Diencephalic and septal structures containing the avian vasotocin receptor (V1aR) involved in the regulation of food intake in chickens, *Gallus gallus*.

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Abstract

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

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

1. Introduction

Behavioral responses in both mammals and birds are determined by internal and external cues [1,2] and sensory integration is necessary in order to effect appropriate behavioral responses that ensure the survival of individuals. Foraging and food intake are behavioral responses initiated by deficiency of energy and nutrients [3-5]. The basic food intake response is perturbed during stress that has a significant effect on growth. Food intake and stress responses are regulated by neurohormones and/or neuromodulators that are synthesized and released by neurons in the hypothalamus of vertebrates [6,7]. Several neurohormones are involved in the regulation of stress [6,8] and some of them, such as corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and pro-opiomelanocortin, are known for their anorectic effect [7,9,10].

In birds, arginine vasotocin (AVT), homologous to mammalian AVP, is a neurohormone that regulates several physiological and behavioral responses [11-15] and its functions have been extensively reviewed [16,17]. Vasotocin containing neurons are almost exclusively present in the preoptic area and hypothalamus including the supraoptic nucleus (SO), preoptic periventricular nucleus (POP), paraventricular nucleus (PVN), periventricular hypothalamic nucleus (PHN) and with few exceptions including dorsolateral thalamus, nucleus of the stria terminalis, and perirotundic area [18,19]. Both parvocellular and magnocellular AVT-ir neurons are involved in neuroendocrine regulation and upon their activation AVT is released either into the median eminence (from parvocellular AVT neurons) or the neurohypophysis (from

magnocellular neurons). Similar to AVP in mammals, AVT regulates the hypothalamic-pituitary-adrenal (HPA) axis [20-22] and is involved in physical, social and psychological stress [23-26]. Vasotocin released in response to stress has a direct effect on the HPA and augments the effect of stress along with CRH [20,21,27]. Being a stress hormone, AVT also appears to affect food intake. Interestingly, both endogenous and exogenous analogs of AVP and AVT have been reported to suppress food intake in both mammals [28,29] and birds [30,74], respectively. Thus, AVT acts as a stress hormone as well as an anorectic agent. Furthermore, rodent studies have shown that food deprivation for an extended period of time significantly reduced AVP expression in the hypothalamus [31,32]. Hence, AVT may play a short term role in the central regulation of food intake and during a long period of food deprivation AVT is expected to be suppressed.

Although, AVT is released via its axonal terminals in the median eminence or the neurohypophysis, the nonapeptide is also found to be released within the brain [33] to modulate central responses. This concept concurs with the effects induced from central administration of analogs of AVT [15,30,74]. Specific functions of AVT in birds are mediated through its four known receptor types. Depending on localization, each receptor type has distinct functions ranging from osmoregulation to reproduction and social bonding to stress [34-36]. Behavioral and physiological roles of the nonapeptide have been extensively studied through the application of agonists or antagonists to specific receptors or using knockout animal models and related techniques (for review see 37).

Several lines of evidence suggest that in mammals food intake is mediated through V1a receptors [29,38,39]. In birds, a specific function of vasotocin receptors in

92 the regulation of food intake remains controversial. Two lines of evidence support the
93 avian central V1aR involvement in food intake. Specifically, the avian V1aR and not
94 V1bR (previously termed the VT4R and VT2R, respectively; for the change in receptor
95 nomenclature see reference 42) was reported in the chicken brain [40,41] including
96 strong V1aR immunoreactivity in glial cells surrounding circumventricular organs and
97 moderate to weak V1aR immunoreactivity in diencephalic structures such as the SO
98 and ventral PHN [41]. Hence, neurons within these structures containing V1aR
99 immunoreactivity are likely involved in the regulation of food intake. Secondly,
100 intracerebroventricular (ICV) administration of a selective V1aR antagonist (SR-49059)
101 [51,52] not only attenuated stress levels but also increased food intake in chicks [42]. It
102 was based on this assumption that anorectic neurohormones, such as CRH and AVT,
103 associated in the initiation of stress response could be blocked by the antagonist
104 resulting in an increase in food intake. Moreover, ICV administration of a V1aR
105 antagonist also augmented food intake induced by neuropeptide Y (NPY) further
106 supporting the premise that anorectic effects of stress neurohormones could be blocked
107 by the V1aR antagonist to facilitate the additional increase in food intake [42].

108 Therefore, a study was conducted to identify V1aR containing brain structures
109 associated with the regulation of food intake. Thus the central role of V1aR on food
110 intake was examined using ICV administration of SR-49059 followed by FOS
111 immunoreactivity in brain structures. As a positive control, neuropeptide Y (NPY), a
112 known potent orexigenic peptide in vertebrates [43,44], was used in order to compare
113 brain structures associated with the regulation of food intake in birds. Furthermore, in
114 the present study because of the changes observed in FOS-ir cell counts following ICV

administration of a V1aR antagonist, dual immunohistochemistry was also performed to identify the signature of neuronal phenotypes.

2. Materials and Methods

Day-old male Cobb 500 chicks were obtained from a commercial hatchery and raised in brooder cages set at 32° C with a weekly 2.5°C reduction in temperature until 21°C was reached and maintained until the end of the experiment. Birds had access to a standard chick starter feed (22% protein, metabolizable energy was 3100 kcal/kg) and water ad libitum. After two weeks of age, birds with similar body weight were randomly selected for the study. All of the procedures in experiments were approved by the University of Arkansas Institutional Animal Care and Use Committee.

