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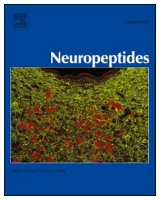

Expression and localization of the neuropeptide Y-Y4 receptor in the chick spleen: mRNA upregulation by high ambient temperature

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ABSTRACT

High ambient temperatures (HT) can increase diencephalic neuropeptide Y (NPY) expression, and central injection of NPY attenuates heat stress responses while inducing an antioxidative state in the chick spleen. However, there is a lack of knowledge about NPY receptor expression, and its regulation by HT, in the chick spleen. In the current study, male chicks were used to measure the expression of NPY receptors in the spleen and other immune organs under acute (30 vs. 40 ± 1°C for 3 h) or chronic (30 vs. 40 ± 1°C for 3 h/day for 3 days) exposure to HT and in response to central injection of NPY (47 pmol, 188 pmol, or 1 nmol). We found that NPY-Y4 receptor mRNA was expressed in the spleen, but not in other immune organs studied. Immunofluorescence staining revealed that NPY-Y4 receptors were localized in the splenic pulp. Furthermore, NPY-Y4 receptor mRNA increased in the chick spleen under both acute and chronic exposure to HT. Central NPY at two dose levels (47 and 188 pmol) and a higher dose (1 nmol) did not increase splenic NPY-Y4 receptor mRNA expression or splenic epinephrine under HT (35 ± 1°C), and significantly increased 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations under HT (40 ± 1°C). In conclusion, increased expression of NPY-Y4 receptor mRNA in the spleen under HT suggest that Y4 receptor may play physiological roles in response to HT in male chicks.

1. Introduction

In chickens, high ambient temperatures (HT) can cause increased brain neuropeptide Y (NPY) mRNA expression (Ito et al., 2015; Tu et al., 2016), and central NPY injection attenuates heat stress responses (Bahry et al., 2017; Eltahan et al., 2017). Additionally, central injection of NPY is associated with reduced expression of heat-shock protein-70 and elevated glutathione synthase mRNA in the spleen, but not in the liver, in chicks, suggesting that central NPY may attenuate the splenic heat stress response (Nishimura et al., 2022). NPY and norepinephrine (NE) contribute to vasoconstriction (Wahlestedt et al., 1990) and NPY reduces renal blood flow via a classical NPY-Y1 receptor (Bischoff et al., 1997). NPY immunoreactive fibers were detected in the cat spleen (Lundberg et al., 1985), and high affinity-binding sites for NPY with receptor characteristics were identified in both blood vessels and the capsule of the pig spleen (Lundberg et al., 1988). Moreover, the splenic NPY-Y1 receptor, which is one of the NPY receptors (NPY-Y1, -Y2, -Y4, -Y5, -Y6, and -Y7), functions in immune regulation in rats and mice (Yu et al., 2022). The contribution of the avian spleen to the immune system as a whole may be more important than it is in mammals because of the

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Table 1
Primers used for real-time PCR.

Gene	Accession no.	Sequences 5′ – 3′ (forward/reverse)	Annealing temperature (°C)	Product size (bp)
NPY-Y1	NM_001031535.1	5′ – TAGCCATGTCCACCATGCA–3′/5′ – GGGCTTGCCTGCTTTAGAGA–3′	60	58
NPY-Y2	NM_001031128.1	5′ – TGCCTACACCCGCATATGG–3′/5′ – GTTCCCTGCCCCAGGACTA–3′	60	58
NPY-Y4	NM_001031555.1	5’ – CCTGCCCTTTCTGACCACAT–3′/5’ – GGGATGCAGTATTGCAGAAGC–3’	58	164
NPY-Y5	NM_001031130.1	5′ – TGATCGGTGGATGTTTGGCA–3′/5′ – AGCCAACGGCCCAAATGATA–3′	60	191
NPY-Y6	NM_001044687.1	5’ – GAACGAGAGCAGGTTGAGTGA–3′/5’ – ACGAGGTGGCACAGTGTAAA–3’	60	173
NPY-Y7	NM_001037824.1	5′ – TGGCCATCTTCAGAGAGTTCC–3/5′ – GGACTGACGTGGTTTTTCAGC–3′	64	208
HSP-70	AY143691.1	5′– GGGAGGACTTTGACAACCGA–3′/5′ – CAAAGCGTGCACGAGTGATG–3′	60	219
GAPDH	NM_204305.2	5’ – CCTCTCTGGCAAAGTCCAAG–3′/5’ – CATCTGCCCATTTGATGTT–3’	58	200

Primers were designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) for neuropeptide Y receptors (*NPY-Y1, -Y2, -Y4, -Y5, -Y6, -Y7*), heat-shock protein-70 *(HSP-70)* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

poorly developed lymphatic vessels and nodes in birds (Kaiser and Balic, 2015). Therefore, it could be speculated that central NPY may regulate splenic functions via NPY receptors in chickens. The three NPY peptides are highly homologous and share an overall sequence identity of 50% between pancreatic polypeptide (PP), peptide YY (PYY) and NPY (Larhammar et al., 1992; Blomqvist and Herzog, 1997; Berglund et al., 2001; Schüß et al., 2024). However, there are no reports of NPY peptides receptor expression in the chicken spleen. Thus, the first aim of the current study was to examine the mRNA expression of NPY receptors in the spleen and other immune organs, and determine the localization of expressed receptor proteins, in the chick spleen.

