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Research paper

Central effect of vasotocin 4 receptor (VT4R/V1aR) antagonists on the stress response and food intake in chicks given neuropeptide Y (NPY)

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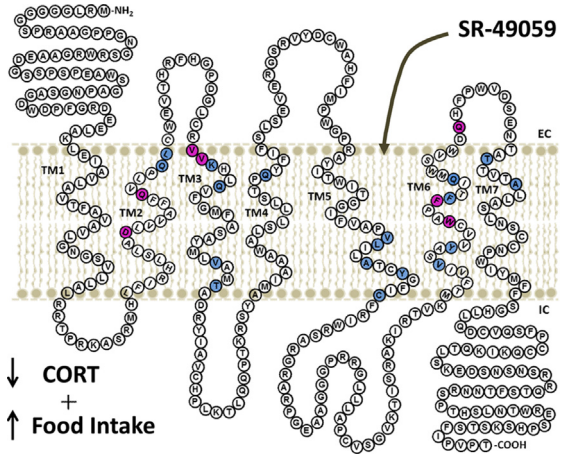
HIGHLIGHTS

- SR-49059 is a centrally effective antagonist for the avian V1aR.
- SR-49059 significantly reduces corticosterone following stress.
- The avian V1aR plays a role in the regulation of food intake in birds.

GRAPHICAL ABSTRACT



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ABSTRACT

Previous studies identified SR-49059 as a most effective antagonist of the avian vasotocin 4 receptor (VT4R) compared to other candidate blockers including the Manning compound using *in silico* 3 dimensional (3D) modeling/docking analysis of the chicken VT4R and an *in vitro* anterior pituitary cell culture study. The present experiments were designed to validate whether SR-49059 and the Manning compound would likewise be effective *in vivo* in blocking the VT4R when applied intracerebroventricularly (ICV) to chicks. Two treatments were tested, a stressor (immobilization) and administration of neuropeptide Y (NPY), a potent orexigenic compound. In the first experiment, birds were given the Manning compound, SR-49059 or physiological saline ICV followed by immobilization stress. Blood samples were taken and corticosterone (CORT) was determined by radioimmunoassay. It was hypothesized that both antagonists would reduce the stress response. A second experiment examined the role of the VT4R in food intake regulation. The Manning compound, SR-49059 or physiological saline was administered prior to NPY and food intake was monitored for 1 h. It was hypothesized that each of the two antagonists coupled with NPY would augment food intake above the intake resulting from saline plus NPY administration. Related to the second experiment was a third that examined the difference between the effect of central

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administration of NPY versus SR-49059 in releasing CORT. Results of the first study showed that the Manning compound or SR-49059 prior to stress decreased CORT levels compared to controls while the second experiment showed that SR-49059 or the Manning compound plus NPY, enhanced food intake above that of the experimental group given saline and NPY. The last study showed that NPY increased plasma CORT above birds given SR-49059 centrally or saline administered controls. Taken together, results suggest that the avian VT4R is involved in the central neuroendocrine stress response as well as functions in appetite regulation by mediating an anorexigenic effect similar to what has been reported in mammals for the V1aR. In conclusion, similar to the past *in silico* and *in vitro* tests, the current *in vivo* experiments showed SR-49059 to be a most efficacious avian vasotocin receptor antagonist. Therefore based upon results of functional tests utilizing a highly specific mammalian antagonist, SR-49059, to the mammalian V1aR that likewise was most effective in blocking the avian VT4R and past reported high sequence homology between the mammalian V1aR and the VT4R, it is recommended that the chicken VT4R be renamed the avian V1aR to facilitate better communication among scientists involved in comparative studies.