2.1. Intracerebroventricular Injections

At 4 weeks of age, a stainless steel cannula was implanted into the lateral ventricle of each bird as previously described [26,27]. Birds were allowed to recover for at least 4 days and the cannula position in each bird was tested with an ICV dose (80ng) of angiotensin II (Human ANG II, Sigma Aldrich, St. Louis, MO), a potent dipsogen [45]. Birds that displayed binge drinking behavior within 3 min were randomly selected and placed in individual cages for the study. Later, birds were injected with one of the three treatments: 1) control (physiological saline 0.9%), 2) V1aR antagonist SR-49059 (250 ng/bird) or 3) NPY (4 µg/bird). Doses of SR-49059 (Sigma-Aldrich, St. Louis, MO) and NPY (Bachem, Torrance, CA) were determined in a previous study [42]. Sterile physiological saline was used as the diluent for preparing SR-49059 or NPY and were administered ICV in 8 µl volume over a minute period (n=5/treatment). Following

ICV administration, birds were returned to their home cages with feeder removed and their behavior was video recorded for 1 hour using a Sony HD PJ430V camcorder. Thereafter, birds had access to food for 1 hour. Birds were then anesthetized with sodium pentobarbital solution (27 mg/kg, i.v.), perfused via carotid arteries using ice cold 0.1 M phosphate buffer (PB) and ice cold Zamboni fixative. The calvarium of each skull was removed and brains were blocked using a stereotaxic instrument (Kopf, Tujunga, CA). The blocked brains were removed from the skull and placed in the same fixative overnight. Thereafter, brains were transferred to a sucrose solution (30% in 0.1M PB) until they sank. After saturation each sample was removed from the sucrose solution and stored at -80° C until sectioned.

2.2. Behavioral Assessments

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

2.3. Immunocytochemistry (ICC)

Brains were sectioned in the coronal plane at 40µm thickness and sections were stored at -20°C in ethylene glycol based cryoprotective solution [75] until use. Immunocytochemistry for FOS was performed as described [26, 46]. Because of changes observed in FOS-ir cell counts in the SO dual FOS/AVT immunocytochemistry

was also performed. Briefly, sections were rinsed several times in 0.02 M phosphate buffered saline (PBS) and treated with 0.2% hydrogen peroxide solution to block peroxidase activity, followed by treatment with 0.4% TritonX-100 for 20 min. Sections were incubated in 5% normal goat serum for 30 min and then with anti-FOS primary antibody raised in rabbit (sc-253, LOT#C2112, Santa Cruz Biotechnology; 1:3000) for at least 24 hours at 4°C and subsequently incubated in goat anti-rabbit secondary antibody (Jackson Immuno Research, 1:500) for 90 min. Sections were then incubated in avidin-biotin complex-horse radish peroxidase (1:5) for another 90 min. Dark-blue staining of FOS was obtained using the nickel-DAB chromogen method. Sections were rinsed in PBS between incubations. Selected sections were incubated with rabbit anti-AVT (1:10000, a gift from Dr. Gray) for at least 48 hours and the above sequence repeated to immunostain AVT neurons. DAB was used as the chromogen in order to stain arginine vasotocin neurons brown.

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

Due to changes in FOS-ir cell counts observed in the SO and the nucleus of the hippocampal commissure (NHpC) dual immunofluorescent labeling was also performed for AVT/V1aR and CRH/V1aR, respectively. Sections were rinsed in 0.02M PBS and then treated with 0.4% TritonX-100 for 20 min. They were then incubated in a blocking

solution (5% normal donkey serum, 0.3% TritonX-100 in PBS) and immediately transferred to a cocktail solution of primary antibodies containing a rabbit polyclonal antibody to chicken vasotocin receptor (1:2000, Code: VT4-1 as described in [41]) and guinea pig anti-AVP (1:8000, T-5048, Lot A03607, Bachem, Torrance, CA), in 0.02M PBS with 1% normal donkey serum, 0.2% Triton X-100 and 0.05% sodium azide. After incubation for at least 40 hrs at 4°C or overnight at room temperature sections were washed and transferred to a cocktail solution of secondary antibodies containing donkey anti-rabbit IgG conjugated with DyLight 594 (1:400, Pierce-Thermo Scientific, Rockford, IL) and donkey anti-guinea pig IgG conjugated with fluorescein (FITC) (1:400, Jackson Immuno Research, West Grove, PA). In order to visualize CRH-ir cell bodies in the NHpC, colchicine was administered ICV as described elsewhere [26]. An immunocytochemical procedure was performed using a cocktail of guinea pig anti-CRH (1:2000, T-5007, Bachem, Torrance, CA) and chicken vasotocin receptor [41] antibodies. Secondary antibodies used were a cocktail of donkey anti-guniea pig IgG conjugated with Cy3 and donkey anti-rabbit IgG conjugated with FITC (1:500, Jackson Immuno Research, West Grove, PA). After completion of each staining procedure sections were washed in PBS, mounted on glass slides and cover slipped using Vectashield (Vector Laboratories, Burlingame, CA).