Exposure of young chicks to HT increases rectal temperature and plasma glucose (GLU) concentrations and reduces food intake and plasma triacylglycerol (TG) concentrations (Chowdhury et al., 2012a, 2012b; Ito et al., 2015). HT also stimulates the production of heat-shock proteins (HSPs) in the spleen and liver in acute heat-exposed chicks (Nishimura et al., 2022). Among the various HSPs, HSPs-70 and -90 are known to be related to the development of thermotolerance in many cell types (Li and Mak, 1989). Monoamines also contribute to various physiological functions, including thermoregulation (Elhussiny et al., 2022), food intake (Ikemoto, 2010), and stress response (Zhang et al., 2003; Zhang et al., 2004). Central injection of NPY can enhance brain dopamine (DA) in heat-exposed chicks (Bahry et al., 2017). However, there is no information on whether central NPY can regulate splenic monoamine concentrations under HT. Moreover, it is also unknown, whether splenic NPY-Y4 receptor expression can be regulated by central NPY. The second aim of the present study was thus to measure the effects of acute and chronic exposure to HT on rectal temperature, food intake, plasma metabolites, and splenic NPY-Y4 receptor mRNA expression. Additionally, we analyzed splenic NPY-Y4 receptor and HSP-70 mRNA and monoamine concentrations following the central injection of NPY.

2. Materials and methods

2.1. Animals

Fertilized eggs (Julia strain; *Gallus gallus domesticus*) were purchased from a local hatchery (Tsuboi Hatchery, Kumamoto, Japan) and placed in an incubator (Rcom Maru Deluxe MAX 380, Autoelex Co., Ltd., Korea) at 37.6 °C with approximately 60% relative humidity. Eggs were auto-turned every hour. On day 19 eggs were transferred to hatching chambers. After hatching, day-old chicks were housed in groups (25 birds/cage) in metal cages (50 × 35 × 33 cm) under control thermoneutral temperature (CT: 30 ± 1°C) and continuous lighting. Feed (Adjust diet; Toyohashi Feed Co., Ltd., Aichi, Japan; metabolizable energy: >12.55 MJ/kg, protein > 23%) and water were provided *ad libitum*. Male chicks were selected by feather identification at 2 days post-hatch and were used for the current study. Male chicks were used in this study because we always used male chicks in our previous studies related to heat challenge and NPY treatment (Ito et al., 2015; Bahry et al., 2017; Eltahan et al., 2017). At 3 days of age, 2 chicks were housed in individual plastic cages (15 × 28 × 13 cm) for acclimatization for 24 h. Body weights were distributed uniformly among treatment groups. This study was conducted following the regulations for animal experiments of Kyushu University and the Japanese law, specifically the Act on Welfare and Management of Animals (Act No. 39 of June 19, 2019). All protocols and procedures were approved by the Institutional Animal Experiment Committees of Kyushu University (Approval No. A22–346–1 and A22–120–1).

2.2. Isolation of total RNA and electrophoresis

Male chicks (6 days old) were used for the collection of diencephalon, liver, spleen, bursa of Fabricius and thymus to examine all NPY-receptor mRNA expressions using RT-PCR. Total RNA was extracted from the diencephalon, liver, spleen, bursa of Fabricius and thymus using RNAiso Plus (TakaRa Bio Inc., Shiga, Japan), according to the manufacturers instructions. cDNA was synthesized using 1 μg of total RNA and PrimeScript™ RT reagent kits with gDNA Eraser (Takara, Shiga, Japan), according to the manufacturers instructions. All primers (Table 1) were tested using routine PCR and gel electrophoresis (TaKaRa PCR Thermal Cycler Dices, Takara, Shiga, Japan). cDNA from the diencephalon, liver, spleen, bursa of Fabricius and thymus tissues was used for routine PCR to confirm the expression of NPY receptors (NPY-Y1, -Y2, -Y4, -Y5, -Y6 and -Y7). RT-PCR was performed for 30 cycles.

2.3. Immunofluorescence staining for NPY-Y4 receptor and hematoxylin and eosin (HE) staining

Spleen samples were collected from chicks under CT (6 days old) and fixed with Bouin's solution and embedded in paraffin. Midsagittal sections of the spleen (5 μm thick) were prepared using a microtome and mounted on MAS-coated glass slides (Matsunami Glass Ind. Ltd. Osaka, Japan). Deparaffinized sections were rinsed with phosphate-buffered saline (PBS, pH 7.4) and treated with 1% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 20 min. After blockage of nonspecific binding components with 1% normal goat serum and 1% BSA in PBS (pH 7.4) containing 0.3% Triton X-100, the sections were immersed overnight at 4 °C in a 1:1000 dilution of the primary antibody raised against chicken NPY-Y4 receptor antigen [C-STMQTEVSKGSLS (C stands for Cys which was introduced in the N-terminal sequence of NPY-Y4 receptor peptide)], which was a custom made rabbit-polyclonal chicken NPY-Y4 receptor antibody (Cosmo Bio Co., Ltd., Japan). The cross activity of the chicken NPY-Y4 receptor antibody was confirmed by an ELISA with a serial dilution of NPY-Y4 receptor antigen (C-STMQTEVSKGSLS) by Cosmo Bio Co., Ltd., Japan (*data not shown*). Results confirmed that the antibody showed a specificity for binding with the chicken NPY-Y4 receptor. The primary immunoreaction was followed by a 120 min incubation with Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific Inc. Waltham, MA, USA) at a dilution of 1:500. Immunofluorescence staining for NPY-Y4 receptor (green) was examined with a fluorescence microscope (NIKON Eclipse Ts2-FL

microscope equipped with the NIKON DS-Fi3-L4 (Nikon Solutions Co., Ltd., Tokyo, Japan)). The control staining, in which the primary antibody was replaced with normal rabbit IgG, did not show any specific reactions.

