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Characterization of the Vasotocin Receptor Subtype 4 (VT4R) in the Brain and Pituitary Gland of the Chicken, Gallus gallus

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of the requirements for the degree of
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By

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ABSTRACT

The present study investigated the distribution of the vasotocin subtype four receptor (VT4R) in brain and pituitary gland of the chicken, *Gallus gallus.* The anterior pituitary cell types associated with the VT4R were also determined. Two polyclonal antibodies were raised in rabbit against a cocktail of peptides, 15 amino acids from the amino terminal region and 17 amino acids from the carboxy terminal region of VT4R receptor. The antibody was validated utilizing the Western blot and immunocytochemistry. A single band at 47KDa utilizing membrane protein extracts of chicken brain and pituitary tissues was shown for the VT4R antibody. A peptide preadsorption or blocking experiment was performed to confirm the specificity of the antibody to the VT4R. Second, the VT4R distribution in brain and pituitary were studied using immunocytochemistry. The VT4R antibody immunostained both neuronal and non-neuronal elements present in the brain. The VT4R was widely distributed throughout the brain. Intense cell body staining was observed in the following neural structures: supraoptic nucleus, paraventricular nucleus, periventricular hypothalamic nucleus, Edinger Westphal and other oculomotor nuclei, nucleus tractus solitarius, hypoglossal nucleus, trigeminal nucleus, sensory nucleus of the trigeminal nerve, nucleus of the abducens nerve, nucleus supraspinalis and magnocellular cochlear nucleus. Intense immunoreactivity of the VT4R in glial cells was evident in circumventricular organs. Radial glial cells in hippocampus, astrocytes in optic tectum and tanycytes in median eminence were prominently immunostained with the VT4R. In addition, the regional distribution of the VT4R was determined in the pituitary gland of the chicken. The VT4R was located in the cephalic lobe of the anterior pituitary. The phenotype of pituitary cells was revealed using dual labelled immunofluorescence microscopy. The VT4R immunoreactive cells were located in both corticotropes and gonadotropes. About 89% and 12% of corticotropes and luteinizing hormone (LH) containing gonadotropes, respectively, displayed

the VT4R. The VT4R was not detected in lactotropes nor somatotropes. Based on its neuroanatomical distribution in brain and colocalization studies of the VT4R in anterior pituitary, the proposed functions of the VT4R was suggested to be associated with stress responses, maintenance of homeostasis and lutenizing hormone release from a subgroup of pituitary gonadotropes. The receptor could also have a plausible role in auditory function and motor regulation of the eye based upon intense immunoreactivity of neurons associated with the fifth and third cranial nerves, respectively.

This thesis is approved for recommendation
to the Graduate Council.

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DEDICATION

This thesis is dedicated to my cute nephew Nitin and my beloved parents Selvam Annamalai and Janaki Selvam.

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I. INTRODUCTION

The survival of a species depends on its adaptative responses to stressful (challenging and threatening) situations (Ulrich-Lai and Herman, 2009). The brain is the key organ that distinguishes threatening and non-threatening situations and determines the physiological responses to situations (McEwen, 2007). The stress response includes the suppression of activities like feeding which are not important for short-term survival. In response to stress, the neuroendocrine and autonomic nervous system together undergo changes to restore the disturbed homeostasis. The sympathetic arm of the autonomic nervous system coupled with activation of the adrenal medulla and the hypothalamic pituitary adrenal axis (HPA) aid in bidirectional communication between brain and body in response to adverse situations (Engelmann et al., 2004). The autonomic nervous system helps in the immediate response to stressors through sympathetic and parasympathetic nerve activity. The preganglionic neurons of the sympathetic nervous system act on the adrenal medulla to release epinephrine and norepinephrine. Under stressful conditions, the animal shows a flight or fight response that includes dilation of pupils, shunting of blood to voluntary muscles and release of energy through gluconeogenesis. The energy is further provided to the animal by stimulating fatty acid release, enhancing glucose utilization and inhibiting protein synthesis (Bear et al., 2001). The slower more prolonged response of stress follows activation of the HPA axis. In response to a stressor, the parvocellular neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus secrete the nine-amino acid peptide, arginine vasopressin (AVP) and 41 amino acid polypeptide, corticotropin releasing hormone (CRH) into the portal circulation. The neuropeptides are transported to anterior pituitary. The corticotropes present in anterior pituitary subsequently secrete adrenocorticotropin releasing hormone (ACTH) in response to binding of the peptides to specific receptors and initiation of specific signal transduction pathways. The hormone, ACTH,

in turn acts on the adrenal cortex in mammals and inter-renal tissue of adrenal glands in birds to produce glucocorticoids, cortisol in humans and corticosterone in birds (Engelmann et al., 2004; Volpi et al., 2004; McEwen, 2007; Aguilera et al., 2008; Kuenzel and Jurkevich, 2009; Ulrich-Lai and Herman, 2009).

Arginine vasopressin (AVP) and corticotropin releasing hormones (CRH) are two critical peptides involved in neuroendocrine regulation. Both hormones act on their target tissues via G protein coupled receptors. In birds, the CRH neurohormone is a 41 amino acid long peptide which is identical to CRH of human and rats. Corticotropin releasing hormone exerts its effects through corticotropin releasing hormone receptor subtypes: CRH1 and CRH2 (Vandenborne et al., 2005; Mikhailova et al., 2007). Arginine Vasotocin (AVT) is the avian counterpart of arginine vasopressin. Avian AVT differs from AVP in the third amino acid of the molecule where isoleucine (Ile) is replaced by phenylalanine (Phe). In mammals, AVP exerts it effects via vasopressin (V) receptor subtypes: V1a, V1b and V2 receptors. Vasopressin 1a and V1b receptors activate phospolipase C through coupling of $G_{\alpha q/11}$ and V2 receptors couple to G_s and activate adenylate cyclase (Birnbaumer, 2000; Robert and Clauser, 2005).

Studies on vasotocin receptors in non mammalian species like frogs (Acharjee et al., 2004), chickens (Tan et al., 2000; Cornett et al., 2003; Baeyens and Cornett, 2006; Gubrij et al., 2005; Jurkevich et al., 2005; Jurkevich et al., 2008), newts Hasunuma et al., 2007; Hasunuma et al., 2010) and fish have been reported (Warne, 2001; Kline et al., 2011). In chickens, the vasotocin receptors are of four known subtypes: VT1R, VT2R, VT3R and VT4R. Molecular studies of the genes have focused on VT1R, VT2R and VT3R. The first receptor, VT1R is present in shell gland (confined to endometrial lining) and brain, involved in reproductive/maternal behavior and contraction of smooth muscles in uterus and proposed to be homologous to the V2 receptor in

humans (Tan et al., 2000). The second receptor, VT2R is present in the cephalic lobe of the anterior pituitary gland. The major function of the VT2R is to release ACTH and corticosterone during stress responses and is proposed to be homologous to the mammalian V1b receptor (Tan et al., 2000; Cornett et al., 2003; Jurkevich et al., 2005; Baeyens and Cornett, 2006). The third receptor, VT3R, has been shown to occur in the endometrium and myometrium of the shell gland indicating a plausible role in stimulating uterine contraction during oviposition. The receptor is proposed to be homologous to the oxytocin receptor in humans (Gubrij et al., 2005). The receptor, VT4R is a recent cloned receptor which shares 69% similarity with the mammalian V1a receptor (Cornett et al., 2007, Genebank submission). The receptor might be involved in stress responses and in regulation of water and blood pressure (Baeyens and Cornett, 2006). To date, a study examining the distribution of the VT4R in neural systems and its functional role in chickens have not yet been reported. A study was therefore conducted to determine if the VT4R is present in brain and pituitary gland of the chicken and possibly associated with the stress response.

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II. LITERATURE REVIEW

Chapter 1

Arginine vasopressin/Arginine vastocin and its receptors

1.1. Arginine vasopressin and its function

1.1.1 Arginine vasopressin

Arginine vasopressin (AVP) is a neuropeptide secreted by neurons of the supraoptic (SON) and paraventricular nuclei (PVN) in the hypothalamus. The vasopressinergic neurons originate from the ventral region of supraoptic nucleus and central region of the paraventricular nucleus of mammals (Vandesande et al., 1977). On proper stimulation, AVP is synthesized and released by one of two types of neurons found in the neural systems: (i) magnocellular system where vasopressin is released into axon terminals in the neural lobe of pituitary and (ii) parvocellular system where axons project to the external zone of the median eminence and AVP is released into the portal circulation on appropriate stimulus activation and transported to the anterior pituitary (Engelmann et al., 2004; Aguilera et al., 2008). Vasopressin is present in both internal and external zones of the median eminence. The magnocellular axons from PVN containing vasopressin pass through the internal zone of median eminence and terminate in the posterior pituitary (Holmes et al., 1986). Herring bodies are neurosecretory bodies found in the posterior pituitary that store vasopressin temporarily and release it into the bloodstream on hypothalamic stimulation (Vandesande et al., 1977). The parvocellular neurons from the paraventricular nucleus project to the external zone of median eminence. The neuropeptides are transported to the pituitary which secretes adrenocorticotropic releasing hormone (ACTH) from corticotropes of the anterior pituitary. The ACTH hormone acts on the adrenal cortex in mammals (Figure. 1)

and adrenal interrenal tissue in birds to release gluocorticoids (Engelmann et al., 2004; Volpi et al., 2004).

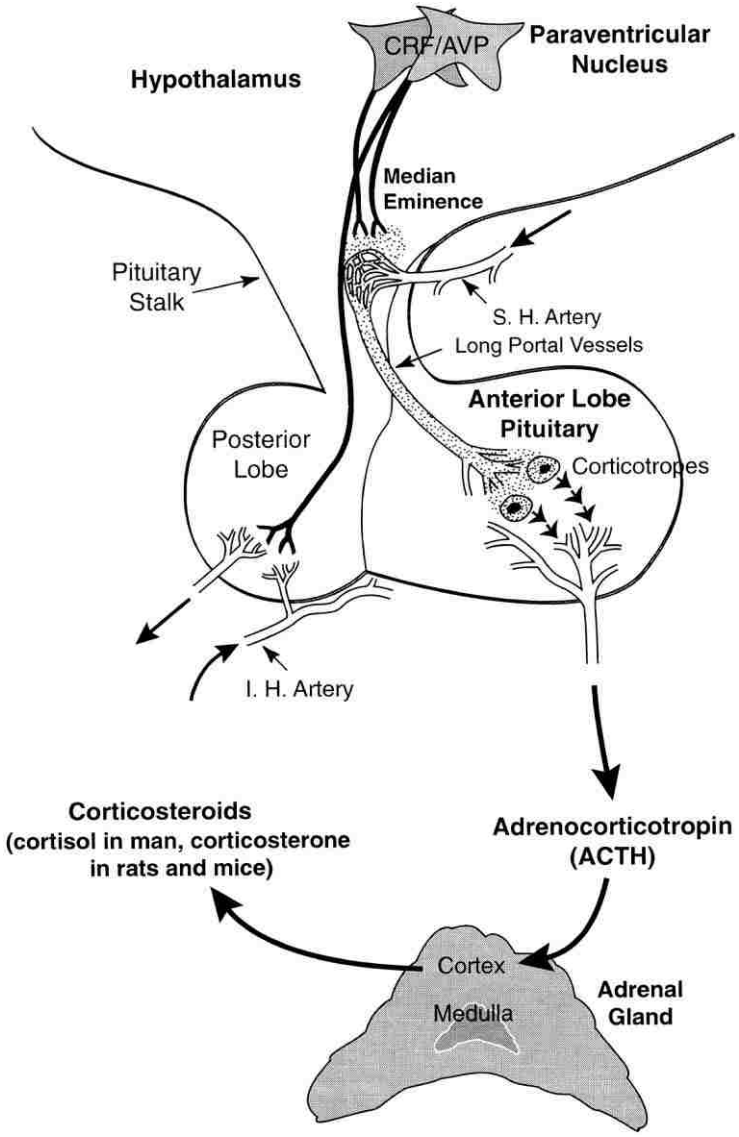

Figure 1: Functional anatomical components of Hypothalamo-pituitary adrenal axis. CRF: Corticotropin releasing factor, AVP: Arginine vasopressin, ACTH: Adrenocorticotropic hormone, I.H.: inferior hypophysial, S.H.: superior hypophysial (Turnbull and Rivier, 1999).

1.1.2 Arginine vasopressin gene:

The vasopressin gene has the following components: a signal peptide, vasopressin precursor, preprovasopressin, neurophysin II and copeptin. Neurophysin II helps vasopressin to be packaged as secretory granules while vasopressin domain (association of vasopressin and neurophysin) aids in trafficking of the prehormone to the secretory pathway. This AVP gene is regulated negatively by glucocorticoids at the protein level (Itoi et al., 2004).

1.1.3 Role of Arginine Vasopressin:

Arginine vasopressin regulates body fluid volume, homeostasis, vasoconstriction and release of ACTH in stress responses (Fahrenholz et al., 1993). Vasopressin plays an important role in hypothalamic pituitary adrenal axis adaptation to chronic stress but not for the sensitization of ACTH responses to a novel stress observed when the animal is in chronic stress (Aguilera et al., 2008). During acute stress, there is an immediate release of CRH and VP from parvocellular neurons of PVN to pituitary. In chronic stress, CRH is released first followed by delayed and prolonged response of VP. Glucocorticoids inhibit CRH followed by VP in chronic stress. In adrenalectomized rats subsequently administered glucocorticoids, the inhibition of the VP gene is preferred over CRH gene during stress responses. Glucocorticoids provide a negative feedback by acting on multiple sites inhibiting VP and CRH actions and indirectly acting on neural pathways of GABAergic neurons. The number of V1bR increases in anterior pituitary during acute stress due to gene transcription of V1bR. The key component in regulating V1bR expression is 5' UTR (untranslated region) of the gene (Volpi et al., 2004). Vasopressin receptors play a critical role in corticotrope function. In mammals, AVP receptors (V1 type) in pituitary are downregulated during chronic osmotic stimulation while upregulated during

repeated intra peritoneal injections or repeated immobilization. The V1 type receptors fluctuates with the number of binding sites during up- or down- regulation of the receptors, however, there is no change in the binding affinity of the receptors (Aguilera et al., 1994). Vasopressin aids in water regulation through the subfornical organ (SFO), choroid plexus, organum vasculosum of the lamina terminalis (OVLT) that are labelled for AVP receptors. The structures SFO and OVLT are involved in thirst control in both rats and birds (Fahrenholz et al., 1993). Arginine vasopressin also plays a behavioral role in aggression, social recognition, affiliation, anxiety and depression, spatial and non-spatial memory (Caldwell et al., 2008). Arginine vasopressin via V1aR acts on lateral septum which inturn receives inputs from olfactory vomeronasal system and thus helps in social recognition. Offspring recognition in sheep and pair bonding in prairie voles are examples of social recognition in animals where AVP has a major impact. The lateral septum also modulates anxiety in mice. (Bielsky and Young, 2004).

Vasopressin/vasotocin function as a neurohormone peripherally by mediating the release of hormones both *in vivo* and *in vitro.* In the ovine pituitary gland, AVP and CRH regulate ACTH secretion *in vitro* (Smith et al., 1993). The release of prolactin (PRL) from anterior pituitary gland was stimulated by AVP/AVT when injected subcutaneously in rats (Blask et al., 1982; Blask et al., 1984) while AVP/AVT stimulated PRL and luteinzing hormone release in *vitro* (Vaughan et al., 1975). Angiotensin II, CRF, and AVP act as positive stimulator for ACTH secretion in rat AtT-20 cell lines. Epinephrine, AVP and CRH stimulate POMC gene expression in a time and dose related manner in rat cells (Aoki et al., 1997). Both AVP and AVT stimulate both α-MSH and ACTH in perfused pituitary cells of ducks. These peptides act synergistically on the corticomelanotropic cells to release hormones (Castro et al., 1986; Castro et al., 1988). The release of prolactin is stimulated by AVT in turkey anterior pituitary cell cultures while

AVT mediates PRL release in chickens (Proudman and Opel, 1988; Jurkevich et al., 2008). In frogs, ACTH release is stimulated by CRF and AVP in anterior pituitary cells (*in vitro)* (Tonon et al., 1986). In trout, CRH and AVT act synergistically to stimulate ACTH release. This synergistic mechanism has an added advantage that there is an increased flexibility over ACTH control as the two peptides are produced by independent genes (Baker et al, 1996). Synergistic increases in the release of plasma corticosterone following injections of AVT and CRH in the chicken have been reported (Madison et al., 2008).

1.2 Arginine vasopressin/vasotocin receptors:

Arginine vasopressin exerts its effect through vasopressin receptors (V) and oxytocin (OT) receptors. Vasopressin receptors are G protein coupled receptors and are divided into three subtypes: V1a, V1b and V2. The vasopressin receptors, V1a and V1b couple to $G_{\alpha q/11}$ GTP binding proteins and activate phosopholipase C (Caldwell et al., 2008). The magnocellular neurons secreting vasopressin display vasopressin receptors, V1a and V1b receptor subtypes (Hurbin et al., 1998; Hurbin et al., 2002).

In non-mammalian vertebrates, the neurohypophysial hormones arginine vasotocin (AVT) and mesotocin (MT) are counterparts of mammalian arginine vasopressin (AVP) and oxytocin (OT), respectively. It was found that the non-mammalian vertebrates have arginine vasotocin (AVT) instead of vasopressin, having an amino acid substitution at the third position with phenylalanine (Phe) being replaced by isoleucine (Ile) (Acher et al., 1970). Arginine vasotocin is involved in various behavioural functions like scent marking, aggression, vocal communication, stress, social recognition and sexual behaviour (Goodson, 1998). The neuropeptides, AVT and MT are activated through G protein coupled receptors (GPCR)

through the phospholipase C/protein kinase C pathway. The receptors belong to the vasotocin/mesotocin subfamily. Vasotocin receptors (VT) have been identified in birds, frogs and newts that are comparable to vasopressin receptors (AVP) of mammals based on conservation of their amino acid residues of the respective peptides across vertebrate species. The comparable nomenclature of mammalian and non-mammalian species is shown in Table 1.

Table 1: Designation of vasopressin/vasotocin (AVP/AVT) receptors and oxytocin/mesotocin (OT/MT) receptors across vertebrate species

CLASS/ SPECIES	AVT/AVP RECEPTOR 1	AVT/AVP RECEPTOR 2	AVT/AVP RECEPTOR 3	OT/MT RECEPTORS	Ref
Human	V1a	V1b (V3)	V2	OT	29, 86
Rat	V1a	V1b (V3)	V2	OT	4,66,71, 95
Chicken	VT4	VT2	VT1	VT3	10,29,40, 52, 53, 84
Frogs	VT1[1]	-[2]	V2[3]	MT[1]	1, 2, 56
Newt[4]	V1a	V1b (V3)	V2	-[5]	42, 43
Fish[6,7]	V1a1	V1a2	V2	-[5]	55, 57

[1] Receptor designation was obtained from *Rana catesbeiana* (Bull frog) and *Rana esculenta* (Edible frog)

[2] A receptor comparable to mammalian V1b has not been identified in frogs.

[3] Receptor designation was obtained from *Hyla Japonica* (Japanese tree frog).

[4] Receptor designation was obtained from *Cynops pyrrhogaster* (Japanese red bellied newt, an amphibian).

[5] A receptor comparable to mammalian oxytocin receptor has not been identified in newts nor fish up to this time.

[6, 7] Receptor designation was obtained from *Epinephelus adscensionis* (rock hind fish) and *Cyprinodon nevadensis amargosae* (pupfish).

1.2.1 Comparable receptors in other vertebrate classes

Several studies have addressed AVP/AVT receptors in both mammalian and non-mammalian species. The widely studied species on VT receptors are birds, amphibia and fishes. The vasotocin receptors are comparable to mammalian vasopressin/oxytocin receptors based on sequence homology, distribution in body organs and function. (Table 2 -6).

Table 2: Amino acid sequence identity of AVP/AVT and OT/MT receptors among selected vertebrates[1] compared to rats.

Species	V1a	% identity	V1b	% identity	V2	% identity	OTR	% identity	Ref
Rats	V1a	100%	V1b	100%	V2	100%	OT	100%	4,66,71, 95
Chicken	VT4	69%	VT2	54%	VT1	43%	VT3	76%	10,29,40 ,52,53, 84
Frog	VT1	58%	-	-	V2	51%	MT	67%	1, 2, 56
Newt	V1a	61%	V1b	50%	V2	59%	-	-	42, 43
Teleost fish	V1a1	53%	V1a2	51%	V2	41%	-	-	55, 57

[1]Rat V1a, V1b, V2 and OT receptors were selected as the standard from which the amino acid sequence of other vertebrate species were compared. The % identity refers to the degree of homology to the rat.

Table 3: Comparison of mammalian V1a receptors with non mammalian species based on receptor distribution in organs and function

Species	Rats	Frog	Newt	Teleost fish
V1aR	V1a	VT1	V1a	V1a1
Sequence homology[1]	80	68	67	59
Organs	Brain, pituitary, liver, kidney	Brain, pituitary	Brain, heart, liver, kidney, bladder, testis, ovary	Brain, pituitary, gills, testis, ovary, liver
Physiology and behavior	Social recognition, anxiety/depression, memory, aggression	ACTH release.	Courtship behavior	Aggression, courtship behavior
Ref	71, 72, 73, 74	1, 2, 56	42, 43	55, 57

[1]The proposed homologous receptor were compared to human V1a receptor (V1aR). The sequence homology refers to the degree of homology to humans.

Note: The avian VT4R shares **69% homology to human V1aR** and proposed to be **homologous to the V1aR in mammals**. The distribution of the VT4R in the brain and pituitary is the objective for this study.

Table 4: Comparison of mammalian V1b receptors with non- mammalian species based on receptor tissue and organ distribution and function

Species	Rats	Chicken	Newt	Teleost fish
V1b	V1b	VT2	V1b	V1a2
Sequence homology[1]	63	47	59	50
Organs	Brain, pituitary	Pituitary	Pituitary	Brain, heart
Physiology and behavior	Regulates ACTH release and CORT levels.	Release of ACTH and prolactin	ACTH release.	Aggression and courtship behavior
Ref	45, 59, 85	29, 52, 53	43, 43	55, 57

[1]The proposed homologous receptors in other vertebrates were compared to the human V1b receptor (V1bR). The sequence homology refers to the degree of homology to humans.

Table 5: Comparison of mammalian V2 receptor with non-mammalian species based on receptor distribution in organs and function

Species	Rats	Chicken	Frog	Newt	Telost fish
V2	V2	VT1	V2	V2	V2
Sequence homology[1]	86	57	52	50	41
Organs	Kidney	Shell gland, uterus	Brain, heart, kidney, urinary bladder	Brain	Gills, heart and kidney
Function	Enhances cAMP mediated aquaporin for water reabsorption	Contraction of uterus muscles	Cutaneous water reabsorption	Osmotic or ionic regulation	Regulated at the hypothalamic level during hyperosmotic conditions
Ref	67	84	56	42, 43	56

[1]The proposed homologous receptors were compared to the human V2 receptor (V2R). The sequence homology refers to the degree of homology to humans.

Table 6: Comparison of the mammalian OT receptor with non-mammalian species based on tissue and organ receptor distribution and function

Species	Rats	Chicken	Frog
OT	OT	VT3	MT
Sequence homology[1]	91	74	67
Organs	Brain, pituitary, mammary gland and uterus	Endometrium, myometrium of shell gland	Brain and pituitary
Function	Release of prolactin	Oxytocic function in reproduction	Controls the activity of pituitary melanotropes
Ref	4	40	1

[1]The proposed homologous receptors based upon amino acid sequence identity to the human OT receptor (OTR). The sequence homology refers to the degree of homology to humans.

Previous studies in our lab focused on the VT2 receptor (VT2R) in chicken which is an avian homolog of V1b receptor (V1bR) in mammals. A vasotocin receptor, little studied in chickens is the VT4R. The VT4R has the highest homology with its mammalian counterpart V1aR when compared to other receptor subtypes. Furthermore, the distribution of the V1aR in several species (Table 3) is widespread throughout the body compared to other three subtypes investigated to date. There have not been published studies on the distribution of receptor subtypes VT1R, VT3R or VT4R in the chicken brain upto this time. Due to the presence of V1aR in rat, fish and the amphibian brain, the avian VT4R is a good candidate for its presence in the chicken brain. The current literature review will focus on distribution of V1aR in mammals and in non-mammalian species.

1.3 V1a receptors:

There are two conserved glycosylation sites within the second and third extracellular loop in V1aR. The receptor V1aR is not o-glycosylated. The two sites that are glycosylated in the N terminus are asparagine (Asn) 14 and Asn 27, while in the second extracellular loop Asn 198 is glycosylated. The glycosylation of the V1aR regulates the level of receptor expression at the cell surface and is responsible for normal receptor expression and efficient folding of proteins. The glycosylation of the receptor, however, is not required for ligand recognition for the intracellular signalling pathway or for trafficking of receptor to the cell surface (Hawtin et al., 2001).

The receptor is present in brain and pituitary of several species. The review addresses the distribution of receptors in pituitary and brain among a few selected species.

1.3.1 Hormones secreted by the pituitary in avian species:

The pituitary gland is connected to the hypothalamus at the base of the brain by the infundibular stalk. The pituitary is classified as neurohypophysis and adenohypophysis beginning at the embryonic level and throughout the adult stage. The two lobes of the pituitary (adenohypophysis and neurohypophysis) are also connected by a network of blood capillaries (Singh and Dominic, 1975). The neurohypophysis is derived from the infundibulum while the adenohypophysis is derived from the Rathke`s pouch. The adenohypophysis in mammals is further differentiated to form the pars distalis (anterior pituitary), the pars intermedia and the pars tuberalis. The neurohypophysis forms the posterior pituitary (pars nervosa), infundibular stalk and median eminence (Scanes, 2000).

The avian pituitary lacks the pars intermedia and hence the adenohypophysis forms the anterior pituitary gland and the pars tuberalis. The anterior pituitary gland is further divided into

two lobes: cephalic lobe and caudal lobe (Mikami, 1986; Shin-ichi, 1986; Mikami and Takahashi, 1987; Scanes, 2000). The anatomy of the hypothalamic-hypophyseal complex and the hormones secreted by anterior pituitary in avian species are summarized in Figure 2.

The anterior pituitary comprises six types of cells: ACTH/α melanocyte stimulating hormone (αMSH) cells, prolactin cells, growth hormone cells, thyrotropin releasing hormone cells and, in contrast to mammals, separate gonadal hormone producing cells, namely lutenizing hormone (LH) and follicle stimulating hormone (FSH) cells. The hormone, pro-opiomelanocortin (POMC) is a huge precursor protein that is confined to the cephalic lobe of pituitary as studied by *in situ hybridization* (Gerets et al., 2000). Its (POMC) derivatives are produced in the anterior pituitary gland that includes an N terminal fragment, a joining peptide, adrenocorticotropin (ACTH) and β endorphin. The adrenocorticotropin further can also be cleaved to form α MSH and corticotropin like intermediate lobe peptide, peptides that in mammals would be produced in the pars intermedia (Berghman et al., 1998). The ACTH cells, prolactin cells and TSH cells are confined to the cephalic lobe while growth hormone is present in the caudal lobe. The hormones, ACTH and α-MSH are produced from the same cell type, hence both are confined to the cephalic lobe and the cells are called corticomelanotropes. They are present in very small clusters of cells or in large masses (Ferrand et al., 1974). Gonadal hormone producing cells, LH and FSH cells, similar to mammals are spread throughout the cephalic and caudal lobes (Puebla-Osorio et al., 2002; Proudman et al., 1999).