2.4. Imaging

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

(Fig.5c) were acquired using a Leica SP8 laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, IL) equipped with the supercontinuum white light laser. To excite FITC and CY3, the laser was tuned to 495 and 550 nm, respectively. Emission bandpass was set to 505-550 nm (FITC) and 560-600 nm (Cy3). Adobe Photoshop CS6 was used to adjust brightness and contrast of digital images.

2.5. Statistical Analysis

One way analysis of variance was used to determine if an overall effect of the treatments was significant and a Tukey HSD post hoc test was used to do a pairwise comparison between treatment groups. For FOS+AVT quantification, a Student's t-test was used to test for significant differences between the saline and V1aR antagonist treatment groups. All data are presented as mean ± SEM and the significance level utilized was $p < 0.05$.

3. Results

3.1. Behavior

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

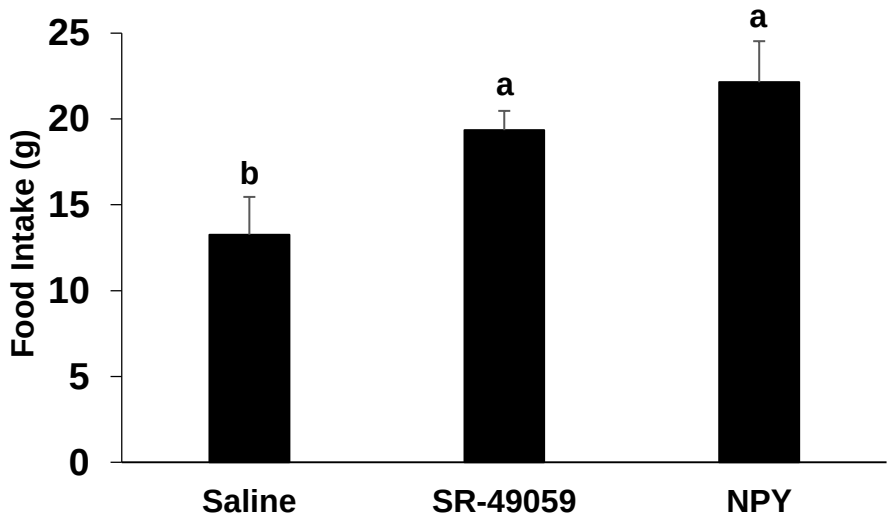


Fig. 1. Central effect of the V1a receptor antagonist (SR-49059), neuropeptide Y (NPY) or saline (control) on food intake. Data are presented as mean±SEM (n=5/group). Significant differences among the groups are shown by different letters.

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

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

p=0.009). In the absence of food, frequency of food search after central injection of SR-49059 was not significantly different compared to controls (p=0.06). In contrast, NPY administered birds showed significantly increased frequency of food searching behavior (Table 1) when compared to controls (p=0.0078).

Table 1.

Time spent drinking, frequency of resting bouts and food search behavior following intracerebroventricular administration of saline, V1a receptor antagonist SR-49059, or neuropeptide Y (NPY).

Group	Drinking Response (sec)		Resting bouts		Food searching behavior	
	Preprandial	**Prandial**	**Preprandial**	**Prandial**	**Preprandial**	**Prandial**
Saline	164±47[b]	130±72[a]	5±2[a]	12±4[a]	5±2[b]	NA
SR-49059	392±27[a]	128±27[a]	8±3[a]	10±2[a]	10±3[b]	NA
NPY	338±63[a,b]	272±88[a]	7±3[a]	13±7[a]	20±4[a]	NA