For the HE staining, midsagittal sections (5 μm thick) of spleen samples were de-paraffinized using xylene and rehydrated through a descending series of ethanol. The HE staining was performed conventionally, and the spleen sections were examined under a NIKON Eclipse Ts2-FL microscope equipped with the NIKON DS-Fi3-L4 (Nikon Solutions Co., Ltd., Tokyo, Japan).

2.4. Drug preparation and intracerebroventricular (i.c.v.) injection

NPY (Porcine, Peptide Institute, Osaka, Japan), was dissolved in a vehicle of 0.85% saline containing 0.1% Evans Blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan). We used porcine NPY because Lundell (2002) reported that this peptide showed similar affinity to chicken NPY-Y1, -Y4 and -Y5 receptors. The control treatment consisted of 0.1% Evans Blue saline solution only, as in previous studies (Tachibana et al., 2006, 2007). Treatments were kept on ice during the experimental period. I.c.v. injections were performed following the method of Davis et al. (1979). Briefly, the head of the chick was inserted in an acrylic device which positioned a hole in a plate overlying the skull immediately over the left lateral ventricle. A micro syringe was then inserted into the left lateral ventricle through the hole and the treatments were injected. The syringe was kept in place for 10 s to prevent overflow. Chicks were not anaesthetized as the i.c.v. injection of 0.85% saline, which was used for control injections, did not affect feeding behaviour (Furuse et al., 1999) or plasma corticosterone concentrations (Saito et al., 2005) compared with non-injected chicks in previous studies. Chicks in the current study appeared unaffected by injection. At the end of the experiment, after euthanasia, the brain was opended and the presence of Evans Blue dye in the lateral ventricle was confirmed. Results from chicks which did not have Evans Blue dye in the lateral ventricle were excluded from the analysis.

2.5. Experimental design

In Experiment 1, ad libitum fed chicks (5 days old; $n = 10$ per group) were kept in their cages (15 × 28 × 13 cm), and cages were placed in a temperature-controlled chamber (Panasonic Electric Co. Ltd. Japan; Catalog number: Panasonic MIR-254) and maintained at either CT (30 ± 1 °C) or an acute heat challenge (HT; 40 ± 1 °C) for 3 h. For a chronic heat challenge, chicks were subjected to treatment temperatures applied for 3 consecutive days for 3 h each day, at the same time each day. The HT was chosen based on our previous study (Ito et al., 2015). For the acute heat challenge, rectal temperatures were measured immediately before subjecting chicks to CT or HT (0 min), then 60 and 180 min later. For the chronic heat challenge, rectal temperatures were measured daily, immediately before and after subjecting the chicks to CT or HT chambers. Rectal temperature was measured using a digital thermometer with an accuracy of ±0.1 °C (Thermalert TH-5, Physitemp Instruments Inc., USA). The thermistor probe was inserted into the colon (rectum) through the cloaca to a depth of 2 cm as reported previously (Ito et al., 2015; Chowdhury et al., 2015; Eltahan et al., 2017; Bahry et al., 2017; Nishimura et al., 2022). At the end of 3 h exposure to HT for both acute and chronic heat challenge, all chicks were immediately euthanized for culling by exposure to isoflurane (Mylan Inc., Tokyo, Japan). Blood was immediately collected through the jugular vein into heparinized tubes and centrifuged at 10,000 ×g for 4 min at 4 °C, then plasma was stored at −80 °C. Chick spleens were collected and snap-frozen in liquid nitrogen and stored at −80 °C for analysis of NPY-Y4 receptor expression by real-time quantitative PCR. Plasma was used for the analysis of metabolites.

In Experiment 2, ad libitum-fed 5 days old chicks were individually separated into plastic boxes (15 × 28 × 13 cm) to acclimatize for 24 h before the start of the experiment. On the day of the experiment, chicks (6 days old; $n = 12$ per group) were intracerebroventricularly injected with 10 μL of either 0, 47, and 188 pmol NPY. Two lower doses (47 and 188 pmol) were chosen because Tachibana et al. (2006) showed that both 188 and 375 pmol NPY reduced plasma GLU. After i.c.v. injection of saline or low dose NPY (47 and 188 pmol), chicks were returned to their cages (15 × 28 × 13 cm) and placed in a temperature-controlled chamber (Panasonic Electric Co. Ltd. Japan; Catalog number: Panasonic MIR-254) under fasting conditions and maintained under HT (35 ± 1 °C) for a 60 min heat challenge. Since the low doses (47 and 188 pmol) were used in the Experiment 2, we chose moderate HT (35 ± 1 °C) and a relatively short time of exposure (60 min) for this experiment based on our previous report where 375 pmol NPY was injected in chicks under 35 ± 1 °C for 60 min (Eltahan et al., 2017). At the end of the experiments, chicks were euthanized and spleen samples were collected, snap-frozen immediately and stored at −80 °C for measurement of NPY-Y4 receptor and HSP-70 mRNA expressions.