1. Introduction

The neuroendocrine regulation of stress in birds, similar to mammals comprises two types of neurons, corticotropin releasing hormone (CRH) and arginine vasotocin (AVT), the latter regarded as homologous to the mammalian neuropeptide, vasopressin. Four G-protein coupled receptors, vasotocin 1–4 (VT1-4R), have been identified in birds for the ligand AVT [1]. Two of the four receptors have been shown located on corticotropes in the anterior pituitary [2–5] and have shown significant changes in gene expression following a psychogenic stressor, immobilization [4–6]. Of the two receptors (VT2R and VT4R), the VT4R has been shown located not only in the anterior pituitary [5] but also in the brain of chickens [7] as well as the brains of the white-throated sparrow and zebra finch [8]. Effective antagonists of the VT4R have been identified, specifically the Manning compound, H-5350 [9,10] and SR-49059 [11]. The most efficacious blocker of the VT4R in chickens was shown to be SR-49059, based upon its binding value utilizing the development of a 3D model of the receptor as well as an *in vitro* blocking assay using a primary avian pituitary cell culture method [11]. We next planned to test the effectiveness of the identified antagonists at the organismal level to attenuate CORT release following stress as well as determine whether the avian VT4R functions as the mammalian V1aR and plays a role in the neuroendocrine regulation of food intake. Specifically, neuropeptide Y (NPY), when administered centrally, is a most potent orexigenic peptide in mammals [12,13] as well as in birds [14–16]. Of interest, mammalian data showed a highly significant release of vasopressin that occurred following administration of NPY within the paraventricular [17] or supraoptic [18] nucleus. It is known that vasopressin in mammals [19–21] and its homologue, AVT in birds significantly reduces food intake [22]. A recent study using vasopressin receptor-deficient ($V1aR^{-/-}$) mice showed enhanced food intake over and above the amount consumed by wildtype mice given the same NPY dose ICV [23]. The conclusion of the authors was that in mammals the AVP/V1aR acts to inhibit the regulation of food intake particularly with respect to the NPY-induced orexigenic action in the rodent central nervous system [23]. We therefore wished to test the hypothesis that in birds, the VT4R functions like the mammalian V1aR in suppressing the stimulatory effect of NPY on food intake in birds.

2. Materials and methods

2.1. Facilities and animals

Male broiler chicks, Cobb 500 were obtained from a commercial hatchery on day of hatch. Chicks were raised in battery brooder cages for their first two weeks and thereafter were randomly distributed to cages (two per cage). Environmental temperature was set at 32 °C from the day of hatching and was dropped 3 °C per week to reach approximately 22–23 °C, where it was maintained until the end of experiments. Birds were fed a standard broiler chick starter diet *ad libitum*. The diet contained 22% crude protein and its metabolizable energy was 3100 kcal/kg. The first three days chicks were exposed to continuous light in order to find food and water. Thereafter, birds were maintained on a daily photoperiod of 16 h of light and 8 h of darkness. All of the procedures and experimental protocols for use in chickens were approved by the University of Arkansas Institutional Animal Care and Use Committee.

2.2. Surgical procedures

At three weeks of age, birds were deeply anesthetized with sodium pentobarbital solution (27 mg/kg, *i.v.*) administered via the brachial vein. Their heads were carefully positioned in a stereotaxic instrument, their beak was closed and secured. The skin covering the top of the skull was severed at midline and parted to expose the calvarium. A dental drill was then used to drill a single hole through the skull and a guide cannula (Plastics One, Roanoke, VA) at the following coordinates (1.0 mm anterior to the lambda suture mark on the skull and 0.8 mm lateral to midline (bird's right) was lowered into the brain and maintained in place using dental cement. The target was the dorsal region of the lateral ventricle in order to perform intracerebroventricular (ICV) injections. The depth of the guide cannula was 3.0 mm from the dorsal reading of the skull. Birds were given a minimum two-day post-surgical recovery period before testing for correct cannula position using the angiotensin II drinking response [24–26]. Birds that drank water, evidenced by continuous pecking at the nipple drinker, within two minutes after injection of angiotensin II confirmed an accurate placement of the cannula and were used in the study.