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

3.2. Quantification of FOS-ir cells

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

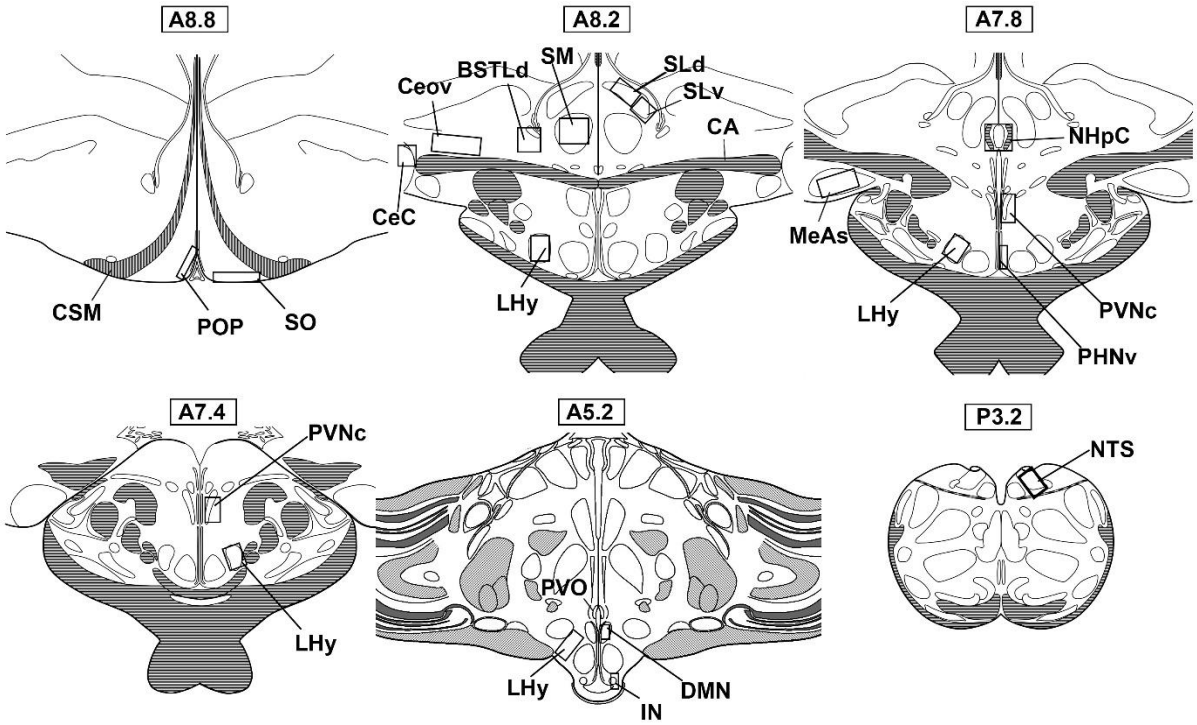


Fig. 2. Modified schematic diagrams from a chick brain atlas [47], representing coronal planes where FOS quantification was performed. Boxed regions in each schematic diagram mark the brain structures quantified following administration of saline, V1a receptor antagonist SR-49059 or NPY. CA – anterior commissure and CSM – corticoseptomesencephalic tract were used as landmarks; BSTLd – dorsolateral bed nucleus of the stria terminalis; CeC - central capsular nucleus; Ceov - oval central amygdalar nucleus; DMN – dorsomedial nucleus; IN – infundibular nucleus; LHy - lateral hypothalamic area; MeAs – subpallial medial amygdalar nucleus (previously known as TnA - nucleus taeniae of the amygdala); NHpC - nucleus of the hippocampal commissure; NTS – nucleus tractus solitarius or nucleus of solitary tract; PHNv - ventral periventricular hypothalamic nucleus; POP – preoptic periventricular nucleus; PVNc – core subnucleus of the paraventricular nucleus; PVO – paraventricular organ; SLd&v – lateral septum (dorsal and ventral); SM – medial septum; SO – supraoptic nucleus.

Table 2.

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

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

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

3.2.1. Supraoptic Nucleus (SO)

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

3.2.2. Avian Central Amygdalar Complex and Medial Amygdala

Among different sub-divisions of the avian central extended amygdalar complex, (CEA) [48], FOS-ir cells with clear boundaries were observed in the dorsal bed nucleus of stria terminalis (BSTLd), central capsular nucleus (CeC) and oval central amygdalar nucleus (Ceov). A significant difference was observed in the BSTLd ($F_{2,11}$ =9.96, p=0.005), but not in the CeC ($F_{2,11}$=0.03, p=0.96) or the Ceov ($F_{2,11}$=1.64, p=0.24). Both SR-49059 and NPY administered birds showed a significant increase in FOS-ir cells in the BSTLd compared to controls (p<0.01 and p<0.05, respectively). No significant difference was observed among the treatments in the subpallial medial amygdala (MeAs) ($F_{2,11}$=1.92, p=0.2).

3.2.3. Septum

Septal regions are often associated with social behavior [34,49] and AVT-ir innervation is especially prominent in the lateral septum of males [19].

3.2.3.1. Nucleus of Hippocampal Commissure (NHpC): The NHpC is located at the midline directly dorsal to the third ventricle, a circumventricular organ and

hypothalamus. Significant differences occurred among experimental groups ($F_{2,11}$=10.88, p=0.004). Specifically, the SR-49059 treated group showed significantly reduced number of FOS-ir cells compared to both controls (p<0.02) and the NPY group (p<0.004).

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

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

3.2.4. Hypothalamus

3.2.4.1. Lateral Hypothalamic area (LHy): Since the LHy extends rostrocaudally for more than 3.5 mm in the chicken brain [50], quantification was conducted at three rostro-caudal levels (Fig. 2). First, at the level of the atlas plate A8.2 covering the anterior commissure, second covering coronal plane A7.8-A7.2 and a third one adjacent to the median eminence best shown in A5.2. At the levels, A8.2 and A5.2, the number of quantified FOS-ir cells were not significantly different among the three groups ($F_{2,11}$=3.85 and $F_{2,11}$=1.73, p>0.05). Nonetheless, at the level A7.8-A7.2, an increase in

the number of FOS-ir cells in the LHy was found for both the SR-49059 and NPY groups compared to controls, $F_{2,11}$=4.37, p=0.04 (Table 2).

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

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

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

3.2.5 Nucleus of Solitary Tract (NTS): A significant difference was observed among the treatment groups ($F_{2,11}$=4.99, p=0.03). An increase in FOS-ir cell numbers in both SR-49059 and NPY treated groups was observed in the NTS, however, only the NPY group showed a significant increase when compared to the saline injected group (p<0.05).