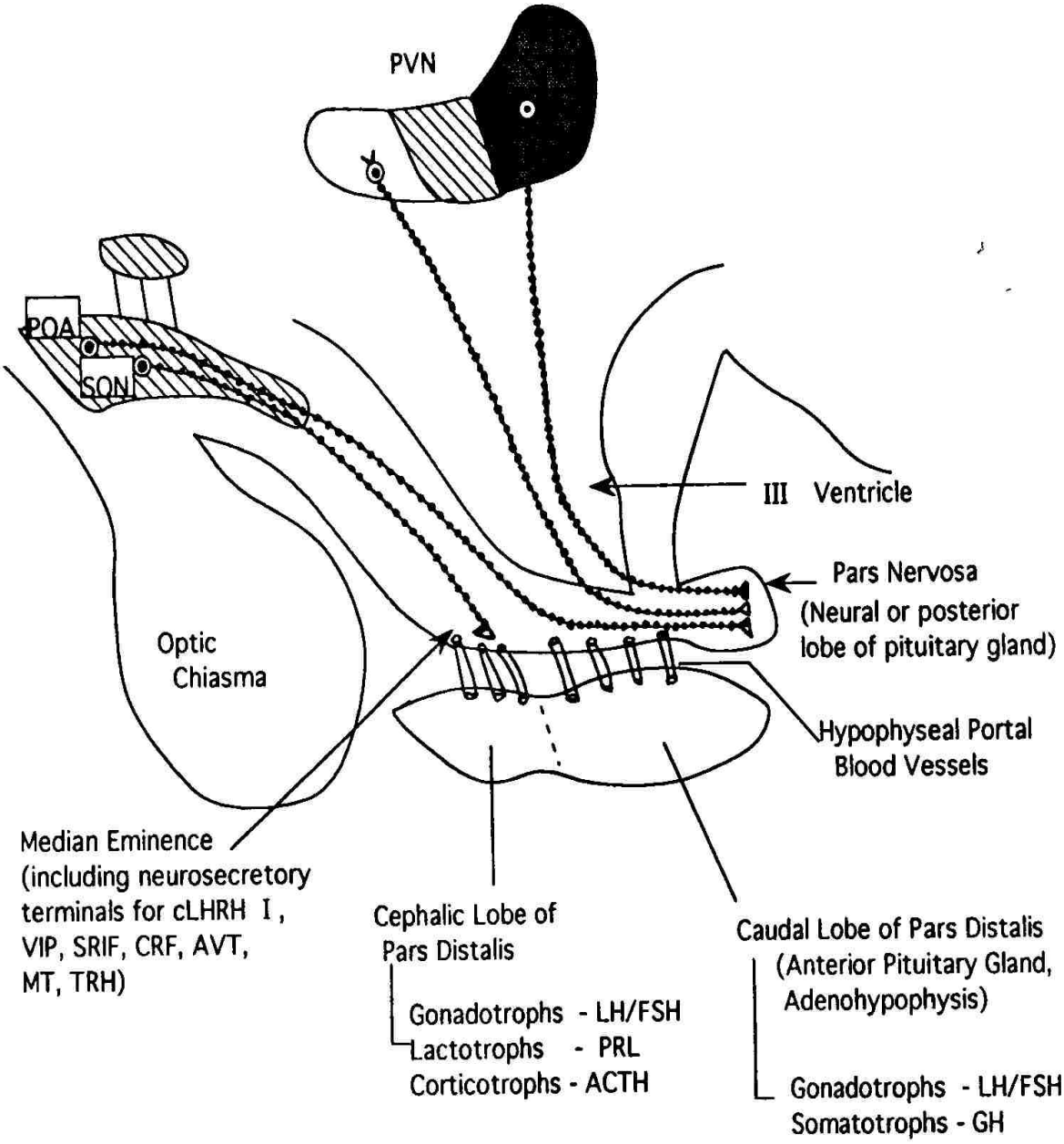

Figure 2: Schematic diagram of the avian hypothalamus and anterior pituitary gland. Location of AVT (solid white shading) and MT (solid black shading) cell bodies based on Bons et al., 1980; (>O•••••••••◄) neurosecretory neuron; (O═══O) blood vessel; POA, Preoptic area; PVN, paraventricular nucleus, SON, supraoptic nucleus; AVT, Arginine vasotocin; CRF, Corticotropin releasing hormone; MT, Mesotocin; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; PRL, Prolactin; ACTH, Adrenocorticotropin hormone; GH, Growth

hormone; cLHRH-I = GnRH-1, Gonadotropin releasing hormone ; VIP, Vasoactive intenstinal peptide; TRH, Thryotropin releasing hormone; SRIF, Somatostatin (Reproduced from (Scanes, 2000). LH/FSH cells are separate pituitary cells while ACTH and αMSH are produced from same cell type.

1.3.2 Distribution of V1a receptor in anterior pituitary among selected vertebrates:

1.3.2.1 V1a receptor in rats:

The V1aR is present in a subset of cells in the anterior pituitary. Immunoreactive V1aRs were associated in cells containing the gonadal hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). This helps in understanding the role of vasopressin in LH/FSH regulating pathways including gonadotropin releasing hormone (GnRH) neurons in brain (Orcel et al., 2002).

1.3.2.2 VT1 receptor in frogs:

The VT1R were present in the distal and intermediate lobes of the amphibian pituitary and helps in the secretion of ACTH from corticotropes in anterior pituitary (Acharjee et al., 2004a; Acharjee et al., 2004b).

1.3.2.3 V1a1 receptor in fish:

The receptor distribution V1a1R in the fish pituitary, *Epinephelus adscensionis* was found in the proximal pars distalis (Kline et al., 2011). A clone of the vasotocin receptor, V1a1R in the sucker fish was obtained utilizing a sequence based upon the human V1aR. The homology of the fish V1a1R to human V1aR was 48% and to the rat V1aR was 60%. The V1a1R was found

in liver, pituitary, and gills. Based on its distribution, the functions of the receptor were suggested to be involved in liver metabolism, ACTH secretion and osmoregulation in bony fish (Mahlmann et al., 1994).

1.4 Distribution of V1a receptor in brain among selected vertebrates:

The V1a receptor is widely distributed throughout the body of vertebrates. Most studies, however, addressed receptor distribution in the brain and its associated functions therefore the latter will be emphasized among a few selected mammals and non-mammalian vertebrates.

1.4.1 V1a receptor in humans:

Arginine vasopresssin receptor (V1aR) was cloned in humans and comprises 418 amino acids that shares 83% similarity to the V1a receptor sequenced in rat liver. It has three glycosylation sites at asparagine (Asn) 14, Asn 17 and Asn 196, eight threonine and seventeen serine phosphorylation sites located in third loop of the transmembrane domain of the receptor (Thibonnier et al., 1994)

1.4.2 V1a receptors in mammals:

In **rats**, the V1a vasopressin receptor is widely expressed in brain (Aoyagi et al., 2009). The mass of the rat V1aR is 44 kDa and comprises 394 amino acids. In the rat brain, autoradiography studies found prominent V1aR binding in olfactory bulb, hippocampus, lateral septum, paraventricular nucleus, ventral tegmental area, raphe nuclei, locus coeruleus, nucleus of the solitary tract, subfornical organ, area postrema and hypoglossal nucleus (Ostrowski et al., 1994); (Ostrowski et al., 1992). Arginine vasopressin through V1aR aids in the regulation of

blood pressure through vascular contractions, arterial baroreceptor reflex, sympathetic nerve activity, water reabsorption, lipid metabolism, protein catabolism and glucose tolerance (Caldwell et al., 2008; Aoyagi et al., 2009)

Campbell et al., 2009 studied the V1aR pattern in two species of **singing mice** *Scotinomys. teguina* and *Scotinomys. xerampleinus.* The V1aR binding was evident in lateral septum, centromedial and ventrolateral nuclei of thalamus, supraoptic nucleus, paraventricular nucleus, amgydala and globus pallidus. No significant binding was observed in hippocampus and olfactory bulb. Receptor binding was higher in forebrain of *S. teguina* while thalamic regions had higher binding with *S. xerampelinus.* There was fiber tract binding in *S. xerampelinus* that was suggested to be V1aR expressed in glial cells or localization of V1aRs in neuronal axons. The high receptor density was observed in periaqueductal gray and anterior hypothalamus (preoptic area) in *S.teguina.* Periaqueductal gray is associated with vocal production in singing mice while anterior hypothalamus is important for partner preference and association. Similarly, strong binding was observed in the medial geniculate nucleus which is associated with auditory function. Based on anatomical evidence, the V1aR plays a functional role in regulating species-specific vocal behavior (Campbell et al., 2009).

Tuco-Tuco is a rodent species found in South America. The V1aR distribution in two closely related species of Tuco-Tuco, *Ctenomys haigi* and *Ctenomys sociabilis* were studied. The V1aR binding was observed in the following neural structures: lateral septum, ventral pallidum, nucleus accumbens, hippocampus, and arcuate nucleus of the hypothalamus (Beery et al., 2008).

The V1aR binding was evident in the brain of golden **hamsters** in the following structures: cerebral cortex (cingulate, insular, retrosplenial, temporal and perirhinal cortex), corpus striatum, lateral septum, amgydala, bed nucleus of stria terminalis, hippocampus, suprachiasmatic nucleus,

medial preoptic nucleus, superior colliculus, area postrema, subfornical organ, nucleus of solitary tract, hypoglossal nucleus, ventral tegmental area, raphe nuclei, paraventricular nucleus and periventricular nucleus (Dubois-Dauphin et al., 1990). In hamsters, the V1aR plays a role in flank marking behavior (Dubois-Dauphin et al., 1990) and aggression (Ferris, 2000). In anterior hypothalamus, dense binding of the V1aR with vasopressin fibers was observed. The neurons secreting vasopressin from the supraoptic nucleus and nucleus circularis reach the medial preoptic nucleus of anterior hypothalamus and releas the peptide thereby aiding in aggression of hamsters (Ferris, 2000)

The receptor subtype, V1aR was cloned in **sheep** with a similarity of 85% and 80% to human V1a receptor and rat V1a receptor amino acid sequence, respectively. The gene encoding 418 amino acids has a Kozak consensus sequence (Kozak consensus sequence is a sequence of mRNA that has the consensus [(gcc)gccRCCAUGG where AUG is a start codon] that helps in translation. The receptor has two N terminal glycosylation sites, Asn 13 and Asn 26, seven threonine and sixteen serine phosphorylation sites in the third loop of the transmembrane domain. The receptor is expressed in brain, testis, aorta, pituitary, kidney, adrenal glands and muscle (Hutchins et al., 1995).

1.4.2 VT1 receptor in amphibia:

In **frogs**, AVT increases mate calling frequency in males while phonotaxis in females. Immunoreactive AVT cells are present in septum; amgydala pars lateralis, magnocellular preoptic area, suprachiasmatic nucleus and hypothalamus (Boyd, 1997). The VT1R was widely distributed in telencephalon, diencephalon and mesencephalon of the brain, though the expression level varies from region to region. A high intensity signal was observed in nucleus

ambens, ventral striatum, lateral pallium, preoptic nucleus and hypothalamic nucleus while a moderate intensity signal was observed in amgydala and ventral thalamic nucleus (Acharjee et al., 2004b; Acharjee et al., 2004a).

The V1a type receptor in **newts** is widespread in telencephalon, diencephalon, mesencephalon and medulla oblongata. The immunoreactive cells for the receptor were observed in the following structures: mitral layer of the olfactory bulb, dorsal and medial pallium, lateral and medial amgydala, bed nucleus of the decussation of the fasciculus telencephali, bed nucleus of stria terminalis, anterior preoptic area, magnocellular preoptic nucleus, suprachiasmatic nucleus, ventral thalamus, dorsal and ventral hypothalamic nucleus, tegmentum, interpeduncular nucleus, median reticular formation and nucleus motorius tegmenti (Hasunuma et al., 2007; Hasunuma et al., 2010).

1.4.3 V1a1 receptor in fish:

In fishes, AVT plays a key role in osmoregulation, seasonal biology and stress response. Gills play a vital role in maintaining water and ion balance in homeostatic environment. This is mediated through V1a receptors present in gills. Gills and renal tissues function together to maintain body fluid homeostasis (Balment et al., 1993; Balment et al., 2006). The receptor distribution of the V1aR in the fish brain, *Epinephelus adscensionis* was observed in the following structures: inner cell layer of the olfactory bulb, entopeduncular nucleus, lateral septal organ, dorsal, intermediate and ventral nucleus of ventral telencephalon, supracommissural nucleus, post commissural nucleus and lateral nucleus of ventral telencephalon, preoptic nucleus, periventricular nucleus, tuberal nucleus, nuclei saccus vasculous, paraventricular organ, preglomular nucleus, mammillary bodies, ventromedial thalamic nucleus, habenular nucleus,

pretectal nucleus, purkinje cells in cerebellum, raphe nuclei and locus coeruleus. Arginine vasotocin (AVT) can act as a neuromodulator or mediate its actions on monoamine neurotransmitter systems through the V1aR. Based on its anatomical distribution it could also be involved in olfaction, vision and learning (Kline et al., 2011)

A 2701 bp transcript produces the 384 amino acid polypeptide, vasotocin receptor (VTR) in the Euryhaline flounder. The AVT receptor is homologous to the mammalian V1a receptor by 62%. The receptor is present in brain, kidney and gill of the flounder (Warne, 2001). The receptor plays a key role in water loss in these fishes by increasing the secretion mechanisms of AVT (Warne et al., 2005).

1.4.4 Functional role of V1a receptors:

The functional roles of V1aR in mice were elucidated to be: social recognition, anxiety, while other tasks like memory and learning effects were more task oriented. Emotionality, however wasn't affected by V1aR activation (Bielsky et al., 2004; Bielsky et al., 2005a). The lateral septum via V1a receptors may play a key role in processing olfactory information received from conspecifics and delivering it to hypothalamus and hippocampus (Bielsky et al., 2005b). A V1aR agonist (Terlipressin) helped in reduction of vascular leakage in hypotensive and sepsis patients (Kampmeier et al., 2010). The receptor also helps in restoring the vasomotor tone and urine output by regulating renal blood flow (Holmes et al., 2011). The receptor also induces vasoconstriction in septic shock patients (Kampmeier et al., 2010). The receptor also regulates the expression levels of clock controlled genes (Li et al., 2009). The V1aR helps to maintain net water balance in brain through aquaporin channels present in cerebral cortex by water reabsorption (Niermann et al., 2001). Under water deprived conditions, V1aR enhances

locomotor activity by activating orexin neurons to locate the sources of water (Tsunematsu et al., 2008; Ulrich-Lai and Herman, 2009). The V1aR targets might play a role in post traumatic brain injury and secondary brain damage (Trabold et al., 2008). Arginine vasopressin (AVP) via V1aR regulates maternal aggression, grooming and licking behavior and maternal behavior in lactating rats (Nephew and Bridges, 2008). The gene AVPR1A encodes for AVP receptor subtype V1aR. The variability of the 5' flanking region of AVPR1A gene affects pair bonding behavior and affiliation in humans (Walum et al., 2008). The prairie vole has two copies of the AVPR1A gene while the montane vole has only one copy. Montane voles have microsatellite elements (repetitive di- and tetra-nucleotide sequences) that aid in transcriptional regulation of the V1aR. In prairie voles, there are short and long alleles of microsatellite elements, the long alleles are correlated with high binding intensities in the lateral septum and olfactory bulb and are associated with behavioral functions like grooming and licking (Nair and Young, 2006). The receptor, V1aR exhibits enormous plasticity that contributes to species differences in voles. (Winslow et al., 1993; Young et al., 1997; Young et al., 1999).

The growth locus QTL present in chromosome 1 in chickens has been under a strong selection process in breeding strategy programs over the past several decades. The QTL (growth) region in chickens has 50 genes including AVPR1a. The AVPR1a gene encodes for the neuroendocrine peptide AVT. This gene is associated with behavioral traits in humans and birds (Goodson et al., 2004; Bielsky et al., 2005b; Walum et al., 2008). A microsatellite allele for homozygosity was selected from the AVPR1a gene located in the growth QTL on chromosome 1 and evaluated to test if the genotype is responsible for social or explorative behavior. Several behavioral tests including a complex environment test, sociality test, mirror test and resident intruder test clearly shows that there is a marked difference in social and emotional behavior in

chickens. The study concluded that there might be an impact on emotionality and sociality of birds when the chicken is selected for growth and breast production during domestication (Wiren and Jensen, 2011).

1.5 Chicken AVT receptors:

The neurohormone AVT effects are mediated through G protein coupled receptors (GPCR). Chicken vasotocin gene is intermediate between the mammalian AVP gene and that of lower vertebrates. Specifically the AVT gene has a C terminal that is not cleaved properly from the neurophysin but glycosylated. The gene encoding vasotocin has a single copy with three exons and two short intervening sequences (Hamann, et al., 1992). Arginine vasotocin receptors are characteristic GPCRs having seven transmembrane domains, an extracellular N terminal, carboxy C terminal and two to three glycosylation sites in the N terminal domain (Baeyens and Cornett, 2006) . The heptahelical membrane proteins activate G proteins to perform effector functions (Birnbaumer, 2000). Functions of vasotocin include osmoregulation, increase in water permeability through AVT dependent aquaporin channels, regulation of blood pressure, oviposition and release of ACTH in response to stress. In addition, it also plays a major role in sexual behavior, social behavior, aggression and vocalization (Baeyens and Cornett, 2006). To date four types of avian VT receptors are known: VT1, VT2, VT3 and VT4 receptors. The receptors are compared to the human AVP receptors in Table 7.

Table 7: Homology of mammalian AVP receptors and chicken AVT receptors based on percentage identity (%) of their respective amino acid sequences

Species	Human V1aR	Human V1bR	Human V2R	Human OTR
Chicken VT1R	48	49	**46**	43
Chicken VT2R	53	**62**	45	50

Chicken VT3R	49	51	44	**74**
Chicken VT4R	**69**	53	43	51

1.5.1 VT1 receptor:

In chicken, the VT1 receptor was the first receptor identified in the vasotocin family and comprised 370 amino acids. The receptor is present in brain and in shell gland (Tan et al., 2000). The neuropeptide AVT via the VT1 receptor activates prostaglandin release in the shell gland resulting in contraction of smooth muscles and facilitating egg parturition (Seth et al., 2004a; Seth et al., 2004b). The receptor is also present in the endometrium in shell gland suggesting its role in contraction of smooth muscles in the uterus for expulsion of the egg. This receptor helps in AVT induced phosphatidylinositol turnover and high intracellular calcium mobilization. The receptor, however, induces uterine contractions during laying cycle as a possible paracrine mechanism. The receptor also has oxytocic-like activity that could play a role in maternal behavior (Tan et al., 2000; Baeyens and Cornett, 2006).

1.5.2 VT2 receptor:

The second receptor subtype identified and cloned was the VT2 receptor comprising 425 amino acids. The VT2R was predominant in the anterior pituitary, however, absent in brain (Cornett et al., 2003; Jurkevich et al., 2005; Baeyens and Cornett, 2006; Jurkevich et al., 2008). The VT2R is widely expressed in the cephalic lobe and scarcely distributed in caudal lobe of anterior pituitary. The receptor VT2R was colocalized with a high percentage of corticotropes followed by a smaller proportion of lactotropes. The main function of the receptor, based largely on its anatomical distribution and molecular interactions is the neuroendocrine regulation of stress responses and in the release of ACTH and corticosterone (Cornett et al., 2003;

Jurkevich et al., 2005; Baeyens and Cornett, 2006; Mikhailova et al., 2007; Jurkevich et al., 2008; Kuenzel and Jurkevich, 2010).

1.5.3 VT3 receptor:

The third receptor identified and cloned was the VT3R. It is 391 amino acids in length and expressed in the endometrium and myometrium of the shell gland. It suggests that this receptor might be involved in oviposition (Gubrij et al., 2005).

1.5.4 VT4 receptor:

The AVP vascular receptor responsible for blood pressure regulation is the V1a vasopressin receptor in mammals. The V1aR is activated through phospatidyinositol and intracellular calcium signalling pathway. It also activates phospholipases A_2, C and D (Peter et al., 1995). Recently, the VT4 gene was sequenced and submitted to GenBank (Cornett, 2007, Genebank submission). The receptor VT4R comprises 419 amino acids and has two exons and one intron. The VT4R shares 69% similarity with V1aR of mammals (Baeyens and Cornett, 2006). The VT4 receptor is a novel receptor identified in chickens with little information available. The present study was designed to determine the VT4 receptor distribution in brain and pituitary gland of chickens.

1.6 Summary:

Avian vasotocin receptors, largely by amino acid sequence identity, have been shown to be comparable to mammalian vasopressin receptors. The VT4R of chickens shares the highest sequence homology with the mammalian V1aR. Most of the studies have focussed on the V1a

receptor brain distribution and its association with social behavior in mammals. The current study focuses on the receptor distribution in the avian brain and anterior pituitary gland.

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III. ORIGINAL STUDIES

Chapter 2

Distribution of the vasotocin subtype four receptor (VT4R) in the anterior pituitary gland of the chicken, *Gallus gallus.*

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Abstract

The neurohormone arginine vasotocin (AVT) in non-mammalian vertebrates is homologous to arginine vasopressin (AVP) in mammals. Its actions are exerted via G protein coupled receptors that belong to the vasotocin/mestocin family. Arginine vasotocin mediates some of its effects through receptors located in anterior pituitary cells. Recently, an avian vasotocin receptor subtype designated VT4R has been cloned which shares 69% sequence homology with a mammalian vasopressin receptor, the V1aR. In the present study, a polyclonal antibody to VT4R was developed and validated to confirm its specificity. The antibody was used to test the hypothesis that the VT4R has a regional distribution and found on specific types of cells in the avian anterior pituitary. Western blotting of membrane protein extracts from pituitary tissues, use of HeLa cells transfected with the VT4R and peptide competition assays have confirmed the specificity of the antibody to the VT4R. Dual labelled immunofluorescence microscopy was utilised to identify pituitary cell types that contained immunoreactive VT4Rs. The receptor was found widely distributed throughout the cephalic lobe of the anterior pituitary demonstrating its regional distribution. Immunoreactive VT4Rs were associated with both corticotrophs and gonadotrophs. Approximately 89% and 12% of immunolabelled corticotrophs and gonadotrophs were shown to be immunoreactive for the VT4R, respectively. In both corticotrophs and gonadotrophs, the colocalisation of cells was present in the cephalic lobe of the pituitary. No immunoreactive VT4Rs were found in somatotrophs or lactotrophs. The high percentage of corticotrophs colocalised with the VT4R suggests a role of the receptor in stress responses.

Keywords: V1aR, stress, cephalic lobe, corticotrophs, gonadotrophs.

1. Introduction:

Homeostatic imbalance leads to activation of the hypothalamic pituitary adrenal axis (HPA) by secreting neuropeptides arginine vasopressin (AVP) and corticotropin releasing hormone (CRH). The neuropeptides are produced in the hypothalamus and transported to the anterior pituitary resulting in secretion of adrenocorticotrophic hormone (ACTH) from cotricotrophs. The ACTH acts on the adrenal cortex to stimulate release of glucocorticoids in humans. In avian species, ACTH stimulates the release and production of corticosterone in adrenal chromaffin tissues (1-6). Chicken CRH is identical to CRH of humans and rats (7, 8). Arginine vasotocin (AVT) is the non-mammalian counterpart of arginine vasopressin (AVP). Avian AVT differs from mammalian AVP in position 3 of the amino acid sequence (phenylalanine to isoleucine substitution in birds). Similar to mammals, avian ACTH contains 39 amino acids. Avian ACTH differs from human ACTH in 8 different positions of the amino acid sequence (positions 15, 20, 27, 28, 29, 30, 31 and 33) (9, 10). There have been several studies in birds on CRH (8, 11-13) and AVT (12-17) emphasizing that both CRH and AVT aid in the regulation of stress reponses by acting synergistically to release ACTH (3, 12, 18-25). The release of ACTH was observed in rats when AVP was microinjected into third ventricle of freely moving rats. Rats subjected to ether stress (acute stress) also showed an increase in ACTH release (26) Arginine vasopressin mediated the release of ACTH in a dose-dependent manner from rat anterior pituitary cells *in vitro* (23, 26, 27). Plasma LH levels increased in response to ether stress in female rats. Nevertheless, injection of AVP antiserum into ether stressed rats resulted in a decline in plasma LH levels (26). Arginine vasopressin stimulated the release of LH from gonadotrophs in rats (28), perfused rat pituitary cells (28, 29) and in cultured hemipituitaries of rats (30), however, intracerebroventricular administration of AVP lowered the level of plasma LH release in male

rats (21). In hamsters, AVP stimulated the release of LH and prolactin from anterior pituitary cell cultures in a dose dependent manner (31). Arginine vasopressin/AVT mediated the release of prolactin in cultured hemipituitaries in vitro (30). An intravenous and subcutaneous injection of AVT/AVP caused an increase in prolactin levels in rats pretreated with estradiol benzoate and progesterone (32, 33). Intracereboventricular injection of AVT increased plasma CORT levels but the response was significantly less than CRF induced plasma CORT levels in chicks (12, 13, 34). The neuropeptides, AVT and CRF at equal concentrations potentiated the release of ACTH from dispersed perfused duck pituitary cells (19, 20). Prolactin secretion was stimulated by AVT in monolayer cultures of turkey anterior pituitary cells (35) and in adult male chickens (36).

Arginine vasopressin/AVT act through G protein coupled receptors to mediate their effector functions. In mammals, the AVP/oxytocin (OT) family of receptors comprises four major subtypes: vasopressin (V) V1a, V1b, V2 and OT receptors. In birds, four receptor subtypes in the vasotocin family have been identified VT1R, VT2R, VT3R and VT4R. The first identified vasotocin receptor, VT1R comprised 370 amino acids. The receptor is present in the shell gland and brain of chickens (37, 38). It appears to be a homolog of the V2 receptor in humans. The VT1 receptor shows only 46% identity with its mammalian homolog and 51% identity with other VT receptor suggests that its an early divergent receptor from the other avian receptors. The receptor plays a role in contraction of smooth muscles in the uterus during oviposition (38). The VT2R is a 425 amino acid protein found predominantly in the cephalic lobe of the avian anterior pituitary but absent in brain. The VT2R is the non- mammalian homolog of V1b receptor (39, 40). The receptor plays a role in mediating the secretion of ACTH from corticotrophs and to a lesser extent prolactin (PRL) from lactotrophs based upon co-localization of the VT2R in corticotrophs and lactotrophs, respectively (36, 37, 39, 40). In several mammalian species

including rats (41), mice (42, 43) and humans (44), the V1bR is the most abundant receptor in pituitary. The major role of the receptor is to facilitate the release of ACTH from the anterior pituitary during stress responses in both mammals and non-mammalian species (36, 40-42, 45). The third cloned receptor, VT3R is a 391 amino acid protein. The receptor is present in the myometrium and endometrium of the shell gland in chickens (37, 46). The non-mammalian counterpart of the oxytocin receptor is suggested to be the VT3R in chickens (46). The receptor might play a role in oviposition by stimulating myometrial contractions in uterus (46).