2.3. Dose and ICV treatments

Antagonists used in this study were H-5350, Manning compound (Bachem, Torrance, CA) and SR-49059 (Sigma-Aldrich, St. Louis, MO). The compounds and doses were selected based upon results of a previous *in vitro* cell culture study [11] and a preliminary ICV injection study. The dose used for both the antagonist compounds was 250 ng (in 0.9% saline).

To test the hypothesis that the stimulatory effect of NPY on food intake is suppressed by V1aR, chicks were administered SR-49059 coupled with NPY (4 μg). Control birds in all of the experiments were given 0.9% physiological saline. All compounds were administered in 4 μl volume using 10 μl Hamilton syringes given over a 1 min period.

2.4. Immobilization stress

Chicks underwent immobilization stress for 30 min or 1 h following ICV injections, which was regarded as an acute stress. Immobilization stress included wrapping the birds in a harness that prevented wing movement and standing. Birds had full access to water during that time period. Control birds were picked up, taken out of their cage and immediately placed back in their home cage.

2.5. Experimental treatment groups

Effects of vasotocin receptor antagonist compounds on immobilization stress (30 min) were tested in the first experiment using the following 4 treatment groups: (1) saline control, (2) saline + stress, (3) Manning compound + stress, and (4) SR-49059 + stress. All birds had cannulae positioned in the lateral ventricle of the brain. Birds were either administered ICV 4.0 μl of physiological saline, 250 ng/4 μl Manning compound, or 250 ng/4 μl SR-49059. The stressed groups were subjected to immobilization stress (see Section 2.4) while the unstressed controls were each taken out of their individual cages and replaced immediately. A blood sample was taken from the brachial vein after the stress period for later determination of plasma corticosterone (n = 8/treatment (trt) group). Experiment 2 comprised 3 treatment groups: (1) saline, (2) the Manning compound, and (3) SR-49059. Each bird was administered either saline, Manning compound or SR-49059 at the same doses as Expt. #1. One hour later food intake was measured (n = 8/trt). Experiment 3 was a dose-response study to establish a sub-maximal food intake response to ICV administered NPY with either physiological saline or NPY at 1, 3, or 7 μg/4 μl; n = 6/trt). Experiment 4 had the same treatment groups as Expt #1 except that in place of a stress, birds were either administered saline ICV or were co-administered with sequential injections of saline + NPY, Manning + NPY, or SR–49059 + NPY. The dose of NPY was 4 μg/4 μl while doses of blockers were the same as Expt #1. Experiment 5 was a 2 × 3factorial experiment: with birds either unstressed or immobilized for 60 min and administered ICV either saline, SR-49059 or NPY. Doses were the same as Expt #1 and blood samples were taken after 60 min for later determination of plasma corticosterone, (n = 6/trt).

2.6. Radioimmunoassay

Plasma samples from experiment 1 (antagonist + the immobilization stress) and experiment 5 (antagonist + NPY) were quantified for corticosterone (CORT) by radioimmunoassay (RIA) [27]. All samples were assayed in duplicate. The primary antibody for CORT was purchased from Fitzgerald Inc. (Concord, MA, USA). The secondary antibody and I^{125} tracer were purchased from MP Biomedicals Inc. (Orangeburg, NY, USA).

2.7. Statistical analysis

One-way ANOVA was used to determine a level of significance among treatment groups for experiments 1–4. The least significant difference (LSD) test was used to determine differences among the means. The fifth experiment was a 2 × 3 factorial study with stress or unstressed groups given either saline, SR49059 or NPY. When significant differences were obtained, the LSD procedure was used to separate means. Data presented are shown as mean ± SEM with a significance level of $p < 0.05$ used throughout all studies.