3.3. Dual Colocalization

3.3.1. FOS and AVT immunoreactivity

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

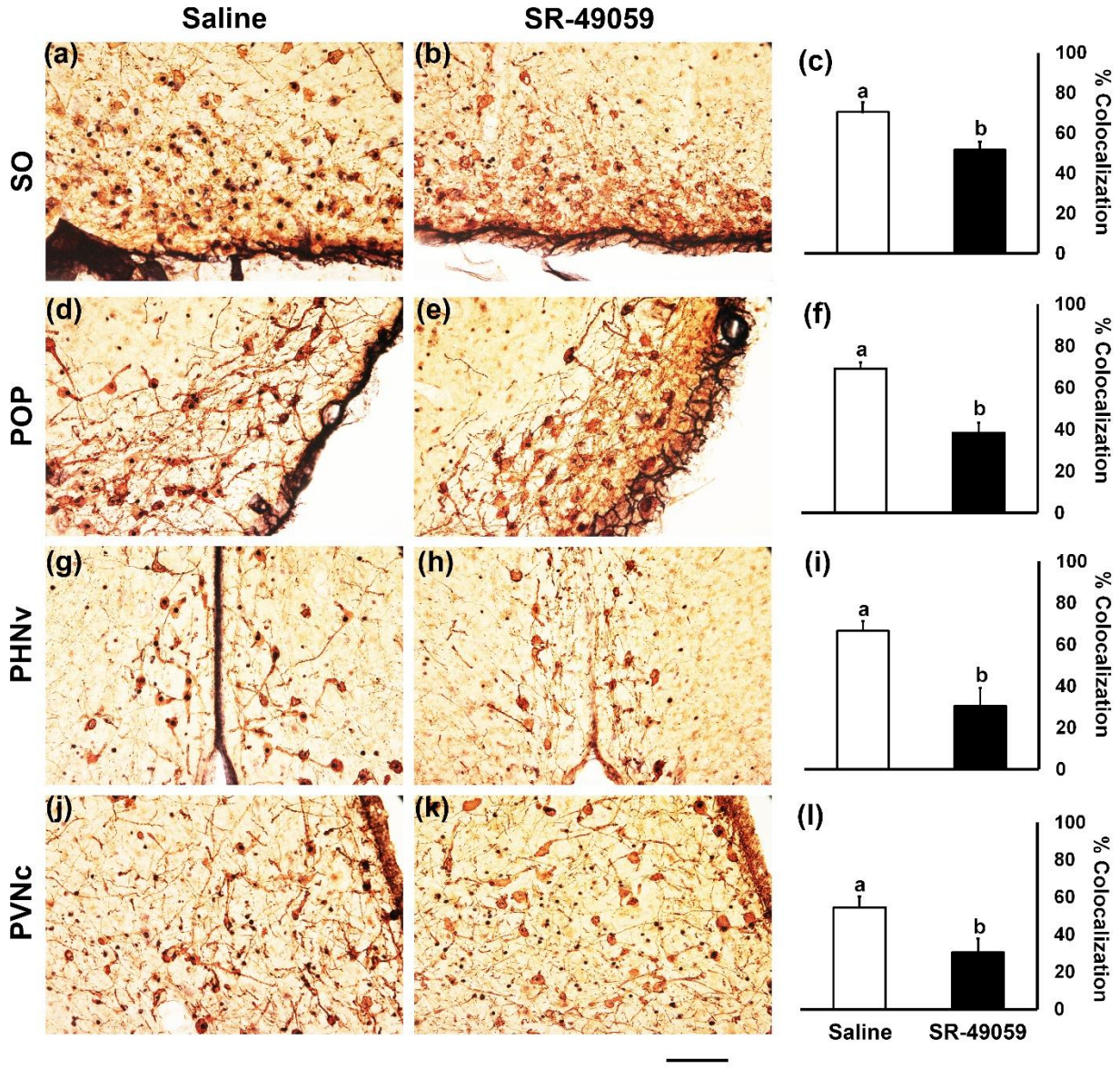
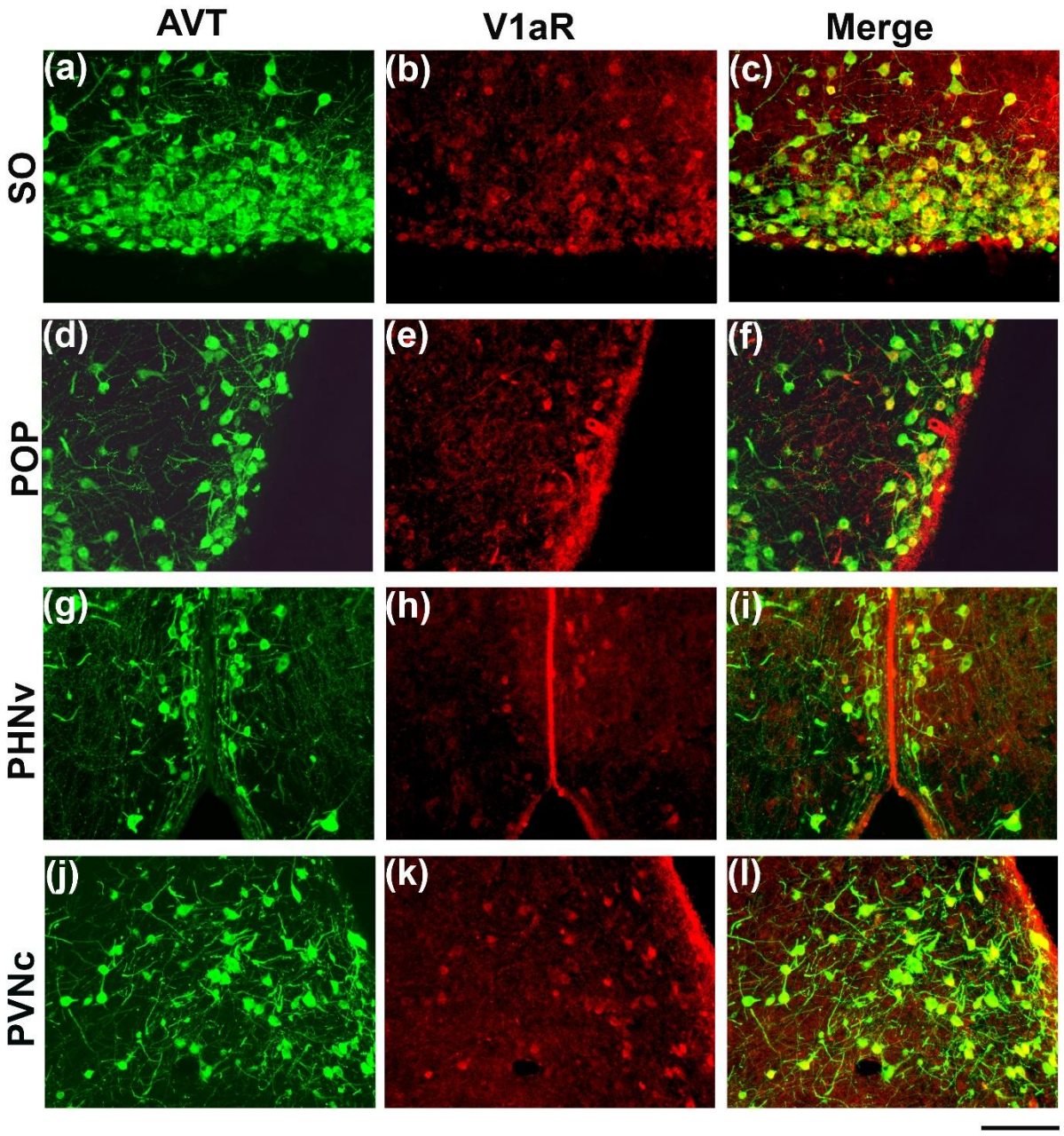