The VT4R in chickens is the non-mammalian counterpart of the V1aR in mammals. The VT4R is a novel vasotocin receptor that has been cloned recently in chickens (Cornett, 2007, GenBank Accession no: XM_001235007). The receptor comprises 419 amino acids and its gene (AVPR1A) is present in chromosome 1(47). The VT4R shares an amino acid sequence homology of 69% when compared to the V1aR in humans (37). The receptor has been suggested to play a role in the regulation of blood pressure (37). Several studies have been done on the V1aR in humans (44), rats (28, 42, 43, 48), mouse (49, 50), frogs (51, 52) and newts (53, 54). In rats, V1aR is present in the anterior pituitary and colocalises with gonadotrophs (28, 42, 43) . A definitive distribution of VT4R in anterior pituitary has not yet been reported in chicken. The avian VT4R has the highest sequence homology to the mammalian V1a receptor (69%) compared to the other three receptors (VT1R (48%), VT2R (53%) and VT3R (49%) (See Table 1 for references). Since the vasotocin system in avian species is involved with the regulation of stress (13, 36, 40, 55), a study was designed to determine whether or not the VT4R occurs in the anterior pituitary of the chicken. The hypothesis tested was that the VT4R is expressed in avian anterior pituitary cells.

2. Materials and methods

2.1 Animals

(a) Organs sampled for antibody validation study: Twenty anterior pituitary glands were obtained from 6-8 week old birds for the extraction of membrane protein. Birds were cervically dislocated and heads placed on ice. The pituitary glands were immediately dissected, frozen on dry ice and stored in Eppendorf vials at -80°C.

(b) Animals utilized for immunohistochemical study: Six adult male broiler birds (24-32 weeks old) were utilised for the study of the VT4R distribution in anterior pituitary. All birds used in the current study were kept individually in cages under a 14h light and 10h dark lighting cycle. Food and water were given *ad libitium*. All animals used in this study were treated in accordance with the protocols approved by the University of Arkansas Institutional Animal Care and Use Committee.

2.2 Antibody production for vasotocin receptor subtype 4 (VT4R)

Polyclonal antibodies to the chicken vasotocin receptor VT4R were raised in rabbit against synthetic peptides. In brief, rabbits were injected with two peptides, one peptide comprising 15 amino acids from the amino terminal region, AGDWDPFGRDEELAC (residues 45-58) and other peptide containing 17 amino acids from the carboxy terminal region, NSNSRRQTSFTNNRSPT (residues 376-392) of the chicken VT4 receptor (Cornett, 2007, GenBank submission). The two peptide sequences selected were unique between VT4 and other vasotocin receptor subtypes (GenBank Accession No: XM_001235007). For ease of conjugation of the peptides to a carrier, a cysteine thiol with a 6 carbon spacer and glutaraldehyde was added

to the C terminal and N terminal, respectively. The peptides were then conjugated to a carrier protein, keyhole limpet Hemocyanin (KLH) by the use of bifunctional reagent m-maleimidobenzoyl N hydroxy succinimide (MBS). The conjugate was used to immunize two rabbits with a booster dose given at 2 week intervals. The bleeds were collected at 52, 56, 73 and 80 days. The antiserum from rabbit 1 obtained at 73 days (bleed 3) gave the best staining pattern in pituitary sections using immunohistochemistry. This antiserum named as VT4#3/63 was subsequently used for future studies. The antibody was developed by 21st Century Biochemicals Inc. (Marlboro, MA).

2.3 Tissue and VT4R cell lysates

Membrane protein fractions from pituitary glands were extracted using a plasma membrane protein extraction kit (Biovision, CA, USA) as per the manufacturer's protocol. Tissues were homogenised with the buffer mix provided and the cellular membrane protein fraction was obtained by centrifugation at 10, 000g for 30min. The pellet was resuspended in equal volumes of upper phase and lower phase buffers provided in the kit. After repeated centrifugations, the plasma membrane protein fraction lysate was obtained. The concentration of the protein lysate was estimated by RC/DC (reducing agent and detergent compatible) protein assay kit II (Biorad, CA, USA).

HeLa cells were seeded onto 10 cm dishes in 10 ml growth medium (10% fetal bovine serum and Eagle`s minimum essential medium) and incubated in a 5% CO_2 incubator. Twenty hours post transfection; the cells were transiently transfected with 2.8µg of cDNA encoding VT4R with expression vector pcDNA3 or 2.8 µg of pGEM7-Z in 50 µl of OPTI-MEM (Gibco, Invitrogen, USA) using Lipofectamine 2000 method (56). The cells were incubated at 37°C in a

5% CO_2 incubator for 4 hours. The transfection medium was removed and replaced with fresh medium (39). Ten days after post-transfection, cells were scrapped from the dishes and resuspended in PBS and the cell count was estimated. Later, the cells were centrifuged at 14,000 rpm for 15 min. The pellets were resuspended in 15 ml of PBS. The cells were aliquoted into smaller volumes and centrifuged at 14,000 rpm for 15min. The supernatant was aspirated, washed with PBS and frozen with liquid nitrogen. The HeLa cell aliquots transfected with the VT4R were prepared by Sandie Jacoby, University of Arkansas for Medical Sciences and shipped to our laboratory.

2.4 Western blotting:

Fifty micrograms of protein from pituitary tissue lysate and 40 µg of VT4R cell lysate were diluted in 6X concentrated Laemmli buffer containing sodium dodecyl sulphate (SDS) and β-mercaptoethanol. Samples were heated for 15 min at 90°C and loaded on 12% SDS polyacrylamide gel and the proteins were separated at 180V for 55 min (BioRad, CA, USA). Proteins were transferred to a nitrocellulose membrane at 0.25A for 70 min (BioRad, CA, USA) using Tris-Glycine HCl buffer pH 9.5. The membrane was immersed in 5% non-fat milk (Sigma, St. Louis, MO, USA) for 30 min to block non-specific binding sites. The membrane was incubated overnight with primary rabbit antiserum against chicken VT4R at a working dilution of 1:1200, rinsed in Tris-buffered saline with 1% Triton-X 100 (TBS-T) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 hr (Santa Cruz Biotechnology, CA, USA). The membrane was washed with TBS-T. An Enhanced Chemiluminescence Kit (Amersham Biosciences, NJ, USA) was utilised to visualise the immunoreactive bands on Kodak Biomax Maximum Resolution Autoradiography film (Carestream Health, PA, USA).

Specificity of the antibody was tested using a peptide competition assay or preadsorption assay with the protein fraction obtained from pituitary tissues. The antibody at a working dilution of 1:1200 was preincubated with increasing amounts of the antigenic peptides (5µg and 50 µg) for 3 hours at room temperature. The peptide-antibody complex was centrifuged at 12,000 rpm for 30 minutes at room temperature. The supernatant was used as a substitute for the primary diluted antiserum normally used in the Western blot.

2.5 Immunohistochemistry

2.5.1 Sample preparation

Birds were anaesthetised with an intravenous injection of sodium pentobarbital (40 mg/kg i.v) and perfused through cartoid arteries with 250 ml heparinized phosphate buffered saline (0.1M PBS with 0.1% sodium nitrite, pH 7.4) immediately followed by fixation using 400 ml of Zamboni`s fixative (4% paraformaldehyde with 15% saturated picric acid in 0.1M PBS buffer pH 7.4). The pituitary glands were dissected from the cranium, postfixed in the same fixative overnight at 4°C, cryoprotected in 30% sucrose in 0.1M PB at 4°C until they sank to the bottom and subsequently frozen in dry ice and stored until sectioned. The pituitary tissues were embedded in Jung OCT medium to form a tissue block (Leica Microsystems, Germany) and sectioned at 10 µm in horizontal/coronal planes on a cryostat (Leica CM 3050S, Leica Microsystems, TX). Sections were collected on double coated gelatinized microscopic glass slides and stored at -80°C in sealed boxes with dessicant until processed for immunohistochemistry.

2.5.2 Bright field immunohistochemistry

Slide-mounted sections were rinsed in PBS to remove fixative. Endogenous peroxidase activity was suppressed using 0.6% hydrogen peroxide followed by cell permeabilization with 0.4% Triton X-100. Sections were placed in 5% normal goat serum (NGS) for 30 min to block non-specific binding sites. Sections were incubated in primary rabbit antiserum against VT4R for 40hrs (dilution 1:3000 in 0.02M PBS with 1% NGS, 0.2% Triton X-100). Sections were incubated with goat anti rabbit IgG (Vector Laboratories, CA, USA) for 90 min followed by Vectastain Elite ABC peroxidase complex incubation for 90 min (dilution 1:5, Vector Laboratories, CA, USA). Immunoreactive VT4R cells were visualized using chromogenic diaminobenzidine nickel complex developed in the presence of glucose oxidase (57). After several rinses of PBS, sections were coverslipped with Histamount (National Diagnostics, CA).

2.5.3 Immunofluorescence

Slide mounted pituitary sections and fixed cultured HeLa cells transfected with the VT4R gene were subjected to immunofluorescence. Sections were rinsed in PBS to remove fixative; cell permeabilization was done using 0.4% Triton X-100 for 20 min. Sections were covered with 10% normal donkey serum (NDS) in TBS (PBS with added 0.2% Triton X-100, pH 7.4) for 30min (Jackson Immunoresearch, PA) to decrease non-specific immunostaining. Later, they were incubated with rabbit antibody against chicken VT4R diluted 1:2000 (in TBS with 1% NDS) for 40 hrs at 4°C. Sections were washed in three changes of PBS followed by incubation with Texas Red donkey anti-rabbit IgG or DyLight 594 donkey anti-rabbit IgG for 90 min at room temperature in a humidified chamber (dilution 1:500 in TBS, Jackson Immunoresearch,

PA). After three changes of PBS, sections were coverslipped with Vectashield or Vectashield with DAPI to counterstain cell nuclei (Vector Laboratories, CA).

2.5.4 Dual labelling immunofluorescence:

The types of pituitary cells immunoreactive for VT4R were determined using a dual labelling immunofluorescence study. This study focussed on the presence of VT4R immunoreactivity in pituitary corticotrophs, gonadotrophs, somatotrophs and lactotrophs. Sections were washed, permeabilized and blocked as described in section 2.5.3. Sections were then incubated with a cocktail of rabbit antiserum raised against chicken VT4R (VT4 #3/63) and mouse monoclonal antibody to chicken proopiomelanocortin, prolactin (PRL), luteinising hormone (LH) or growth hormone (GH). All primary antibodies, source and dilutions that were employed in this study are listed in Table 2. Sections were incubated with either of the above cocktail for 40-45 hrs at 4°C (1% NDS, 0.2% Triton X-100 in PBS). It was followed by three rinses in PBS 10 min each and incubation with a cocktail of Texas Red donkey anti-rabbit IgG and DyLight 488 donkey anti-mouse IgG diluted at 1:500 (PBS with 0.2% Triton X-100) for 90 min at room temperature in a humidified chamber. All secondary antibodies used in this study were obtained from Jackson Immunoresearch, PA. The antibodies were of multiple labelling grades that were solid phase adsorbed to reduce cross reactivitiy to IgG of other species. Immunolabelled sections were coverslipped with Vectashield or counterstained by Vectashield with DAPI (Vector Laboratories, CA USA).

2.6 HeLa cell transfection with chicken VT4R gene using Lipofectamine:

To validate that the antibody is specific to VT4R in cells, immunocytochemistry was performed on HeLa cells containing the vector pcDNA3-VT4 and pcDNA3-VT1, pcDNA3-VT2, pcDNA3-VT3 respectively. In brief, the HeLa cells were seeded onto a four-chamber slide (Lab Tek-II Chamber Slide system, Rochester, NY) at a concentration of $2X10^5$ cells per chamber in 1 ml growth medium. The growth medium contains 10% fetal bovine serum and Eagle`s esssential minimum medium in the absence of antibiotics. Twenty four hours later, the cells were transiently transfected with 2.8 µg of expression vector pcDNA3 containing cVT4R, cVT1R, cVT2R or cVT3R in 1 µl of Lipofectamine 2000 (Invitrogen, CA, USA) in 50 µl OptiMEM (Gibco, Invitrogen, CA, USA) per chamber. Cells were incubated in a 5% CO_2 incubator. In addition, pCDNA3 containing cVT2R was tagged with Yellow Fluorescent Protein (YFP). Two chambers of HeLa cells were left nontransfected to serve as negative controls with each subtype. The transfection efficiency of cells was about 75-80%. Forty eight hours later, the slides were rinsed with PBS pH 7.4, fixed with 4% paraformaldehyde in 0.1M PBS, pH 7.4 for 15min at room temperature, briefly rinsed again with PBS, dried overnight at 4°C and freezed at -80°C in sealed slide boxes. The slides were processed for immunocytochemistry as described in section 2.5.3 as a confirmatory test for antiserum specificity. The cell nuclei were counterstained using DAPI.

2.7 Antiserum specificity tests:

The peptide used as an antigen was preadsorbed with the antibody produced as a specificity control. Antiserum in a working dilution 1:1200 was preincubated with 8 µg and 50 µg of the peptide for 3 hrs at room temperature and centrifuged for 30 min at 14,000 rpm. The supernatant

obtained was substituted for primary antiserum on brain sections processed for immunofluorescence labelling as described in section 2.5.3. Additional controls included omission of primary or secondary antibody from all immunocytochemical protocols described above. Additionally, in dual labelling protocols, the specificity tests included the omission of one of the primary or one of the secondary antibodies.

2.8 Image Analysis

Sections were examined using an Axioplan Fluorescence Research Microscope (Carl Zeiss, USA). Images were captured using a Nikon Eclipse 90i upright scanning laser confocal microscope (Nikon systems, USA). The image adjustments for confocal images were controlled by Fiji software version 1.45r. The brightness and contrast were adjusted in Adobe Photoshop CS5.

The proportion of corticotrophs and gonadotrophs colabelled for the VT4R was quantified using three animals. Two sections (per animal) sectioned near the middle of the cephalic lobe were chosen for analysis. Each histological section was scanned using epifluorescence illumination at 40x magnification, switching between GFP and Rhodamine filters. Based on random sampling, six regions were imaged in each section using Axiovision product suite 4.0.6 (Carl Zeiss, USA). The cells were counted within six 165µm X 165µm fields per section per animal. Corticotroph or gonadtroph immunoreactive cells were counted in each region provided, and cells single labelled and colabelled for the VT4R were tabulated. All data were recorded as means ± one standard deviation. The proportion of dual labelled corticotrophs and gonadotrophs were calculated.

3. Results

3.1 Antiserum specificity:

(a) Western blot:

The specificity of the rabbit VT4R #3/63 antiserum was tested using the Western blot procedure and membrane protein extracts of chicken pituitary tissues. The antibody revealed a robust single band at 46-47KDa with pituitary tissue and HeLa cells transfected with an expression vector encoding chicken VT4R cDNA. The absence of the 46-47KDa band was observed in Hela cells transfected with an expression vector pGEM7Z lacking the VT4R (Fig 2A). Preadsorption of the antibody with 5µg of immunogenic peptides attenuated the intensity of the band while the staining was completely abolished by the antiserum preadsorbed with 50 µg of immunogenic peptides (Fig 2B).

(b) Immunocytochemistry:

In coronal sections of pituitary, VT4R immunoreactive receptors were observed in the cephalic lobe of the anterior pituitary (Fig 3A). The preadsorption of antibody with 8 µg of peptide significantly reduced the immunostaining of cells while absence of VT4R immunoreactivity was observed with 50 µg of peptide preadsorbed with the antibody (Fig 3B and 3C).

HeLa cells transfected with VT receptors were used as a final test to validate the specificity of the antibody. Rabbit VT4R #3/63 antibody revealed immunoreactivity in cultured HeLa cells transfected with an expression vector encoding chicken VT4R cDNA. Almost all cells had immunoreactivity displayed throughout the whole cell profile (Fig 4A and 4B) while few cells had VT4Rs internalized (Fig 4B). Absence of VT4R immunoreactivity was observed in HeLa

cells transfected with plasmid pGEM7Z lacking VT4R (Fig 4C), expression vector encoding chicken VT1R cDNA (Fig 4D), VT2R cDNA (Fig 4E) and VT3R cDNA (Fig 4F).

3.2 Regional distribution of VT4R in anterior pituitary:

The vasotocin receptor, VT4R immunoreactive cells were present predominantly in the cephalic lobe of the anterior pituitary gland, although a few cells were scattered in the caudal lobe of anterior pituitary (Fig 5, 5A and 5B). The VT4R immunoreactive receptor was arranged either as a group of cells clustered together (Fig 5A, 5B and 5C) or scattered individual cells (Fig 5A, 5C, 6D). Immunolabelled VT4Rs were round or oval in shape (Fig 5C, 6C and 6D) and were associated with plasma membrane and more prominently in the cytoplasm (Fig 5B, 5C, 6C).

3.3 VT4R in corticotrophs:

Dual immunofluorescence labelling for the VT4R and POMC revealed that a large population of corticotrophs contained immunoreactive VT4Rs. These cells were shown distributed either as a group of 8-10 cells or as scattered, individual cells observed strictly in the cephalic lobe of anterior pituitary. Pro-opiomelanocortin (POMC) cells containing VT4Rs occurred at the periphery and in the cytoplasm of each cell (Fig 7A, 7B and 7C). The proportion of POMC cells containing VT4R varied from 88% to 91% with a mean of 89.2%. The proportion of VT4Rs containing POMC varied from 70% to 74% with a mean of 72%.

3.4 VT4R in gonadotrophs labelled for luteinising hormone (LH):

Gonadotrophs containing LH were present throughout the cephalic and caudal lobes in the avian anterior pituitary. Dual labelling of the VT4R and LH revealed a minor population of gonadotrophs immunoreactive for the VT4R. Colocalised cells were observed only in the cephalic lobe of the anterior pituitary. Colocalisation of the VT4R in LH immunoreactive cells was shown mostly in the peripheral regions of the cells (Fig 7D, 7E and 7F). The percentage of LH cells containing VT4R ranged from 11% to 13% with an average of 12%.The proportion of VT4Rs containing LH ranged from 15 to 17% with an average of 16.2%.

3.5 VT4R in lactotrophs/somatotrophs:

Dual labelling of VT4R was completed with somatotrophs and lactotrophs. Neither lactotrophs nor somatotrophs had immunoreactive VT4R in their cells. Lactotrophs and VT4R are both present in the cephalic lobe of anterior pituitary. Nevertheless, there were no immunolabelled cells that contain both VT4R and lactotrophs (Fig 8).

With regard to somatotrophs, there was a difference in regional distribution of the receptor and hormone in the anterior pituitary. Somatotrophs are present in the caudal lobe of pituitary while the receptor is present in cephalic lobe. Examination of the region around the anatomical boundary of the two lobes wherein the distribution overlapped did not have any somatotrophs containing the VT4R (Fig 9).

4. Discussion

4.1 Antiserum specificity:

In order to investigate the distribution of the VT4R in the pituitary gland, a polyclonal antibody was raised in rabbit against a cocktail of two peptides. The specificity of the VT4R #3/63 antibody was evaluated by four major techniques: Western blot using pituitary tissues, Western blots on transfected cells, immunohistochemisty, peptide competition or preadsorption assays with Western blots and with immunohistochemistry (58-60). The receptor protein VT4R was detected from membrane protein extracts at 47 KDa utilising a Western blot. The predicted size of the receptor based upon its molecular weight of 419 amino acids encoding the receptor is 46.2 KDa which is in good agreement with the estimated mass of the band. The absence of a band at 47 KDa in non transfected HeLa cells and its disappearance when the VT4 #3/63 antiserum was preadsorbed with the cocktail of synthetic peptides against which the antibody was raised lends support that the antibody specifically recognizes only the VT4R (Fig. 2).

As the primary goal of the antibody was to be employed in immunocytochemistry, the antibody was tested for its specificity with sections of pituitary and cell cultures. The antibody revealed immunoreactive cells in the cephalic lobe while the immunoreactivity was absent when preadsorbed with a cocktail of peptides (Fig 3). In addition, HeLa cells transfected with the VT4R had immunoreactive receptors present in the cytoplasm including its peripheral regions and the absence of immunolabelled cells in cultures transfected with other VT receptors subtypes (VT1R, VT2R, VT3R) (Fig 4). Results confirmed specificity of the antibody to the VT4R protein.

4.2 Anatomical distribution of VT4R in pituitary

Similar to mammals, the avian pituitary gland comprises an anterior and posterior pituitary. The organization of anterior pituitary, however, is different from mammals. As suggested by Wingstrand (1951) the anterior pituitary is subdivided into cephalic and caudal lobes (61). Two separate sets of portal capillaries serving the anterior and posterior regions of the anterior pituitary suggested the possible separation of functional groups of pituitary cells in the cephalic and caudal lobes of anterior pituitary, respectively, of the white-crowned sparrow, *Zonotrichia leucophrys gambelii* (62). The division of the anterior pituitary into two lobes has been confirmed in other avian species (63, 64). The VT4R shown to be distributed regionally; predominantly in the cephalic lobe (Fig 6A and 6B) provides further evidence for a functional division of the avian anterior pituitary as well as our past data addressing the distribution of the VT2R (36,40). Most of the cells in cephalic lobe were labelled for the VT4R. Present data showing the great abundance of the VT4R in the cephalic lobe, the major lobe of the avian anterior pituitary, as well as previously demonstrated abundance of the VT2R in the cephalic lobe (36, 40) are in good agreement with high expression of VT2Rs and VT4Rs mRNAs (Kang and Kuenzel, unpublished data) and suggest that the these receptors are likely the most abundant vasotocin receptors present in the avian pituitary gland.

4.3 VT4R in corticotrophs/melanotropes:

The present study provides strong evidence that in male chickens, the VT4R are colocalised with corticotrophs and gonadotrophs. Virtually 89.2% of cells that contained POMC were labelled for VT4R. Anatomical results support previous studies that AVT stimulates the release of ACTH (12, 34, 36). The receptor, VT4R is also present in neurons of supraoptic nuclei

(SON), paraventricular nucleus (PVN) and internal (iz) and external zones (ez) of median eminence (ME) (65). The VT4R located in hypothalamic neural structures associated with stress (65) and colocalised with corticotrophs in the anterior pituitary, suggests that the VT4R is involved in neuroendocrine regulation of stress responses. The VT2R in chickens is also colocalised in a high percentage of corticotrophs (36, 40) suggesting an interaction of both the VT2R and VT4R in the stress response.

Proopiomelanocortin (POMC) is a big precursor protein that produces hormones including ACTH and α-MSH by cleavage of prohormone molecules (66, 67). It has been shown that α-MSH and ACTH are colocalised in same cells in anterior pituitary in chickens as well as possess the VT2R (36). Similarly, the corticotrophs that contain the VT4R likewise contain α- MSH suggesting that the VT4R not only mediates the release of ACTH but also α-MSH (36, 40).

4.4 VT4R in gonadotrophs:

Gonadotrophin releasing hormone type one (GnRH-1) is the primary neurohormone for the release of gonadotropins from the anterior pituitary (68-70). There has been evidence suggesting that AVP mediates the release of LH in mammals. In rats, AVP stimulates the release of LH and FSH hormones (26, 28). Administration of AVP/AVT subcutaneously (71) causes a reduction in the plasma levels and release of LH in rats (21, 71). Administration of AVP intracerebroventricularly lowered the plasma levels of LH release (21) while ether stressed animals showed a concomitant increase in the plasma levels of LH release in adult rats (26). Arginine vasopressin augments the release of LH in hamsters in a dose dependent manner *in vitro* (31). In rats, almost all V1aR labelled cells were gonadotropes suggesting that V1aR stimulates the release of LH (28). The presence of V1aR on gonadotropes implies that a direct

action of AVP on these cells is plausible (28). Certainly, AVT/AVP exerts various and controversial actions on gonadotropes according to species, mode of administration and neurotransmitter molecules (72, 73). In the present study, 12% of cells contain both VT4R and LH immunoreactive cells. It would be of interest to determine whether the VT4R on gonadotrophs function to stimulate or inhibit the release of LH from those cells.

4.5 VT4R in lactotrophs/somatotrophs:

In the present study, we have not found immunoreactive VT4Rs located either in lactotrophs nor somatotrophs. Immunohistochemical results presented here suggest that AVT do not mediate growth hormone or prolactin release via VT4R in chickens. Previous anatomical studies suggest that the VT2R may regulate the release of prolactin from lactotrophs in birds (36). Arginine vasopressin, mediates the release of prolactin *in vivo* and *in vitro* in rats (30, 32, 33) and in hamsters (31).

4.6 Similarities between mammalian V1aR and avian VT4R:

The vasotocin receptor, VT4R shares 69% sequence homology with mammalian V1aR (Table 1). This receptor (VT4R) has the highest sequence homology with its mammalian counterpart (V1a) compared to other vasotocin receptors. In chickens, VT2R and VT4R are most abundant receptors of the vasotocin family of receptors in the anterior pituitary. In agreement with these findings, V1bR and V1aR in mammals are the most abundant receptors in pituitary (28, 43). In chicken, 89% of corticotrophs and 12% of LH containing gonadotrophs contain the VT4R. In rats, the V1a receptor is found in 100 % of gonadotrophs (28) and is not found in corticotrophs (28).

In mammals, the V1a receptor is found in the neural structures of the brain including paraventricular nucleus, hippocampus, median eminence, area postredema and subfornical organ (42, 43). Behaviorally, the V1aR mediates effects of AVP on social recognition and pair bonding (74, 75, 76-78), regulates maternal aggression, grooming and licking behavior and maternal behavior in lactating rats (79). The role of V1aR in stress responses and reproduction are under studied. Similarly, the VT4R is present in telencenphalon, diencephalon and mesencephalon of brain and preliminary data suggest its involvement in stress pathways due to its distribution in the supraoptic nuclei (SON), paraventricular nucleus (PVN) and median eminence (ME) (65). Very limited information is available on the functional role of VT4R in stress responses and social/reproductive behavior. Based on similarities in sequence homology, distribution in anterior pituitary gland and brain, the VT4R proposes to be the avian homolog of the mammalian V1a receptor subtype.

5. Concluding remarks

This study investigated the presence of VT4R in anterior pituitary. The VT4R was predominantly present in the cephalic lobe of the anterior pituitary gland in chicken. The VT4R was shown to be present in corticotrophs and gonadotrophs (LH containing cells) and not found in somatotrophs or lactotrophs. The overall profile of VT4R in anterior pituitary gland and high percentage of corticotrophs containing the VT4R suggest its involvement in the regulation of stress. The VT4R also appears to play a role in the regulation of LH.