In Experiment 3, feeding and acclimatization of chicks were similar as describe above in Experiment 2. On the day of the experiment, chicks (6 days old; n = 12 per group) were intracerebroventricularly injected with 10 μL of either 0 or 1 nmol NPY. The dose was chosen based on our previous studies where 375 pmol reduced body temperature under CT but not under HT (Bahry et al., 2017; Nishimura et al., 2022). Therefore, we considered that a higher dose (1 nmol) may be more effective under HT. Chicks were i.c.v. injected saline or the high dose (1 nmol of NPY) and placed in the above-mentioned chambers at CT (30 ± 1 °C) or HT (40 ± 1 °C) for 120 min. Since 1 nmol NPY was a relatively high dose, we measured its effect under a high HT (40 ± 1 °C) for a relatively long time of exposure (120 min). At the end of the experiments, birds were euthanized and spleen samples were collected, snap frozen immediately and stored at −80 °C for measurement of NPY-Y4 receptor and HSP-70 mRNA expressions and monoamine concentrations in the 0 and 1 nmol NPY groups.

2.6. Real-time quantitative PCR of NPY-Y4 receptor and HSP-70

Total RNA was extracted from the spleen and used to produce cDNA as aformentioned (see section 2.2). To quantify the expression of NPY-Y4 receptor and HSP-70 in the spleen, real-time quantitative PCR was performed using Stratagene MX 3000P (Agilent Technologies, Tokyo, Japan) with a denaturation step at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 5 s, and a primer-specific temperature for 30 s. The primer sequences are listed in Table 1. Relative mRNA expression was calculated using the delta-delta CT method, with GAPDH used as the reference gene (Schmittgen and Livak, 2008). A melting curve analysis was performed after PCR to confirm the amplicon specificity.

2.7. Analysis of monoamine concentrations in the spleen

The concentrations of monoamines including DA, epinephrine (E), NE, 3-methoxy-4-hydroxyphenylglycol (MHPG), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were analyzed in the spleen using a high-performance liquid chromatography electrochemical detector Stand-Alone System (HTEC-510; Eicom Co. Ltd., Kyoto, Japan) as previously described (Elhussiny et al., 2022). The spleen samples were homogenized in 5 volumes of 0.2 M perchloric acid solution containing 0.01 mM ethylenediaminetetraacetic acid disodium salt and incubated on ice for 30 min for deproteinization. The homogenates were centrifuged at 20,000 ×g for 15 min at 4 °C and then the supernatants were filtered through 0.2 μm hydrophilic polytetrafluoroethylene filters to remove tissue debris (Millipore, Bedford, MA, USA). The external standard solution consisted of 100 pg/μL of each monoamine, serially diluted with 0.01 N HCl. The mobile phase (consisting of 0.1 M sodium acetate, 0.1 M citric acid, 100 mg/mL sodium 1-octane sulfonate, and 5 mg/mL disodium ethylenediaminetetraacetic acid and methanol) was perfused at a flow rate of 0.5 mL/min. 30 μL of the standard solutions

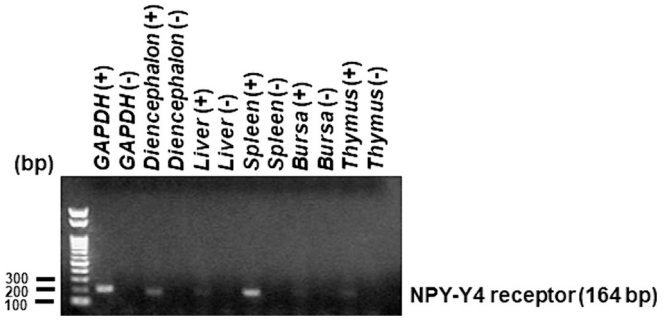

Fig. 1. RT-PCR analysis of chicken NPY-Y4 receptor mRNA expression in the diencephalon, liver, spleen, bursa of Fabricius and thymus.
cDNA corresponding to 1 μg total RNA extracted from each tissue was used for a PCR reaction, and the PCR product was applied on a lane. Lanes labeled different tissues (−) were left without the template as negative controls. The extreme left lane shows the ladder followed by the RT-PCR for GAPDH as an internal control, and diencephalon (+) as a positive control. RT-PCR experiments were repeated five times using independently extracted RNA samples from different chicks and produced similar results.

and brain filtrate were injected using an autosampler. The splenic monoamines were separated using a 150 mm × 3 mm octadecylsilane column (Eicompak SC-5ODS; Eicom, Kyoto, Japan) at 25 °C and detected at an applied potential of +0.75 V versus an Ag/AgCl reference analytical electrode. Electric current (nA) changes were recorded on a computer using an interface system (Power Chromver 2.3.2.J: AD Instruments, Tokyo, Japan). The splenic monoamine concentrations were calculated using the area under each peak and expressed as pg/mg wet tissue.

2.8. Analysis of plasma metabolites

GLU, TG, uric acid (UA), and total cholesterol (TCHO) were analyzed in plasma samples using a Dri-Chem 7000 system (Fuji Medical System Co., Ltd., Japan; Kimura et al., 1991).

