurons. Rostrally, CRH-ir neurons occupy the dorsal part of the NHpC and a dense cluster of CRH-ir

neurons started to appear at the level of the anterior commissure A8.2, (Fig 1a) and extends caudally to cover a total distance of approximately 1mm where the subseptal organ protrudes into the third ventricle (Fig 1b). The CRH neurons disappear just before the septum separates from the thalamic region.

A comparative analysis of CRH-ir neurons in the NHpC, BSTL and PVN revealed that CRH-ir neurons in these brain regions have distinct morphological differences (Fig 2). In the NHpC CRH-ir neurons are multipolar in nature with two or more processes (Fig 2a), while CRH-ir parvocellular neurons are mostly multipolar in the BSTL (Fig 2b) and bipolar in the PVN (Fig 2c). Perikarya size analysis of CRH-ir neurons showed that cells in the NHpC are significantly larger compared to those in the BSTL and PVN ($p < 0.05$, Fig 2d). The mean diameter of CRH-ir perikarya in the NHpC was found to be around 13-14 μm.

3.2. Vasotocinergic system in the NHpC

The NHpC CRH-ir neurons were found to occur in close proximity to a dense array of AVT-ir terminals (Fig 3). In some brains these terminals are present beginning at the level of the anterior commissure and are observed posteriorly at the level where the septum separates from the thalamic region (Fig 6). In the same coronal planes, glial cells immunoreactive for the V1aR receptors have processes that project horizontally from the brain midline (Fig 3b).

3.3. Effects of AVT on CRH expression in the NHpC

Brain slices, containing the NHpC, treated with AVT for 24 h showed a significant increase in CRH mRNA levels compared to controls treated with serum free media ($p < 0.05$, Fig 4). On the other hand, co-treatment of AVT (agonist) and SR49059 (antagonist) for 24 h significantly decreased CRH mRNA levels with respect to AVT treatment alone (Fig 4).

3.4. Neurotrophic factors in the NHpC

An attempt to identify factors associated with signaling between V1aR in glia and CRH-ir neurons resulted in identification of brain derived neurotrophic factor (BDNF) immunoreactivity in the NHpC (Fig 5a). Based upon immunocytochemical information, gene expression of BDNF was measured in the cultured sample (Fig 5c). Similar to CRH gene expression, BDNF mRNA levels were found increased following AVT treatment for 24 h and a cocktail of AVT and SR49059 downregulated BDNF gene expression levels compared to AVT treatment ($p < 0.05$). Furthermore, presence of the BDNF receptor TrkB was identified in the glia (Fig 5b) and the pattern of immunostaining was similar to that of V1aR-ir glia of the NHpC (Fig 3b).

4. Discussion

4.1. Anatomical implications of CRH neurons in the NHpC

Novel characteristics of CRH-ir neurons in the NHpC involved in the neuroendocrine regulation of stress were investigated in this study. Results from the immunocytochemical and morphometric analysis revealed a distinct morphology of CRH-ir neurons in the NHpC that is different from CRH-ir neurons in other brain regions including the PVN. Previous studies have shown that the NHpC contains CRH immunoreactivity (Péczely and Antoni, 1984; Wang and Millam, 1999; Richard et al., 2004; Goodson et al., 20004), however, use of colchicine was needed to visualize clear immunoreactivity within perikarya. As a result, these CRH-ir neurons were found to be large, oblong and multipolar in nature covering a significant rostral-caudal distance (1 mm) from the preoptic region until the separation of the septum from thalamus. Further, these neurons occur dorsal to CRH-ir neurons in the PVN and contain vasotocin

terminals (Fig 3). Concomitantly, the present study concurs with the morphology of CRH-ir neurons in other brain structures, particularly in the BSTL and PVN that have been described in earlier studies (Józsa et al., 1984; Yamada and Mikami, 1985; Kovács et al., 1989; Ball et al., 1989; Richard et al., 2004).

4.2. Functional implications of CRH neurons in the NHpC

Results from the current study suggest that AVT modulates activity of CRH-ir neurons in the NHpC via V1a-ir glial cells. Past studies identified V1aR immunoreactive glia coexisting with CRH-ir neurons in the NHpC (Nagarajan et al., 2016) and AVT-ir terminals occur in the entire rostrocaudal extent of the NHpC. However, the origin of these AVT-ir terminals remains unknown. Using sequential sections past immunocytochemical studies traced the origin of these AVT-ir terminals from the mBST AVT-ir neurons (Jurkevich et al., 1996), while a tract tracing method revealed that neurons could be traced from the medial preoptic nucleus (Berk and Butler, 1981; Balthazart et al., 1994; Riters and Alger, 2004) and PVN (Berk and Finkelstein, 1983), whose phenotypic natures are not known. However, vasotocin terminals in the NHpC could possibly originate from the medial preoptic nucleus or the PVN for the following reason. Vasotocin nerve terminals observed in the NHpC appear to originate from AVT-ir magnocellular neurons located in the periventricular hypothalamic nucleus that occurs in close proximity to the medial preoptic nucleus (Fig 6) whose neuronal connection were traced to end in the NHpC. Irrespective of the origin of AVT-ir terminals, we postulated that these terminal fields release AVT resulting in subsequent V1a glia - CRH neuronal interaction within the NHpC. This possibility is demonstrated in this *in vitro* study where AVT (an endogenous agonist of V1aR) increased CRH gene expression in slices containing the NHpC. Support for this finding is further substantiated from utilizing an antagonist to V1aR that blocked the stimulating effect of

AVT on CRH gene expression. This result is consistent with reduced FOS cells observed in the NHpC following intracerebroventricular administration of the same antagonist (Nagarajan et al., 2016). Thus, AVT appears to act as a neuromodulator in the NHpC to facilitate activity of CRH neurons.

Apart from activation of CRH by AVT shown in this in vitro study and by AVP from other studies (Bernardini et al., 1994), the nonapeptide was found to induce CRH release though V1 receptor subtypes from adrenal gland cells (Mazzocchi et al., 1997). Thus, AVT/AVP could affect the expression and release of CRH though V1 receptors, both centrally and peripherally. However, the mechanism though which AVP induces CRH secretion from adrenal medulla (Mazzocchi et al., 1997) or V1 receptors present in the PVN of the murine hypothalamus (Bernardini et al., 1994) is likely though direct action of AVP on V1 receptors present on the cell membrane. In contrast, we observed V1a receptors are present in glia and not on CRH-ir neurons in the NHpC. Hence, we attempted to identify a factor that could be involved in signaling the information from V1aR-ir glia to CRH-ir neurons. Interestingly, we found that gene expression of BDNF occurs in parallel to CRH gene expression in cultured slices. Moreover, immunoreactivity of BDNF and its receptors TrkB were found in the NHpC. However, the latter was found only in glia lining the midline, while the former was found in few glia lining the midline as well as within cells in dorsal region of the NHpC (Fig 5a and 5b). Available evidence shows that AVP and its potent analog are effective in inducing BDNF mRNA levels (Zhou et al., 1997). Additionally, BDNF expression increased by osmotic stress (Castren et al., 1995) and restraint stress (Givalois et al., 2004) suggest that BDNF is also involved in the regulation of the stress pathway. Thus, BDNF could be involved in glia-neuron communication in the NHpC, although this possibility warrants further investigation.

4.3. Possible roles of CRH in the NHpC within the avian brain

The NHpC appears to integrate neural and humoral signals, since it receives projections from several forebrain and brain stem structures as well as posterior extent of the NHpC contains a circumventricular organ. Having reciprocal interseptal connections, (e.g. to and from the lateral and medial septum (Atoji and Wild, 2004; Montagnese et al., 2008)), it is possible that the NHpC could play a role in the stress pathway associated with social behavior. For example, when roosters display dominance-subordinate behavior and mating behavior an increase in FOS cells was observed in the NHpC and lateral septum suggesting that the stress component of the NHpC could possibly be influenced by the social behavior network (Xie et al., 2010; Goodson, 2004; O'Connell and Hofmann, 2011). Likewise, past tract tracing methods demonstrated direct projections from the PVN to the NHpC (Berk and Finkelstein, 1983), where the former nucleus is known to be involved in endocrine as well as autonomic functions, could also contribute to activation of the NHpC CRH neurons. Furthermore, the NHpC also receive projections from the nucleus of the solitary tract and the dorsal motor nucleus of the vagus (Arends et al., 1988; Montagnese et al., 2008) implying that visceral sensory information could be relayed to the NHpC. Close structural proximity of the subseptal organ, a circumventricular organ, to the NHpC also implies a possible role in physiological homeostasis. Finally, based on the results from the present study it is possible to suggest that the NHpC is in part modulated by the vasotocinergic system. Thus, CRH neurons in the NHpC appear to respond to multiple stimuli, where the vasotocinergic system could be associated with providing physiological stimuli to the NHpC.

In conclusion, the present study showed that 1) morphometric data of CRH-ir neurons in the NHpC showed that their perikarya are larger than CRH-ir neurons present in the PVN and 2)

AVT-ir terminals and V1aR-ir glia occur within the NHpC where AVT acts as a neuromodulator to increases CRH mRNA levels by modulating CRH neuronal activity. Overall, the results from the study suggest that CRH-ir neurons in the NHpC are in part regulated by AVT via a glial-neuronal interaction.

Table 1. Primary antibodies used for immunocytochemical analysis.

Antibody	Dilution	Source
Guinea pig anti-CRH	1:2000	T-5007, Bachem.
Rabbit anti-V1a	1:600	RRID:AB_2336062, Selvam et al., 2015
Guinea pig anti-AVP	1:10000	T-5048, Bachem.
Rabbit anti-BDNF	1:600	AB1534SP, EMD Millipore Corp.
Rabbit anti-TrkB	1:500	sc-12, Santa Cruz Biotechnology, Inc.

Table 2. Primer sets used for gene expression analysis.

Gene	Genbank ID	Forward/Reverse primers	Product size
AVT	NM_205185	F- 5'CCTTCCCCGAACGCATAG3' R-5'GGGCAGTTCTGGATGTAGCAG3'	117bp
CRH	NM_001123031	F-5'GCCCACAGCAACAGGAAAC3' R-5'GTGATGGCTCTGGTGCTGAC3'	98bp
GAPDH	NM204305	F-5'CTTTGGCATTGTGGAGGGTC3' R-5'ACGCTGGGATGATGTTCTGG3'	128bp
BDNF	NM_001031616	F-5'GACATGGCAGCTTGGCTTAC3' R-5'GTTTTCCTCACTGGGCTGGA3'	167bp

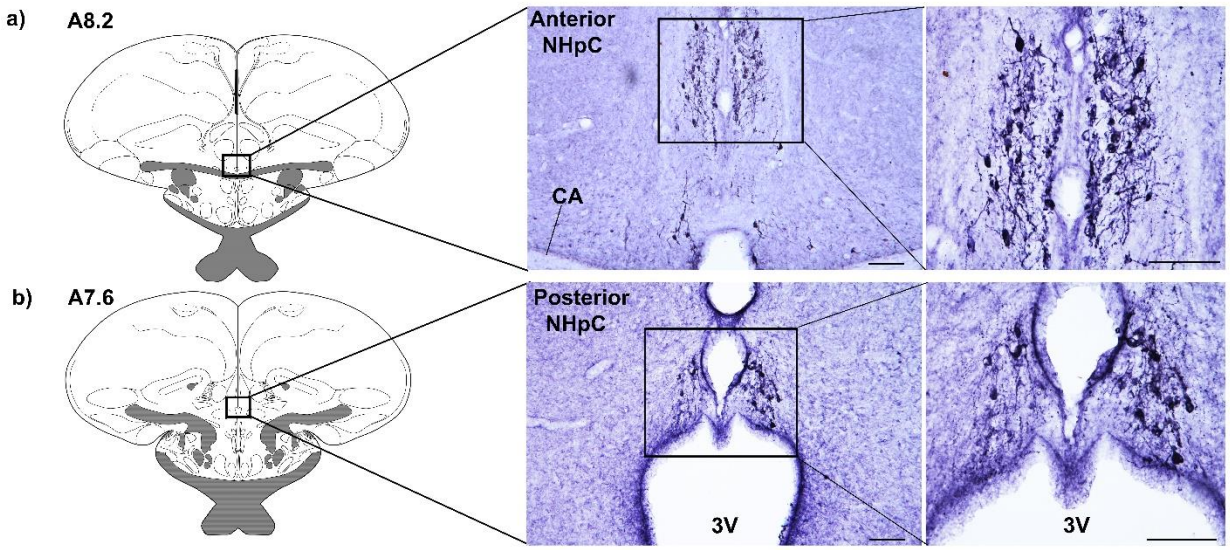

Fig. 1. Corticosterone releasing hormone immunoreactive neurons present in the nucleus of the hippocampal commissure (NHpC) of the chick brain after colchicine injections. a) Dense populations of CRH-ir neurons are present in the dorsal region of the NHpC from the level of the anterior commissure (atlas plate A8.2) until b) atlas plate A7.6 just before the septum separates from the thalamus. CA – anterior commissure, 3V – third ventricle. Scale bar – 100 µm.