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List of tables:

Table 1: Amino acid identity (%) of chicken VT4R compared with mammals and other members of vasotocin/oxytocin, vasotocin/mesotocin-isotocin receptor family to human vasopressin V1a receptor (V1aR)

Receptor	Accession No.	Species	Identity (%)
Vasopressin V1a	U19906	*Homo sapiens* (human)	100
Vasopressin V1a	Z11690	*Rattus norvegicus* (rat)	80
Vasopressin V1a	D49730	*Mus musculus* (mice)	82
Vasopressin V1a	L41502	*Ovis aries* (sheep)	82
Vasopressin V1a	GU954352	*Microtus montanus* (Meadow vole)	83
Vasopressin V1a	AF069304.2	*Microtus ochrogaster* (Priarie vole)	82
Vasotocin VT4	**EU124684.1**	***Gallus gallus* (chicken)**	**69**
Vasotocin V1a	XP 002187321	*Taeniopygia guttata* (Zebra finch)	66
Vasotocin VT	AY277924.1	*Rana catesbeiana* (Bull frog)	68
Vasotocin V1a	GU945196.1	*Taricha granulosa* (Rough skin newt)	68
Vasotocin V1a	AB274037	*Cynops pyrrhogaster (Red bellied newt)*	67
Vasotocin	AF184966	*Platichthys flesus* (flounder)	63
Vasotocin	AF517936	*Haplochromis burtoni* (cichlid)	63
Vasotocin	X76321	*Catostomus commersoni* (white sucker)	61
Vasotocin V1a1	HQ662334.1	*Epinephelus adscensionis* (rock hind)	61
Vasotocin V1a1	GQ981412	*Cyprinodon nevadensis amargosae* (pupfish)	59
Vasopressin V1b	L37112	*Homo sapiens* (human)	54
Vasopressin V1b	D45400	*Rattus norvegicus* (rat)	50
Vasopressin V1b	AF098867	*Mus musculus* (mice)	53
Vasotocin VT2	**AY008272**	***Gallus gallus* (chicken)**	**53**
Vasotocin V1b	XP 002189484	*Taeniopygia guttata* (Zebra finch)	54
Vasotocin V1b	XP 003212983	*Meleagris gallopavo* (Turkey)	53
Vasotocin V1b	EF567079.1	*Taricha granulosa* (Rough skin newt)	52

Vasotocin V3/V1b	AB284503	*Cynops pyrrhogaster* (Red bellied newt)	53
Vasotocin V1a2	HQ141396	*Epinephelus adscensionis* (rock hind)	63
Vasotocin V1a2	GQ981413	*Cyprinodon nevadensis amargosae* (pupfish)	60
Vasopressin V2	L22206	*Homo sapiens* (human)	42
Vasopressin V2	Z11932	*Rattus norvegicus* (rat)	41
Vasopressin V2	NM019404	*Mus musculus* (mice)	41
Vasotocin VT1	**AF147743**	***Gallus gallus* (chicken)**	**48**
Vasotocin V2	XP 002195418	*Taeniopygia guttata* (Zebra finch)	46
Vasotocin V2	XP 003202046	*Meleagris gallopavo* (Turkey)	47
Vasotocin V2	ABQ 23253	*Rana catesbeiana* (Bull frog)	45
Vasotocin V2	BAF 38755	*Cynops pyrrhogaster* (Red bellied newt)	43
Oxytocin OT	X64878	*Homo sapiens* (human)	54
Oxytocin OT	U15280	*Rattus norvegicus* (rat)	49
Oxytocin OT	X87986	*Ovis aries* (sheep)	51
Oxytocin OT	U82440	*Macaca mulatta* (rhesus monkey)	52
Vasotocin VT3	**AY833434**	***Gallus gallus* (chicken)**	**49**
Mesotocin	AY277925.1	*Rana catesbeiana* (Bull frog)	48
Mesotocin	DQ186599.1	*Taricha granulosa* (Rough skin newt)	49
Isotocin	X87783	*Catostomus commersoni* (white sucker)	47
Isotocin	GQ981415	*Cyprinodon nevadensis amargosae* (pupfish)	53

Table 2: List of antibodies employed in the immunohistochemical study (p for polyclonal, m for monoclonal)

Target antigen	Code	Antigen	Host	Dilution	Source
Vasotocin receptor (VT4R)	VT4R #3/63	Synthetic peptides corresponding to 15 amino acids from amino terminal and 17 amino acids from carboxy terminal region of chicken VT4 receptor	Rabbit (p)	1:2000	21st Century Biochemicals Inc.
Adrenocorticotropic hormone (ACTH)	16D9	Native Hypophysial chicken proopiomelanocortin	Mouse(m)	1:2000	Berghman et al.1998; Gerets et al. 2000
Prolactin	VIIA2	Synthetic peptide PD2 corresponding to 59-67 of chicken prolactin	Mouse(m)	1:6000	Berghman et al. 1992
Growth hormone (GH)	IH7	Native hypophysial chicken GH	Mouse(m)	1:6000	Berghman et al. 1998; Zheng et al. 2006
Luteinising hormone (LH)	IIIC7E10	Native hypophysial chicken LH	Mouse(m)	1:5000	Berghman et al. 1993

Figure legends:

Figure 1: (A) Amino acid sequence of the VT4R with the two peptides synthesized for antibody shown in red (N terminal) and blue color (C-terminal). (B) Amino acid sequence alignment of the carboxy terminal region of the VT4R compared with V1aR in other species indicating the specificity of the immunogenic site of the VT4R. (C) Amino acid sequence alignment of the amino terminal region of the VT4R with the synthesized peptide.

Figure 2: Characterization of the antibody against chicken vasotocin receptor VT4 (VT4R) using Western blot analysis. (A) VT4R protein extracts labeled with the antibody shows a single band of 47KDa as predicted with a molecular weight marker (M) in HeLa cells transfected with chicken VT4R (C1) and avian pituitary tissues (P). This band was absent in HeLa cells transfected with an empty expression vector (C2). (B) Specificity control of the antibody using the peptide competition or preadsorption assay in extacts from the chicken pituitary (P) tissues. The antibody at working dilution of 1:2000 was preincubated with 5 µg (P1) and 50 µg (P2) of antigenic peptides prior to use in the Western blot.

Figure 3: Antiserum specificity of chicken vasotocin receptor (VT4R) using the peptide competition or preadsorption assay in sections of chicken anterior pituitary gland. Coronal sections of pituitary showing cells labeled by VT4R #3/63 at a working dilution of 1:2000 (A). Antibody at 1:2000 dilution was preadsorbed with 8 µg (B) and 50 µg (C) of the antigenic peptides prior to use in immunocytochemistry. Scale bars 5µm.

Figure 4: Immunocytochemical detection of chicken vasotocin receptor in tranfected HeLa cells. Confocal image of HeLa cells transfected with chicken VT4R cDNA (A). VT4R shown in red with nuclei counterstained with DAPI (blue). Higher magnification of the VT4Rs in cytoplasm (B). HeLa cells transfected with an empty pGEM7z expression vector lacking VT4R (C), VT1R cDNA (D), VT2R cDNA (E) and VT3R cDNA (F). Nuclei are counterstained with DAPI. Scale bars 8 µm (C, D, E, F), 10 µm (B), 20µm (A).

Figure 5: VT4R shown in cells in the cephalic lobe of anterior pituitary. Brightfield images showing different arrangement of VT4Rs as individual cells (A, B) or clusters (C, D). Scale bars 20µm (A), 10 µm (B, C), 5 µm (D).

Figure 6: Avian pituitary cells containing the VT4R and distributed in cell clusters as well as in isolated, individual cells. (A) Low power fluorescence image of anterior pituitary showing VT4R confined to cells in the cephalic lobe (Cp) and scarce immunostaining in caudal lobe (Cd). The asterisk shows the boundary between the Cp and Cd lobes. (B) Higher magnification of VT4R in the cephalic lobe of anterior pituitary. Confocal images at higher magnification showing different arrangements of clusters of cells (C) or individual cells labelled with the VT4R (D) in the cephalic lobe of anterior pituitary. Scale bars 300µm (A), 50µm (B), 10µm (C), 5µm (D).

Figure 7: Confocal microscopic images showing two types of hormone containing pituitary cells with and without the VT4R. (A) Cells in cephalic lobe immunostained for VT4R (red), (B) Cells in cephalic lobe stained for proopiomelanocortin (POMC) (green), (C) the merged image of cells

showing colocalisation of POMC and VT4R. (D) Cells immunostained for VT4R (red), (E) Cells immunostained for luteinising hormone (LH), (F) Merged image of cells colocalised with VT4R and LH. The colocalised cells appear to be yellow in color in the superimposed image. Scale bars 10µm (A-C) and 10µm (D-F).

Figure 8: Cells in anterior pituitary immunostained for VT4R and prolactin (PRL). Low power confocal image of cells containing the VT4R (A), PRL immunoreactive cells (B) and merged image of VT4R and PRL immunoreactive cells (C). Scale bars 60µm.

Figure 9: Confocal photomicrographic images at the interface of cephalic and caudal lobe for Growth hormone (GH) and VT4R immunoreactive cells stained in anterior pituitary. Cells stained for VT4R (A), GH immunoreactive cells (B) and merged image of GH and VT4R containing cells (C). Scale bars 10µm.

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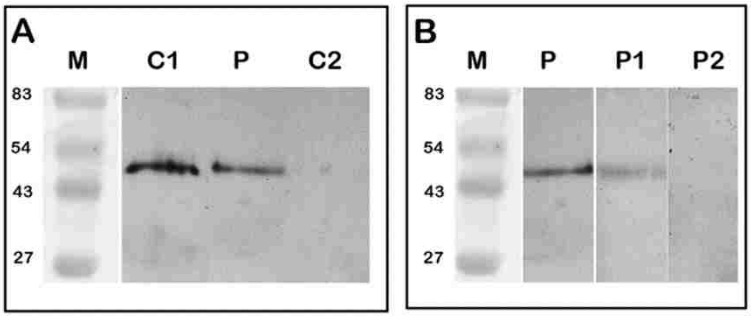
Figure 2:



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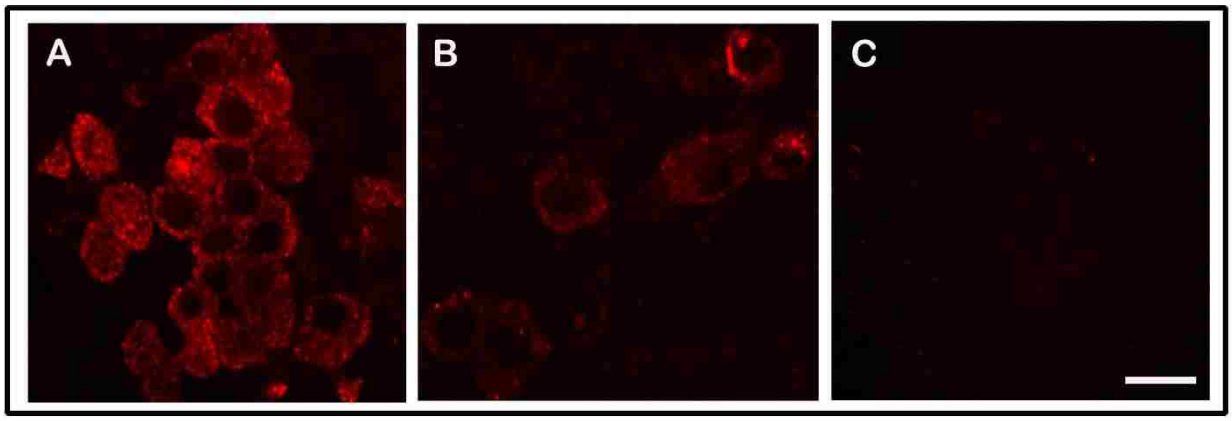

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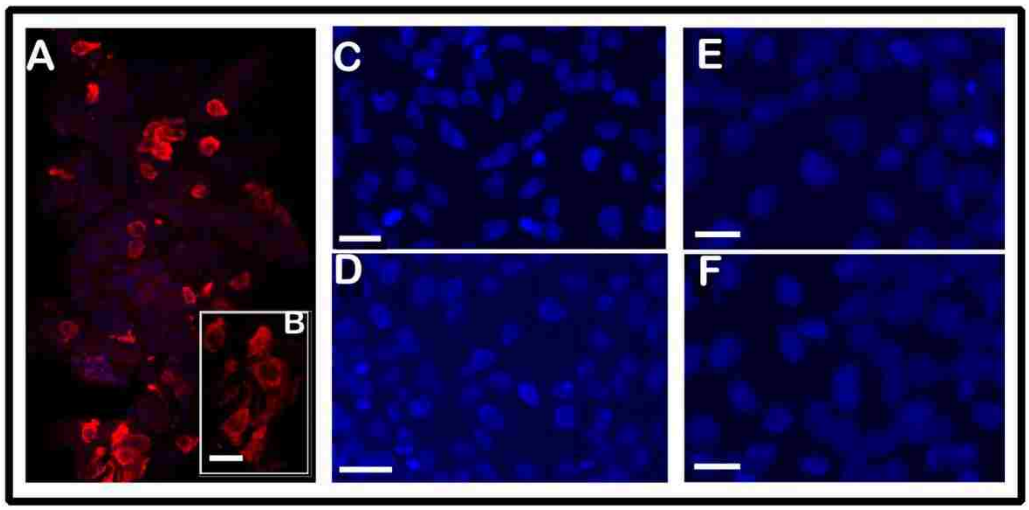

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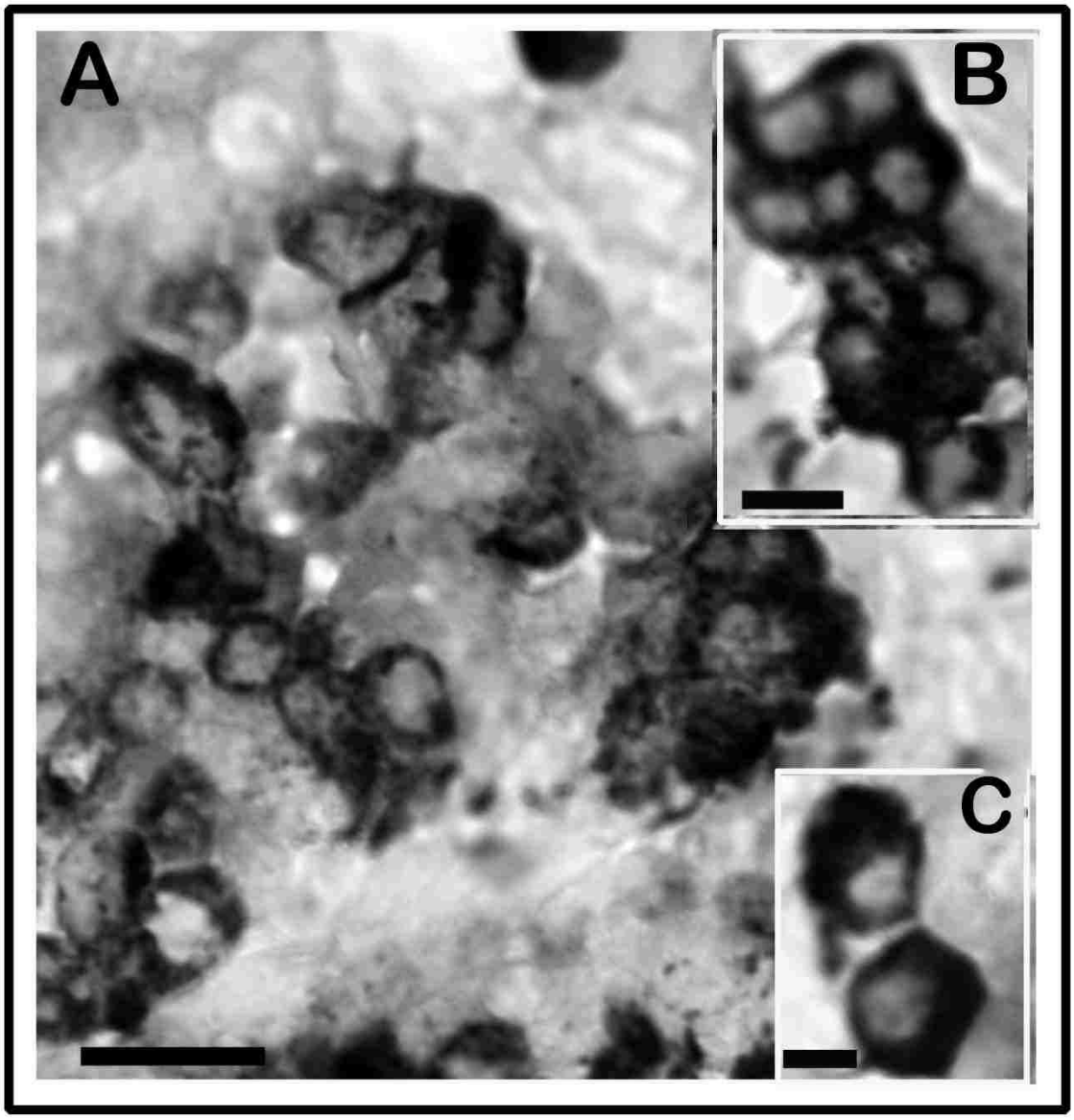

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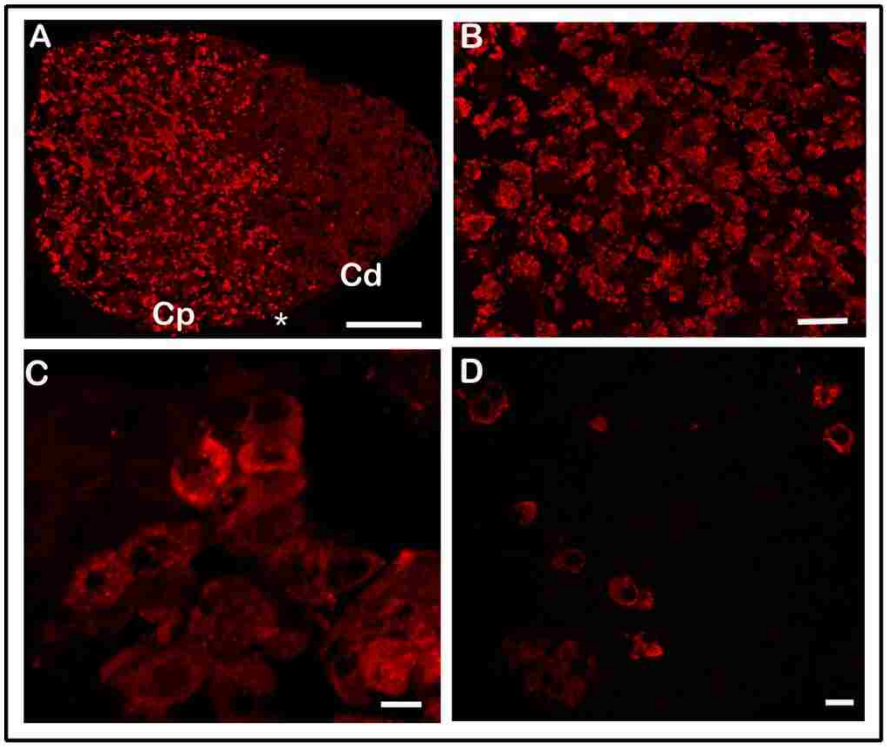

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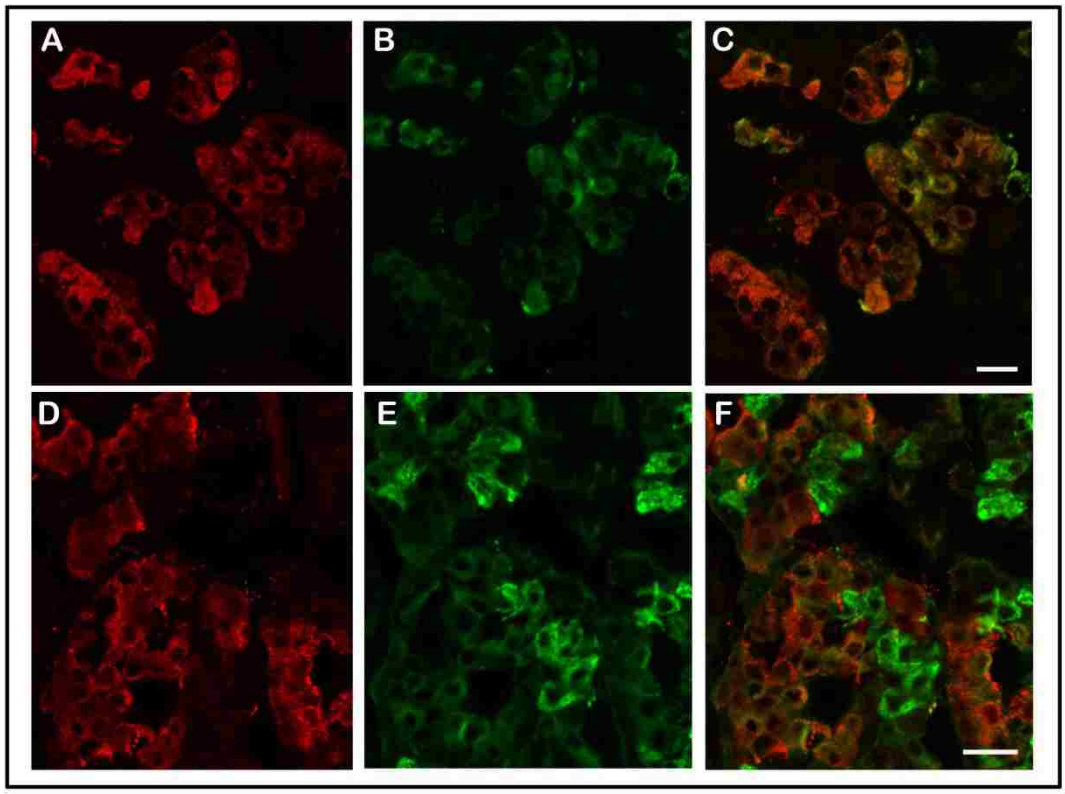

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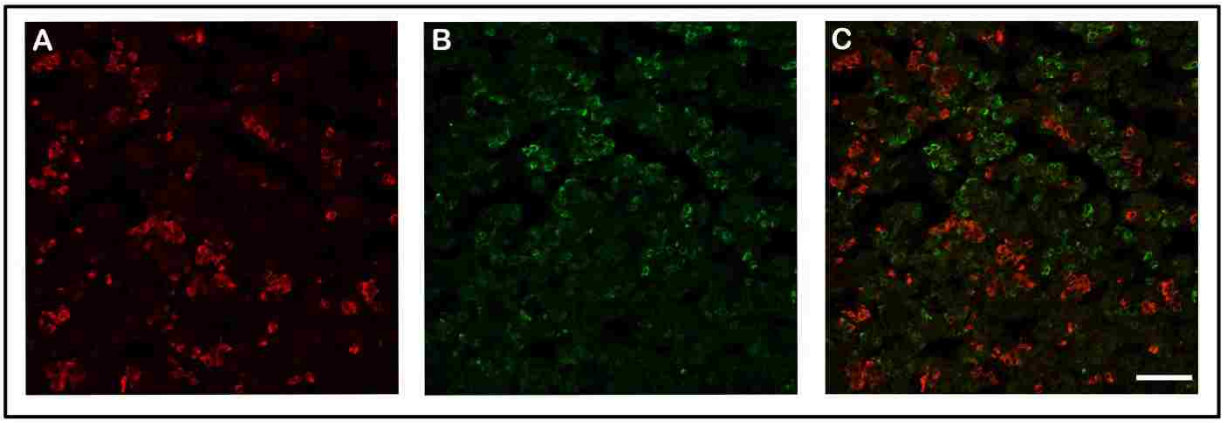
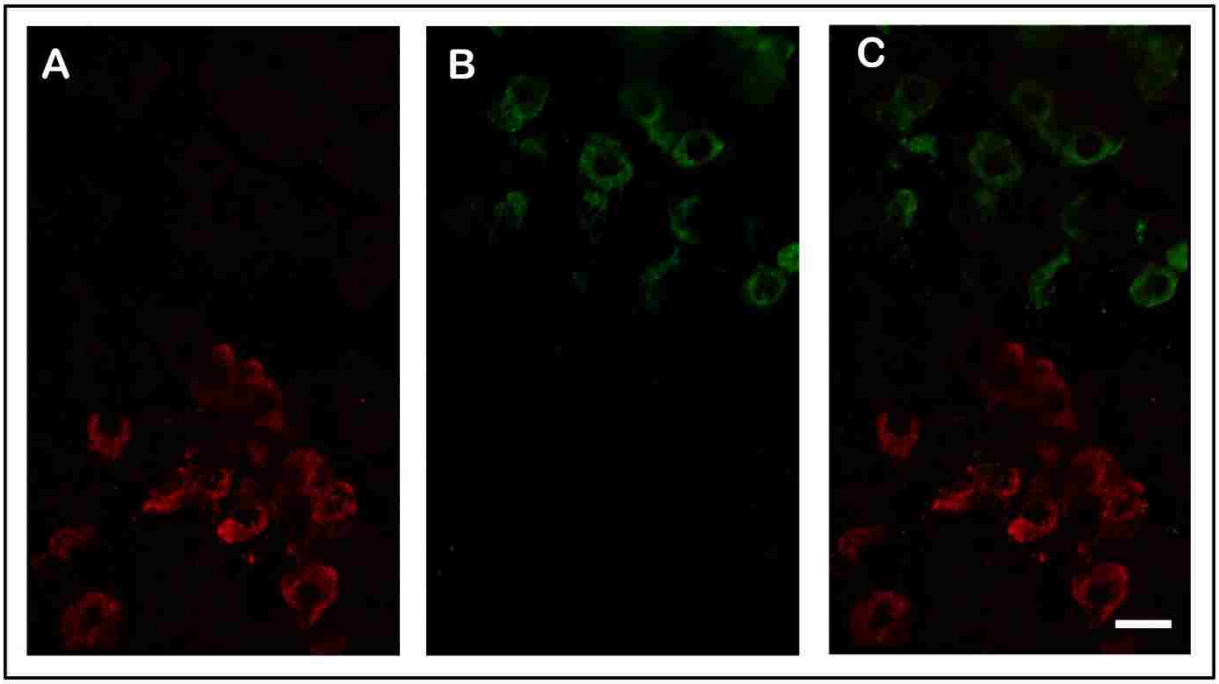


Figure 9:



Chapter 3

Vasotocin receptor subtype 4 (VT4R) distribution in the male brain of the chicken, *Gallus gallus*

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Abstract

Arginine vasopressin (AVP) and its non-mammalian homolog arginine vasotocin mediate their effects through G protein coupled receptors. In birds, AVT mediates its effects through four vasotocin (VT) receptor subtypes: VT1R, VT2R, VT3R, and VT4R. Previous studies have shown that the VT2R is absent in brain and abundant in pituitary gland. The VT4R in chickens shares 69% amino acid homology with its mammalian counterpart V1aR, a receptor found in the mammalian brain. Therefore the present study focussed on the VT4R to determine whether it occurs in the avian brain. The aim of the study was to investigate the distribution of the VT4R in the chicken brain using a polyclonal antibody developed against the amino terminal region of the receptor. The antiserum specificity was validated using Western blot, peptide blocking assays and immunocytochemistry. Results indicate that the VT4R is widespread in brain regions. Intense VT4R immunoreactive perikarya were found in the paraventricular nucleus, supraoptic nucleus, oculomotor nucleus, Edinger Westphal nucleus, magnocellular cochlear nucleus and nucleus of the solitary tract. Glial cells were intensely immunoreactive for the VT4R in hippocampus, forebrain and in circumventricular organs. Data suggest that the VT4R may play a role in central control of water balance, regulation of stress responses and sensory functions like olfaction. Further, the VT4R is also associated with the auditory and visual system.