2.9. Statistical analysis

Rectal temperature, food intake, plasma metabolites, and monoamine data were analyzed by two-way ANOVA followed by Tukey-Kramer tests only when interactions were significant. NPY-Y4 receptor and HSP-70 expressions were analyzed by *t*-tests when only two groups

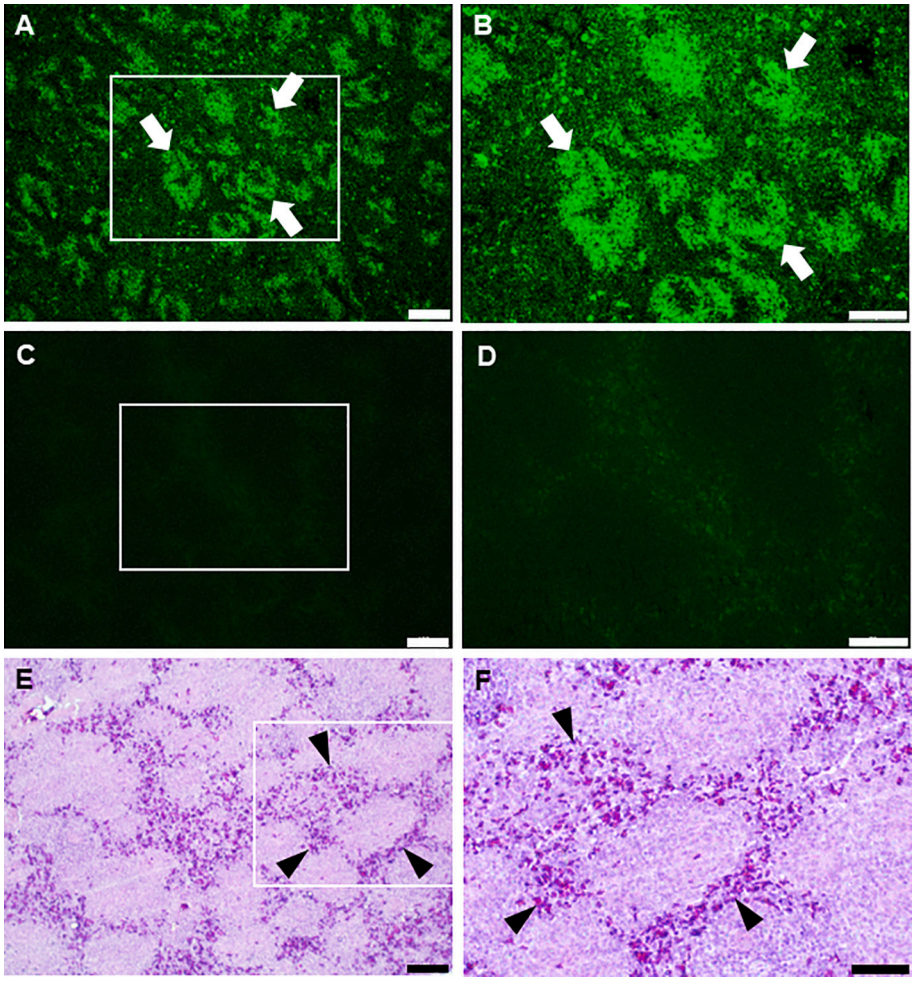

Fig. 2. Cellular localization of NPY-Y4 receptor protein by immunofluorescence staining in the chick spleen.
Arrows indicate NPY-Y4 receptor staining (green) in the spleen pulp-like structures of a mid-sagittal section (A). A higher magnification of the highlighted area in A clearly shows NPY-Y4 immunoreactive tissues in splenic pulps (B). Immunoreactions were not observed in sections without the NPY-Y4 receptor antibody (C, D). The highlighted area in C is shown with a higher magnification in D. Arrowheads in hematoxylin and eosin-stained sections (E and F) indicate red blood cells in the chick spleen. The highlighted area in E is shown with a higher magnification in F. Spleen samples (n = 5) were collected from chicks (6 days old) under a control thermoneutral temperature. Scale bars represent 100 μm (A, C and E) and 70 μm (B, D and F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