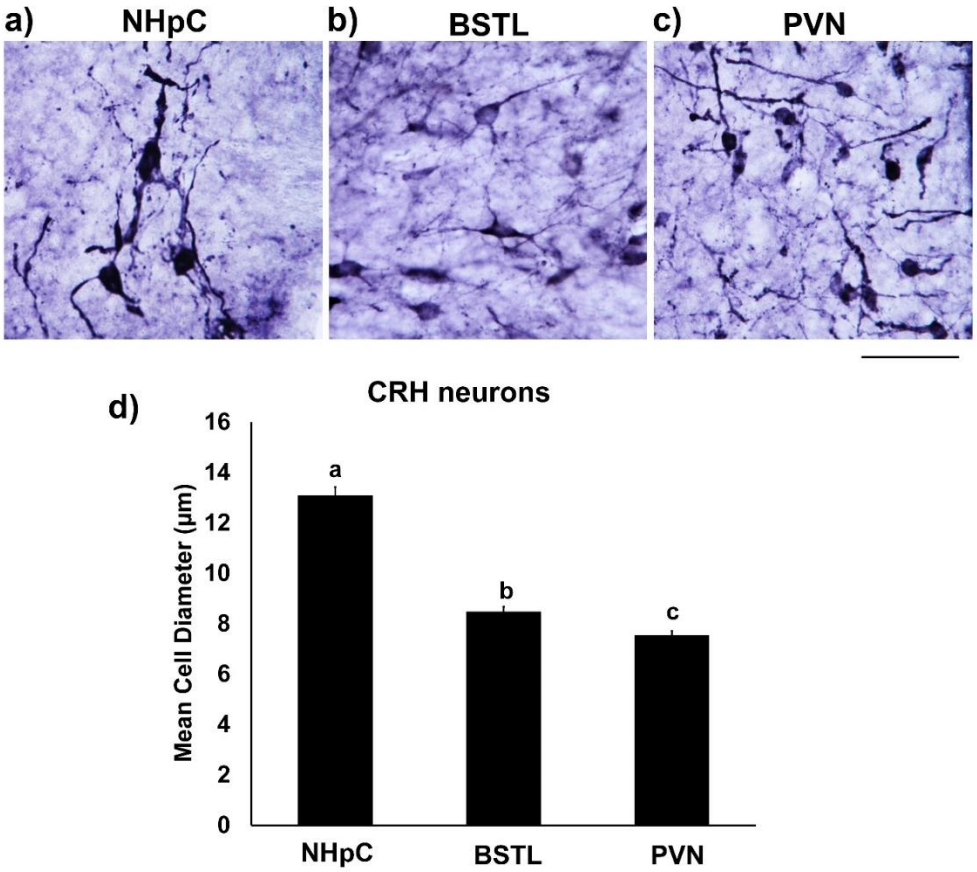

Fig. 2. Comparative analysis of CRH-ir neurons in the nucleus of the hippocampal commissure (NHpC), lateral bed nucleus of the stria terminalis (BSTL) and paraventricular nucleus (PVN). a) Neurons in the NHpC are their perikarya are multipolar, while neurons in the BSTL b) and PVN c) are multipolar and bipolar, respectively. Scale bar - 50 µm. d) Graph showing differences in perikarya size of CRH-ir neurons in the NHpC, BSTL and PVN (n = 100 neurons per region of interest). Different letters in the histogram represent significant differences among the cell groups.

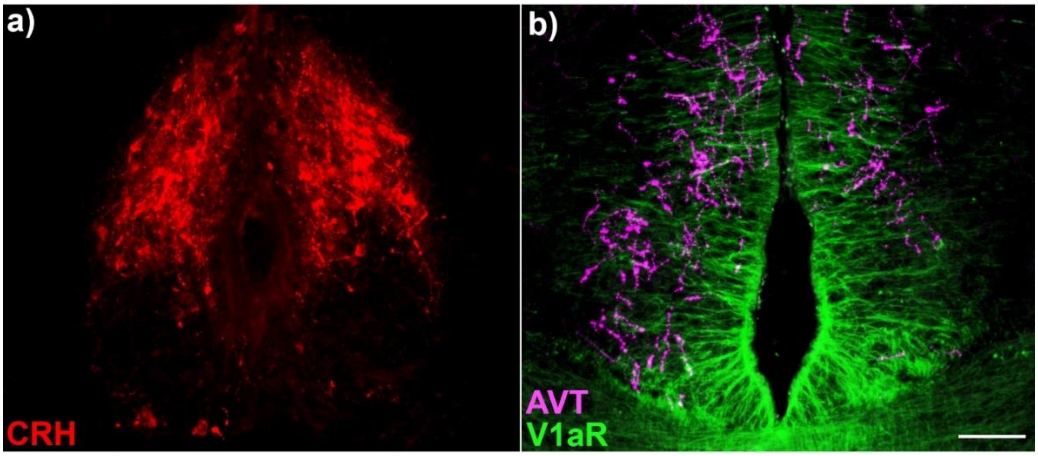

Fig. 3. Presence of the vasotocinergic system in close proximity to CRH-ir neurons in the nucleus of the hippocampal commissure (NHpC). a) CRH-ir neurons (red) in the dorsal region of the NHpC. b) Vasotocin immunoreactive terminals (magenta – pseudo color) and V1a receptor immunoreactivity (green) in glial cells lining the midline represented at the same plane as a). Scale bar – 100 µm.

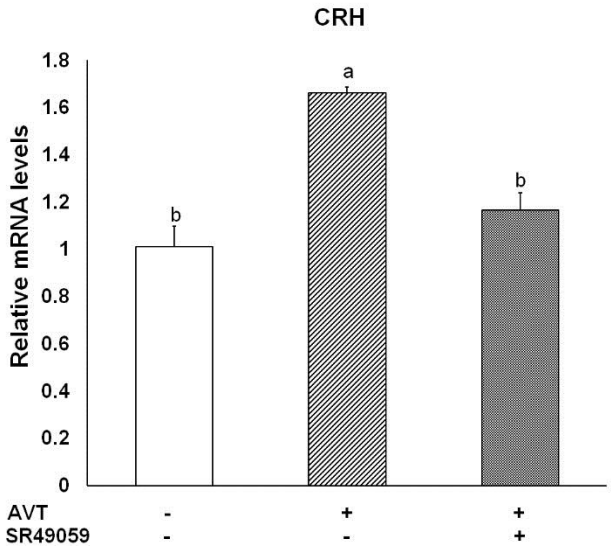

Fig. 4. Effect of vasotocin (AVT) and vasotocin 1a (V1a) receptor antagonist (SR49059) in slices containing the nucleus of the hippocampal commissure. Relative expression of CRH mRNA levels are shown after AVT treatment or a combination of AVT and SR49059 for 24 h compared to controls. n = 6. Different letters in the histogram represent significant differences among the treatment groups.

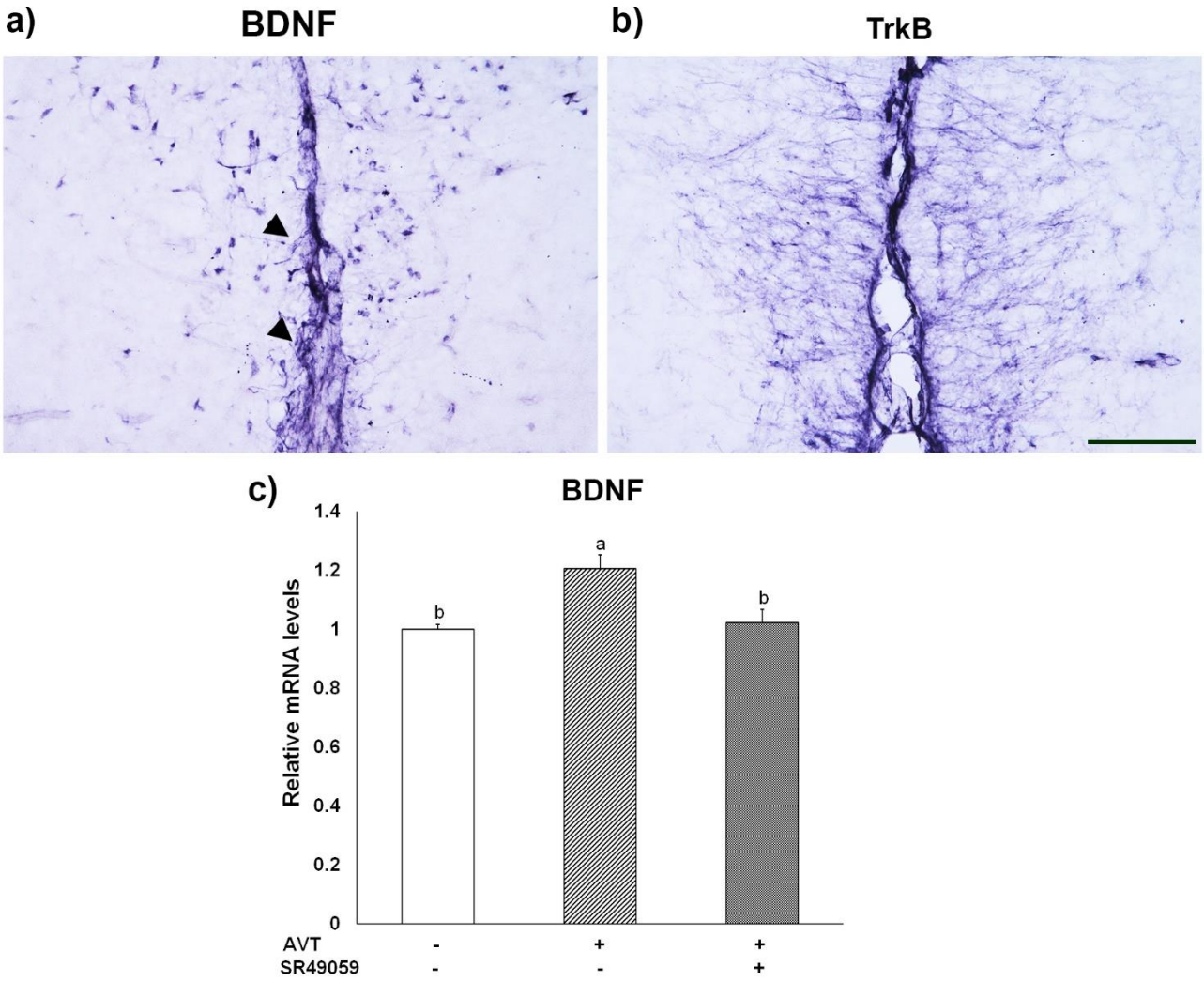

Fig. 5. Characterization of brain derived neurotrophic factor (BDNF) in the nucleus of the hippocampal commissure (NHpC). a) Localization of BDNF in glia lining the brain midline (arrow heads) and in glia dispersed in the dorsal region of the NHpC. b) Localization of BDNF receptor TrkB in glia lining the midline. Scale bar - 100 µm. c) Effect of vasotocin (AVT) and vasotocin 1a (V1a) receptor antagonist (SR49059) on gene expression of BDNF in slices containing the NHpC for 24 h. n=6. Different letters in the histogram represent significant differences among the treatment groups.