Keywords: V1aR, glial cells, circumventricular organs, stress, visual system

1. Introduction

The hypothalamic pituitary adrenal axis (HPA) is activated due to homeostatic disturbances by secreting the neuropeptide arginine vasopressin (AVP) and corticotropin releasing hormone (CRH). The former, in mammals, is a nine amino acid peptide arginine vasopressin (AVP) produced in paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and transported from the median eminence to the anterior pituitary. The peptides act on corticotropes of the anterior pituitary resulting in the secretion of adrenocorticotropic hormone (ACTH) which inturn acts on adrenal glands to produce glucocorticoids, cortisol in some mammals and corticosterone in birds, rats and mice. The avian neuropeptide, arginine vasotocin (AVT), is considered homologous to the mammalian peptide arginine vasopressin (AVP) (Landgraf and Neumann, 2004). Human AVP differs from avian AVT at the third position of its amino acid sequence (a phenylalanine to isoleucine substitution). The neuropeptide AVP/AVT exhibits a wide range of biological actions, functioning as a neurohormone peripherally and as a neurotransmitter/neuromodulator centrally. In mammals, AVP is involved in physiological functions such as maintenance of blood pressure, electrolyte and osmotic balance, thermoregulation and stress responses (Aguilera et al., 2008; Aoyagi et al., 2009; Bielsky et al., 2005a; Bielsky et al., 2005b; Engelmann et al., 2004; Volpi et al., 2004) and also involved in behaviours including social recognition, anxiety (Bielsky et al., 2004a; Bielsky et al., 2004b), maternal care and attachment (Nair et al., 2006), affiliation and pair bonding (Insel et al, 1996; Liu et al., 2001; Winslow, 1993; Winslow et al., 2004; Young et al., 1997; 1999), and aggression (Ferris et al., 2000; Francis. 2002). In non-mammalian vertebrates, AVT is involved in scent marking, mate calling, phonotaxis (defined as the ability to show orientation toward sound) and vocal production (Boyd et al., 1997; Do-Rego et al., 2006), courtship behaviour

(Hasunuma et al., 2010), osmoreguation (Balment et al., 1993; 2006), aggression (Goodson, 1998), stress responses and sexual behavior (Jurkevich et al., 1997; Robinzon et al., 1988).

The neuropeptides AVP/AVT act through multiple receptor subtypes in brain and pituitary to mediate its effects. The vasopressin receptors are G protein coupled receptors and belong to the vasopressin/oxytocin receptor family that is classified into four subtypes: vasopressin receptors (V) V1a, V1b and V2 and oxytocin receptors (OT). The distribution of receptors and their functions have been identified in mammals (Table 1) (Birnbaumer, 2000; Peter et al., 1995). Similar to mammals, birds also have four vasotocin receptor subtypes in their family to mediate the functions of AVT, mesotocin and other related peptides. Vasotocin receptors in non-mammalian vertebrates are proposed to be homologous to vasopressin/oxytocin receptors in mammals. Four avian receptors in the vasotocin family have been identified: VT1R, VT2R, VT3R, VT4R (Baeyens & Cornett, 2006) (Table 2). The second avian receptor, VT2R, has been proposed to be homologous to the mammalian V1b receptor. The VT2R is found predominantly in the cephalic lobe of the anterior pituitary and plays a major role in release of ACTH in stress responses and to lesser extent in the release of prolactin. The receptor, however, was not detected in brain using immunohistochemistry or Northern blot (Cornett et al., 2003; Jurkevich et al., 2005; Jurkevich et al., 2008).

The fourth receptor, VT4R, the most recently cloned vasotocin receptor has an amino acid sequence that is 69% homologous (Table 2) with that of the mammalian V1a receptor (Cornett, 2007, unpublished). Its gene has been shown to be present in chromosome 1 of the chicken (Scarbrough et al., 2003). Studies have been done on sequence comparisons of vasotocin receptors in non-mammalian vertebrates with that of the human V1aR. Data suggest that in amphibia (Acharjee et al., 2004; Mahlmann et al., 1994; Hasunuma et al., 2007), fishes (Kline et

al., 2011; Warne, 2001) and rats (Ostrowski et al., 1994 ; Morel et al., 1993; Ostrowski et al., 1992; Szot et al., 1994) the V1aR is homologous to the V1aR of humans (Table 2). It is of interest to determine if the VT4R occurs in the chicken brain and, if so, whether its distribution is similar to that of the rat where V1aR mRNA has been located in olfactory lobes, hippocampus, hypothalamic, mesencephalic and rhomboencephalic regions and in cerebellum (Ostrowski et al., 1992; 1994). Research studies have indicated that the distribution of the V1aR varies greatly among mammalian species and is linked to their sociality. For instance, the neural distribution of V1aR differs between monogamous voles and polygamous voles. This difference in distribution is associated with pair bond formation and maternal care that are exclusive behaviors for monogamous voles (Bielsky et al., 2004; Insel et al., 1994; Leung et al., 2009; Wang et al., 1997; Young et al., 1999; 1997). The remarkable difference in V1aR distribution in the ventral pallidum and arcuate nucleus in a solitary and social species of a rodent, Tuco-Tuco, is critical for maintenance of a specific social behavior in the species (Beery et al., 2008). Further, the high expression of V1aR in the periaqueductal gray and medial geniculate nucleus in singing mice *Stenomys teguina* favors vocal communication while the high expression in the lateral thalamus and mediodorsal thalamus favors spatial working memory in *S. xeramphelinus* (Campbell et al., 2009). Thus, the interspecies and intraspecies difference in V1aR distribution may explain the variations in social behavior among different species. It is therefore of considerable interest to study the neural distribution of the proposed homologue of the V1aR and its possible association with stress or social behavior in avian species as well. The objective of the present study is to test the hypothesis that the VT4R is present throughout the brain using an antibody directed against a partial peptide sequence produced from the VT4 receptor gene sequence in *Gallus gallus*.

2. Materials and methods:

2.1 Animals

2.1. a. Antibody validation study:

Ten septal regions, ten hypothalamic regions and twenty anterior pituitary tissues were obtained from 6-8 week old broilers for extraction of membrane protein. Birds were decapitated and heads placed on ice. The brain was removed from the skull and the following brain regions and pituitary dissected (septum, hypothalamus and anterior pituitary) and frozen in dry ice. The dissected brain regions and pituitaries were stored in vials at -80°C.

2.1.b. Receptor distribution in Brain:

Ten male broiler birds in the age group of 24-32 weeks were raised in order to sample their brains to identify VT4R distribution throughout the brain. All birds were caged individually under 14h light and 10h dark lighting cycle and food and water were provided *ad libitum*. All animal procedures used in this study were conducted in compliance with protocols approved by the University of Arkansas Institutional animal care and use committee.

2.2 Antibody generation specific to vasotocin receptor subtype 4 (VT4R):

Two peptides, AGDWDPFGRDEELAC corresponds to N terminal 15 amino acids (residues 45-59) and NSNSRRQTSFTNNRSPT corresponds to C terminal 17 amino acids (residues 376-392) of chicken VT4R were synthesized (Cornett, 2007, GenBank submission). Polyclonal rabbit antiserum to the chicken vasotocin receptor VT4R was raised against the two peptides. Sequences of peptides were chosen to minimize their similarity with other known vasotocin receptor subtypes or any other avian protein (Figure 1; GenBank accession no: XM_001235007).

A cysteine thiol and a 6 carbon spacer were added to the C-terminal and glutaraldehyde to the N-terminal peptide for easier conjugation to the carrier protein (ovalbumin) by the use of m-maleimido benzoyl N-hydroxy succinimide (MBS). The synthetic peptide was coupled to ovalbumin and this conjugate was employed to immunize two rabbits with a two week interval between boosters. The rabbits were bled 10 days post immunization, starting after the third booster administration.

2.3 Affinity purification of VT4R specific antibodies:

The synthetic N terminal and C-terminal peptide were coupled individually to the free sulfhydryl group of its C-terminal cysteine residue of iodoacetyl gel (Biorad, CA, USA) at a ratio of 1mg of peptide per 1ml of Gel in Tris/EDTA buffer for use as an immunoadsorbent. For the isolation of peptide-specific antibodies, 47 ml of serum were mixed with the immunoadsorbent (50ml). The non-specific antibodies were rinsed away with 60 ml of salt buffer. Antibodies specific to the peptides were eluted with 100mM Glycine-HCl buffer pH 2.5 and immediately neutralized with 1M Tris buffer pH 9.5. The concentration of eluate (N-terminal and C-terminal antibody) was quantified individually using absorbance at 280 nm. The production and affinity purification of the antibody was completed by 21st Century Biochemicals (Marboro, MA). Immune serum obtained from both rabbits produced adequate staining pattern on brain sections. The best immunocytochemical result for brain sections was obtained from rabbit 2 (#4364) N terminal peptide (termed as VT4 #1) after 4 or more booster injections.

2.4 Membrane protein extraction and Western blotting:

Septal region, hypothalamic region and pituitary tissues were used for the extraction of plasma membrane protein using plasma membrane protein extraction kit (Biovision, CA, USA). Briefly, the tissues were homogenized with a buffer mix provided in the kit. The total cellular membrane proteins were pelleted at 10,000 g for 30 min at 4°C. The pellet was resuspended in 200 µl of upper phase buffer and lower phase buffer. The plasma membrane proteins were obtained in pellets after centrifugation at 1,000 g for 5 min followed by a final spin at 20,000 g for 10 mins. Protein concentration in each homogenate was determined using RC/DC Protein Assay kit-II (Biorad, CA, USA). The protein lysates of concentration 40µg was diluted using a ratio 1:1 with 6X Laemmli sample buffer containing SDS and β-mercaptoethanol and heated for 15min at 90°C.

Samples were loaded onto a 12% sodium dodecyl sulphate-polyacrylamide gel. The separation of protein takes place at 180V for 55min (Biorad, CA, USA). The proteins were transferred to a nitrocellulose membrane (0.25A for 70 min) using Tris-Glycine buffer pH 9.5. Non-specific binding to the membrane was achieved by treating the membrane with 5% non-fat milk for 30mins. All antibodies were diluted in tris buffered saline (TBS) buffer with 5% non-fat milk. Each membrane extract was incubated with an antibody raised in rabbit against VT4R at a dilution of 1:1000 overnight at 4°C. Simultaneously, anti-rabbit VT4R IgG against the N-terminal peptide was preincubated in blocking buffer for 3 hrs at room temperature with serial amounts of peptide (5 µg and 20 µg). The antibody-peptide mixture was centrifuged at 14,000 rpm for 30 min. The supernatant served as the primary antiserum. Membranes were washed with TBS for three changes. Later the membrane fraction was incubated with horseradish peroxidase conjugated with goat anti-rabbit IgG (dilution 1:12,500, Santa cruz Biotechnology, CA, USA)

for 1hr. Bands were visualized using Pierce Super Signal Femto Maximum Sensitivity substrate kit (Pierce, Thermoscientific, USA) according to the manufacturer`s instructions followed by exposure to Kodak Biomax Maximum Resolution (MR) autoradiography film (Carestream Health, USA). Images were scanned and compiled in Adobe Photoshop CS5.

2.5 Detection of VT4R in cell lysates:

The chicken VT4R subtype was transiently transfected with 2.8 µg of expression vector pCDNA3 containing VT4R cDNA or pGEM7Z lacking VT4R using Lipofectamine 2000 method (Invitrogen, CA, USA) (Davis et al., 2000). The cells were cultured in growth medium containing 10% Fetal bovine serum (FBS) and Eagle`s minimum essential medium (MEM) at 37°C and incubated in a 5% CO_2 incubator. Twenty four hours later, the medium was removed and replaced with fresh medium. Seven to ten days after post transfection, the VT4R cells were scrapped off from the plates and resuspended in PBS. Cells were centrifuged at 14,000rpm for 15min. The pelleted cells were then rinsed with PBS, supernatant removed and frozen in liquid nitrogen. The cell count for cells containing VT4R was estimated to be $6.5X10^7$ per 1.5ml. The pCDNA3-VT4R vector served as a positive control while pGEM7Z plasmid served as a negative control. The membrane protein from cell lysates was extracted using a plasma membrane protein extraction kit (Biovision, CA, USA). Western blotting was performed on cell lysates as described in section 2.4.

2.6 Immunohistochemistry

2.6.1 Tissue collection:

Birds were anaesthetized by administering sodium pentobarbital intravenously (40mg/kg i.v). Birds were perfused through carotoid arteries with 250ml of heparinized phosphate buffer (0.1M PB with 0.1% sodium nitrite, pH 7.4) followed immediately by 400ml of Zamboni`s fixative pH 7.4 (4% paraformaldehyde in 0.1M PB, 15% saturated picric acid). Brains were blocked in a stereotaxic instrument (Kopf Instruments, CA) and placed in the same fixative for overnight postfixation at 4°C. Brains were then placed in 30% sucrose solution (in 0.1M PB buffer) at 4°C until the brains sank to the bottom, then frozen in dry ice, sealed in parafilm and stored at -80°C until processed for immunohistochemistry.

Brains were embedded in Jung tissue freezing medium (Leica Instruments, Germany). Four sets of 40 µm thick coronal sections were sectioned using a cryostat. (Leica CM 3050S, Leica Microsystems, Frisco, TX) and preserved in cryoprotective solution pH 7.2 at -20°C. The collected sections covered the brain structures located in the stereotaxic anterior-posterior plates A14.8 to P4.0 of a chick brain atlas (Kuenzel and Masson, 1988). One set was used to map the distribution of the VT4R while three sets were kept as backups.

2.6.2 Immunohistochemistry:

Sections were rinsed in several changes of 0.02M PBS to remove the cryoprotective solution. It was then treated with 0.6% hydrogen peroxide to quench endogenous peroxidase activity and permeabilized with 0.4% Triton X-100. After rinsing with PBS, sections were incubated with 5% normal goat serum for 30min to block non-specific binding sites. Sections were incubated for 40hrs at 4°C with VT4R antibody raised in rabbit (at a dilution of 1:2000 in 0.02M PBS with 1%

NGS, 0.2% Triton X-100 and 0.1% sodium azide). Sections were incubated for 90min with biotinylated goat anti-rabbit IgG (dilution 1:500 in 0.02M PBS with 0.2% Triton X-100) (Vector laboratories, CA, USA) followed by incubation with Vectastain Elite ABC peroxidase complex diluted 1:5 for 90 min (Vector laboratories, CA, USA). The VT4R immunoreactive cells were visualized using glucose-oxidase diaminobenzidine nickel (Shu et al., 1988). The reaction was stopped in a 2-3min short rinse with acetate buffer. Sections were rinsed in PBS for two changes, rinsed with deionized water, mounted onto clean glass slides and coverslipped with Histamount (National Diagnostic Laboratories, Atlanta, USA).

2.6.3 Antisera specificity control:

Rabbit antiserum VT4 #1 was preadsorbed with the N terminal VT4R peptide used to immunize the rabbits. Antiserum in working dilution 1:2000 was preincubated with increasing amounts of peptide (10µg and 50µg) for 3hr at room temperature. The peptide-antibody complex was centrifuged at 14,000 rpm for 30min and the supernatant applied to brain sections. Sections were processed for immunohistochemistry as described in section 2.6.2. Additional controls included omission of primary and secondary antibodies from the immunohistochemical protocol described in section 2.6.2.

2.7 Transfection of HeLa cells with VT4R:

HeLa cells were plated onto a four chamber slide (Lab Tek-II chamber slide system, Rochester, NY) at a concentration of $2X10^5$ cells per chamber in 1ml of growth medium (10% Fetal bovine serum and Eagle`s minimum essential medium), cultured at 37°C in a humdified 5% CO_2 incubator overnight. After twenty fours later, cells were transiently transfected with

2.8µg of expression vector pCDNA3 containing a cDNA encoding the chicken VT4 receptor in 50µl Opti MEM per chamber or pGEM7-Z lacking VT4R, using Lipofectamine 2000 (Invitrogen, CA, USA) method (Davis et al., 2000). Forty eight hours post transfection, the cells were fixed with 4% paraformaldehyde (in 0.1M PBS, pH 7.4) for 15min at room temperature. The cells were then rinsed with PBS and air dried at 4°C and kept in air-tight boxes until processed for immunocytochemistry. To determine whether the antibody is able to specifically recognize cells containing the VT4R in brain, immunocytochemistry was performed on HeLa cells transfected with cDNA encoding VT1R, VT2R or VT3R cloned into pCDNA3 in addition to pCDNA3-VT4 cells. The transfected HeLa cells plated onto four chamber slides were prepared by Sandie Jacoby (University of Arkansas for Medical Sciences).

2.7.1 Immunofluorescence

The tranfected HeLa cells were subjected to immunofluorescence. Slides with cells were rinsed in PBS and permeabilized with 0.4% Triton X-100. To block the nonspecific binding sites, cells were treated with 10% normal donkey serum (NDS) (Jackson immunoresearch, PA) for 30min in a humidified chamber. Slides were then incubated with rabbit antiserum VT4 #1 (at a dilution of 1:500 in TBS with 1% NDS) for 40hrs in a humidified chamber at 4°C. After a brief rinsing in PBS, slides were incubated with Dylight 594 donkey anti rabbit IgG (dilution 1:400, Jackson Immunoresearch, PA, USA) for 90min in a humidified chamber. The slides were rinsed in three changes of PBS followed by a short rinse with deionized water. The slides were coverslipped with Vectashield mounting medium (Vector Laboratories, CA). Cell nuclei were counterstained with DAPI.

2.8 Imaging

Sections were observed using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, NY). Digitized images were taken using a CCD camera (Orca-ER camrea, Hamamatsu, NJ) and simple PCI software (Compix Imaging systems, Cranberry Township, PA). The photomicrographs were prepared in Adobe Photoshop CS5. Digitized schematic diagrams of the atlas of the domestic chick brain (Kuenzel and Masson, 1988) were used to map the anatomical structures identified by VT4R immunoreactive cells. The brightness and contrast was adjusted in Adobe Photoshop CS5. No image transformation was performed.

3. Results

3.1 Antiserum specificity:

(a) Western blot:

The specificity of the antibody VT4#1 was validated using the Western blot technique with membrane protein extracts from chicken hypothalamus, septal regions and pituitary tissues. The antibody presented a strong band at 45-47KDa with the chicken tissues as estimated by a molecular weight ladder. The 47KDa band was also present in HeLa cells transfected with the expression vector encoding chicken VT4R cDNA, however, was absent in non-transfected HeLa cells (expression vector pGEM7z lacking VT4R) (Fig 2A). In the peptide competition experiment, the antibody preadsorbed with 5µg of peptide significantly reduced the intensity of band while 20 µg of peptide eliminated the band completely (Fig 2B).

(b) Immunocytochemistry:

The specificity of the antibody was further validated utilizing a peptide competition assay with coronal brain sections. The antibody VT4 #1 was preadsorbed with 10 µg of the peptide used to produce the antibody. The procedure reduced the intensity of staining drastically while eliminated the staining completely when preadsorbed with 50 µg of the same peptide (Fig 3).

Further, the antibody VT4 #1 was tested on HeLa cells transfected with VT receptor subtypes to confirm the specificity of the antibody. The VT4 #1 antibody had intense staining with HeLa cells transfected with chicken VT4R cDNA. Almost all cells immunolabelled for VT4R had a whole cell profile (Fig 4A, 4B, 4C). About 50% of cells had imunoreactivity in the cell body and processes (Fig 4B). The cell body staining was present in or about the plasma membrane of the cells (Fig 4B, 4C). Absence of immunoreactivity with the antibody VT4 #1 was found with HeLa cells transfected with plasmid pGEM7Z (Fig 4D), and HeLa cells transfected with expression vectors encoding VT1R cDNA (Fig 4E), VT2R cDNA (Fig 4F) and VT3R cDNA (Fig 4G).

3.2 Distribution of VT4R in brain:

The VT4 #1 antibody was used on coronal brain sections to determine the distribution of VT4R in male chicken brains. The abbreviations shown on schematic diagrams of brain sections are shown in Table 3. The distribution of VT4R immunoreactive perikarya and VT4R immunoreactive glial cells are summarized in Figure 5. The VT4R is widely distributed in brain and an abundant receptor present in chicken brain. The VT4R immunostains both neurons and glia. The VT4R distribution is organized in the following figures and tables into VT4R

immunoreactivity identified in perikarya followed by VT4R immunoreactivity shown in glial cells.

3.2.1. VT4R immunoreactive perikarya:

3.2.1.1 Telencephalic regions:

In the globus pallidus, numerous cells expressed a strong immunoreactivity with the VT4R antibody (Fig 8D). Dense VT4R immunoreactive cell bodies and processes were seen with the periventricular preoptic nucleus. Intense VT4R perikarya were observed in the fiber tracts of the lateral forebrain bundle (Fig 5C-5D).

3.2.1.2 Thalamic regions:

In thalamic regions, the immunostaining of VT4R varies from weak to strong. The weak immunoreactivity was observed in the lateral habenular nucleus. A moderate immunoreactivity of VT4R was observed in nucleus dorsomedialis posterior thalami, nucleus dorsointermedius posterior thalami, nucleus dorsolateralis anterior thalami, nucleus geniculatus lateralis (GLv) and in nucleus reticularis superior (RSd). An intense immunoreactivity was found in nucleus rotundus (Fig 5E-5H).

3.2.1.3 Hypothalamic regions:

In general, the hypothalamic regions were intensly stained with VT4R. The structures that line the third venticle namely the paraventricular, periventricular and supraoptic nuclei had intense VT4R immunoreactivity in their perikarya and in cell processes (Fig 5E-G, 6).

3.2.1.4 Mesencephalic regions:

Strongly labeled immunoreactive VT4R perikarya were found in dorsomedial, dorsolateral and ventral portions of oculomotor nuclei and Edinger Westphal nucleus (Fig 5I, 89B). Intense VT4R immunoreactivity was observed in the magnocellular isthmus nucleus while weak staining was observed in parvocellular isthmus nucleus, central gray and intercollicular nucleus. Moderate immunoreactive cells were found in mesencephalic nucleus (MLd and MPv) (4I-4K).

3.2.1.5 Pontine regions:

Strong immunoreactive VT4R perikarya were found in magnocellular cochlear nucleus (MCC), laminar nucleus (La) (Fig 5N, 10B) and lateral reticular nucleus paragigantocellularis (RpgI) (Fig 5N). Moderate staining was seen with cranial nerves V, VI, VII in the structures namely sensory nucleus of principal trigerminal nerve (nPrV), motor nucleus of trigerminal nerve (MnV) (Fig 11C, 11D), nucleus of abducens nerve (nVI) (Fig 9D), dorsal (MnVIId; Fig 11E) and ventral portion of motor nuclei of facial nerves (MnVIIv) (Fig 5K-L). The presence of VT4R in these cranial nerves might aid in somatic sensation of the face, facial expressions and movement of muscles for mastication (Fig 11) (Bears et al., 2000; Paxinos et al., 2004).A weak immunoreactivity of VT4R was observed in subceruleus nucleus (SCd and SCv), leminiscus lateral nucleus (LLd and LLi), lateral pontine and medial pontine nuclei (Fig 5K-5L).

3.2.1.6 Cerebellum:

Intense labelled VT4R immunoreactive perikarya were observed in internal and intermedial cerebellar nuclei (CbI and CbIM). Purkinje cells of cerebellum were very weakly stained with VT4R (Fig 5L-O, 12B, 12D).

3.2.1.7 Medulla:

The subtrigerminal reticular nucleus (RST), medial and lateral vestibular nuclei (VeD, VeL; Fig 10C), nucleus of solitary tract and nucleus supraspinalis were strongly immunostained with VT4R. The staining was characteristic of cell body with long densely stained processes (Fig 5M-P). The cranial nerves namely glossopharyngeal nucleus, motor nucleus of vagal nerve and hypoglossal nucleus were also immunostained for VT4R very strongly (Fig 5M-P). The presence of VT4R in hypoglossal nucleus suggests that it might provide motor innervation to the muscles of tongue (Paxinos et al., 2004).

The intensity of VT4R perikarya in neuronal structures are shown in Table 4(a).

3.2.2 VT4R immunoreactive glia:

3.2.2.1 Telencephalic regions:

In the olfactory bulb, there were intense immunoreactive of glial cells in layers of olfactory ventricles including internal plexiform layer, external plexiform layer, mitral cell layer and glomerular layer. The glial cells move mediolaterally and mediodorsally from the ventricles and terminate either in bulbus olfactorius or in granule cell layer of olfactory ventricles (Fig 5A, 7A). Numerous small round cells were immunostained in nucleus basorostralis pallii and entopallium that lacked a distinguishable cell processes. They are termed as glial elements due to the smaller size of glia compared to neurons. The glial elements could be radial glia in nature. Some glial cells originating from the ventricles travel towards the hyperpallial structures including hyperpallium apicale, lamina frontalis superior and lamina frontalis suprema in forebrain (Fig 5A-5C) and also gets terminated in these regions. The glial cells in lateral septum were densely stained for VT4R, which originate from the walls of the lateral ventricle. Glial cells originating

from the ventral lateral walls of the lateral ventricle send long processes laterally to different parts of the brain that line the third ventricle while the dorsal lateral walls of the lateral ventricle send the processes to the pallium and striatum (Fig 5C-F, 7B). There were few glial elements observed in the medial septum. The glial cells in the hippocampus originate predominantly from the medial dorsal wall of the lateral ventricle. The VT4R immunoreactive glial cells in hippocampus are characterized by a small densely packed cell body and long protruding processes. The radial glial cells originating from dorsal portion of Hp move medially to midline while the glial cells originating from ventral portion of Hp reach different parts of the brain. The long processes are observed in glial cells on ventral side rather than on dorsal side (Fig 5D-F, 6E).

3.2.2.2 Diencephalic regions:

Very densely packed VT4R immunoreactivity of glial cells was observed in the inferior hypothalamic and infundibular hypothalamic nuclei. The cell bodies are small and stained very intensely while the processes are very long, protruding fibres from the third ventricle moving laterally (Fig 5G-H, 7F). The glial cells that are stained in this region could be tanycytes. The medial portion of interstitial nucleus and mesencephali nucleus of central gray (GCt) also had glial cell VT4R immunoreactivity.

3.2.2.3 Mesencephalic regions:

The periventicular nucleus of stratum griesum had intense short glial cells immunostained for VT4R moving laterally to other regions of the brain (Fig 5I-J).

3.2.2.4 Cerebellum:

The molecular layer of cerebellum has intense glial cell staining of VT4R characteristic of Berghmann glial cells. Such glial cells run perpendicular to the cerebellar surface (Fig 5M-O, 12C).

3.2.2.5 Medulla:

The central canal in the medulla has very dark immunostaining of VT4R in glial cells and the staining is observed around the fourth ventricle (Fig 5P). The central canal continues to the spinal cord.