Fig. 3. Dual immunocytochemistry of FOS and arginine vasotocin (AVT) in magnocellular neurons within different hypothalamic structures following central administration of saline (a,d,g and j) and vasotocin V1a receptor antagonist, SR-49059 (b,e,h, and k). Scale bar - 100 µm. Graphs on the right (c,f,i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

3.3.2. V1aR and AVT immunoreactivity

Since the SO, POP, PHNv and the PVNc showed a significant decrease in FOS-ir cell counts following SR-49059 treatment, a dual immunofluorescence assay for AVT and V1aR was conducted to determine the co-localization of the V1aR in AVT neurons in all four structures. Although, V1aR immunoreactivity was not as prominent as AVT immunoreactivity (a threefold difference in the exposure time was used to take digital images of V1aR in Fig. 4 b,e,h and k compared to images of AVT-ir in Fig. 4 a,d,g and j) many AVT-ir magnocellular neurons in the SO, POP, PHNv and the PVNc are colocalized with V1aR (Fig. 4a-l). The POP, PHNv and PVNc also contain V1aR-ir glial cells lining the third ventricle (Fig. 4d-l). Overall, the SO seemed to contain more AVT neurons colocalized with the V1aR than the POP, PHNv and PVNc.



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372 **Fig. 4.** Colocalization of arginine vasotocin (AVT) and its receptor (V1aR) in
373 magnocellular neurons present in the supraoptic nucleus (SO), preoptic periventricular
374 nucleus (POP), ventral periventricular hypothalamic nucleus (PHNv) and paraventricular
375 nucleus core (PVNc). Arginine vasotocin-ir is show in green - stained with FITC (a,d,g
376 and j) and V1aR-ir is shown in red - stained with DyLight 594 (b,e,h and k). The merged
377 images are shown in the panel on the right side (c,f,i and l). Scale bar - 200µm.

3.3.3. V1aR and CRH immunoreactivity

The reduced number of FOS-ir cells in the NHpC following SR-49059 administration (Table 2, Fig. 5a and 5b) prompted us to perform dual immunofluorescent labeling for V1aR and CRH. Interestingly, a large number of CRH-ir cells coexist with V1aR-ir glial cells in the dorsal part of the NHpC (Fig. 5c).