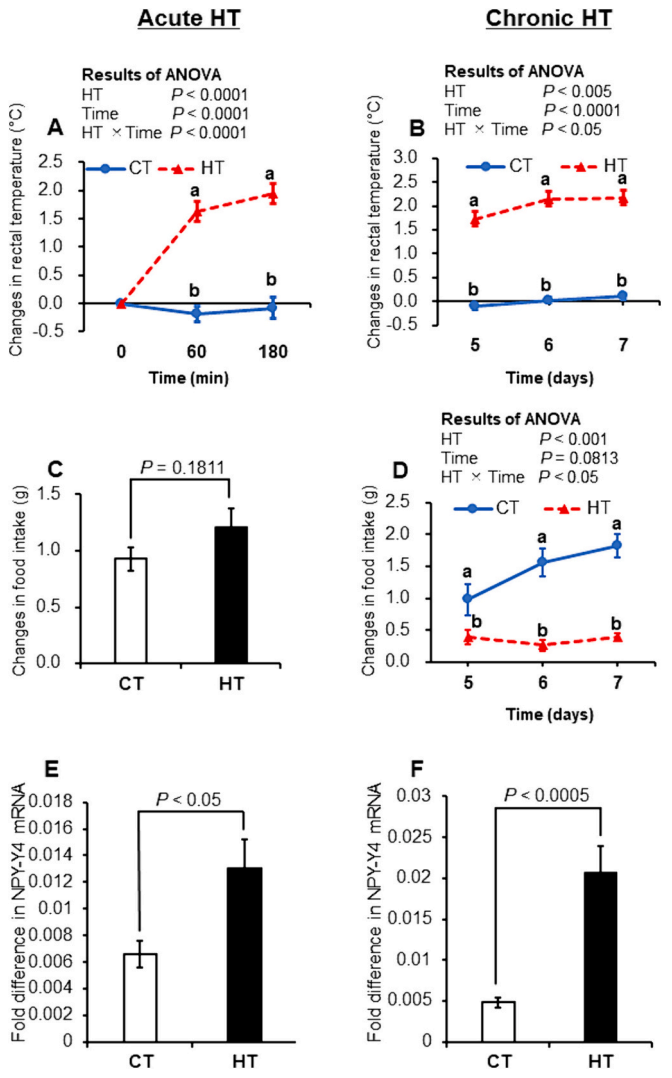

Fig. 3. Changes in rectal temperature (A, B), food intake (C, D), and splenic NPY-Y4 receptor mRNA (E, F) in chicks exposed to a control thermoneutral temperature (CT; 30 ± 1 °C) or a high ambient temperature (HT; 40 ± 1 °C for 3 h for acute and 40 ± 1 °C for 3 h/d for 3 d for chronic exposure).
Values are mean ± SEM of the 9 chicks in each group. Different lowercase letters in some panels (A, B and D) indicate significant differences between groups at $P < 0.05$.

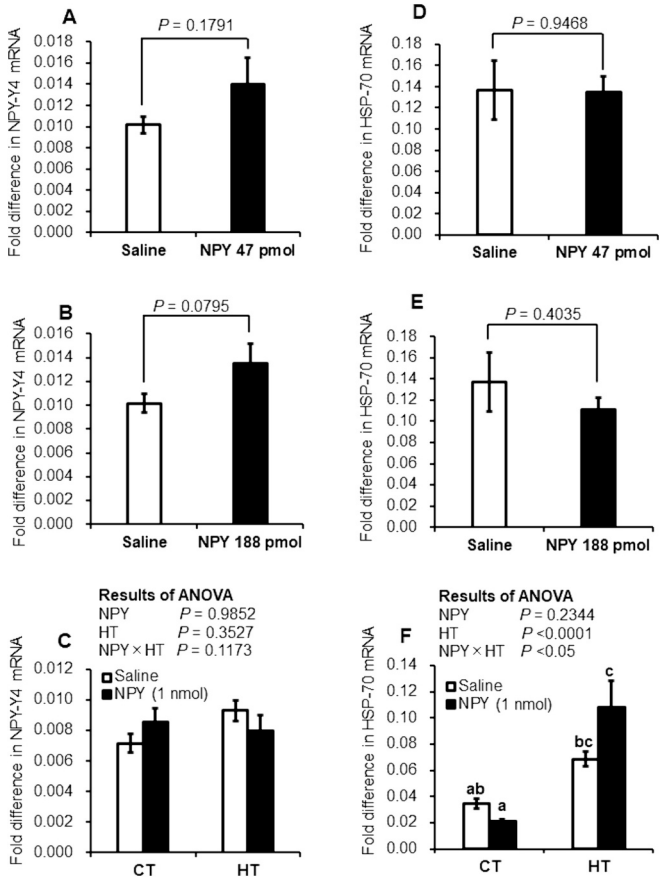

Fig. 4. Changes in NPY-Y4 receptor (A, B) and HSP-70 (D, E) mRNA in the spleen when chicks were exposed to high ambient temperature (HT: 35 ± 1 °C) for 60 min.
Changes in NPY-Y4 receptor (C) and HSP-70 (F) mRNA expression in the spleen when chicks were exposed to either a control thermoneutral temperature (CT: 30 ± 1 °C) or HT (40 ± 1 °C) for 120 min. The number of chicks used in each group was 10. Values are means ± SEM. Different lowercase letters in a panel (F) indicate significant differences between groups at $P < 0.05$.

were compared, or using two-way ANOVA with respect to temperature and NPY. Values were presented as means ± SEM. Statistical analyses were performed using Stat View (version 5, SAS Institute, Cary, USA, 1998). All data were subjected to a Thompson's rejection test, as described by Kobayashi and Pillai (2013), to eliminate outliers ($P < 0.01$), and the remaining data were used for analysis.

3. Results

3.1. Expression of NPY-Y4 receptor mRNA in the spleen

All NPY receptors (NPY-Y1, -Y2, -Y4, -Y5, -Y6 and -Y7) mRNA expression were found in the diencephalon (*data showed only for Y4 in* Fig. 1); however, only NPY-Y4, but not other NPY receptors, was expressed in the spleen while no other NPY receptors were found in other immune tissues studied (Fig. 1). The expression of mRNA encoding for chicken NPY-Y4 receptor fragments with a size of 164 bp product, between nucleotides 674 and 510, was detected by RT-PCR from the diencephalon, and spleen (Fig. 1). NPY-Y4 receptor expression bands were found for the spleen and diencephalon, with no expression in the liver, bursa of Fabricius or thymus. Chicken GAPDH was used as an internal control, with expression of mRNA encoding chicken GAPDH in the diencephalon (200 bp).