3.2.2.6 Circumventricular organs:

The VT4R immunoreactivity was observed in all circumventricular organs (CVOs) (Fig 5C-P, 12). The lateral septal organ (LSO) identified in birds (Kuenzel and van Tienhoven, 1982) is the most anterior CVO. It was shown to be composed of two components, a medial (LSOm) and lateral (LSOl) portion (Walsh et al., 1997; Kuenzel and Blahser, 1994). Of interest is that both portions have glial cells immunostained for VT4R as the glia are located at the base of the lateral ventricle and are continuous around the medial wall of the lateral ventricle and move around the base of the VL and up the lateral wall of the VL. The glia along the medial wall of the lateral ventricle originate in the septum and their processes move medially into the LSOm. Glia along the lateral wall of the VL originate in the pallidum and their processes move away from ventricle to different regions of the brain including pallidum and striatum (Fig 13D). The second CVO encountered is the organum vasculosum of the lamnia terminalis (OVLT). The VT4R immunolabelled glia are intensely stained along the wall of the third ventricle and their processes

move laterally from midline into the preoptic and hypothalamic regions. As originally described by Dellmann et al., (Dellmann et al., 1964) and clarified by the distribution of gonadotropin-releasing hormone sub-type 1 (GnRH-1) neurons (Kuenzel and Golden, 2006), the OVLT moves dorsally and passes in front of the anterior commissure. As it moves over the anterior commissue it appears to merge with the nucleus of the hippocampal commissure (HpC) formerly called the bed nucleus of the pallial commissure (NCPa) (Fig 13E). Posteriorly, the OVLT-HpC complex becomes a third CVO, the subseptal organ (SSO) that has a finger-like projection from the roof of the third ventricle into the chamber of the third ventricle. It has VT4R immunolabelled glia whose processes enter the septum and dorsal thalamus (Fig 13F).

Choroid plexus is a complex group of blood vessels that resides in the enlarged ventral horns of the lateral ventricles that merge with the dorsal region of the third ventricle. Choroid plexus is tightly connected by epithelial cells which encloses a loosely connected by capillaries and is responsible for the secretion of cerebrospinal fluid (CSF) (Strazielle et al., 2000; Strazielle et al., 2005). The plexus is densely immunostained with VT4R antibody suggesting a large number of ependymal glial cells associated with the capillaries comprising the plexus (Fig 13C). The next circumventricular organ, paraventricular organ forms a bilateral small groove on the walls of the third ventricle. It lies caudal to the paraventricular nucleus and extends ventrocaudally from the mid hypothalamic regions to medial mammilary nucleus (Kuenzel and van Tienhoven 1982; Vigh, 1970; Guglielmone, 1995). Paraventricular organ has short glial fibres immunolabelled for VT4R characteristic of ependymal cells (Fig 13H).

Median eminence is an important circumventricular organ which is the ventral most region of the hypothalamus. It forms the floor of the third ventricle. Its dense immunostaining suggests a large number of VT4Rs occur in glia known as tanycytes. Both internal zone and external zone

of median eminence are immunolabeled. It has radially directed processes of glia (proposed tanycytes) that originate at the base of the third ventricle and curve ventrally toward the base of the brain (Fig 13I).

The pineal gland is devoid of neurons and has glia and pinealocytes. Glial cell staining (proposed to be ependymal cells) of VT4R was observed in pineal gland (Fig 13J). Pineal gland in birds is found to be involved in the release of melatonin (Natesan et al., 2002) and in control of daily and seasonal rhythms (Csernus et al., 2006).

The subcommissural organ (SCO) lies in posterior portion of third ventricle and leads to the cerebral aqueduct. Glial cells immunoreactive of VT4R were observed proposed to be ependymal and hypependymal glial cells. The processes are long projecting fibers running from the walls of the ventricle into midbrain regions (Fig 13G). The SCO might participate in the circulation of cerebrospinal fluid (CSF) or clearance of compounds from CSF though the exact pathway is unknown (Rodriguez et al., 1998).

The Subtrochelar organ (STO) is an organ found in the chicken brain (Kuenzel and van Tienhoven, 1982) that has specialized ependymal cells. These ependymal cells are immunoreactive for VT4R. The organ possesses the form of alphabet 'V' located in posterior walls of the mesencephalon. The apex of the structure has a bulb shaped projection which has intense glial cells stained for VT4R. The arms of the organ are directed rostrolaterally, which possess small cell bodies along the walls of the organ while the cell processes project to the apex of the organ (Fig 13K).

Area postrema is present in the fourth ventricle and is heavily stained VT4Rs present in glial cells (suggestive of modified ependymal cells). The cell processes are very dense staining of

VT4R. The processes of the glial cells move from the area postrema into the perivascular space while few processes extend beyond and reach the central canal. It helps in the baroreflex and cardiovascular control as well as the autonomic control of systems involved in feeding and mechanism (Price et al., 2008; Fig 13L).

The intensity of VT4R immunoreactivity in glia is summarized in Table 4(b).

4. Discussion

4.1 Antiserum specificity:

To determine the distribution of VT4R in brain, a polyclonal rabbit antiserum was developed against 15 amino acids from amino terminal region of the VT4R. The antibody was validated before use in determining the receptor distribution. The antibody was validated utilizing three different techniques: a Western blot on chicken tissues and transfected cells, peptide blocking experiments using both Western blots and immunocytochemistry and immunocytochemistry on HeLa cells transfected with VT receptor subtypes (Bordeaux et al., 2010; Burry, 2000; Michel et al., 2009; Rhodes and Trimmer, 2006). The Western blot showed that the VT4R protein was detected with a mass of 47KDa in extracts of chicken tissues. The estimated band size is of reasonable agreement with the observed band as the receptor is calculated to be 46.2KDa based on its molecular weight comprising 419 amino acids (Fig 2A). The band was absent in non transfected HeLa cells (Fig 1A). When the antibody was preincubated with peptide prior to use in Western blot, the band at 47KDa disappeared (Fig 2B).

The VT4 #1 antibody immunostained neurons and glia using coronal brain sections and imunocytochemistry. The staining was abolished when the peptide-antibody complex (50 µg of peptide to 5 µg of antibody) was used as a control for immunocytochemistry (Fig 3). Additional

controls included the omission of primary and secondary antibodies from the protocol of immunocytochemistry (data not shown). In order to ascertain the antibody recognizes specifically VT4R and not other subtypes, immunocytochemistry was done on cell cultures. The results clearly indicate an intense staining of the cell body and processes with cells transfected with expression vector encoding chicken VT4R cDNA (Fig 4A, 4B, 4C). Absence of immunoreactivity was found in non transfected HeLa cells (Fig 4D), transfected cells with expression vector encoding chicken VT1R cDNA (Fig 4E), VT2R cDNA (Fig 4F) and VT3R cDNA (Fig 4G).

4. 2 Distribution of VT4R in brain:

The current study describes the distribution of VT4R in the male chicken brain. The receptor, VT4R, is widely distributed in the brain from olfactory bulbs to caudal medulla (Fig 5). The VT4R is an abundant receptor in the avian brain. The intensity of staining varies from weak to intense (Table 4). The expression of the VT4R was observed in neuronal cell bodies as well as in glia. It also immunostained an endocrine gland, the pineal, and choroid plexus which lack neurons. This indicates that AVT mediates its effect through VT4Rs in neurons as well as non-neuronal elements which suggests that the VT4R may play a role in various physiological functions in birds. The VT4R has high expression levels in supraoptic, paraventricular, Edinger Westphal, oculomotor and nucleus solitary tract nuclei. All circumventricular organs in the chicken were immunostained for VT4R in glial cells.

The VT4R shares 69% sequence homology with mammalian V1a receptors and 65%-85% homology with the proposed V1a receptors identified in non mammalian vertebtrates (See Table 2). In rats, V1a receptors were identified in the following structures: olfactory bulb,

hippocampus, paraventricular nucleus, cerebellum, raphe nuclei, choroid plexus, subfornical organ, area postrema, solitary tract nucleus, pineal gland and lateral habenular nucleus (Morel et al., 1993; Ostrowski et al., 1992; Ostrowski et al., 1994; Szot et al., 1994). The present study also showed the presence of VT4R immunoreactivity in comparable structures. Nevertheless regions like suprachiasmatic nucleus, bed nucleus of stria terminalis, amgydala, ventromedial nucleus had V1a immunoreactivity in rats but not in avian species. In contrast, the VT4R is distributed throughout the circumventricular organs, in cranial nerves V, VI, VII and VIII and in neural structures including Edinger Westphal nucleus, oculomotor nucleus, dorsal and medial portion of vestibular nucleus and magnocellular cochlear nucleus. Comparing the receptor distribution of VT4R with fishes (Kline,R.J. 2011), newts (Hasunuma,I. 2010; Hasunuma,Itaru 2007) and songbirds (Leung et al., 2009; 2011) revealed that there are comparable structures that are immunoreactive for VT4R such as layers of olfactory lobes, hippocampus, paraventricular nucleus, molecular layer of cerebellum, hypoglossal nucleus and nucleus of solitary tract, potentially indicating that the V1aR distribution for AVT/AVP has been relatively conserved among different species.

4.3 Potential roles of VT4 receptors:

Based on wide distribution of VT4R, its AVT mediated functions can be elucidated. The presence of immunoreactive VT4R perikarya in neuronal structures namely parventricular nucleus, supraoptic nucleus and presence of glial cells immunolabelled for VT4R in hippocampus and median eminence indicates that the VT4R might play a role in stress responses (Aguilera et al., 1994; Engelmann et al., 2004; Holmes et al., 1986). The VT4R is also present in corticotropes in the cephalic lobe of the pituitary (see chapter on pituitary gland) and therefore

helps in the release of ACTH. These data lends support that the VT4R is associated with stress responses (Figure 6).

Social recognition in mammals begins with the detection of the pheromones from the conspecific. The pheromones are detected by vomeronasal organ (VNO) which projects to the accessory olfactory bulb (AOB) and the olfactory epithelium projects to the main olfactory bulb (MOB). The AOB and MOB project to higher brain regions including piriform cortex (Cpi), medial amgydala (MeA), lateral septum (LS) and hippocampus (Hp) that are associated with AVP mediated social recognition or memory processes. It is suggested that the pheromone signals are released by major urinary proteins (MUPs) to the olfactory bulb while AVP modulates these signals in higher brain regions in order to generate a specific response (Ferguson et al., 2002; Bielsky et al., 2004). In contrats to mammals, the olfactory system in birds lacks the VNO and AOB. Hence, the olfactory signals are received by MOB and project to piriform cortex, the most dominant pathway in birds, which is also associated with AVP mediated social recognition (Jones, 1997; Kuenzel,1989; Gomez, 2008; Albers, 2011). Previous studies in rats have shown that the olfactory lobes aid in olfaction and process the olfactory information to lateral septum of the brain thereby aiding in social recoginition (Bielsky et al., 2005). In mammals, V1a receptors is abundant in lateral septum, ventral pallium and periaqueductal gray, lesions in these areas abolish social recognition or pair bonding formation, anxiety and aggressive behavior (Bielsky et al., 2004; Bielsky et al., 2005; Liu et al., 2001). The presence of VT4R immunoreactive glial cells in olfactory ventricles, lateral septum and hippocampus might aid in processing information or sense from the olfactory lobes to higher brain regions. It also suggests that the VT4R could be associated with social recognition and/or memory processes (Fig 7).

Many structures have been identified for classical monoamine neurotransmitter systems that had high and moderate levels of VT4R immunoreactivity including the raphe nucleus, a serotonin producing region; ventral tegmental area and substantia nigra, a dopamine producing region; area postrema producing both serotonin and noradrenaline (Bear et al., 2000; Paxinos et al., 2004; Rink and Wullimann, 2002). Therefore it could be possible that VT4Rs mediate AVT actions on these neurotransmitter systems (Fig 8).

The basal ganglia consist of neocortex, striatum, globus pallidus, substantia nigra, subthalamic nucleus and ventral lateral nucleus. The substantia nigra and caudate putamen of striatum serves as input to basal ganglia while globus pallidus serves as an output to thalamic nucleus. The basal ganglia in mammals aids in motor control (planning, coordination and execution of movements) and learning (Reiner et al., 2005; Bear et al., 2001). The direct pathways of basal ganglia aids in releasing motor movement while the indirect pathway of basal ganglia aid in inhibiting the motor movement. In the direct pathway, the substantia nigra excites the putamen neurons by releasing dopamine. The GABAergic neurons in putamen inhibit the globus pallidus neurons which releases the cells in ventral lateral nucleus. The ventral lateral nucleus gets activated and releases glutamate neurons to cortex which projects its neurons to motor area of the cortex and aid in motor movement. Both globus pallidus and substantia nigra contain dopamine and vasopressin. The vasopressin might serve as excitatory input to substantia nigra and hence could be associated with dopaminergic pathways of this region and hence in motor control (Reiner et al., 2005; Paxinos et al., 2004; Cross et al., 1983). Based on this evidence, we suggest that the VT4R mediates the effects of vasotocin in motor movements, which is further supported by the presence of VT4R in globus pallidus and substantia nigra (Fig 8).

The visual system in avian brain includes both tectofugal and thalamofugal pathways. The sensory fibers project to outer layers of optic tectum (TeO). This tectum projects bilaterally to the thalamic structure, nucleus rotundus (RoT) which further projects to entopallium formerly called ectostriatum. Entopallium cells project to entopallial belt (Ep) which inturn projects to different forebrain structures (Gunturkun, 2000; Kuenzel 1989). The presence of VT4R in the structures optic tectum, entopallium and in nucleus rotundus suggests that the receptor might be involved in vision. This is further supported by the presence of VT4R in lateral geniculate (GLv) nucleus, the major target in the thalamofugal pathway that transmits visual information (Bear et al., 2000). Further, the oculomotor nucleus, accessory oculomotor nucleus and the abducens nucleus receive projections from pretectum that functions to regulate constricution and dilation of the pupil size (Dubbeldam, 2000). The VT4R expression in structures like Edinger Westphal nucleus, abducens nucleus and oculomotor nucleus indicates a potential role in movement of eye (extraocular) muscles, control of the pupils and provides parasympathetic control of pupil size. Therefore, it is possible that VT4R is involved in visual pathways for vision and in reflex pathways mediating pupillary contriction (Fig 9).

The cochlear afferents enter magnocellular cochelar nucleus (MCC) which projects to the laminar nucleus (La). The laminar nucleus further projects to nucleus of lateral leminiscus in the midbrain. The midbrain nucleus has different projections. The dorsal and ventral portion of lateral leminiscus projects to the mesencephalic nucleus and to nucleus ovoidalis while the intermedial leminiscus projects to the basalrostral palli nuclei in the forebrain (Arends et al., 1988; Necker, 2000). In mammals, these structures are involved in auditory pathways which suggest that VT4R might play a role in hearing and balance (Phillips et al., 1988). In avian brain, VT4R expresssion was observed in the magnocellular cochelaris nucleus, dorsal and

intermedial lateral leminiscus (LLd and LLi), dorsal mesencephalic nucleus (MLd), nucleus ovoidalis (OV) and basalrostral palli nuclei formerly called nucleus basalis (Bas) suggesting its association with the ascending auditory pathway (Fig 10).

The circumventricular organs (CVOs) are specialized structures positioned around the margin of the ventricles in the midline of the brain. All CVOs lack the blood brain barrier (Ganong et al., 2000). Their location in relation to the ventrical cavities are to be noted. The circumventricular organs organum vasculosum lamina terminalis, pineal gland and median eminence line the ventricular recesses while other CVOs including sub-septal organ, paraventricular organ, subcommissural organ, subtrochlear organ and area postrema are present at the confluence between the ventricles. Further, the CVOs are classified as secretory CVOs (ME) and sensory CVOs (OVLT, SSO, APa). The secretory CVOs releases neurohormones into the blood because of the special permeability of fenestrated capillaries (Duvernoy, 2007) while the sensory CVOs are sensible to blood borne substances and transmit this information to the central nervous system (Johnson et al., 1993; Johnson et al., 1984). Previous studies in mammals have shown that the CVOs is involved in pathways controlling the CSF osmolarity and pressure (Duvernoy, 2007). In birds, the sensory CVOs are involved in thirst control and water balance (Fahrenholz et al., 1993). The presence of VT4R glial cells in circumventricular organs lateral septal organ, organum vasculosum lamina terminalis and area postrema might help in body fluid regulation, autonomic regulation and endocrine function (by releasing hormones into the bloodstream). The VT4R in tanycyte type of glial cells in median eminence suggests that it aids in the release of hormones into the hypophysial portal system. The immunoreactive VT4Rs in subcommissural organ, subtrochlear organ, paraventricular organ might play a role in the secretion or circulation of CSF. To sum up, the immunoreactive VT4R in circumventricular

organs are associated with the CSF osmolarity control, fluid balance, autonomic and endocrine functions (Fig 13).

Considering the robust distribution of VT4R in brain, the most probable functions of the receptor have been identified based upon VT4R immunoreactivity in neurons and glia. Distinctive areas associated with a wide range of functions including behavior, physiological functions like regulation of water balance, stress responses, sensory functions such as vision, and hearing have been identified.

5. Conclusion:

The present study provides the evidence of extensive VT4R distribution in the chicken brain. The receptor is observed in neurons and glia and found in olfactory, auditory and visual systems and in stress responses.Based on VT4R immunoreactivity, the following functions of the receptor have been proposed: neuroendocrine function of stress responses, social behavior, regulation of CSF and sensory functions like vision and hearing. The functional studies have to be carried out in avian species to understand the possible relationship of VT4R to each of the particular function described above.

6. References

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List of tables

Table 1: Comparison of rat vasopressin/oxytocin and chicken vasotocin receptors to human vasopressin/oxytocin receptors

Species	Rats	Homologue in Chicken	Rats	Homologue in Chicken	Rats	Homologue in Chicken
Subtype	V1b	VT2	V2	VT1	OT	VT3
Sequence homology[1]	63	47	86	57	91	74
Organs	Brain, pituitary	Pituitary	Kidney	Shell gland, uterus	Brain, pituitary, uterus	Endometrium and myometrium of shell gland
Physiology and behavior	ACTH release	ACTH and prolactin release	Water reabsorption	Oviposition	Release of prolactin	Endometrial contractions in the uterus

[1] The receptor subtypes proposed homologous to human vasopressin subtypes based upon their amino acid sequence identity to human V1aR.

Table 2: Amino acid identity (%) of chicken VT4R compared with mammals and other members of vasotocin/oxytocin, vasotocin/mesotocin-isotocin receptor family to human vasopressin V1a receptor (V1aR)

(A) Receptor	Accession No.	Species	Identity (%)
Vasopressin V1a	U19906	*Homo sapiens* (human)	100
Vasopressin V1a	Z11690	*Rattus norvegicus* (rat)	80
Vasopressin V1a	D49730	*Mus musculus* (mice)	82
Vasopressin V1a	L41502	*Ovis aries* (sheep)	82
Vasopressin V1a	GU954352	*Microtus montanus* (Meadow vole)	83
Vasopressin V1a	AF069304.2	*Microtus ochrogaster* (Priarie vole)	82
Vasotocin VT4	**EU124684.1**	***Gallus gallus* (chicken)**	**69**
Vasotocin V1a	XP 002187321	*Taeniopygia guttata* (Zebra finch)	66
Vasotocin VT	AY277924.1	*Rana catesbeiana* (Bull frog)	68
Vasotocin V1a	GU945196.1	*Taricha granulosa* (Rough skin newt)	68
Vasotocin V1a	AB274037	*Cynops pyrrhogaster (Red bellied newt)*	67
Vasotocin	AF184966	*Platichthys flesus* (flounder)	63
Vasotocin	AF517936	*Haplochromis burtoni* (cichlid)	63
Vasotocin	X76321	*Catostomus commersoni* (white sucker)	61
Vasotocin V1a1	HQ662334.1	*Epinephelus adscensionis* (rock hind)	61
Vasotocin V1a1	GQ981412	*Cyprinodon nevadensis amargosae* (pupfish)	59
Vasopressin V1b	L37112	*Homo sapiens* (human)	54
Vasopressin V1b	D45400	*Rattus norvegicus* (rat)	50
Vasopressin V1b	AF098867	*Mus musculus* (mice)	53
Vasotocin VT2	**AY008272**	***Gallus gallus* (chicken)**	**53**
Vasotocin V1b	XP 002189484	*Taeniopygia guttata* (Zebra finch)	54
Vasotocin V1b	XP 003212983	*Meleagris gallopavo* (Turkey)	53
Vasotocin V1b	EF567079.1	*Taricha granulosa* (Rough skin newt)	52
Vasotocin V3/V1b	AB284503	*Cynops pyrrhogaster* (Red bellied newt)	53
Vasotocin V1a2	HQ141396	*Epinephelus adscensionis* (rock hind)	63
Vasotocin V1a2	GQ981413	*Cyprinodon nevadensis amargosae* (pupfish)	60

(B) Receptor	Accession No.	Species	Identity (%)
Vasopressin V2	L22206	*Homo sapiens* (human)	42
Vasopressin V2	Z11932	*Rattus norvegicus* (rat)	41
Vasopressin V2	NM019404	*Mus musculus* (mice)	41
Vasotocin VT1	**AF147743**	***Gallus gallus* (chicken)**	**48**
Vasotocin V2	XP 002195418	*Taeniopygia guttata* (Zebra finch)	46
Vasotocin V2	XP 003202046	*Meleagris gallopavo* (Turkey)	47
Vasotocin V2	ABQ 23253	*Rana catesbeiana* (Bull frog)	45
Vasotocin V2	BAF 38755	*Cynops pyrrhogaster* (Red bellied newt)	43
Oxytocin OT	X64878	*Homo sapiens* (human)	54
Oxytocin OT	U82440	*Macaca mulatta* (rhesus monkey)	52
Oxytocin OT	X87986	*Ovis aries* (sheep)	51
Oxytocin OT	U15280	*Rattus norvegicus* (rat)	49
Vasotocin VT3	**AY833434**	***Gallus gallus* (chicken)**	**49**
Mesotocin	AY277925.1	*Rana catesbeiana* (Bull frog)	48
Mesotocin	DQ186599.1	*Taricha granulosa* (Rough skin newt)	49
Isotocin	X87783	*Catostomus commersoni* (white sucker)	47
Isotocin	GQ981415	*Cyprinodon nevadensis amargosae* (pupfish)	53

Table 3: Abbreviations

APa	Area postrema	IP	Nucleus interpeduncularis
Bas	Nucleus basorostralis pallii	Ipc	Nucleus isthmi, pars parvocellularis
BO	Bulbus olfactorius	IS	Nucleus interstitialis
CbI	Nucleus cerebellaris internus	HA	Hyperpallium apicale
CbIM	Nucleus cerebellaris intermedius	La	Nucleus laminaris
CC	Canalis centralis	LC	Nucleus linearis caudalis
DIP	Nucleus dorsointermedius posterior thalami	LFS	Lamina frontalis superior
DLAmc	Nucleus dorsolateralis anterior thalami	LFSM	Lamina frontalis suprema
DMP	Nucleus dorsomedialis posterior thalami	LLd	Nucleus lemnisci lateralis, pars dorsalis
DMA	Nucleus dorsomedialis anterior thalami	LLi	Nucleus lemnisci lateralis, pars ventralis
E	Entopallium	LSO	Lateral septal organ
EW	Nucleus of Edinger Westphal	MCC	Nucleus magnocellularis cochlearis
GCt	Substantia grisea centralis	ME	Median eminence
GLv	Nucleus geniculatus lateralis, pars ventralis	MLd	Nucleus mesencephalicus lateralis
LFB	Lateral forebrain bundle	Mn V	Nucleus motorius nervi trigemini
HL	Nucleus Habenularis lateralis	MnV IId	Nucleus motorius nervi facialis, pars dorsalis
Hp	Hippocampus	Mn VIIv	Nucleus motorius nervi facialis, pars ventralis
Ico	Nucleus intercollicularis	Mn X	Nucleus motorius dorsalis nervi vagi
IH	Nucleus inferioris hypothalami	HpC	Hippocampal commissure(formerly the bed nucleus of pallial commissure)
Imc	Nucleus isthmi, pars magnocellularis	nPrV	Nucleus sensorius principalis nervi trigemini
IN	Nucleus infundibuli hypothalami	nVI	Nucleus nervi abducentis
IO	Nucleus isthmo-opticus	nIX-X	Nucleus nervi glossopharyngei et nucleus motorius dorsalis nervi vagi
nXII	Nucleus nervi hypoglossi	SCd	Nucleus subceruleus dorsalis
OMdl	Nucleus nervi oculomotorii pars dorsolateralis	SCE	Stratum cellulare externum
OMdm	Nucleus nervi oculomotorii pars dorsomedialis	SCv	Nucleus subceruleus ventralis
OMv	Nucleus nervi oculomotorii pars ventralis	SCO	Subcommissural organ
OS	Nucleus olivaris superior	SG	Substantia gelatinosa Rolandi
OV	Nucleus ovoidalis	SGP	Stratum griesum periventriculare
OVLT	Organum vasculosum lamina terminalis	SLu	Nucleus semilunaris
PHN	Nucleus periventricularis hypothalami	SNc	Substantia nigra
PL	Nucleus pontis lateralis	SOe	Nucleus supraopticus, pars externus
PM	Nucleus pontis medialis	SOv	Nucleus supraopticus, pars ventralis

POP	Nucleus preopticus periventricularis	SL	Nucleus septalis lateralis
GP	Globus pallidus	SM	Nucleus septalis medialis
PVN	Nucleus paraventricularis magnocellularis (Paraventricular nucleus)	SpL	Nucleus spiriformis medialis
PVO	Paraventricular organ	SS	Nucleus supraspinalis
R	Nucleus raphe	SSO	Subseptal organ
RgC	Nucleus reticularis gigantocellularis	STO	Subtrochlear organ
ROT	Nucleus Rotundus	VeD	Nucleus vestibularis descendens
RPgc	Nucleus reticularis pontis caudalis	VeL	Nucleus vestibularis lateralis
RpgI	Nucleus reticularis paragigantocellularis lateralis	VeM	Nucleus vestibularis medialis
RSd	Nucleus reticularis superior	VeS	Nucleus vestibularis superior
RST	Nucleus reticularis subtrigerminalis	VTA	Ventral tegmental area
Ru	Nucleus ruber	VO	Ventriculus olfactorius
S	Nucleus tractus solitarii	VL	Ventriculus lateralis

Table 4

(a) Intensity of VT4R immunoreactivity in neurons

Region	Intensity
Telencephalic regions	
LFB: Lateral forebrain bundle	++
POP: Nucleus preopticus periventricularis	+++
GP:Globus pallidus	+++
Thalamic regions	
DLAmc: Nucleus dorsolateralis anterior thalami	+++
DMA: Nucleus dorsomedialis anterior thalami	++
DMP: Nucleus dorsomedialis posterior thalami	++
DIP: Nucleus dorsointermedius posterior thalami	++
HL: Nucleus Habenularis lateralis	+
GLv: Nucleus geniculatus lateralis	++
ROT: Nucleus Rotundus	++
RSd: Nucleus reticularis superior	++
SCE: Stratum cellulare externum	++
Hypothalamic regions	
PHN: Nucleus periventricularis hypothalami	+++
PVN: Paraventricular nucleus	+++
SOe/Sov: Nucleus supraopticus (externus, ventralis)	++
Mesencephalic regions	
GCt: Substantia grisea centralis	+++
ICo: Nucleus intercollicularis	+++
Imc: Nucleus isthmi, pars magnocellularis	+++
IP: Nucleus interpeduncularis	++
Ipc: Nucleus isthmi, pars parvocellularis	++
MLd: Dorsal nucleus mesencephalicus lateralis	++
MPv: Ventral nucleus mesencephalicus profundus	++
VTA: Ventral tegmental area	++
Pontine regions	
EW: Nucleus of Edinger Westphal	+++
IO: Nucleus isthmo-opticus	+++
La: Nucleus laminaris	+++
LLd/LLi: Nucleus lemnisci lateralis (dorsalis, ventralis)	+
MCC: Nucleus magnocellularis cochlearis	+++
Mn V: Nucleus motorius nervi trigemini	++
Mn VIId: Nucleus motorius nervi facialis, pars dorsalis	++
Mn VIIv: Nucleus motorius nervi facialis, pars ventralis	++
nVI: Nucleus nervi abducentis	+++
nPr V: Nucleus sensorius principalis nervi trigemini	++
OMdl: Nucleus nervi oculomotorii pars dorsolateralis	+++
OMdm: Nucleus nervi oculomotorii pars dorsomedialis	+++
OMv: Nucleus nervi oculomotorii pars ventralis	+++

OS: Nucleus olivaris superior	++
PL/PM: Nucleus pontis (lateralis, medialis)	+
R: Nucleus raphe	+
RPgc: Nucleus reticularis pontis caudalis	+++
RpgI: Nucleus reticularis paragigantocellularis lateralis	+++
SCd/SCv: Nucleus subceruleus (dorsalis, ventralis)	+
SNc: Substantia nigra	++
Cerebellar regions	
Purkinje cell layer	+
Cerebellar nuclei	+++
Medullary regions	
MnX: Nucleus motorius dorsalis nervi vagi	++
nIX-X: Nucleus nervi glossopharyngei et nucleus motorius dorsalis nervi vagi	+++
nXII: Nucleus nervi hypoglossi	+++
RST: Nucleus reticularis subtrigerminalis	++
S: Nucleus tractus solitarii	++
SS: Nucleus supraspinalis	+++
VeD: Nucleus vestibularis descendens	+++
VeL/VeM: Nucleus vestibularis (lateralis, medialis)	+++
VeS: Nucleus vestibularis superior	++

Intensity in neurons was evaluated: Oculomotor neurons (+++, strong), isthmus nucleus (++, moderate), pontis nucleus (+, weak)

(b) Intensity of VT4R immunoreactivity in glial cells

Region	Intensity
Telencephalic regions	
Bas: Nucleus basorostralis pallii	+++
BO: Bulbus olfactorius	++++
E: Entopallium	+++
HA: Hyperpallium apicale	+++
LFS:Lamina frontalis superior	+++
LFSM: Lamina frontalis suprema	+++
HpC: Hippocampal commissure, formerly NCPa	++++
OVLT: Organum vasculosum lamina terminalis	++++
SL: Nucleus septalis lateralis	+++
SM: Nucleus septalis medialis	+++
VO: Ventriculus olfactorius	++++
Thalamic regions	
OV: Nucleus ovoidalis	+++
P: Pineal gland	++++
PC: Choroid plexus	++++
SCO: Subcommissural organ	++++
SGP: Stratum griesum periventriculare	+++
SSO: Subseptal organ (subfornical organ)	++++
Hypothalamic regions	
IH: Nucleus inferioris hypothalami	++++
IN: Nucleus infundibuli hypothalami(arcuate nucleus)	++++
IS: Nucleus interstitialis	+++
PVO: Paraventricular organ	++++
ME: Median eminence	++++
Pontine regions	
STO: Subtrochlear organ	++++
Cerebellar regions	
Molecular layer	+++
Medullary regions	
APa: Area postrema	++++
CC: Canalis centralis	++++

Intensity in glial cells was evaluated: Molecular layer of cerebellum (+++, strong) and circumventricular organs (++++, intense).