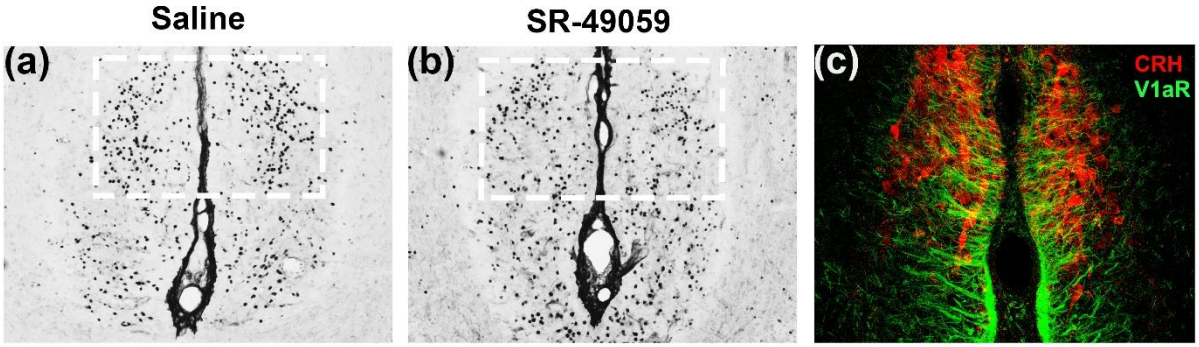

Fig. 5. FOS immunoreactivity in the nucleus of hippocampal commissure (NHpC) following intracerebroventricular administration of saline (a) and vasotocin V1a receptor antagonist SR-49059 (b). The panel on the right (c) shows a confocal image of the dorsal part of the NHpC containing a cluster of corticotropin releasing hormone (CRH) - ir neurons engulfed by processes of V1aR-ir glial cells. The image (c) represents maximum projection of a z-stack of 24 confocal images taken 0.8 µm apart. Scale bar - 200 µm.

4. Discussion

4.1. Behavioral effects

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

V1aR antagonist was observed in the pre-prandial state. However, V1R antagonist administered peripherally did not affect water intake in rodents [29]. The V1aR antagonist effect on water intake observed in the present study may represent the behavioral compensation for appetite induced by the V1aR antagonist in the absence of food. This assumption is further substantiated by the drinking behavior in V1aR antagonist-treated birds that did not increase in the presence of food (prandial drinking). Furthermore, a conspicuous increase in food intake was observed following the V1aR antagonist treatment. As expected, the NPY dose used in this study induced foraging and food intake as reported in a previous avian study [44]. Since, the amount of time spent drinking water in NPY treated birds was increased, the current study also supported the dipsogenic effect of NPY previously documented in rodents [43]. Although, the increase in food intake in antagonist treated birds was not as prominent as after NPY administration, the food intake response was significantly higher than controls.

4.2. Food Intake and FOS immunoreactivity

Neuroanatomical results from the present study suggest a role of the vasotocin receptor V1aR in food intake. Specifically, the increase in food intake following central administration of a selective receptor antagonist SR-49059 fostered the use of FOS immunoreactivity to determine changes in the number of FOS-ir cells and identify brain structures in the septum, diencephalon and caudal brainstem. The increased food intake response following ICV administration of the V1aR antagonist resulted in significant activation of several brain structures, such as the PVNc, LHy, DMN, BSTL, SM and NTS, similar to results from central administration of NPY. In mammals, FOS

studies have shown that food intake also activates several similar brain structures including the PVN, LHy, DMN, central amygdalar nucleus, SO and NTS [54-56]. Increased FOS cell counts in several brain structures presented in this study could have resulted from the effects of compounds administered and food consumed, perhaps involving a complex neural circuitry associated with the treatments and possibly from satiety signals [73,76]. Nonetheless, neuroanatomical studies suggest that the site of action of neurohormones depends on receptor localization. Although an increase in FOS-ir cell number was observed in the PVN, DMN, LHy, BSTLdl, MS, and NTS of the V1aR antagonist treated birds, the receptor localization study did not identify V1aRs in these brain structures in chickens [41], with one exception involving magnocellular neurons of the PVN. In contrast, two brain structures, the NHpC and SO, containing V1aRs showed reduced FOS cell counts following the V1aR antagonist treatment, revealing the antagonist effect in reducing the activity of the two structures. It is possible that both the SO and NHpC could be involved in satiety signals from the food consumed leading to higher FOS-ir cell counts in saline and NPY treated birds [54-56].

The role of the PVNc, LHy and DMN in regulation of food intake in mammals is particularly well documented. Classical techniques such as tract tracing methods in mammals have shown that the PVNc, LHy, DMN and BSTL have direct projections to the NTS and dorsal motor nucleus of the vagus and are involved in the regulation of food intake, particularly by modulating the autonomic nervous system [64-66]. In birds it has been shown that the PVNc, LHy and BSTL also project to the NTS and dorsal motor nucleus of the vagus [67-70]. Based on the hodological information from the PVNc, LHy, BSTL and NTS, it has been proposed that food intake in mammals and

445 birds is controlled by similar brain structures [9]. It is important to note that reciprocal
446 connections between brain structures where FOS was quantified in this study, including
447 BSTL [48,64,69], SM [71,72], DMN [65], PVN [68], LHy [48] and NTS [73,76], could also
448 play a substantial role in the regulation of food intake.

449 *4.3. Attenuation of neuronal activity by V1aR antagonist*

450 Four avian brain structures, the SO, POP, PHNv, and PVNc containing AVT
451 neurons appear to be involved in the regulation of food intake. The SO, in particular,
452 showed low numbers of FOS-ir cells in birds treated with the V1aR antagonist
453 compared to the saline control and NPY treated birds (Table 2) and resulted in
454 subsequent dual immunocytochemical studies. The V1aR antagonist-treated birds
455 showed decreased FOS-ir expression specifically in AVT-ir magnocellular neurons in
456 the SO, POP, PHNv and PVNc. Similar to rodent studies [53], it was also found that
457 AVT-ir magnocellular neurons, but not parvocellular neurons, in these structures contain
458 V1aR immunoreactivity. Coincidentally, no difference was found in AVT-ir parvocellular
459 neurons in the PVNc following V1aR antagonist treatment (see supplementary
460 material). Since magnocellular neurons in the mammalian SO and PVN were reported
461 to have a role in satiety signaling and metabolic challenges [54-57], blocking V1aR
462 present in AVT-ir magnocellular neurons by the V1aR antagonist could have
463 suppressed the effects induced by AVT [51,52,58], thereby reducing the satiety signal
464 [30,74].