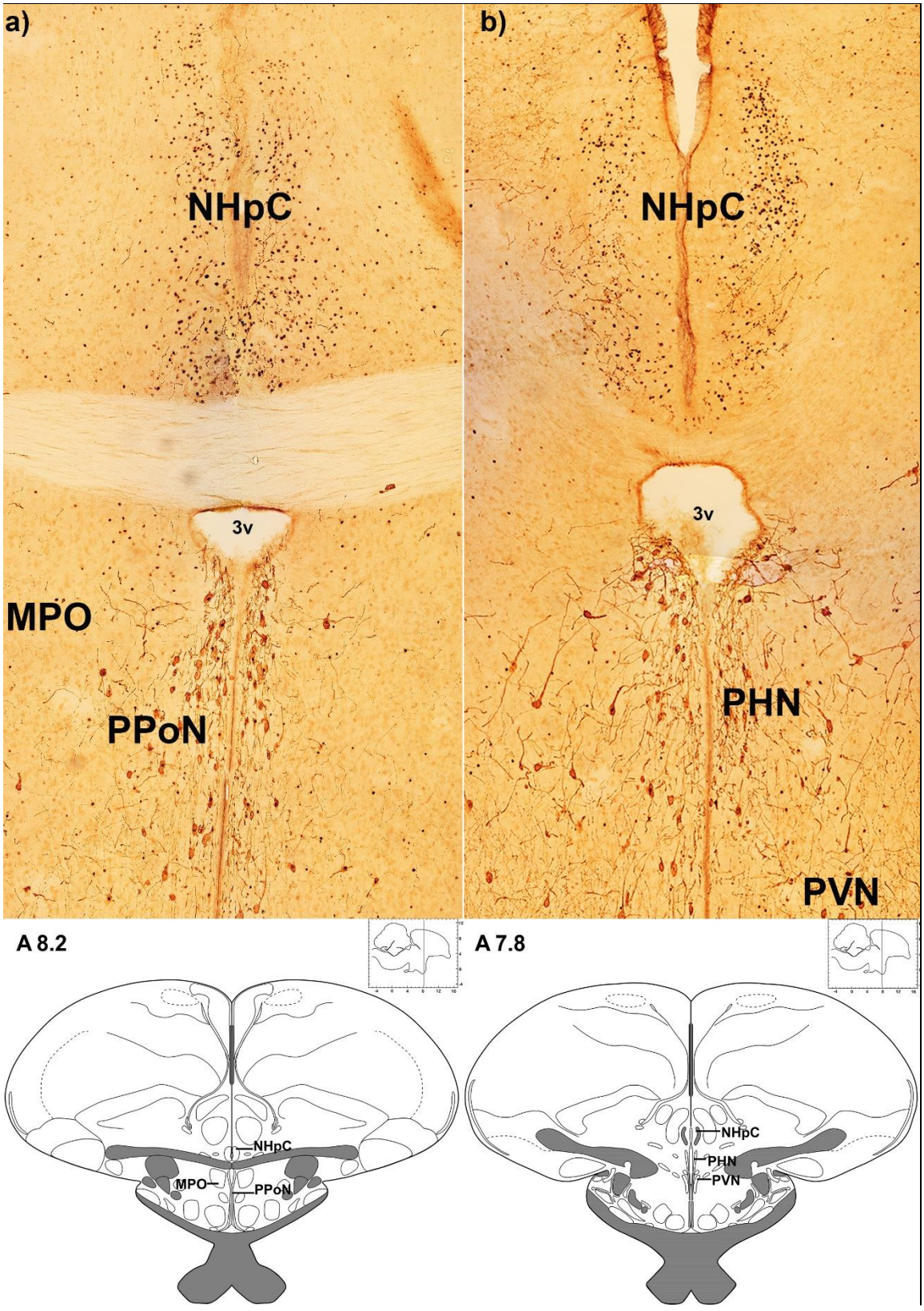
a)
NHpC
3v
MPO
PPoN
b)
NHpC
3v
PHN
PVN
A 8.2
NHpC
MPO
PPoN
A 7.8
NHpC
PHN
PVN

Fig. 6. Possible projections of vasotocin neurons from the periventricular region (PPoN/PHN) into NHpC. a) Represents an anterior section at the level A8.2 and b) represents a posterior section at the level A7.8. Diaminobenzidine was used as a chomogen to stain vasotocin-ir neurons (brown color) in the PPoN, PHN and PVN. Vasotocin-ir terminals can also be seen in the NHpC along with FOS-ir cells (black – Ni-DAB stain). Abbreviations: NHpC - the nucleus of the hippocampal commissure, MPO – medial preoptic nucleus, PPoN – periventricular preoptic nucleus, PHN – periventricular hypothalamic nucleus, PVN – paraventricular nucleus and 3v – third ventricle.

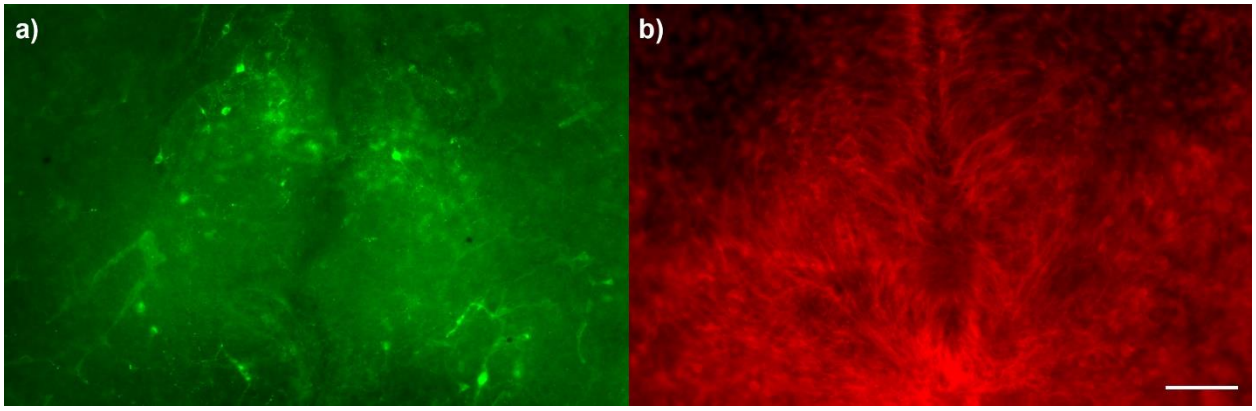

Fig. 7. Immunostaining for (a) CRH and (b) V1a receptors were performed in slices cultured for 9-10 d to test viability and presence of CRH-ir neurons and V1a-ir glia in the NHpC. Image J was used to stack fluorescence digital images at 2 µm interval.

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Chapter 5

Diencephalic and septal structures containing V1a receptors involved in the regulation of food intake.

A part of this chapter was published in "Nagarajan, G., Jurkevich, A., Kang, S. W., & Kuenzel, W. J. (2016). Diencephalic and septal structures containing the avian vasotocin receptor (V1aR) involved in the regulation of food intake in chickens, *Gallus gallus*. Physiol. Behav. 164, 268-276."

Abstract

Recently, it was found that the avian central vasotocin receptor (V1aR) is associated with the regulation of food intake. To identify V1aR-containing brain structures regulating food intake, a selective V1aR antagonist SR-49059 that induced food intake was administrated intracerebroventricularly in male chickens followed by detection of brain structures using FOS immunoreactivity. Particularly, the hypothalamic core region of the paraventricular nucleus, lateral hypothalamic area, dorsomedial hypothalamic nucleus, a subnucleus of the central extended amygdalar complex [dorsolateral bed nucleus of the stria terminalis], medial septal nucleus and caudal brainstem [nucleus of the solitary tract] showed significantly increased FOS-ir cells. On the other hand, the supraoptic nucleus of the preoptic area and the nucleus of the hippocampal commissure of the septum showed suppressed FOS immunoreactivity in the V1aR antagonist treatment group. Further investigation revealed that neuronal activity of arginine vasotocin (AVT-ir) magnocellular neurons in the supraoptic nucleus, preoptic periventricular nucleus, paraventricular nucleus and ventral periventricular hypothalamic nucleus and most likely corticotropin releasing hormone (CRH-ir) neurons in the nucleus of the hippocampal

commissure were reduced following the antagonist treatment. Dual immunofluorescence labeling results showed that perikarya of AVT-ir magnocellular neurons in the preoptic area and hypothalamus were colabeled with the V1aR. Within the nucleus of the hippocampal commissure, CRH-ir neurons were shown in close contact with V1aR-ir glial cells. Results of the present study suggest that the V1aR plays a role in the regulation of food intake by modulating neurons that synthesize and release anorectic neuropeptides in the avian brain.

Keywords: vasotocin, SR-49059, antagonist, magnocellular neurons, hypothalamus.

1. Introduction

Behavioral responses in both mammals and birds are determined by internal and external cues (Paul et al., 2008; Wingfield, 2008) and sensory integration is necessary in order to effect appropriate behavioral responses that ensure the survival of individuals. Foraging and food intake are behavioral responses initiated by deficiency of energy and nutrients (Krikwood, 1991; Schwartz et al., 2000; Richard, 2003). The basic food intake response is perturbed during stress that has a significant effect on growth. Food intake and stress responses are regulated by neurohormones and/or neuromodulators that are synthesized and released by neurons in the hypothalamus of vertebrates (Johnson et al., 1992; Kalra et al., 1999). Several neurohormones are involved in the regulation of stress (Johnson et al., 1992; Pacak and Palkovits, 2001) and some of them, such as corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and pro-opiomelanocortin, are known for their anorectic effect (Kalra et al., 1999; Kuenzel, 1994; Leibowitz and Wortley, 2004).

In birds, arginine vasotocin (AVT), homologous to mammalian AVP, is a neurohormone that regulates several physiological and behavioral responses (Sturkie and Lin, 1966; Kihlstrom

and Danninge, 1972; Braun and Dantzler, 1974; Arad and Skadhauge, 1984; Voorhius et al., 1991) and its functions have been extensively reviewed (Goodson and Bass, 2001; Jurkevich and Grossmann, 2003; Cornett et al., 2013). Vasotocin containing neurons are almost exclusively present in the preoptic area and hypothalamus including the supraoptic nucleus (SO), preoptic periventricular nucleus (PPoN), paraventricular nucleus (PVN), periventricular hypothalamic nucleus (PHN) and with few exceptions including dorsolateral thalamus, nucleus of the stria terminalis, and perirotundic area (Panzica et al., 1988; Jurkevich et al., 1997). Both parvocellular and magnocellular AVT-ir neurons are involved in neuroendocrine regulation and upon their activation AVT is released either into the median eminence (from parvocellular AVT neurons) or the neurohypophysis (from magnocellular neurons). Similar to AVP in mammals, AVT regulates the hypothalamic-pituitary-adrenal (HPA) axis (Castro et al., 1986; Romero et al., 1998; Kuenzel and Jurkevich, 2010) and is involved in physical, social and psychogenic stress (Sharp et al., 1995; Jaccoby et al., 1997; Goodson and Evan, 2004; Chapter 2). Vasotocin released in response to stress has a direct effect on the HPA and augments the effect of stress along with CRH (Castro et al., 1986; Romero et al., 1998; Madison et al., 2008). Interestingly, both endogenous and exogenous analogs of AVP and AVT have been reported to suppress food intake in both mammals (Meyer et al., 1989; Ikemiura et al., 2004) and birds (Tachibana et al., 2004; Masunari et al., 2016), respectively. Thus, AVT acts as a stress hormone as well as an anorectic agent. Furthermore, rodent studies have shown that food deprivation for an extended period of time significantly reduced AVP expression in the hypothalamus (Osaga et al., 1991; Burlet et al., 1992). Hence, AVT may play a short term role in the central regulation of food intake as an anorectic agent and during a long period of food deprivation, AVT expression is expected to be suppressed.