Figure legends:

Figure 1: (A) Amino acid sequence of the VT4R showing the N terminal peptide sequence highlighted in red. (B) Amino acid alignment of the synthesized peptide with the chicken VT4R.

Figure 2: Antiserum specificity of chicken vasotocin receptor (VT4R) was determined using Western Blot analysis. (A) The antibody shows a single band at 47kDa as predicted by molecular weight marker (M) in hypothalamic region (H), septal region (SL), pituitary tissues (P) and in HeLa cells transfected with expression vector encoding the chicken VT4R cDNA (C2). The absence of band was found with HeLa cells transfected with pGEM7Z plasmid (C1). (B) Specificity test of antibody using peptide blocking experiments in membrane protein extracts from the chicken hypothalamic tissues (H). The antibody at 1:1000 dilution was preadsorbed with 5 µg (P1) and 20 µg (P2) of immunogenic peptides prior to application in the Western Blot.

Figure 3: The antibody was validated with a peptide competition assay in brain tissue sections using immunohistochemistry . Sections A and D show the paraventricular nucleus and median eminence, respectively, immunostained for neurons and glia using the VT4R antibody at 1:2000 dilution. The antibody was preadsorbed with 10 µg of peptide (B, E) and 50 µg of peptide (C, F). The peptide competition assay was performed with perikarya (A-C) and glial cells (D-F). Scale bars: 12 µm (B, C), 50µm (A), 100 µm (E), 300 µm (D, F).

Figure 4: Immunohistochemical detection of chicken vasotocin receptor (VT4R) in transfected HeLa cells. HeLa cells transfected with chicken VT4R as detected by antibody VT4 #1 (A). VT4R immunolabelled in the cytoplasm of cell profiles (B, C). VT4R immunoreactive cells and processes are stained in red and nuclei counterstained with DAPI (C). The absence of

immunoreactivity in HeLa cells transfected with pGEM7Z (D), HeLa cells transfected with expression vector encoding chicken VT1 cDNA (E), VT2 cDNA (F) and VT3 cDNA (G). Scale bars 10µm (A, B, C), 15 µm (G), 20µm (D, E), 35µm (F).

Figure 5: Schematic representation of VT4R immunoreactivity in coronal brain sections arranged from rostral to caudal. The distribution of VT4R are illustrated as perikarya () and glial cells (). The plates are modified from Kuenzel and Masson, 1988. See Table 3 for abbreviations and Table 4 for intensity of staining observed in different regions of the brain. Scale bars: 1cm.

Figure 6: Densely immunoreactiveVT4R in hypothalamic neurons and in glia. Schematic diagram of chicken coronal plates modified from Kuenzel and Masson, 1988 (A, B). VT4R immunoreactive perikarya in supraoptic nucleus (C), paraventricular nucleus (D). and glial cells in hippocampus (E). Scale bars: 50 µm.

Figure 7: VT4R immunoreactive glial cell staining in olfactory lobes, dorsal septum and mediobasal hypothalamus. A-C. Schematic diagrams of coronal plates A13.8 to A5.6 modified from Kuenzel and Masson, 1988. D. Intense VT4R immunoreactivity of glial cells in olfactory lobes surrounding the olfactory ventricle. E. Dense glial elements present in the dorsal septum moving horizontally and in nucleus inferioris hypothalami and nucleus infundibuli hypothalami (F). Scale bars: 300 µm (D), 50 µm (F), 20 µm (E).

Figure 8: Dense immunoreactive VT4Rs in neurons associated with motor pathways. Schematic diagrams of coronal plates A13.4 to A3.6 (A, C). Intense immunoreactivity in globus pallidus (B) and substantia nigra (D). Scale bars: 50 µm (B), 12 µm (D).

Figure 9: VT4R immunostaining in neurons of the third and sixth cranial nerves. Schematic diagram of coronal plates A3.4 (A) and P0.4 (C). Intense immunoreactive VT4Rs in Edinger Westphal nucleus (B), oculomotor nucleus sub-nuclei (B) and abducens nucleus (D). Scale bars: 100 µm (B), 12 µm (D).

Figure 10: Dense immunoreactive VT4Rs in neurons associated with the eighth cranial nerve. Schematic diagram of coronal plate P1.0 (A). Dense neuronal staining in magnocellular cochlear nucleus (B) and moderate immunostaining in the dorsal portion of the vestibular nucleus (C). Scale bars: 50 µm (B, C).

Figure 11: VT4R immunoreactivity in neurons involved in beak function. Schematic diagram of coronal sections A 0.8 and A 0.2 (I, A, B). Intense VT4R perikarya staining in the basorostral pallial nucleus (II). Moderate immunoreactivity of VT4R in neurons of the principal nucleus of the trigerminal nerve (C), motor nucleus of trigerminal nerve (D), dorsal portion of motor nucleus of facial nerve (E). Scale bars: 100 µm (C), 50 µm (D, II), 12 µm (E).

Figure 12: Photomicrograph of VT4R immunoreactive staining in the cerebellum. Diagrammatic representation of cerebellum in coronal plate A3.4 (A) of the chick brain atlas. Photomicrograph of VT4R immunoreactive perikarya of purkinje cells (B), Berghmann glial cell immunostained with VT4R in cerebellum (C) and VT4R immunolabelled cells in nucleus cerebellaris internalis (CbI) (D). Scale bars: 100 µm (B), 50 µm (C, D).

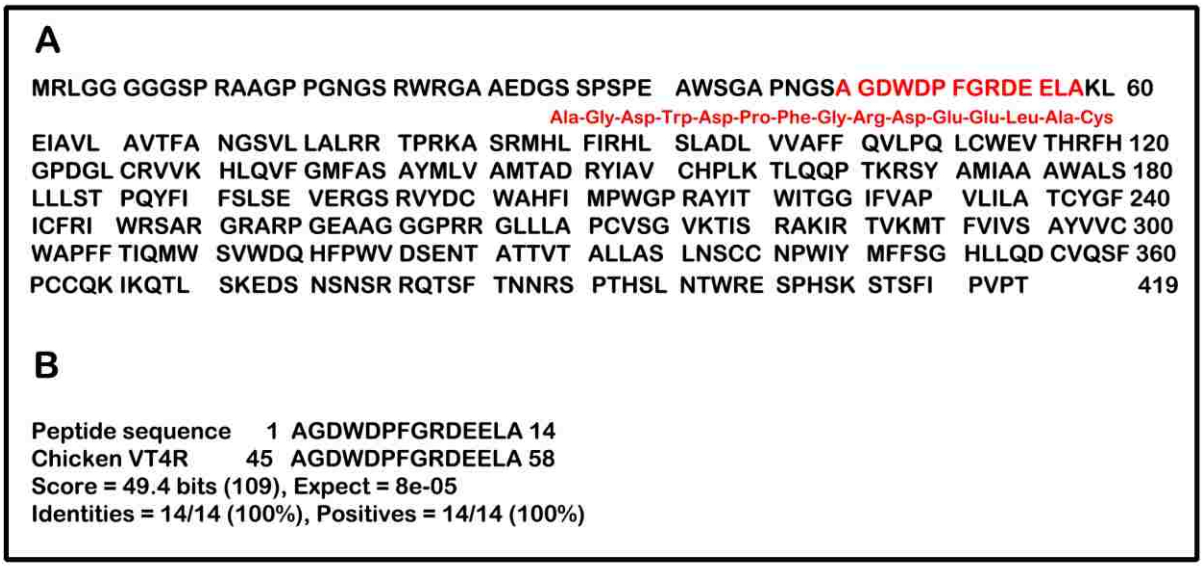
Figure 13: Photomicrographs of VT4R immunoreactivity in circumventricular organs.

(A) Schematic plate deciphering the circumventricular organs in chicken (Reproduced from Kuenzel, 1982) (B) Schematic plate A6.2 showing the choroid plexus of chick brain atlas, modified from Kuenzel and Masson, 1988. (C) Intense glial cell labelling in choroid plexus

(PCV-III) with VT4R. (D) VT4R immunoreactive glial cells observed in lateral septal organ (E) organsum vasculosum lamini terminalis and ventral to the nucleus of the Hippocampal commissure (HpC) formerly known as the nucleus of bed pallial commissure. (F) High maginification of VT4R glial cells stained in subseptal organ (SSO). (G) Low magnification image of VT4R immunolabelled in subcommissural organ (SCO). (H) High magnification image of paraventricular organ (PVO) showing the VT4R immunoreactivity. (I) Low power image of median eminence (ME) showing VT4R immunoreactive tanycytes. (J) Photomicrograph of pineal gland (PIN) showing glial cells immunolabelled for VT4R. Low magnification photomicrographs of subtrochlear organ (STO) (K) and Area postrema (APa) (L) showing VT4R immunoreactivity with subependymal glial cells. Scale bars: 50 µm (C-F), 100 µm (G, I, L), 12 µm (H, J), 300 µm (K).

List of figures:

Figure 1:



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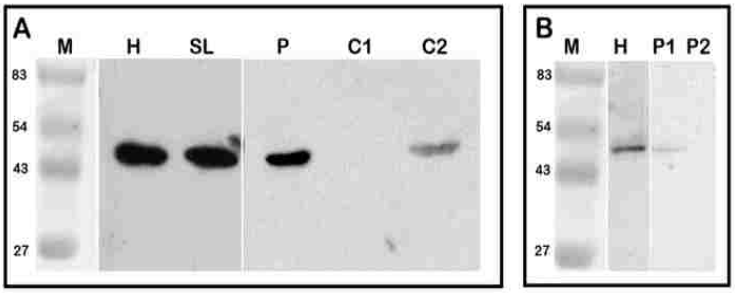

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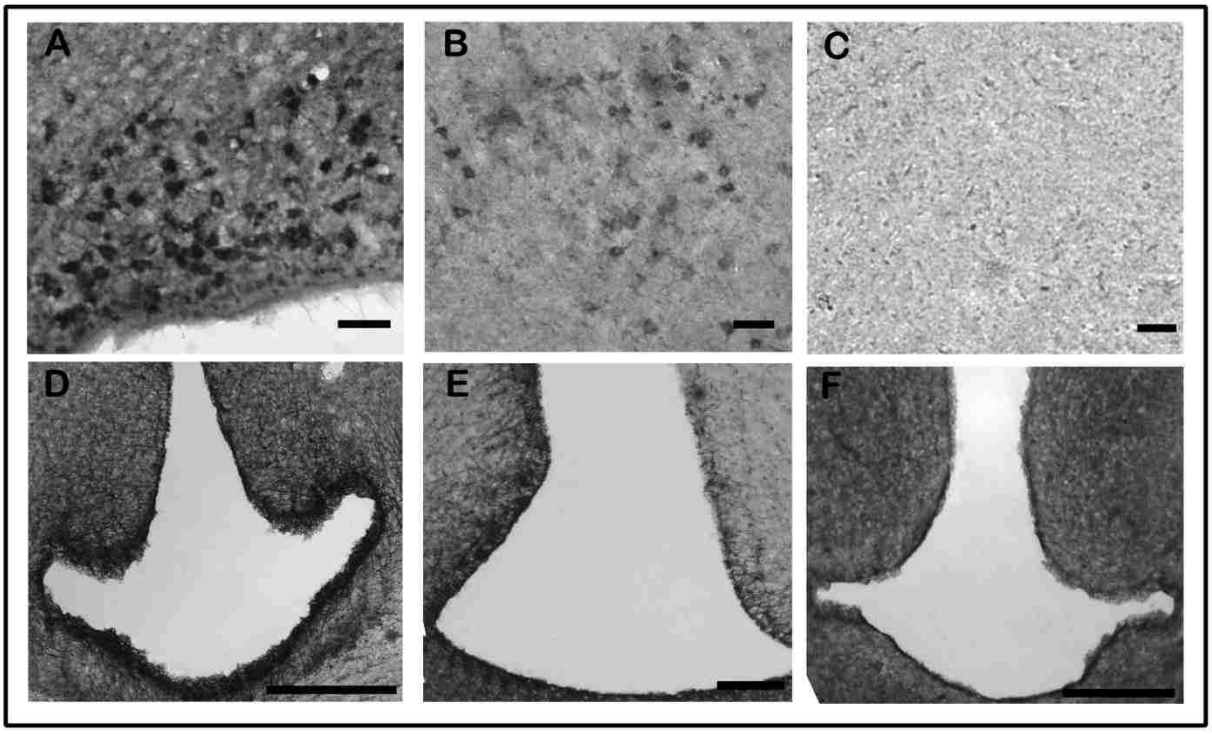

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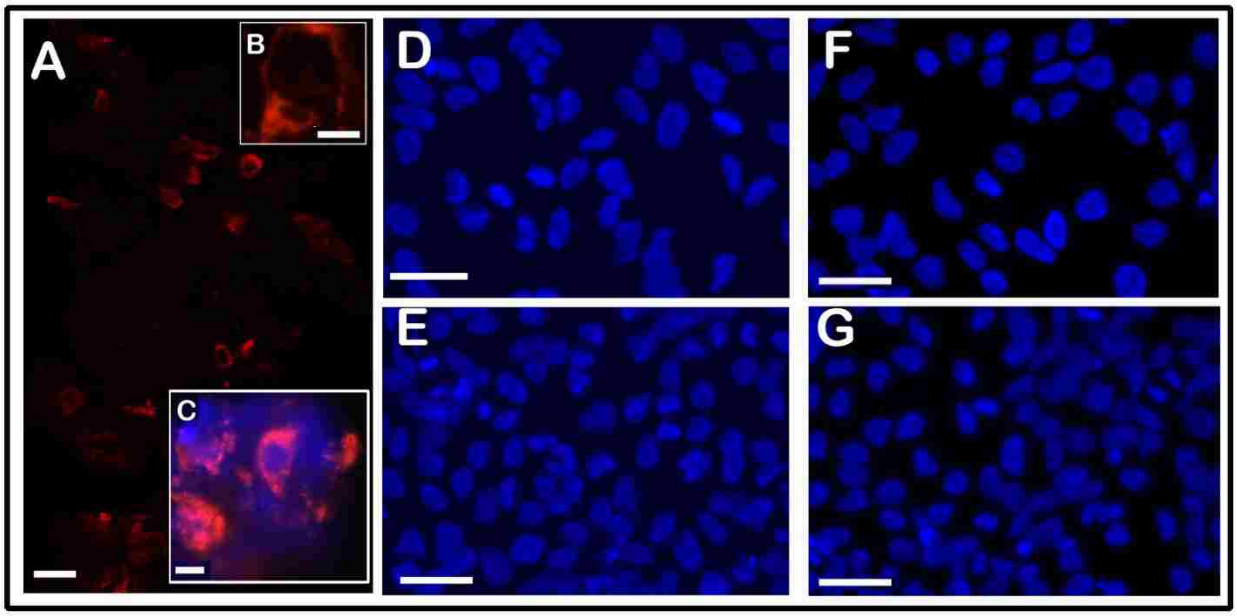

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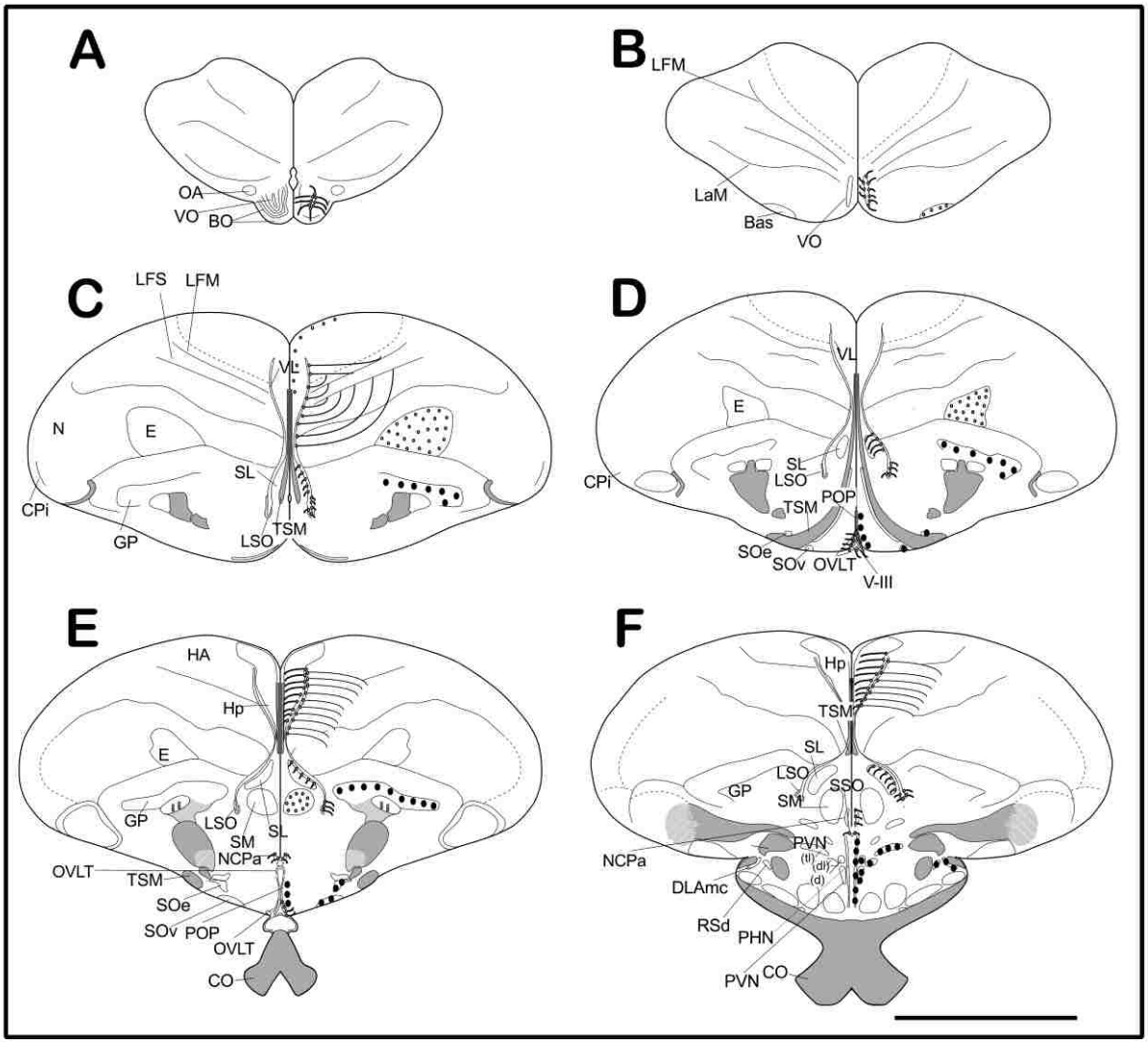
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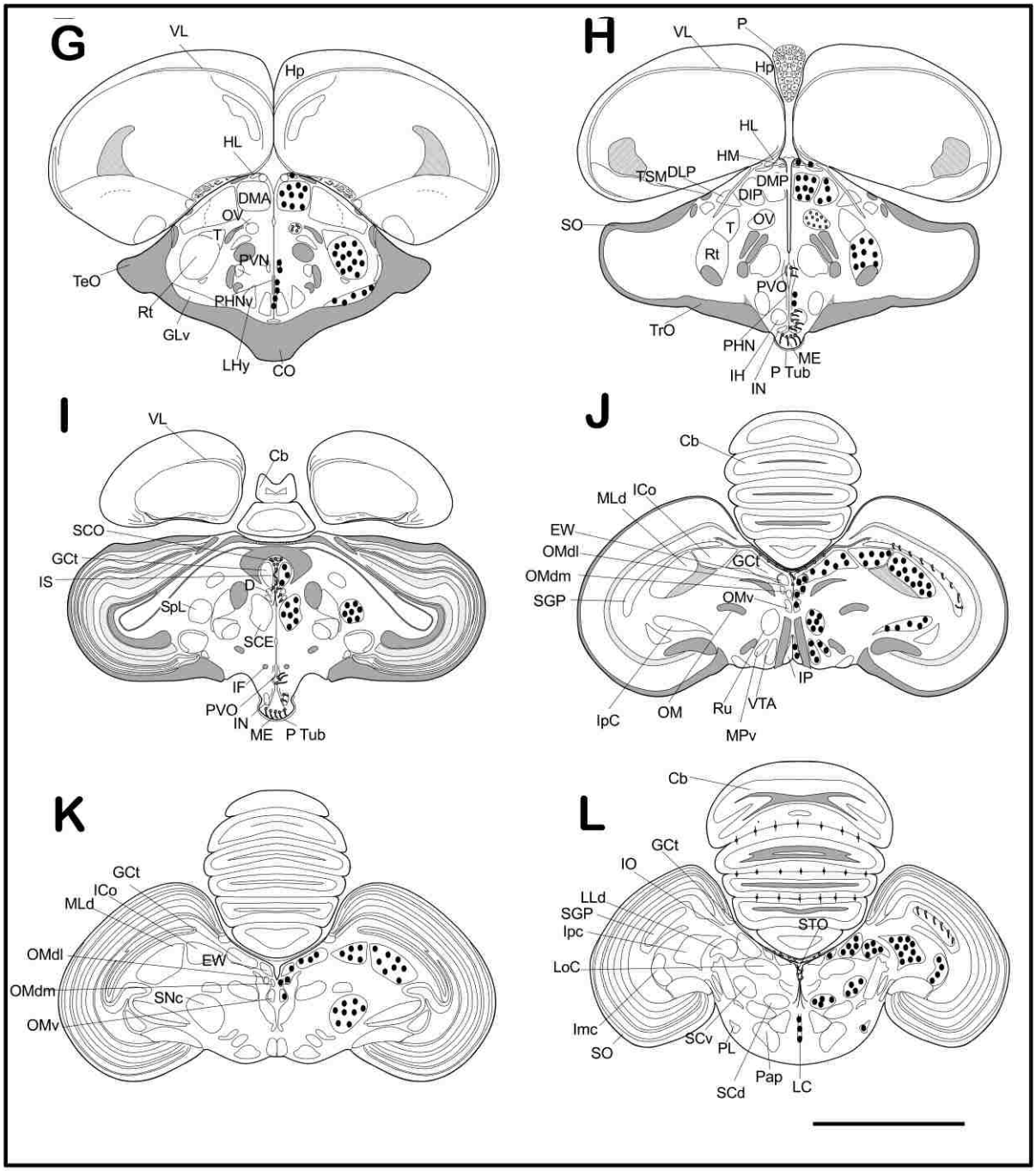
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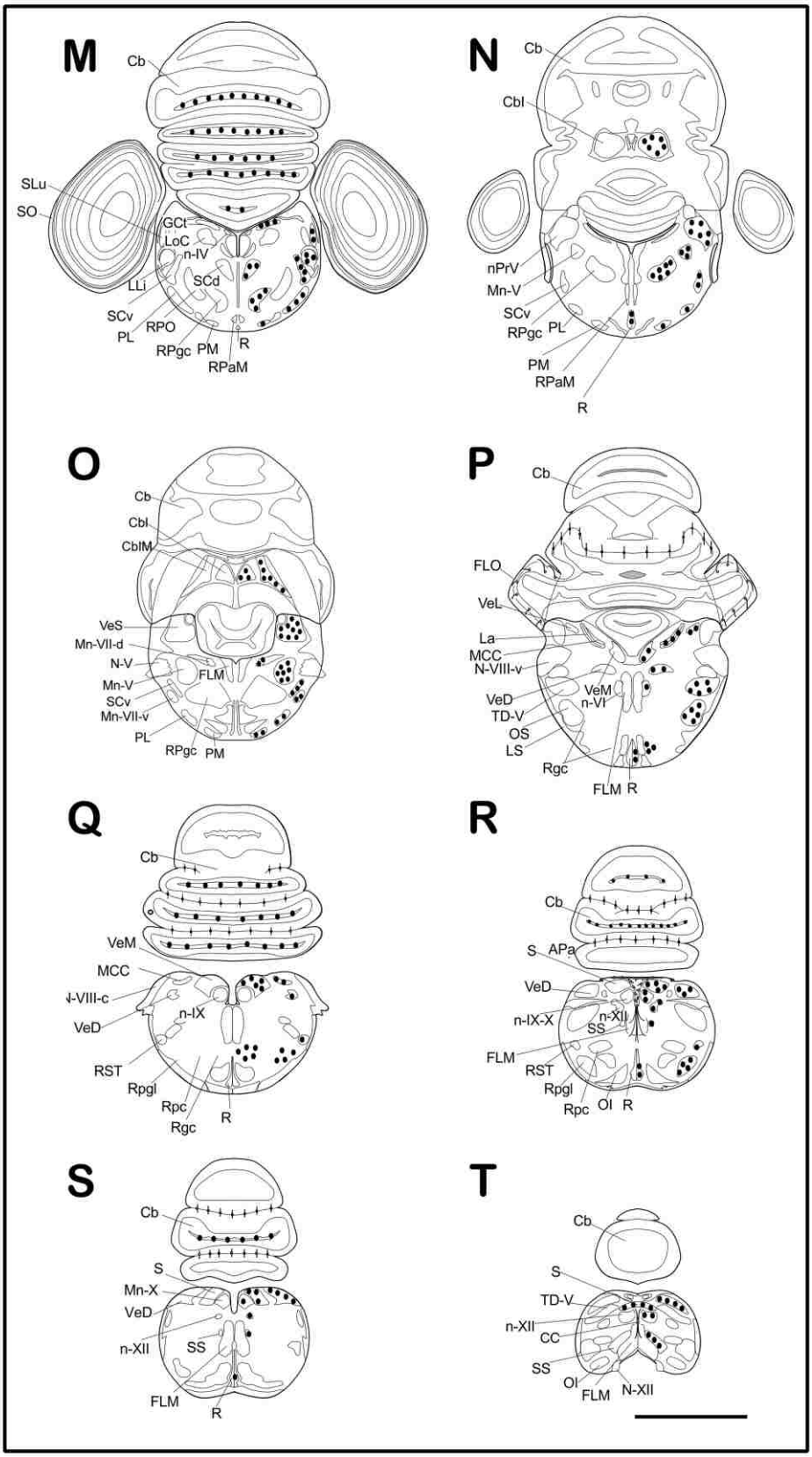
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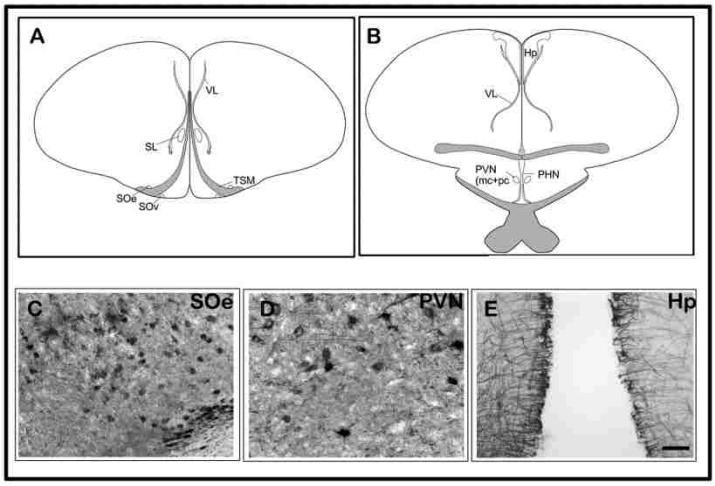