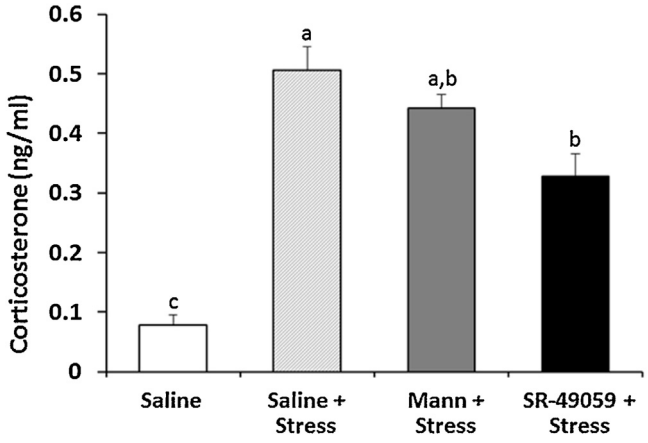

Fig. 1. Plasma corticosterone (CORT) response to immobilization stress following central pre-administration of physiological saline (4 μl), the Manning compound or SR-49059 (250 ng/4 μl). Blood was sampled after 30 min of immobilization stress. Plasma CORT concentration was determined by radioimmunoassay, error bars = 1 SEM; n = 8 birds/group. Different letters above each histogram show means significantly different ($p < 0.05$).

3. Results

3.1. Central effect of avian V1aR antagonists on plasma CORT

Two V1aR antagonists were tested to determine their effectiveness in suppressing the release of corticosterone (CORT) in the acute, stress model we use routinely in our laboratory [4]. Results showed that the psychogenic stressor significantly increased CORT levels compared to unstressed chicks given physiological saline ICV, while both the Manning compound (H-5350) and SR-49059 administered prior to imposing the stress test, reduced plasma CORT. However, SR-49059 was more effective and significantly reduced the release of the stress hormone below the stressed control group given saline ICV (Fig. 1).

3.2. Central effect of avian V1aR antagonists on food intake

To determine the effect of the two antagonists on food intake, a second group of cannulated birds were either injected ICV with either physiological saline, the Manning compound or SR-49059 and food intake was measured during the subsequent 1 h period. Results showed that both the Manning compound and SR-49059 significantly increased food intake compared to saline-treated controls (Fig. 2A). It is well known that NPY is a potent orexigenic compound in mammals [12,13] and birds [14–16]. An objective was to determine whether blocking the VT4R would result in augmented food intake greater than the expected orexigenic effect of NPY administered alone. Therefore a third experiment was first performed to determine an effective dose of NPY that would significantly increase food intake, however, we wished not to maximize food intake in order to determine whether or not co-administration of an antagonist to the VT4R plus NPY would augment food intake above the effect of NPY alone. A dose-response experiment was completed and a dose of 3 μg NPY was sufficient to increase food intake significantly more than saline injected controls (Fig. 2B). We elected to utilize a 4 μg NPY dose in a subsequent experiment to insure that food intake would always exceed the intake of the saline administered controls.

A fourth experiment utilized cannulated birds where treatment groups were co-administered NPY and saline, NPY and the Manning compound, or NPY and SR-49059 ICV. An additional group comprised saline injected controls. Food intake was significantly elevated in the NPY + saline treatment compared to saline injected