Although, AVT is released via its axonal terminals in the median eminence or the neurohypophysis, the nonapeptide is also found to be released within the brain (Landgraf et al., 1999) to modulate central responses. This concept concurs with the effects induced from central administration of analogs of AVT (Voorhuis et al., 1991; Tachibana et al., 2004; Masunari et al., 2016). Specific functions of AVT in birds are mediated though its four known receptor types. Depending on localization, each receptor type has distinct functions ranging from osmoregulation to reproduction and social bonding to stress (Kelly et al., 2011; Cornett et al., 2013; Grozhik et al., 2014). Behavioral and physiological roles of the nonapeptide have been extensively studied though the application of agonists or antagonists to specific receptors or using knockout animal models and related techniques (for review see Koshimizu et al., 2012).

Several lines of evidence suggest that food intake in mammals is mediated though V1a receptors (Ikemura et al., 2004; Langhans et al., 1991; Aoyagi et al., 2009). In birds, a specific function of vasotocin receptors in the regulation of food intake remains controversial. Two lines of evidence support the avian central V1aR involvement in food intake. Specifically, the avian V1aR and not V1bR (previously termed the VT4R and VT2R, respectively; for the change in receptor nomenclature see reference Kuenzel et al., 2016) was reported in the chicken brain (Jurkevich et al., 2005; Selvam et al., 2015) including strong V1aR immunoreactivity in glial cells surrounding circumventricular organs and moderate to weak V1aR immunoreactivity in diencephalic structures such as the SO and ventral PHN (Selvam et al., 2015). Hence, neurons within these structures containing V1aR immunoreactivity are likely involved in the regulation of food intake. Secondly, intracerebroventricular (*i.c.v.*) administration of a selective V1aR antagonist (SR-49059) (Serradeil-Le Gal et al., 1993; Jayanthi et al., 2014) not only attenuated stress levels but also increased food intake in chicks (Kuenzel et al., 2016). It was based on this

assumption that anorectic neurohormones, such as CRH and AVT, associated with the initiation of the stress response could be blocked by the antagonist resulting in an increase in food intake. Moreover, *i.c.v.* administration of a V1aR antagonist also augmented food intake above that induced by neuropeptide Y (NPY) further supporting the premise that anorectic effects of stress neurohormones could be blocked by the V1aR antagonist to facilitate the additional increase in food intake (Kuenzel et al., 2016).

Therefore, a study was conducted to identify V1aR containing brain structures associated with the regulation of food intake. Thus the central role of the V1aR on food intake was examined using *i.c.v.* administration of SR-49059 followed by FOS immunoreactivity in brain structures. As a positive control, neuropeptide Y (NPY), a potent orexigenic peptide in vertebrates (Levin and Morley 1984; Kuenzel et al., 1987), was used in order to compare brain structures associated with the regulation of food intake in birds. Furthermore, in the present study because of the changes observed in FOS-ir cell counts following *i.c.v.* administration of a V1aR antagonist, dual immunohistochemistry was also performed to identify the signature of neuronal phenotypes. Finally, in a separate experiment, to determine whether FOS cells were activated as a consequence of food intake following *i.c.v.* administration of the V1aR antagonist, food deprived and refed birds were sampled for FOS immunocytochemistry and blood chemistry.

2. Materials and methods

Day-old male Cobb 500 chicks were obtained from a commercial hatchery and raised in brooder cages set at 32° C with a weekly 2.5°C reduction in temperature until 21°C was reached and maintained until the end of the experiment. Birds had access to a standard chick starter feed

(22% protein, 3100 kcal/kg metabolizable energy) and water ad libitum. After two weeks of age, birds with similar body weight were randomly selected for the study. All of the procedures in experiments were approved by the University of Arkansas Institutional Animal Care and Use Committee (#16043).

2.1. Intracerebroventricular injections

At 4 weeks of age, a stainless steel cannula was implanted into the lateral ventricle of each bird as previously described (Chapter 2, Materials and Methods). Birds were allowed to recover for at least 4 d and the cannula position in each bird was tested with an *i.c.v.* dose (80ng) of angiotensin II (Human ANG II, Sigma Aldrich, St. Louis, MO), a potent dipsogen (Maney and Wingfield, 1998). Birds that displayed binge drinking behavior within 3 min were randomly selected and placed in individual cages for the study. Later, birds were injected with one of the thee treatments: 1) control (physiological saline 0.9%), 2) V1aR antagonist SR-49059 (250 ng/bird) or 3) NPY (4 µg/bird). Doses of SR-49059 (Sigma-Aldrich, St. Louis, MO) and NPY (Bachem, Torrance, CA) were determined in a previous study (Kuenzel et al., 2016). Sterile physiological saline was used as the diluent for preparing SR-49059 or NPY and were administered *i.c.v.* in 8 µl volume over a minute period (n=5/treatment). Following *i.c.v.* administration, birds were returned to their home cages with feeder removed and their behavior was video recorded for 1 h using a Sony HD PJ430V camcorder. Thereafter, birds had access to food for 1 h. Birds were then anesthetized with sodium pentobarbital solution (27 mg/kg, *i.v.*), perfused via carotid arteries using ice cold 0.1 M phosphate buffer (PB) and ice cold Zamboni fixative. The calvarium of each skull was removed and brains were blocked using a stereotaxic instrument (Kopf, Tujunga, CA). The blocked brains were removed from the skull and placed in the same fixative overnight. Thereafter, brains were transferred to a sucrose solution (30% in

0.1M PB) until they sank. After saturation each sample was removed from the sucrose solution and stored at -80° C until sectioned.

2.2. Behavioral assessments

Video records were used to manually score the following behavioral parameters during the first h: (1) foraging or frequency of food searching behavior, (2) resting bouts, and (3) amount of time spent drinking water. Because water was provided though drinkers attached to the water lines passing though all cages, volume of water consumed by each bird could not be recorded. During the second h of post-injection, food intake was measured to the nearest 0.1g.

2.3. Food deprivation and refeeding

At 5 weeks of age, birds were randomly selected for the following treatment groups 1) controls on a diet fed ad libitum (Control), 2) 24 h of food deprivation (24 h FD) and 3) 1h refeeding after 24 h of food deprivation (24 h FDR) (n=7-8/ group). Blood samples were collected from the brachial vein of each bird for measurements of plasma osmolality and glucose levels. Brain samples were collected as described before.

2.4. Immunocytochemistry

Brains were sectioned in the coronal plane at 40µm thickness and sections were stored at -20°C in ethylene glycol based cryoprotective solution (Watson et al., 1986) until use. Immunocytochemistry for FOS was performed as described (Chapter 2, Material and Methods). Because of changes observed in FOS-ir cell counts in the SO, dual FOS/AVT immunocytochemistry was also performed. Briefly, sections were rinsed several times in 0.02 M phosphate buffered saline (PBS) and treated with 0.2% hydrogen peroxide solution to block peroxidase activity, followed by treatment with 0.4% TritonX-100 for 20 min. Sections were incubated in 5% normal goat serum for 30 min and followed by anti-FOS primary antibody raised in rabbit (sc-253, LOT#C2112, Santa Cruz Biotechnology; 1:3000) for at least 24 h at

4°C. Subsequently, sections were incubated in goat anti-rabbit secondary antibody (Jackson Immuno Research, 1:500) for 90 min. Sections were then incubated in Avidin-biotin complex-horse radish peroxidase (1:5) for another 90 min. Dark-blue staining of FOS was obtained using the nickel-DAB chomogen method. Sections were rinsed in PBS between incubations. Selected sections were incubated with rabbit anti-AVT (1:10000, a gift from Dr. Gray) for at least 48 h and the above sequence repeated to immunostain AVT neurons. DAB was used as the chomogen in order to stain arginine vasotocin neurons brown.

Quantification of FOS-ir cells in defined brain structures were performed using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD). Three to four alternate sections were chosen for each brain structure per bird selected for quantification. The number of FOS-ir cells and/or FOS + AVT double labeled cells were bilaterally quantified in defined areas in each section, and the mean number of immunoreactive cells per structure per bird was calculated.

Due to changes in FOS-ir cell counts observed in the SO and the nucleus of the hippocampal commissure (NHpC) dual immunofluorescent labeling was also performed for AVT/V1aR and CRH/V1aR, respectively. Sections were rinsed in 0.02M PBS and then treated with 0.4% TritonX-100 for 20 min. They were then incubated in a blocking solution (5% normal donkey serum, 0.3% TritonX-100 in PBS) and immediately transferred to a cocktail solution of primary antibodies containing a rabbit polyclonal antibody to chicken vasotocin receptor and guinea pig anti-AVP (1:8000, T-5048, Lot A03607, Bachem, Torrance, CA), in 0.02M PBS with 1% normal donkey serum, 0.2% Triton X-100 and 0.05% sodium azide. After incubation for at least 40 h at 4°C or overnight at room temperature sections were washed and transferred to a cocktail solution of secondary antibodies containing donkey anti-rabbit IgG conjugated with DyLight 594 (1:400, Pierce-Thermo Scientific, Rockford, IL) and donkey anti-guinea pig IgG

conjugated with fluorescein (FITC) (1:400, Jackson Immuno Research, West Grove, PA). In order to visualize CRH-ir cell bodies in the NHpC, colchicine was administered *i.c.v.* as described in Chapter 2. An immunocytochemical procedure was performed using a cocktail of guinea pig anti-CRH (1:2000, T-5007, Bachem, Torrance, CA) and chicken vasotocin receptor antibodies. Secondary antibodies used were a cocktail of donkey anti-guniea pig IgG conjugated with Cy3 and donkey anti-rabbit IgG conjugated with FITC (1:500, Jackson Immuno Research, West Grove, PA). After completion of each staining procedure sections were washed in PBS, mounted on glass slides and cover slipped using Vectashield (Vector Laboratories, Burlingame, CA).

2.5. Imaging

Bright field and dual fluorescence digital images in Fig. 3, 4, 5a and 5b were acquired using a Zeiss Imager M2 microscope (Carl Zeiss Microscopy, LLC., Thornwood, NY) with attached CCD camera (Hamamatsu, Orca ER, Bridgewater, NJ) and Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD). Adobe Photoshop CS6 was used to adjust brightness and contrast of digital images.

2.6. Plasma osmolality and glucose

Plasma osmolality was measured using the freezing point depression method (Model 3250 Osmometer, Advanced Instruments, Inc). Plasma glucose was determined using Express Plus Biochemistry Analyzer (Ciba Corning Diagnostics Corp.). All samples were assayed in duplicate.

2.7. Statistical analysis

For behavioral analysis and FOS+AVT quantification one way analysis of variance was used to determine if an overall effect of the treatments was significant and a Tukey HSD post hoc test or student's t-test was used to do a pairwise comparison between treatment groups. All data are presented as mean ± SEM and the significance level utilized was $p < 0.05$.

3. Results

3.1. Behavior

A significant treatment effect on food intake was observed in birds subjected to *i.c.v.* injection, $F_{2,14} = 5.33$, $p<0.02$ (Fig. 1). Birds injected with the V1aR antagonist SR-49059 showed increased food intake compared to saline injected birds ($p<0.04$). A more pronounced feeding response was observed following NPY administration compared to controls ($p<0.006$).