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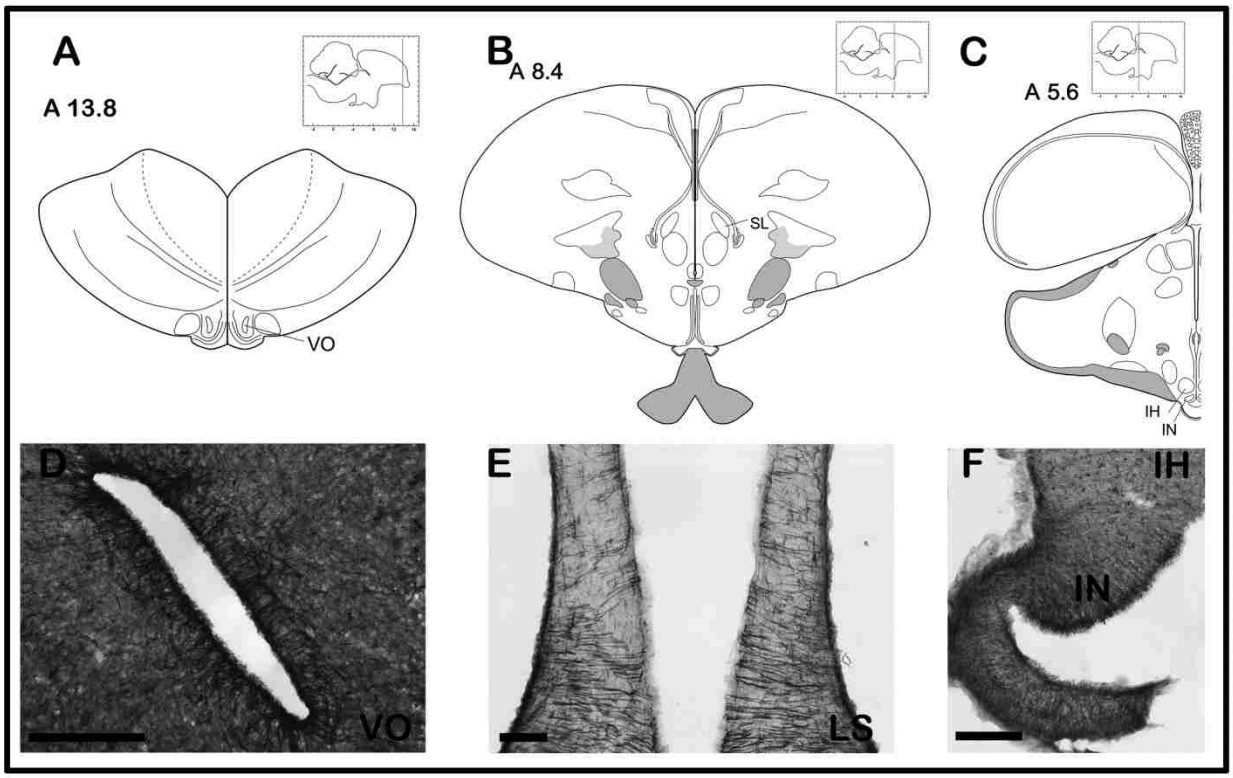

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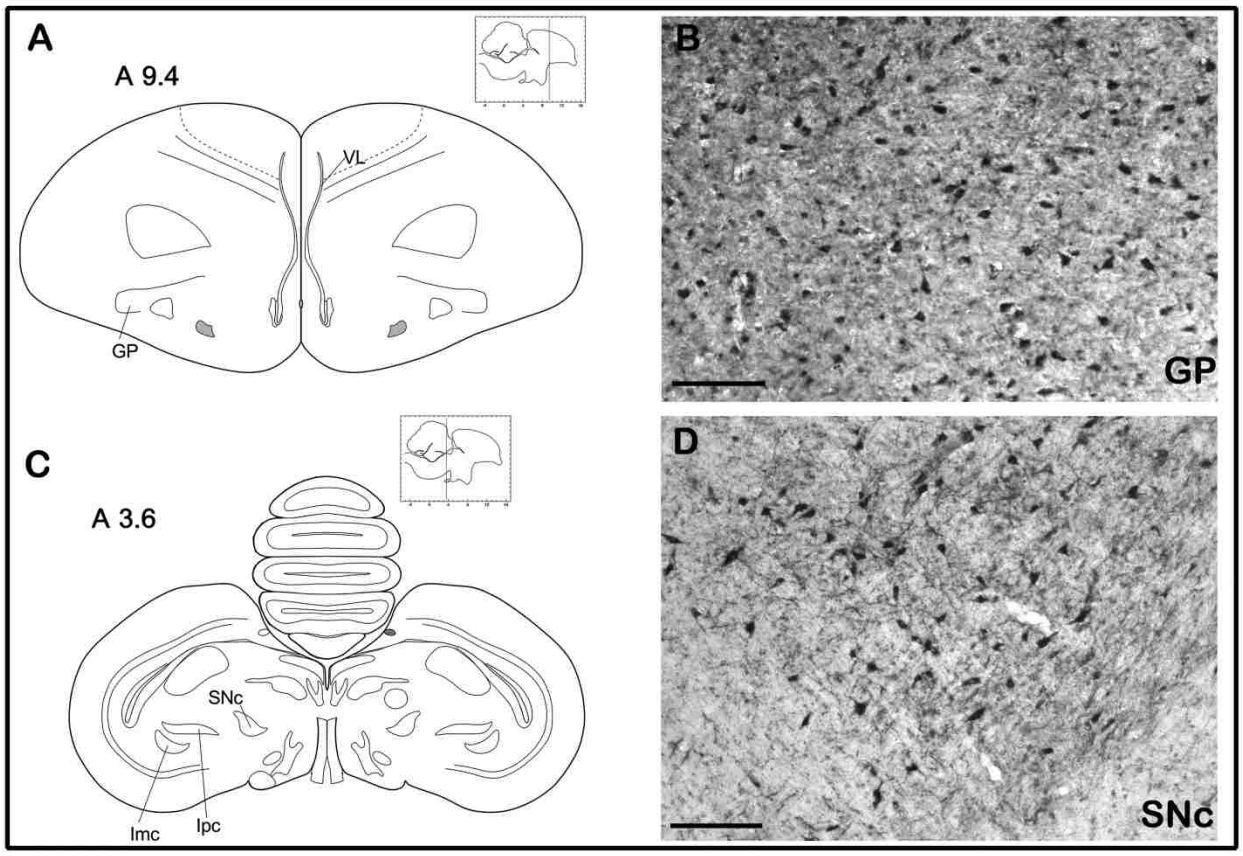

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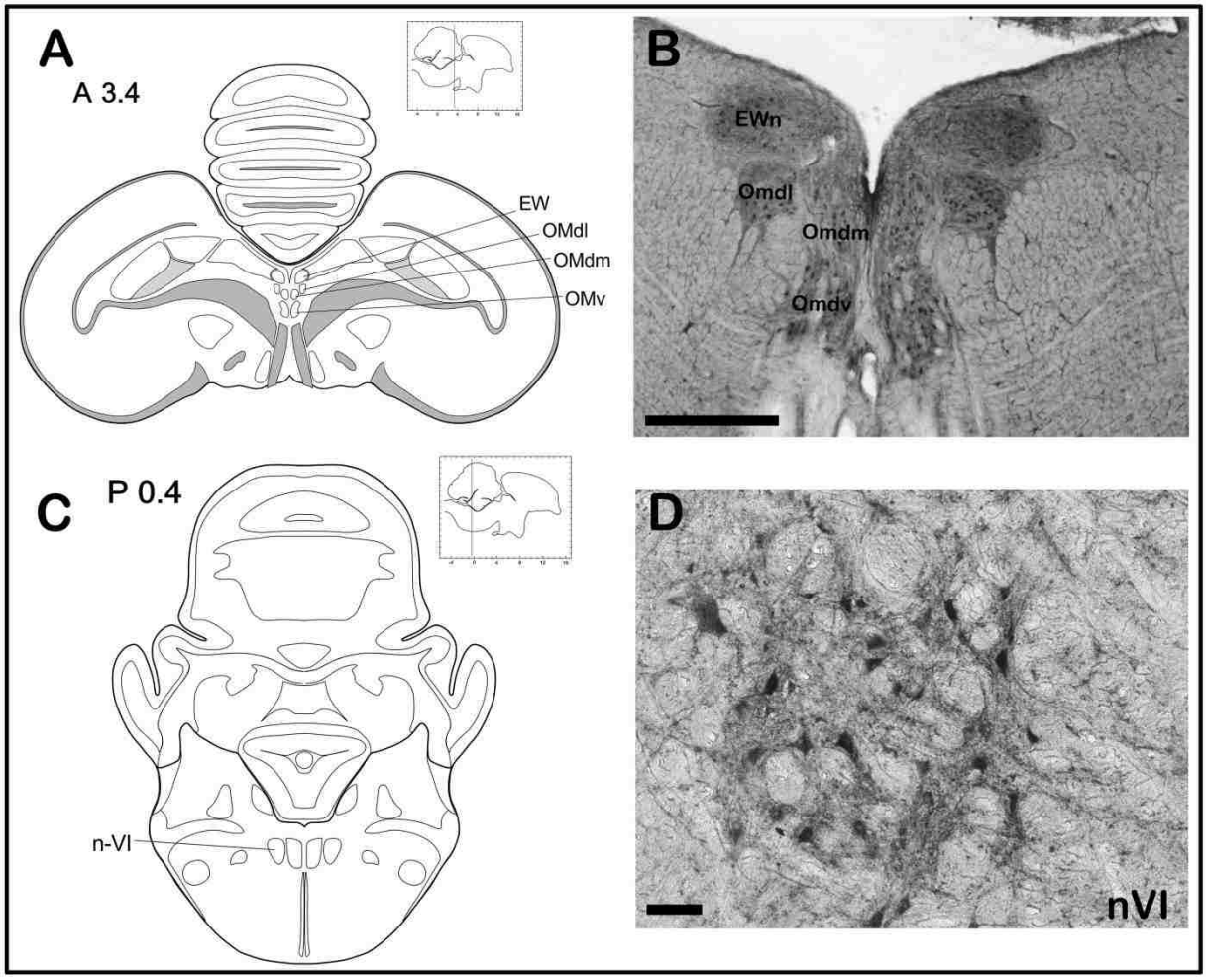

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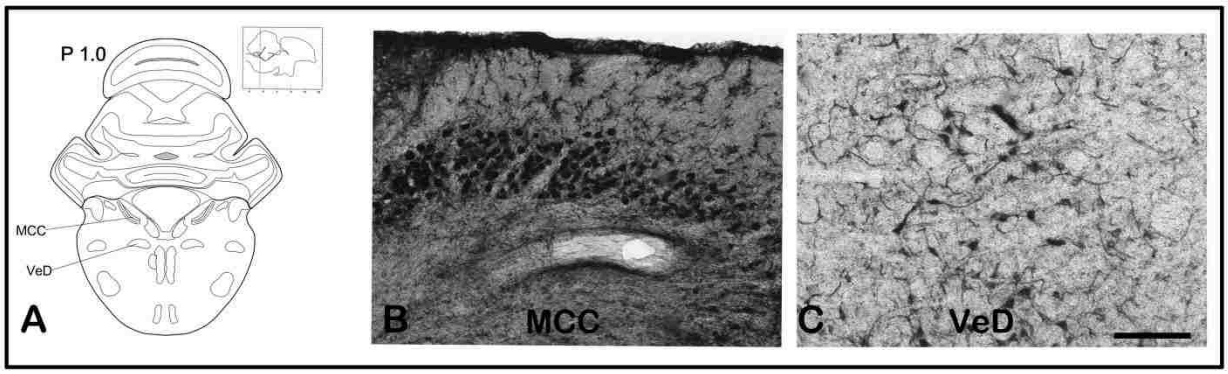

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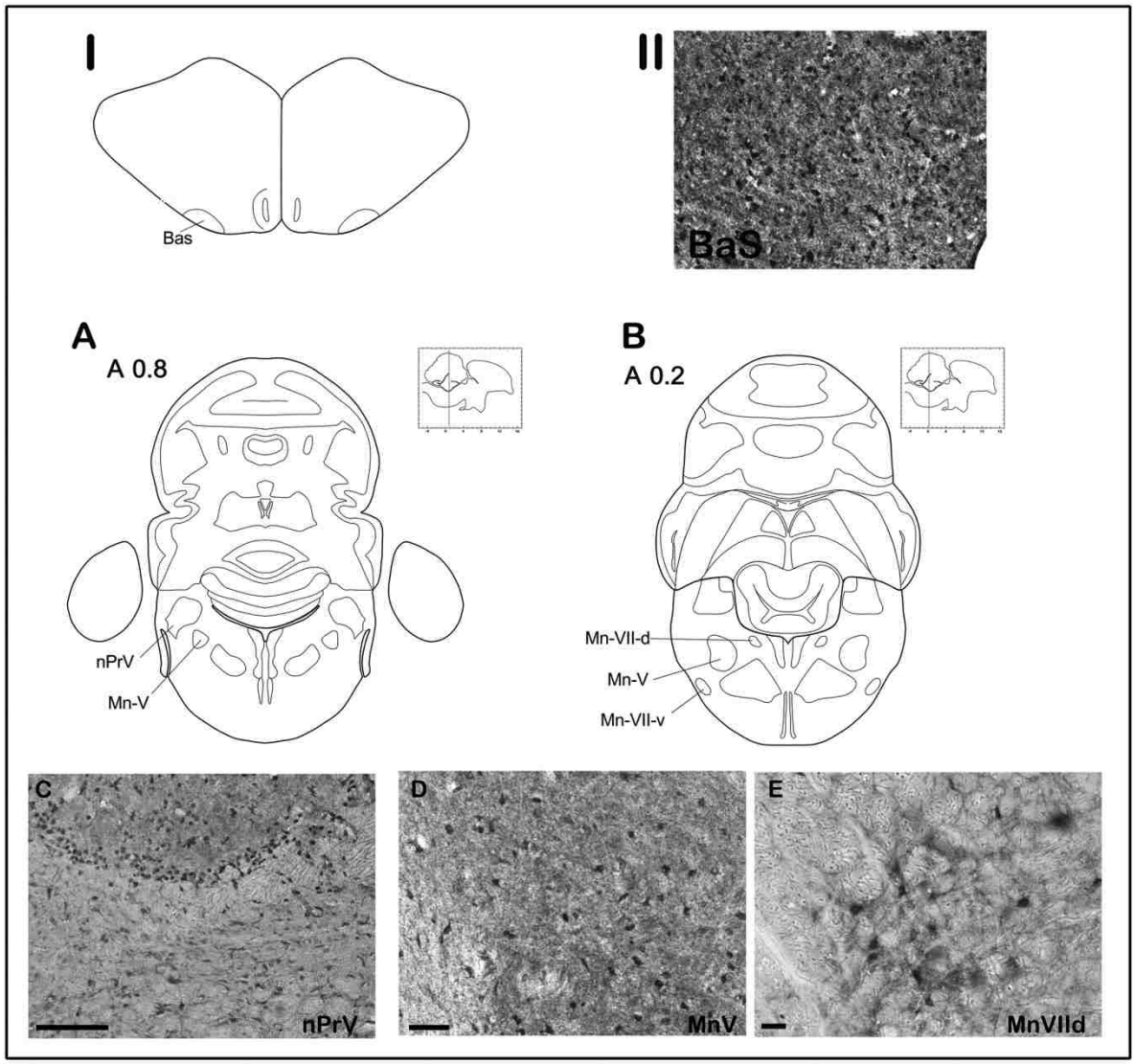
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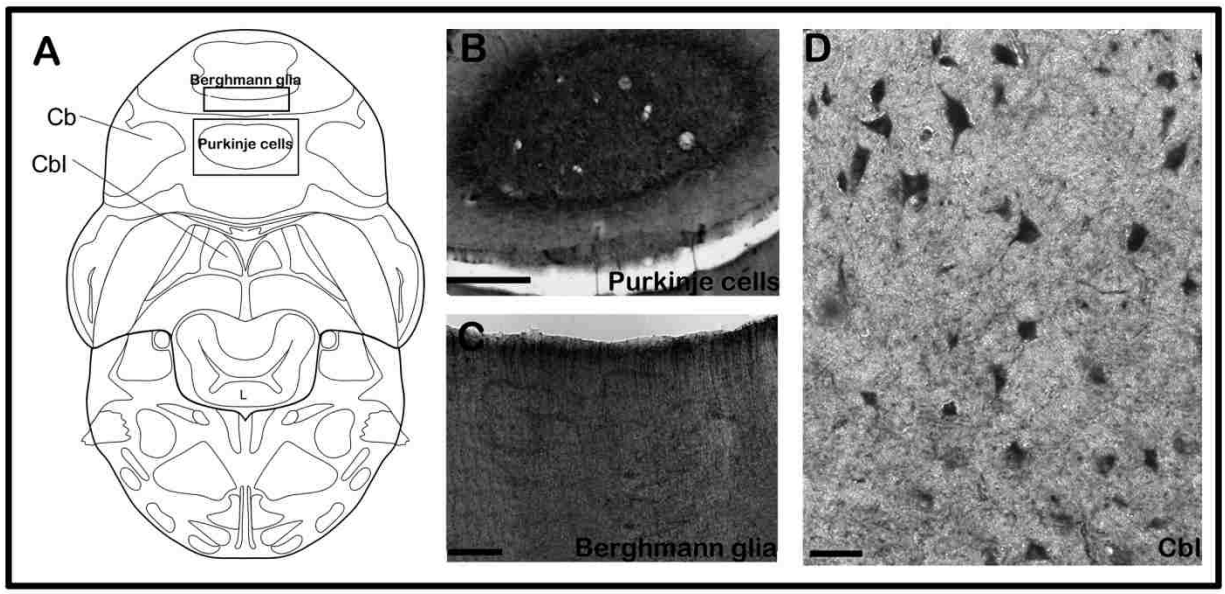
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Purkinje cells
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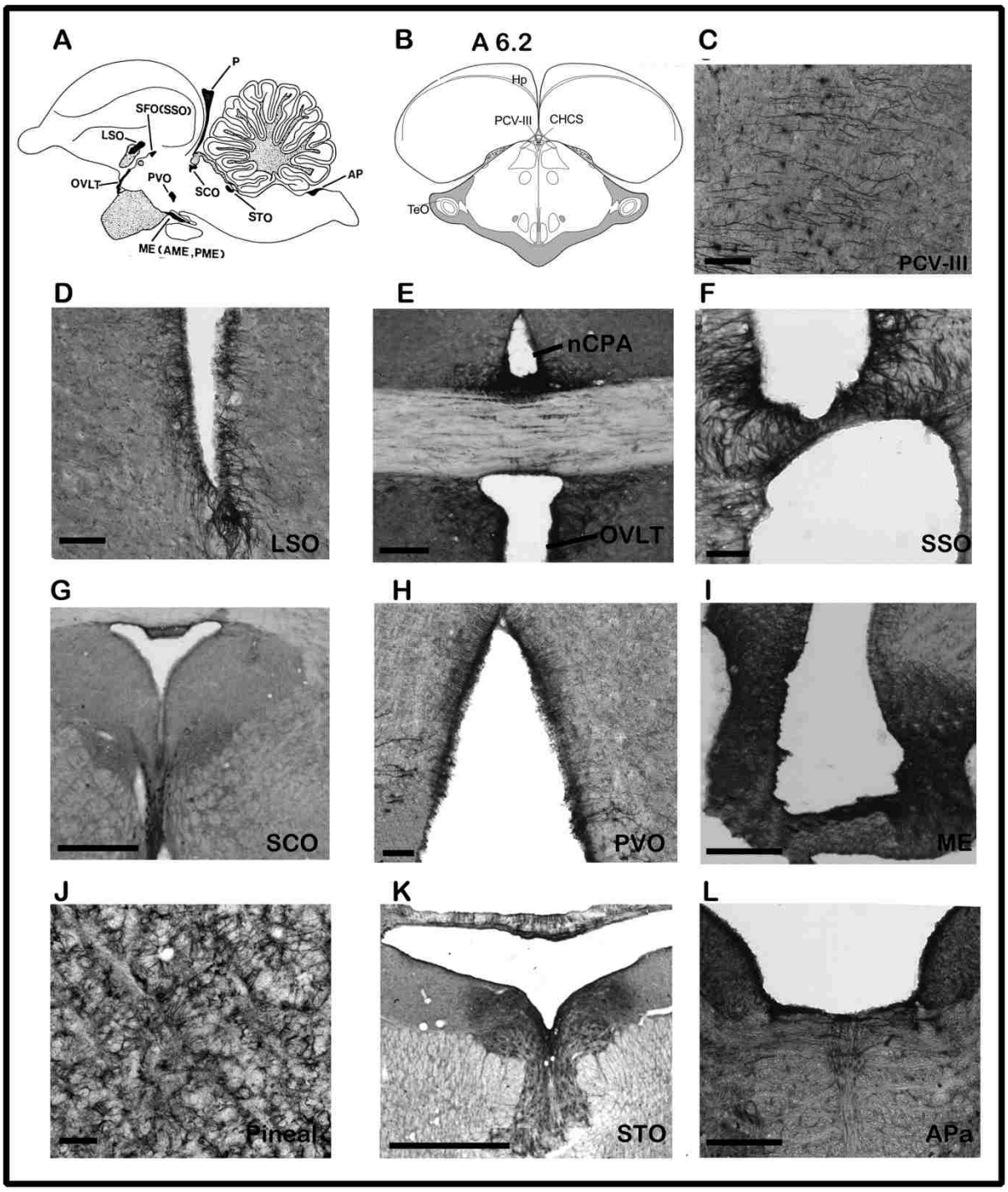
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CHCS
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PCV-III
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PVO
I
ME
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Pineal
K
STO
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IV. CONCLUSIONS:

The most recently cloned chicken vasotocin receptor, VT4R shares 69% amino acid homology with its mammalian counterpart, V1aR, a vasopressin receptor found in the mammalian brain. The current study utilized an antibody to the VT4R and the technique of immunocytochemistry in order to ascertain the distribution of the VT4R in the avian brain and pituitary gland. The VT4R is present primarily in the cephalic lobe of the anterior pituitary and is associated with corticotropes and gonadotropes. Of interest is that the homologous receptor in rats, the V1aR, of rats is co-localized only in gonadotropes and not in corticotropes. The distribution of VT4R in brain is widespread similar to mammals. Some of the neurons densely stained with VT4Rs were observed in the paraventricular nucleus, supraoptic nucleus, Edinger Westphal nucleus, oculomotor nucleus, magnocellular cochlear and hypoglossal nuclei. The striking contrast among the avian brain and mammalian brain was the densely stained VT4R in glial cells of the avian brain. The functions of receptor were suggested to be associated with the regulation of stress responses, body water regulation and involvement with nuclei associated with the eighth and third cranial nerves. In conclusion, the present study determined the distribution of VT4R in brain and pituitary gland of the chicken and addressed the possible functions of the receptor. A major, proposed function of the VT4R in chickens is to serve in stress related neural pathways. Chickens could serve as an animal model to determine the mechanism the VT4R plays in the stress pathway of birds.