3.2. Immunofluorescence staining for NPY-Y4 receptor in the spleen

As shown in Fig. 2 (A, B), NPY-Y4 receptor immunoreactivity was found in the splenic pulp-like structures. The negative control without an NPY-Y4 receptor primary antibody did not have such immunostaining (Fig. 2C, D), although very weak nonspecific immunoflurosense was found in the negative control, which seemed to be an autofluorescence of red blood cells that distributed in the circumference of splenic pulps (splenic sinusoid) as indicated in in the HE staining (Fig. 2E, F). Identification of NPY-Y4 receptor protein in the splenic pulp will enable to clarify the co-localized cell(s) with Y4 receptor in a future study.

3.3. Changes in rectal temperature, food intake and splenic NPY-Y4 receptor mRNA expression under acute and chronic HT

Rectal temperature and food intake results are shown in Fig. 3 A – D. Rectal temperatures increased after both acute ($P < 0.0001$) and chronic ($P < 0.005$) exposure to HT (Fig. 3A, B). There were significant interactions between HT and time ($P < 0.0001$ in acute exposure; $P < 0.005$ in chronic exposure) for rectal temperatures, indicating that rectal

Table 2
Effects of intracerebroventricular injection of NPY (1 nmol) on monoamines concentrations in the spleen of chicks.

Monoamines	Saline		NPY		*P* value		
	CT	HT	CT	HT	NPY	HT	NPY × HT
E	77 ± 7	62 ± 4	59 ± 4	82 ± 9	NS	NS	$P = 0.05$
NE	1050 ± 53	1050 ± 80	1110 ± 66	1040 ± 77	NS	NS	NS
MHPG	356 ± 7[a]	419 ± 13[b]	327 ± 8[a]	441 ± 11[b]	NS	$P < 0.0001$	$P < 0.05$
MHPG/NE	0.34 ± 0.02	0.42 ± 0.04	0.31 ± 0.03	0.45 ± 0.04	NS	$P < 0.005$	NS
5-HT	2400 ± 400	860 ± 200	2700 ± 511	690 ± 130	NS	$P < 0.0001$	NS
5-HIAA	740 ± 88	356 ± 23	550 ± 50	360 ± 50	NS	$P < 0.0001$	NS
5-HIAA/5-HT	0.38 ± 0.07	0.64 ± 0.01	0.30 ± 0.07	1.00 ± 0.43	NS	$P = 0.0598$	NS

The number of chicks used in each group was 7–10. Values are means ± SEM in pmol/mg wet tissue. CT, control thermoneutral temperature (30 ± 1 °C); HT, high ambient temperature (40 ± 1 °C); NPY, neuropeptide Y; E, epinephrine; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; Different superscript letters indicate significant differences at $P < 0.05$; NS, not significant.

temperatures increased during acute than chronic exposure to HT. Food intake was significantly ($P < 0.001$) decreased by chronic, but not acute, exposure to HT (Fig. 3C, D). There was a significant ($P < 0.05$) interaction between HT and time for food intake under chronic exposure to HT. Chicken NPY-Y4 receptor mRNA expression was increased in the spleen during acute ($P < 0.05$) and chronic ($P < 0.0005$) HT challenge (Fig. 3E, F). Current results on increased NPY-Y4 receptor mRNA further suggest to examine the expression of brain NPY and its family peptides under acute and chronic HT challenge to understand the causal relationships between NPY family peptides and splenic Y4 receptor.

3.4. Changes in splenic expression of NPY-Y4 receptor, HSP-70 and monoamine concentrations by central NPY injection

Chicken NPY-Y4 receptor mRNA expression was not affected by either dose of NPY (47, 188 pmol and 1 nmol) during CT or HT (Fig. 4 A–C). The HT caused a significant increase ($P < 0.0001$) in HSP-70 mRNA expression in NPY-treated birds (Fig. 4F). NPY and HT also had a significant interaction ($P < 0.05$), which is consistent with central NPY injection leading to an increase in splenic HSP-70 mRNA under HT compared to CT.

Splenic monoamine concentration changes are shown in Table 2. There were no significant effects of central NPY or HT on splenic NE. Splenic MHPG ($P < 0.0001$) concentrations and MHPG/NE ratios ($P < 0.005$) were increased under HT. Central NPY and HT had a significant interaction ($P < 0.05$), indicating that central NPY increases splenic MHPG concentration under HT but decreases it under CT. Splenic 5-HT ($P < 0.0001$) and 5-HIAA concentrations were decreased ($P < 0.0001$) under HT, but were not affected by central NPY (Table 2). Future research will reveal how central NPY regulates catecholamine metabolism in the chick spleen.

3.5. Plasma metabolites

Exposure to HT significantly increased plasma GLU ($P < 0.0001$) and TCHO ($P < 0.001$) concentrations, and decreased plasma TG ($P < 0.005$) and UA ($P < 0.05$) concentrations. Chronic exposure caused a significant ($P < 0.005$) decrease in plasma TG and TCHO concentrations (Table 3).

4. Discussion

In this study, NPY receptor expression was characterized to determine if NPY may act on the spleen and other immune tissues. Interestingly, mRNA for the NPY-Y4 receptor was detected in the chick spleen, but not in other immune tissues. In addition, we detected NPY-Y4 receptor mRNA in the diencephalon, which includes the hypothalamus. It has been reported that brainstem NPY-Y4 receptor was upregulated by refeeding in 48 h food-deprived rats (Yahya et al., 2006), and diencephalic NPY-Y4 receptor may be involved with feeding regulation in chickens (Ando et al., 2001). However, functions of NPY-Y4 receptor in the spleen in chickens remain unknown, although it was reported that NPY-Y5, -Y6, and -Y7 receptors mRNA expression was increased in the diencephalon by central injection of NPY, and NPY-Y5 receptor was partially involved with thermoregulation in chicks (Eltahan et al., 2017). In the current study, NPY-Y4 receptor mRNA expression was increased in the spleen by HT, which suggests that the NPY-Y4 receptor may have a role in responding to heat challenges in the spleen.