The amount of time spent by each bird drinking water was determined along with two other behavioral measures (Table 1). There was a significant effect of treatments on the amount of time spent drinking water before the presentation of food (pre-prandial) ($F_{2,11}=6.05$, $p<0.02$), however, the effect of treatment after access to food (prandial) was not significant ($F_{2,10}=1.27$, $p=0.33$). Interestingly, both SR-49059-injected and NPY-injected birds showed an increase in drinking time prior to presentation of food when compared to controls. On the other hand, after access to food (prandial), neither the SR-49059 nor the NPY-injected birds showed a significant increase in their drinking response when compared to controls.

The number of resting bouts showed no significant difference among the treatment groups ($F_{2,11}=0.08$, $p=0.91$), however, birds rested less in the absence of food when compared to time during the presence of food (Table 1). The effects of treatment on frequency of food searching behavior was found to be significantly different ($F_{2,10}=8.75$, $p=0.009$). In the absence

of food, frequency of food search after central injection of SR-49059 was not significantly different compared to controls (p=0.06). In contrast, NPY administered birds showed significantly increased frequency of food searching behavior (Table 1) when compared to controls (p=0.0078).

3.2. Quantification of FOS-ir cells

Quantification of FOS-ir cells was performed in different structures of the brain following central administration of the SR-49059, NPY or saline to identify structures activated or attenuated by each treatment. Cell counts are summarized in Table 2 and defined brain structures with FOS-ir cells were quantified are shown in the schematic diagram (Fig. 2).

3.2.1. Supraoptic nucleus (SO)

Number of FOS-ir cells in the SO, which contains AVT magnocellular neurons, showed significant differences among the treatment groups ($F_{2,11}$=4.2, $p<0.05$) and FOS-ir cell count was significantly lower in SR-49059-treated birds compared to controls ($p<0.05$) and the NPY treatment group ($p<0.05$). Dual immunohistochemical staining for FOS and AVT further revealed a significant reduction in colocalization of FOS in AVT-ir magnocellular neurons following SR-49059 treatment (Fig. 3a-c).

3.2.2. Avian central amygdalar complex and medial amygdala

Among different sub-divisions of the avian central extended amygdalar complex, (CEA) (Vicario et al., 2014), FOS-ir cells with clear boundaries were observed in the dorsal bed nucleus of stria terminalis (BSTLd), central capsular nucleus (CeC) and oval central amygdalar nucleus (Ceov). A significant difference was observed in the BSTLd ($F_{2,11}$ =9.96, p=0.005), but not in the CeC ($F_{2,11}$=0.03, p=0.96) or the Ceov ($F_{2,11}$=1.64, p=0.24), Table 2. Both SR-49059 and NPY administered birds showed a significant increase in FOS-ir cells in the BSTLd compared to

controls ($p<0.01$ and $p<0.05$, respectively). No significant difference was observed among the treatments in the subpallial medial amygdala (MeAs) ($F_{2,11}=1.92$, $p=0.2$).

3.2.3. Septum

Septal regions are often associated with social behavior (Xie et al., 2010; Kelly et al., 2011) and AVT-ir innervation is especially prominent in the lateral septum of males (Jurkevich et al., 1997).

3.2.3.1. Nucleus of Hippocampal Commissure (NHpC): The NHpC is located at the midline directly dorsal to the third ventricle, a circumventricular organ and hypothalamus. Significant differences occurred among experimental groups ($F_{2,11}=10.88$, $p=0.004$). Specifically, the SR-49059 treated group showed significantly reduced number of FOS-ir cells compared to both controls ($p<0.02$) and the NPY group ($p<0.004$). Interestingly, compared to food deprived birds controls and refed birds showed increased FOS-ir cells in the NHpC (Fig 7a and b).

3.2.3.2. Lateral Septum (SL): Abundant FOS immunoreactivity was observed in the lateral septum with two clear FOS-ir cell groups, the SLd and SLv (Fig. 2). No significant differences were detected among the thee treatment groups in the SLd ($F_{2,11}=1.03$, $p=0.4$) or SLv ($F_{2,11}=1.36$, $p=0.3$).

3.2.3.3. Medial Septum (SM): Although the number of FOS-ir cells in the medial septum was lower than in the two subdivisions of the SL, a significant difference in FOS-ir cells was observed following the treatment ($F_{2,11}=5.92$, $p=0.022$). Both treatment groups showed increased FOS-ir cell numbers in the SM, however, only the NPY-injected group showed a significant increase ($p<0.05$) compared to controls.

3.2.4. Hypothalamus

3.2.4.1. Lateral Hypothalamic area (LHy): Since the LHy extends rostrocaudally for more than 3.5 mm in the chicken brain (Kuenzel, 1982), quantification was conducted at thee rostro-caudal levels (Fig. 2). First, at the level of the atlas plate A8.2 covering the anterior commissure, second covering coronal plane A7.8-A7.2 and a third one adjacent to the median eminence best shown in A5.2. At the levels, A8.2 and A5.2, the number of quantified FOS-ir cells were not significantly different among the thee groups ($F_{2,11}$=3.85 and $F_{2,11}$=1.73, $p>0.05$). Nonetheless, at the level A7.8-A7.2, an increase in the number of FOS-ir cells in the LHy was found for both the SR-49059 and NPY groups compared to controls, $F_{2,11}$=4.37, p=0.04 (Table 2).

3.2.4.2. Paraventricular Nucleus core (PVNc): The PVNc is present medially and close to the third ventricle and showed an overall significant treatment difference ($F_{2,11}$=7.55, p=0.0118). Both the SR-49059 ($p<0.05$) and NPY (p=0.015) groups showed significantly elevated numbers of FOS-ir cells compared to the control group.

3.2.4.3. Dorsomedial Nucleus (DMN): FOS-ir cells at the coronal level of A5.2 (Fig 2), located ventrolaterally from the paraventricular organ (PVO), can be defined with a clear circular area after FOS staining. A significant overall treatment effect was observed ($F_{2,11}$=6.41, p=0.0217). The number of FOS-ir cells was significantly increased both in the SR-49059 and NPY groups as compared to the controls ($p<0.05$).

3.2.4.4. Infundibular Nucleus (IN): In this avian homolog of the mammalian arcuate nucleus there was no significant difference among the treatment groups ($F_{2,11}$=2.109, $p>0.05$).

3.2.4.5. Nucleus of Solitary Tract (NTS): A significant difference was observed among the treatment groups ($F_{2,11}$=4.99, p=0.03). An increase in FOS-ir cell numbers in both SR-49059

and NPY treated groups was observed in the NTS, however, only the NPY group showed a significant increase when compared to the saline injected group ($p<0.05$).

3.3. Dual colocalization

3.3.1. FOS and AVT immunoreactivity

Dual FOS and AVT immunohistochemistry was utilized to determine if the activity of AVT-containing neurons in specific hypothalamic structures changed in response to SR-49059 treatment. Structures examined included the SO, PPoN, ventral PHN and core sub-nucleus of the PVN. In the SO, significantly less ($p=0.017$) magnocellular AVT-ir neurons showed colocalization with FOS after SR-49059 administration compared to the control group (Fig. 3a-c). Likewise, percent of magnocellular AVT-ir neurons co-labeled with FOS had decreased following the SR-49059 treatment in the PPoN, PHNv and the PVNc, near the wall of the third ventricle, (PPoN, $p<0.001$; PHNv, $p=0.011$; PVNc, $p=0.034$; Fig. 3 d-l). Interestingly, FOS positive AVT neurons were also observed in the SO of birds provided with food compared to unfed or food deprived birds (Fig 7a).

3.3.2. V1aR and AVT immunoreactivity

Since the SO, PPoN, PHNv and the PVNc showed a significant decrease in FOS-ir cell counts following SR-49059 treatment, a dual immunofluorescence assay for AVT and V1aR was conducted to determine the co-localization of the V1aR in AVT neurons in all four structures. Although, V1aR immunoreactivity was not as prominent as AVT immunoreactivity (a theefold difference in the exposure time was used to take digital images of V1aR in Fig. 5 b, e, h and k compared to images of AVT-ir in Fig. 5 a, d, g and j) many AVT-ir magnocellular neurons in the SO, PPoN, PHNv and the PVNc are colocalized with V1aR (Fig. 5a-l). The PPoN, PHNv and PVNc also contain V1aR-ir glial cells lining the third ventricle (Fig. 5d-l). Overall, the SO

seemed to contain more AVT neurons colocalized with the V1aR than the PPoN, PHNv and PVNc.

3.3.3. V1aR and CRH immunoreactivity

The reduced number of FOS-ir cells in the NHpC following SR-49059 administration (Table 2, Fig. 6a and b) prompted us to perform dual immunofluorescent labeling for V1aR and CRH. Interestingly, a large number of CRH-ir cells coexist with V1aR-ir glial cells in the dorsal part of the NHpC (Fig. 6c).

3.4. Plasma osmolality and glucose

Plasma osmolality and glucose levels were measured in birds provided with food (controls or refed birds, FDR) and without food (food deprived birds, FD). Zero h samples represent birds on ad lib food. An overall significant change was observed among the treatment groups (osmolality: $F_{2,19} = 11.48$, $p < 0.0007$, Fig 7b and glucose: $F_{2,19} = 46.28$, $p < 0.0001$, Fig 7c). Compared to birds on feed, plasma osmolality and glucose were significantly decreased in food deprived birds for 24 h ($p < 0.05$). When birds provided with food for 1 h, following 24 h food deprivation, plasma osmolality and glucose levels were normalized compared to controls ($p > 0.05$), however, was significantly higher than food deprived birds ($p > 0.05$).

4. Discussion

4.1. Behavioral effects

To understand the differences in behavioral responses among different treatment groups regarding feeding behavior, frequency or bouts of food searching behavior, drinking responses and resting behavior, in the absence of food were scored. The V1aR antagonist treatment neither affected food searching behavior nor resting state of birds. Interestingly, an increase in time

spent drinking water following *i.c.v.* administration of the V1aR antagonist was observed in the pre-prandial state. However, V1R antagonist administered peripherally did not affect water intake in rodents (Ikemura et al., 2004). The V1aR antagonist effect on water intake observed in the present study may represent the behavioral compensation for appetite induced by the V1aR antagonist in the absence of food. This assumption is further substantiated by the drinking behavior in V1aR antagonist-treated birds that did not increase in the presence of food (prandial drinking). Furthermore, a conspicuous increase in food intake was observed following the V1aR antagonist treatment. As expected, the NPY dose used in this study induced foraging and food intake as reported in a previous avian study (Kuenzel, et al., 1987). Since, the amount of time spent drinking water in NPY treated birds was increased, the current study also supported the dipsogenic effect of NPY previously documented in rodents (Levine and Morley, 1984). Although, the increase in food intake in antagonist treated birds was not as prominent as after NPY administration, the food intake response was significantly higher than controls.

4.2. Food intake and FOS immunoreactivity

Neuroanatomical results from the present study suggest a role of the vasotocin receptor V1aR in food intake. Specifically, the increase in food intake following central administration of a selective receptor antagonist SR-49059 fostered the use of FOS immunoreactivity to determine changes in the number of FOS-ir cells and identify brain structures in the septum, diencephalon and caudal brainstem. The increased food intake response following *i.c.v.* administration of the V1aR antagonist resulted in increased activation of several brain structures, such as the PVNc, LHy, DMN, BSTL, SM and NTS, similar to results from central administration of NPY. In mammals, FOS studies have shown that food intake also activates several similar brain structures including the PVN, LHy, DMN, central amygdalar nucleus, SO and NTS (Li et al., 1994;

Johnstone et al., 2006; Timofeeva et al., 2005). Likewise, food consumption in birds lead to the activation of cells in several brain structures including the SO and NHpC (Fig 7). Hence, increased FOS cell counts in several brain structures presented in this study could have resulted from the effects of compounds administered and food consumed, perhaps involving a complex neural circuitry associated with the treatments and possibly from satiety signals (Arends et al., 1988; Katz and Karten, 1983). Nonetheless, neuroanatomical studies suggest that the site of action of neurohormones depends on receptor localization. Although an increase in FOS-ir cell number was observed in the PVN, DMN, LHy, BSTLdl, SM, and NTS of the V1aR antagonist treated birds, the receptor localization study did not identify V1aRs in these brain structures in chickens (Selvam et al., 2015), with one exception involving magnocellular neurons of the PVN. In contrast, two brain structures, the NHpC and SO, containing V1aRs showed reduced FOS cell counts following the V1aR antagonist treatment, revealing the antagonist effect in reducing the activity of the two structures. It is possible that both the SO and NHpC could be involved in satiety signals from the food consumed (Fig 7a) and thus resulting in higher FOS-ir cell counts in saline and NPY treated birds (Li et al., 1994; Johnstone et al., 2006; Timofeeva et al., 2005).