465 The second interesting finding from this study is the reduced number of FOS-ir cells
466 in the NHpC of the V1aR antagonist-treated birds. Several lines of evidence show that
467 this structure is associated with physical, psychological and social stress responses in

avian species [23,26,49]. It was recently hypothesized that the NHpC in the avian brain is associated with the neuroendocrine regulation of stress due to the presence of CRH neurons [26]. The significant reduction in FOS-ir cells following V1aR antagonist treatment compared to controls may represent decreased activity of CRH neurons. Importantly, the present study shows that CRH-ir neurons in the NHpC are in close contact with glial cells that contain the V1aR (Fig. 5c) and administration of the V1aR antagonist could have attenuated CRH neuronal activity via V1aR-ir glial cells. Currently, it has been widely accepted that glial cells modulate activity of hypothalamic neurons [59-62]. However, the plausible cross talk between V1aR-ir glia and CRH neurons remains to be determined. Nonetheless, blocking V1a receptors in glia by the antagonist treatment appear to suppress the activity of CRH neurons in the NHpC.

5. Conclusion

Behavioral and neuroanatomical results from the present study suggest a role of V1aRs in appetite control. Particularly, based upon receptor localization V1aRs could regulate food intake by modulating neuronal activity of AVT-ir magnocellular neurons in the preoptic and hypothalamic structures and CRH-ir neurons in the NHpC. Attenuation of the anorectic effects by AVT [30,74] and CRH [63] release could be responsible for the increased food intake shown by V1aR antagonist treated birds. These interactions are summarized in Fig. 6.

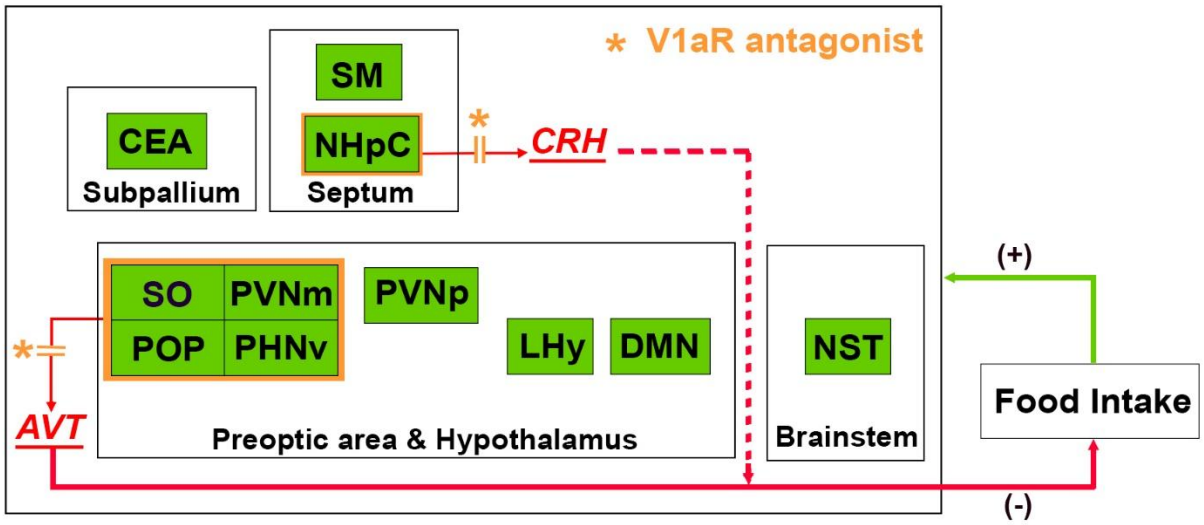

Fig 6. Schematic representation of a possible model of brain structures associated with food intake. Food intake activates several structures (shown in green) in the hypothalamus, septum, subpallium and brainstem representing feedback signals (solid green line). Activation of neurons following food intake could result in the release of anorectic peptides such as CRH and AVT from specific brain structures (NHpC, SO, POP, PHN and PVN) providing an inhibitory signal affecting further consumption of food (solid and dotted red lines). Central administration of a V1a receptor antagonist blocks the V1aR present in the SO, POP, PHN and PVNm (shown in amber) resulting in less anorectic (AVT) peptide release. Indirect evidence suggests that CRH release from the NHpC could be likewise attenuated following administration of the V1aR antagonist (dotted red line). Thus, the V1aR antagonist treated birds showed increase in food intake perhaps by inhibiting anorectic effects of AVT and CRH in the brain. (+) stimulatory effects and (-) inhibitory effects. Subscripts: v - ventral, p - parvocellular, m - magnocellular. Complete structure names are listed in Fig 2.

Acknowledgments

The authors would like to thank Megan Hamilton, Aman Alphonse, S. Emily Jacobson, and Donna Sue Delozier for providing technical assistance while conducting experiments. The authors would also like to thank Dr. David Gray for providing anti-AVT antibody and Dr. Sami Dridi for providing the secondary antibody (donkey anti-rabbit

conjugated with DyLight 594). This research was supported by NSF grant (IOS - 0842937) and an Arkansas Biosciences Institute grant.

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