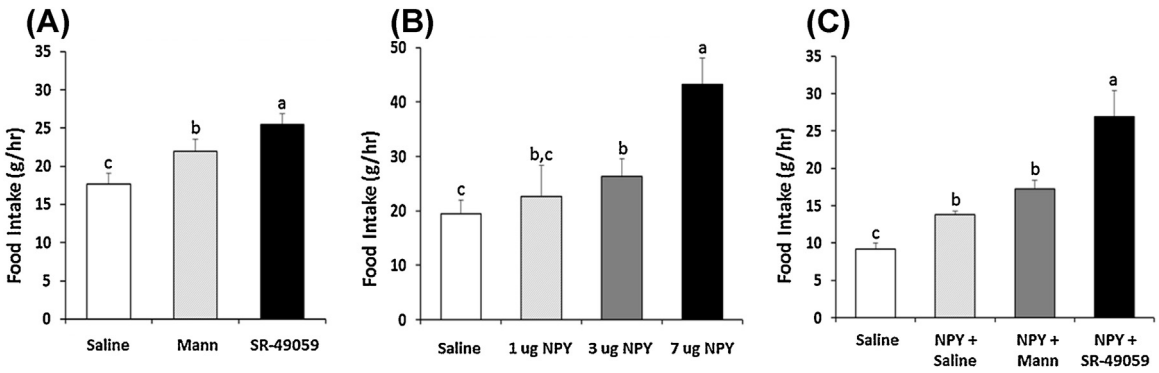

Fig. 2. A. One hour food intake (g/hnr) response following saline (4 μl), Manning compound (250 ng/4 μl), or SR-49059 (250 ng/4 μl) injected intracerebroventricularly (ICV). Significant differences are denoted by different letters ($p < 0.05$). Histograms show means, error bars indicate 1 SEM, n = 8 birds/group). B. Dose-response experiment to determine a neuropeptide Y (NPY) dose that would consistently and significantly increase food intake above saline administered controls, n = 6 birds/group. C. Co-administration of NPY (4 μg/4 μl) and either, saline (4 μl), Manning compound or SR-49059 (250 ng/4 μl) ICV and their effects on 1 h food intake. Significant differences among means denoted by different letters ($p < 0.05$, histograms show means, error bars indicate 1 SEM, n = 8 birds/group).

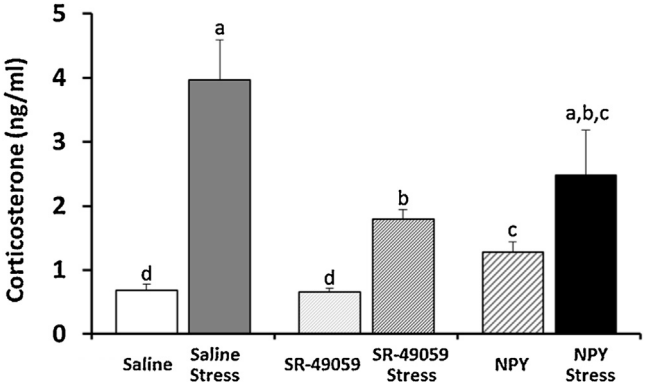

Fig. 3. Plasma corticosterone (CORT) levels in male chicks following ICV administration of saline (4 μl), SR-49059 (250 ng/4 μl) or NPY (4 μg/4 μl). A second set of three groups of chicks were administered ICV one of the same 3 treatments as previously described followed by immobilization stress for 1 h. CORT concentrations (ng/ml) were measured by radioimmunoassay. Significant differences among means are denoted by different letters ($p < 0.05$, n = 8 birds/group).

controls. Co-administration of the Manning compound and NPY increased food intake above the NPY + saline group, however, it was not significantly different. The NPY plus SR-49059 group nearly doubled their intake above the NPY treatment group (Fig. 2C).

3.3. Difference between the effect of NPY and V1aR antagonist on the stress response

To determine whether NPY administration elicited CORT release and if SR-49059 was effective in reducing the CORT response to immobilization stress plus NPY given centrally, three sets of cannulated birds were prepared and administered saline, SR-49059 or NPY. Within each set, one group was subjected to immobilization while its paired group was not stressed. The blocker SR-49059 was effective in significantly reducing CORT release in immobilized birds compared to stressed birds pretreated with saline (Fig. 3). The unstressed NPY treated group was shown to have CORT levels significantly higher than the unstressed SR-49059 treated group as well as the unstressed saline controls (Fig. 3). The group subjected to both ICV NPY administration as well as immobilization had lower levels of CORT compared to birds given saline followed by immobilization, however, the NPY/stress group was not different from either the saline + stress nor the stressed group given SR-49059.