The role of the PVNc, LHy and DMN in regulation of food intake in mammals is particularly well documented. Classical techniques such as tract tracing methods in mammals have shown that the PVNc, LHy, DMN and BSTL have direct projections to the NTS and dorsal motor nucleus of the vagus and are involved in the regulation of food intake, particularly by modulating the autonomic nervous system (Van der Kooy et al., 1984; Luiten et al., 1987; Thompson et al., 1996). In birds it has been shown that the PVNc, LHy and BSTL also project to the NTS and dorsal motor nucleus of the vagus (Kuenzel, 2000; Berk and Finkelstein, 1983; Berk, 1987; Atoji et al., 2006). Based on the hodological information from the PVNc, LHy,

BSTL and NTS, it has been proposed that food intake in mammals and birds is controlled by similar brain structures (Kuenzel, 1994). It is important to note that reciprocal connections between brain structures where FOS was quantified in this study, including BSTL (Vicario et al., 2014; Van der Kooy et al., 1984; Berk, 1987), SM (Montagnese et al., 2004; 2008), DMN (Luiten et al., 1987), PVN (Berk and Finkelstein, 1983), LHy (Vicario et al., 2014) and NTS (Arends et al., 1988; Katz and Karten, 1983), could also play a substantial role in the regulation of food intake.

4.3. Attenuation of neuronal activity by V1aR antagonist

Four avian brain structures, the SO, PPoN, PHNv, and PVNc containing AVT neurons appear to be involved in the regulation of food intake. The SO, in particular, showed low numbers of FOS-ir cells in birds treated with the V1aR antagonist compared to the saline control and NPY treated birds (Table 2) and resulted in subsequent dual immunocytochemical studies. The V1aR antagonist-treated birds showed decreased FOS-ir expression specifically in AVT-ir magnocellular neurons in the SO, PPoN, PHNv and PVNc. Similar to rodent studies (Hurbin et al., 2002), it was also found that AVT-ir magnocellular neurons, but not parvocellular neurons, in these structures contain V1aR immunoreactivity. Coincidentally, no difference was found in AVT-ir parvocellular neurons in the PVNc following V1aR antagonist treatment (Fig. 4). Since AVP magnocellular neurons in the mammalian SO and PVN were reported to have a role in satiety signaling and metabolic challenges (Li et al., 1994; Johnston et al., 2006; Timofeeva et al., 2005; Briski and Brandt, 2000; Mandelblat-Cerf et al., 2017), blocking V1aR present in AVT-ir magnocellular neurons by the V1aR antagonist could have suppressed the effects induced by AVT (Serradeil-Le Gal et al., 1993; Jayanthi et al., 2014; Landgraf and Neumann, 2004), thereby reducing the satiety signal (Tachibana et al., 2004; Masunari et al., 2016).

The second interesting finding from this study is the reduced number of FOS-ir cells in the NHpC of the V1aR antagonist-treated birds. Several lines of evidence show that this structure is associated with physical, psychological and social stress responses in avian species (Sharp et al., 1995; Xie et al., 2010). It was recently hypothesized that the NHpC in the avian brain is associated with the neuroendocrine regulation of stress due to the presence of CRH neurons (Chapter 2). The significant reduction in FOS-ir cells following V1aR antagonist treatment compared to controls may represent decreased activity of CRH neurons. Importantly, the present study shows that CRH-ir neurons in the NHpC are in close contact with glial cells that contain the V1aR (Fig. 6c) and administration of the V1aR antagonist could have attenuated CRH neuronal activity via V1aR-ir glial cells. Currently, it has been widely accepted that glial cells modulate activity of hypothalamic neurons (Ojeda et al., 2008; Brown et al., 2013; Deschepper, 1998; Garcia-Segura and McCarthy, 2004). However, the plausible cross talk between V1aR-ir glia and CRH neurons remains to be determined. Nonetheless, blocking V1a receptors in glia by the antagonist treatment appear to suppress the activity of CRH neurons in the NHpC.

In conclusion, behavioral and neuroanatomical results from the present study suggest a role of V1aRs in appetite control. Particularly, based upon receptor localization, V1aRs could regulate food intake by modulating neuronal activity of AVT-ir magnocellular neurons in the preoptic and hypothalamic structures and CRH-ir neurons in the NHpC. Attenuation of the anorectic effects by AVT (Tachibana et al., 2004; Masunari et al., 2016) and CRH (Denbow et al., 1999) release could be responsible for the increased food intake shown by V1aR antagonist treated birds. These interactions are summarized in Fig. 8.

Table 1. Behavioral responses in chickens following intracerebroventricular administration of saline, V1a receptor antagonist SR-49059, or neuropeptide Y (NPY).

Group	Drinking Response (sec)		Resting bouts		Food search	
	Pre-prandial	**Prandial**	**Pre-prandial**	**Prandial**	**Pre-prandial**	**Prandial**
Saline	164 ± 47^{b}	130 ± 72^{a}	5 ± 2^{a}	12 ± 4^{a}	5 ± 2^{b}	NA
SR-49059	392 ± 27^{a}	128 ± 27^{a}	8 ± 3^{a}	10 ± 2^{a}	10 ± 3^{b}	NA
NPY	$338\pm63^{a,b}$	272 ± 88^{a}	7 ± 3^{a}	13 ± 7^{a}	20 ± 4^{a}	NA

Data are presented as mean±SEM (n=3-4/group). Data for each behavioral category within a column with different superscript letters are significantly different ($p<0.05$). NA – data not available.

Table 2. Quantification of FOS-ir cells in different brain structures after central administration of saline, vasotocin receptor V1a antagonist SR-49059 or neuropeptide Y (NPY).

Structures	Area (mm^2)	Saline	SR-49059	NPY
SO	0.09	81±7[a]	56±6[b]	73±4[a]
BSTLd	0.11	22±3[b]	42±5[a]	38±7[a]
Ceov	0.14	72±11[a]	99±15[a]	83±11[a]
CeC	0.08	33±2[a]	38±6[a]	38±6[a]
MeAs	0.19	197±27[a]	198±8[a]	191±28[a]
SLd	0.2	253±52[a]	328±27[a]	338±53[a]
SLv	0.2	230±36[a]	284±34[a]	312±35[a]
SM	0.18	67±17[b]	115±22[a,b]	155±14[a]
LHy(A8.2)	0.08	29±2[a]	33±3[a]	36±3[a]
LHy(A7.8-7.2)	0.22	70±2[b]	89±6[a]	93±19[a]
LHy(A5.2)	0.1	39±9[a]	47±8[a]	48±3[a]
NHpC	0.06	149±16[a]	91±10[b]	167±8[a]
PVNc	0.05	85±11[b]	143±8[a]	152±18[a]
DMN	0.15	105±13[b]	171±25[a]	162±3[a]
IN	0.11	63±6[a]	69±4[a]	53±5[a]
NTS	0.14	47±9[b]	74±9[a,b]	82±8[a]

Data are represented as mean±SEM (n = 4 birds/group). Significant differences are shown by different superscript letters ($p<0.05$) within a row and underlined. Structures name are listed in Fig 2.

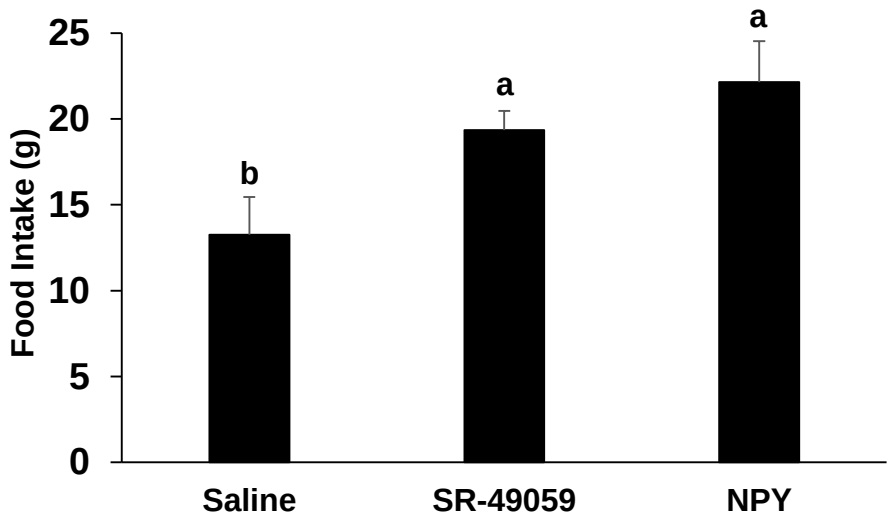

Fig. 1. Central effect of the V1a receptor antagonist (SR-49059), neuropeptide Y (NPY) or saline (control) on food intake. (n = 5 birds/group). Significant differences among the groups are shown by different letters.

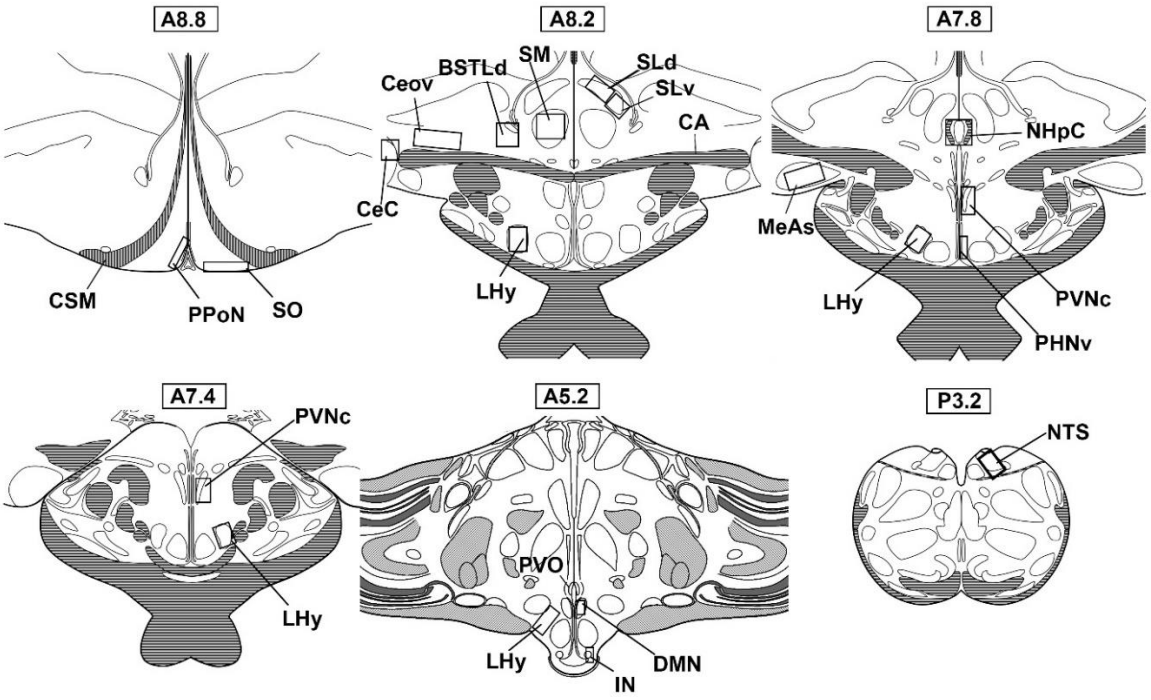

Fig. 2. Modified schematic diagrams from a chick brain atlas (Kuenzel and Masson, 1988), representing coronal planes where FOS quantification was performed. Boxed regions in each schematic diagram mark the brain structures quantified following administration of saline, V1a receptor antagonist SR-49059 or NPY. CA – anterior commissure and CSM – corticoseptomesencephalic tract were used as landmarks; BSTLd – dorsolateral bed nucleus of the stria terminalis; CeC - central capsular nucleus; Ceov - oval central amygdalar nucleus; DMN – dorsomedial nucleus; IN – infundibular nucleus; LHy - lateral hypothalamic area; MeAs – subpallial medial amygdalar nucleus (previously known as TnA - nucleus taeniae of the amygdala); NHpC - nucleus of the hippocampal commissure; NTS – nucleus tractus solitarius or nucleus of solitary tract; PHNv - ventral periventricular hypothalamic nucleus; POP – preoptic periventricular nucleus; PVNc – core subnucleus of the paraventricular nucleus; PVO – paraventricular organ; SLd&v – lateral septum (dorsal and ventral); SM – medial septum; SO – supraoptic nucleus.

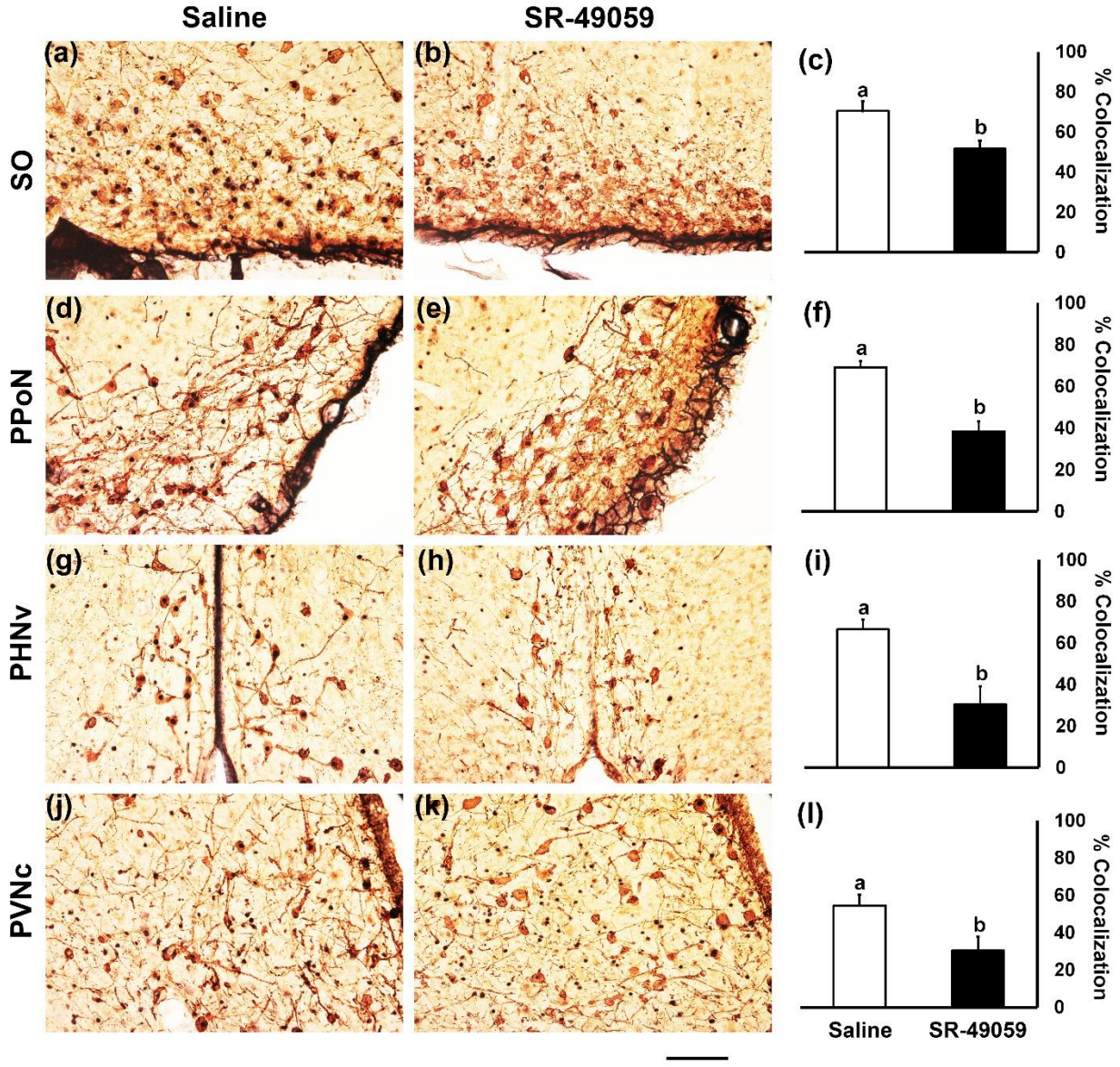

Fig. 3. Dual immunocytochemistry of FOS and arginine vasotocin (AVT) in magnocellular neurons within different hypothalamic structures following central administration of saline (a, d, g and j) and vasotocin V1a receptor antagonist, SR-49059 (b, e, h, and k). Scale bar - 100 µm. Graphs on the right (c, f, i and l) show percentage of AVT-ir magnocellular neurons containing FOS immunoreactivity in the supraoptic nucleus (SO), preoptic periventricular nucleus (PPoN), ventral periventricular hypothalamic nucleus (PHNv) and paraventricular nucleus core (PVNc). n=5 birds/group. Results are presented as mean ± SEM. Significant differences between saline and SR-49059 treatment in each brain structure are indicated by different letters.

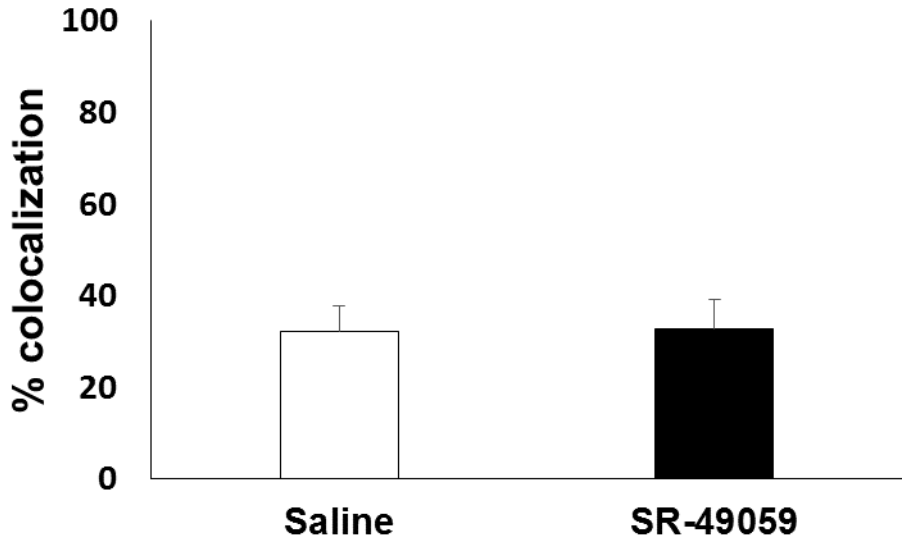

Fig. 4. Colocalization of FOS and AVT in parvocellular neurons of the core region of the paraventricular nucleus following central administration of the V1aR antagonist (SR49059). Data are presented as mean ± SEM (n=4 birds/group).

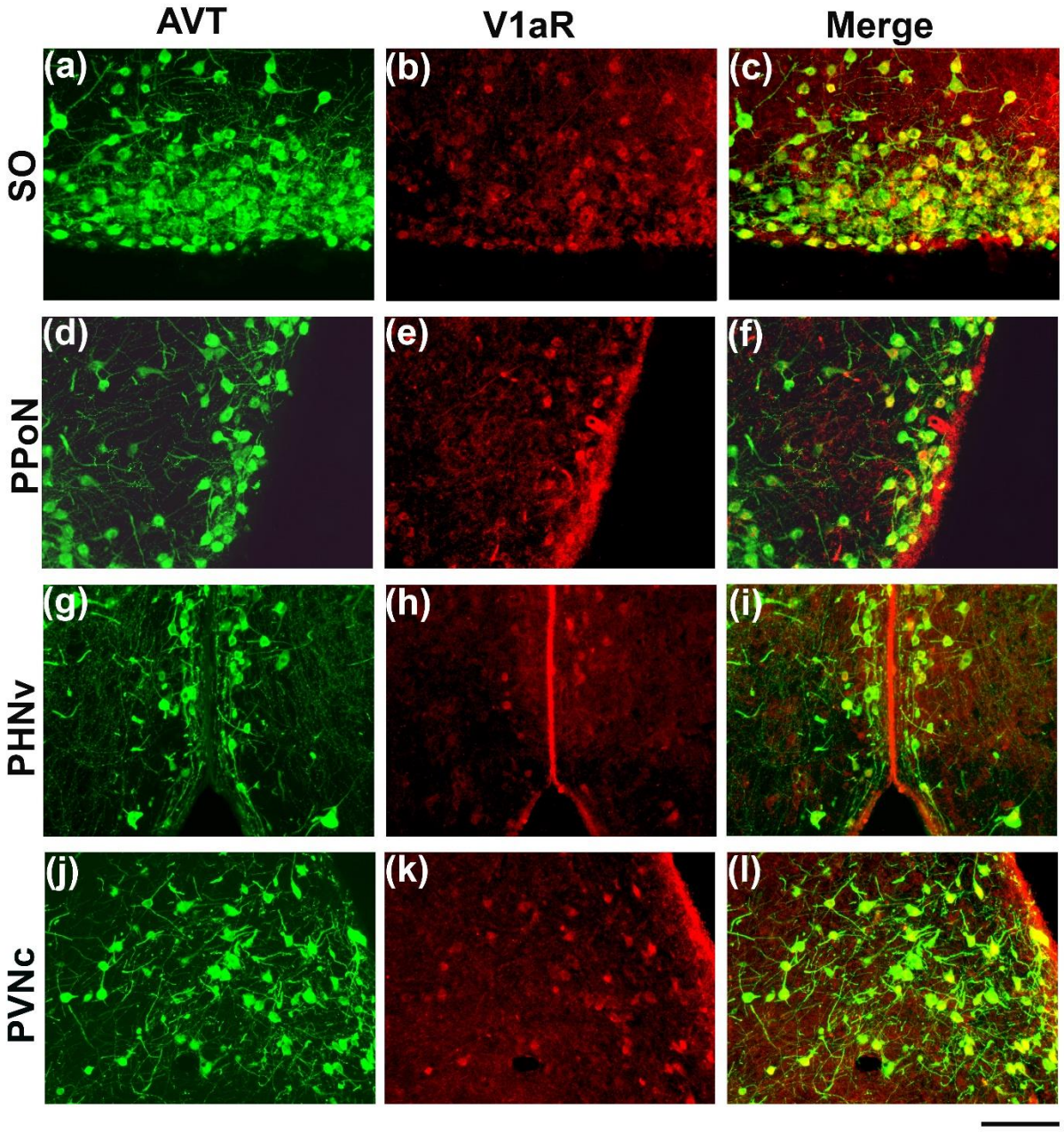

Fig. 5. Colocalization of arginine vasotocin (AVT) and its receptor (V1aR) in magnocellular neurons present in the supraoptic nucleus (SO), preoptic periventricular nucleus (PPoN), ventral periventricular hypothalamic nucleus (PHNv) and paraventricular nucleus core (PVNc). Arginine vasotocin-ir is show in green - stained with FITC (a, d, g and j) and V1aR-ir is shown in red - stained with DyLight 594 (b, e, h and k). The merged images are shown in the panel on the right side (c, f, i and l). Scale bar - 200µm.