4. Discussion

Data obtained in broiler chicks, *Gallus gallus*, four to five weeks of age, have shown that administration of antagonists of the mammalian vasopressin V1aR have likewise been effective in blocking the avian VT4R that has been proposed to be equivalent to the mammalian V1aR based upon its sequence homology [28], Genbank ACCN ABV24997) and key amino acid residues at specific sites that confer specificity to vasopressin-like residues [8,29]. Indeed Jayanthi et al. (2014) showed that molecular models of both the Manning compound and SR-49059 were effective in displaying effective binding values in an analysis utilizing a 3D computer-based model built for the avian VT4R as well as an *in vitro* assay comprising chicken pituitary cells where the same two receptor blockers inhibited the expression of POMC hnRNA. Of interest was that SR-49059 was found most effective in the *in silico* test as well as in the cell culture study. Similarly, the *in vivo* study suggests that SR-49059 is more effective in reducing the stress response following immobilization (Fig. 1).

With respect to food intake, ICV administration of either the Manning or SR-49059 blocker resulted in significantly increased food intake compared to the saline control group. Importantly co-administration of either NPY + the Manning compound or NPY + SR-49059 resulted in enhanced food intake compared to the NPY treatment group alone. Notably, the combination of NPY + SR-49059 resulted in a synergistic increase in food intake.

The study of Aoyagi et al. [23] utilizing V1aR-deficient mice ($V1aR^{-/-}$) showed that giving NPY ICV resulted in a significant increase in food intake compared to wild-type mice. Their conclusion was that the arginine vasopressin V1aR signal acts as an inhibitory system to suppress the NPY orexigenic action via the central nervous system and that blockade of the V1aR lead to increased food intake due to enhanced NPY sensitivity [23]. Our data utilizing an effective antagonist for the mammalian V1aR, SR-49059 [30] has shown it to be efficacious for blocking the chicken VT4R *in vitro* [11] and *in vivo* results of the current study. Due to the significant decrease in plasma CORT when SR-49059 was administered prior to immobilization compared to stressed birds given saline ICV, and the increased CORT plasma levels in birds given NPY compared to saline or SR-49059 administered control groups suggest that SR-49059 effectively blocked the VT4R and attenuated its activation due to inhibition of the binding of neuropeptides released by the stress procedure immobilization or central injection of the peptide NPY. Since the major neuropeptides associated with the stress response in birds and mammals are CRH and AVT/AVP and past studies in rodents showed that administration of NPY into

hypothalamic nuclei resulted in significant elevations of vasopressin [17,18], suggest strongly that AVT is released following stress or central administration of NPY in avian species. Notably, AVT given centrally significantly reduces food intake in birds [22,31].

Due to previous genomic data showing sequence similarity between the mammalian V1aR and avian VT4R [28], the role of the VT4R in the stress response of birds [1,4,6], the presence of the V1aR/VT4R in the anterior pituitary of mammals [32] and birds [5] and both the V1aR and VT4R present in the mammalian [33] and avian brain [7,8] include sufficient data to recommend that the nomenclature preciously utilized for the avian VT4R be changed to the avian V1aR in order to facilitate better communication among comparative biologists/neuroscientists.

In conclusion, data suggest that the avian V1aR in the brain not only functions in the avian neuroendocrine axis in regulating the stress response but may also function to regulate feeding, particularly in partially inhibiting the orexigenic response initiated by NPY. Since the mammalian V1aR has been shown to be involved in the regulation of food intake [23] and the avian V1aR is playing a comparable role in food regulation, a candidate, vertebrate V1aR may likewise function similarly among other classes of vertebrates. The procedure of utilizing ICV administration of available antagonists developed for the mammalian V1aR coupled with administration of NPY may be an effective means of screening candidate blockers, *in vivo*, for determining a specific antagonist for a proposed homologous, yet uncharacterized V1a-like receptor, in other vertebrate species.

Acknowledgments

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