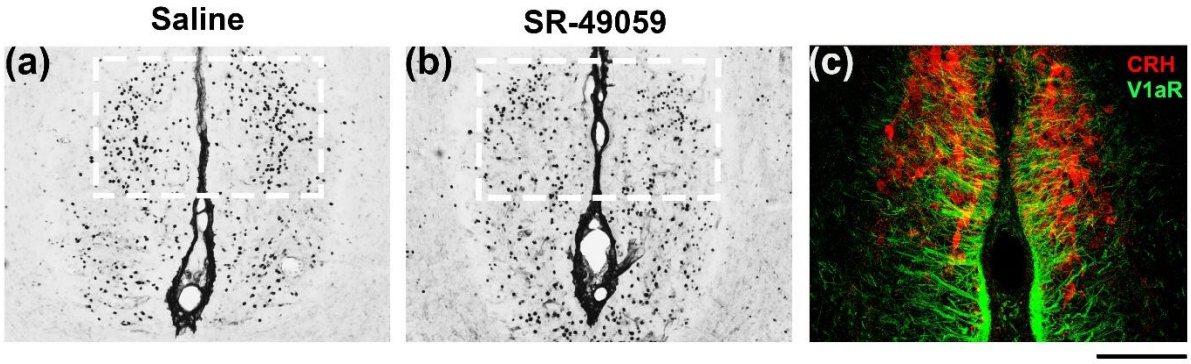

Fig. 6. FOS-ir cells in the nucleus of hippocampal commissure (NHpC) following a) intracerebroventricular administration of saline and b) vasotocin V1a receptor antagonist SR-49059. c) V1a receptor –ir glia (green) and CRH neurons (red) are present in the NHpC (confocal image credits to Dr. Alexander Jurkevich, University of Missouri). Scale bar - 200 µm.

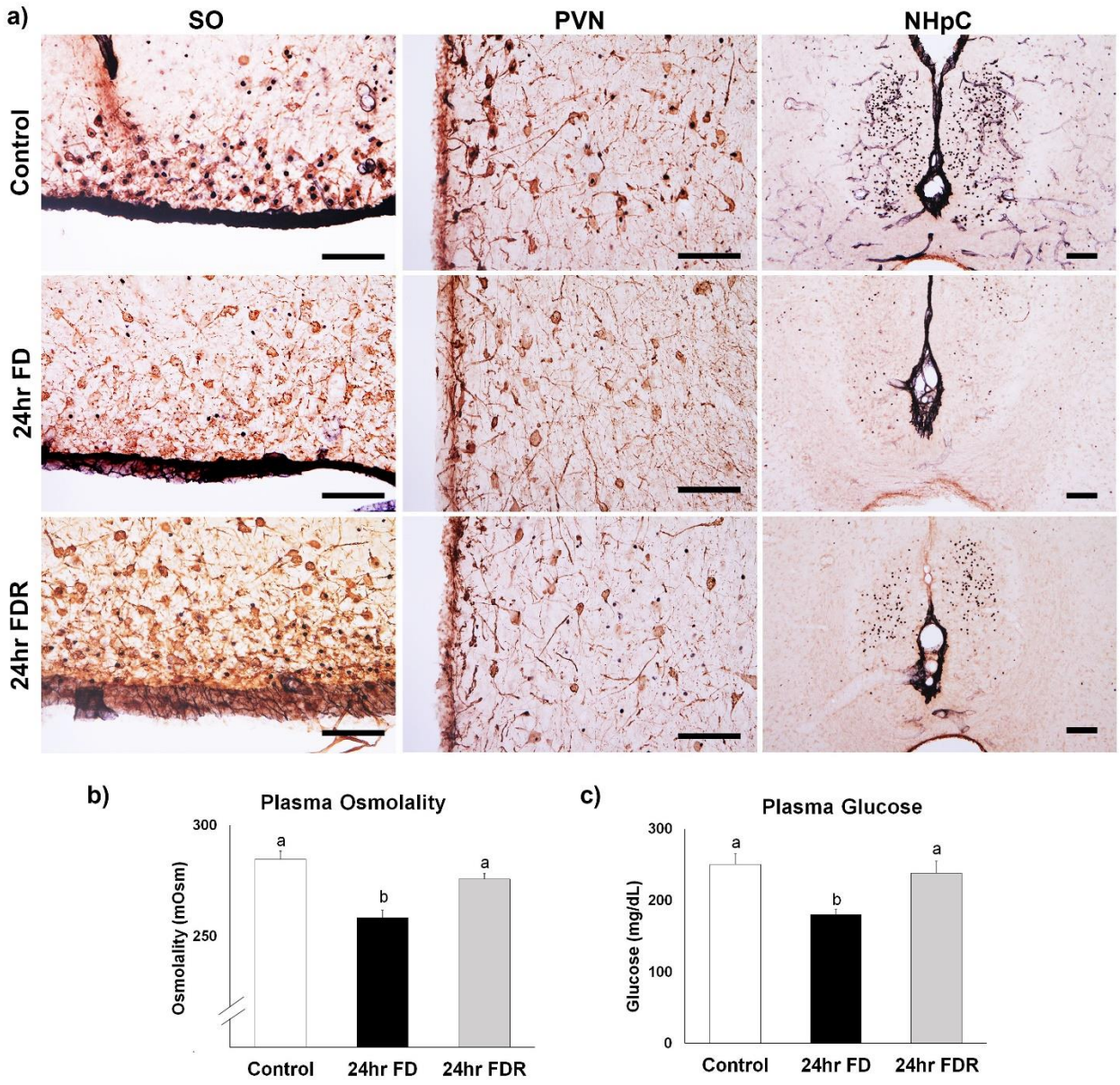

Fig 7. Effects of 24 h food deprivation (FD) and/or 1h refeeding (FDR) in specific regions of the brain and in plasma osmolality and glucose. a) Fos-ir cells in the NHpC and in AVT neurons of the SO and PVN following food deprivation for 24 h or food deprivation for 24 h and refeeding for 1 h. Scale bar – 100 µm. b) Plasma osmolality levels and c) plasma glucose levels in food deprived birds (black) and refed birds (gray) compared to controls (n=7-8/group).

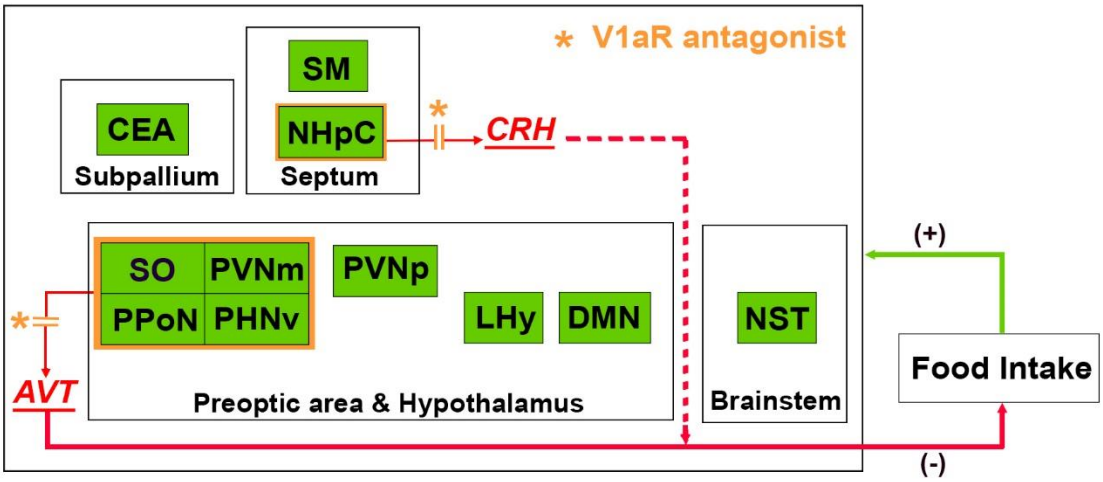

Fig 8. Schematic representation of a possible model of brain structures associated with food intake. Food intake activates several structures (shown in green) in the hypothalamus, septum, subpallium and brainstem representing feedback signals (solid green line). Activation of neurons following food intake could result in the release of anorectic peptides such as CRH and AVT from specific brain structures (NHpC, SO, PPoN, PHN and PVN) providing an inhibitory signal affecting further consumption of food (solid and dotted red lines). Central administration of a V1a receptor antagonist blocks the V1aR present in the SO, PPoN, PHN and PVNm (shown in amber) resulting in less anorectic (AVT) peptide release. Indirect evidence suggests that CRH release from the NHpC could be likewise attenuated following administration of the V1aR antagonist (dotted red line). Thus, the V1aR antagonist treated birds showed increase in food intake perhaps by inhibiting anorectic effects of AVT and CRH in the brain. (+) stimulatory effects and (-) inhibitory effects. Subscripts: v - ventral, p - parvocellular, m - magnocellular. Complete structure names are listed in Fig 2.

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Conclusion

Functional roles of arginine vasotocin (AVT) neurons in the neuroendocrine regulation of stress and food intake were discovered from a series of experiments performed for this dissertation research. The first set of experiments identified that acute and repeated restraint stress activated AVT-ir parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus. Of particular interest, activation of these parvocellular AVT neurons was associated with increased corticosterone concentration (stress hormone) resulting from acute and chonic stress. In a second set of experiments, it was also found that expression of AVT in the PVN occurs only in the later phase of a stress period, where CRH initiates activation of the HPA axis and AVT contributes to its elevated maintenance over an extended period of time. Hence results from these two studies suggest that AVT plays a major role in the stress pathway of poultry as well as in other avian species to facilitate or maintain stress levels under prolonged stress conditions. Although sources of neural inputs to that initiate the HPA response are different, activation of AVT neurons in the PVN likely results from different types of stressors. This is noted from both restraint stress and food deprivation stress, where the former could be associated with stress induced by crowding/stock density and the latter could be associated with management methods (e.g. longer transport time from the hatchery to the farm or feed restriction utilized in broiler breeders) practiced in the industry. Finally, results from a set of experiments suggest that AVT not only facilitates the HPA axis, but also modulates activity of CRH neurons in a stress responsive neural structure (NHpC) in the brain. Hence, AVT appears to act as a neuromodulator within the brain and augment stress responses by further activating the HPA axis. However, under what stressful conditions AVT modulates activity of CRH neurons within the NHpC needs further investigation!

Experiments from this dissertation research not only identified the neuroendocrine role of AVT in the stress response, but also suggested a possible role of AVT in the regulation of food intake in poultry. Food consumption and satiety signals seem to activate AVT magnocellular neurons in hypothalamus and CRH neurons in the septum though multi-synaptic neuronal pathways as shown by FOS activation in several brain structures. Activity of AVT and CRH neurons appears to be facilitated by V1a receptors (one of the vasotocin receptor subtypes in birds), likely involving autocrine and paracrine regulation. Thus, food intake activates anorexigenic neuropeptide containing neurons, where vasotocin receptors (V1aR) likely play a role in arresting further consumption of food and facilitating satiety signals within the avian brain.

Hence, this dissertation research supports the phenomenon that distinct types of AVT neurons in the avian brain are involved in different types of neuroendocrine regulation. Overall, AVT not only has an important role in physiological homeostasis such as water balance or cardiovascular functions but also is associated with the stress pathway and food intake regulation within the chicken brain. Implications of the finding from this basic research should have a significant impact on understanding neuroendocrine regulation in poultry.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Wayne Kuenzel

FROM: Craig N. Coon, Chairman

DATE: 2/15/16

SUBJECT: IACUC Approval

Expiration Date: Feb 4, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 16043 "Neuroendocrine studies addressing stress, reproduction and behavior in poultry".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Feb 4, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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