Gao et al. (2017) reported that NPY-Y4 receptor expressed in the spleen of adult chickens with a very low expression of NPY-Y1 and Y-6 receptors. Moreover, He et al. (2016) reported that NPY-Y2 receptor also expressed in the spleen of Lohmann layer chickens. However, NPY-Y1, -Y2 and -Y6 receptors expressions were not detected in the spleen or other immune organs in the current study. Therefore, it could be thought that the splenic NPY receptor expressions may be related with the age since we have used neonatal chicks in the present study.

It is also important to know whether the corresponding NPY-Y4 receptor protein is expressed in the chick spleen. Therefore, we conducted immunofluorescence staining for the NPY-Y4 receptor using a custom-made chicken NPY-Y4 receptor antibody. NPY-Y4 receptor immunolocalization was observed in pulp-like tissue in the chick spleen (Fig. 2 A, B). The NPY-Y4 receptor-immunopositive tissue shape was somewhat circular with a hollow center resembling spleen pulp, and was probably red pulp as described by Kaiser and Balic (2015). The distribution of nonspecific immunoreactivity in the negative control (Fig. 2C, D) was similar to the distribution of red blood cells observed in HE-stained sections (Fig. 2E, F), so NPY-Y4 receptor may be localized in splenic red pulp rather than blood cells. It was reported that shapes resembling circles with a hole in the center, similar to a pulp, were immune positive with chicken $CD57^{+}$ in the spleen (Mast and Goddeeris, 1998). Although the type of immune cells co-localizing with NPY-Y4 receptor-expressing cells should be identified in future studies, to the best of our knowledge,

Table 3
Concentrations of plasma metabolites in acute or chronic HT-exposed chicks.

Metabolites	Acute		Chronic		*P* value		
	CT	HT	CT	HT	Type of exposure	HT	Type of exposure × HT
Glucose (mg/dl)	251 ± 4	282 ± 5	244 ± 6	283 ± 9	NS	$P < 0.0001$	NS
Triacylglycerol (mg/dl)	75 ± 8	47 ± 7	45 ± 7	34 ± 2	$P < 0.005$	$P < 0.005$	NS
Uric acid (mg/dl)	9.4 ± 0.6	8.4 ± 0.5	9.6 ± 0.4	7.5 ± 0.7	NS	$P < 0.05$	NS
Total cholesterol (mg/dl)	176 ± 4	199 ± 9	155 ± 6	181 ± 3	$P < 0.005$	$P < 0.001$	NS

The number of chicks used in each group was 7–10. Values are means ± SEM. CT, control thermoneutral temperature; HT, high ambient temperature; NS, not significant.

localization of NPY-Y4 receptor in the spleen has been identifed in this study for the first time in chickens.

Young layer chicks are particularly vulnerable to HT, as evidenced by increased rectal temperatures and reduced food intake following exposure to HT (Chowdhury et al., 2012a, 2012b, 2014; Ito et al., 2015). The current study showed that layer chicks were more heat-stressed by the chronic exposure to HT than the acute exposure (3 h per day for 3 days compared with 3 h) since food intake was only decreased by the chronic exposure to HT. Previously, it was shown that diencephalic NPY mRNA expression was increased and food intake was decreased following an acute exposure to HT (40 ± 1 °C for 5 h) in young layer chicks (Ito et al., 2015). In addition, we reported that the expression of hypothalamic gonadotropin-inhibitory hormone (GnIH) precursor mRNA, an orexigenic neuropeptide (Tachibana et al., 2005; McConn et al., 2014), was increased in HT-exposed (35 ± 1 °C for 24- or 48-h) chicks (Chowdhury et al., 2012a). Bahry et al. (2018) further reported that brain GnIH mRNA was increased in fed, but not fasted, heat-exposed (40 ± 1 °C for 1- or 5-h) chicks. It was suggested that voluntary reduction in food intake, but not to fasting, under heat-stress conditions caused to increase brain GnIH expression during HT (Chowdhury et al., 2012a; Bahry et al., 2018). Similarly, it could be thought that brain NPY, which is an orexigenic neuropeptide, might have been induced under chronic heat challenge in the current study when food intake was voluntarily declined. However, further study on in situ hybridization for NPY in the brain to dissect the contribution of NPY to this decreased food intake under HT would be insightful.

Chronic exposure to HT may stimulate, to a greater extent than acute exposure, increased secretion of brain NPY or other peptides in the 'PP fold' peptide family (Blomqvist and Herzog, 1997; Michel et al., 1998). This family consists of NPY, PYY and PP. Their biological functions include major control mechanisms that regulate energy homeostasis, emotion, and anxiety (Zhang et al., 2011; Tasan et al., 2016; Zhang et al., 2019). The role of the NPY-Y4 receptor in the regulation of energy homeostasis was demonstrated though studies investigating the role of PP. While NPY-Y4 receptor can be activated by all 3 ligands of the NPY family, pharmacological studies have provided evidence that PP has the highest affinity for the NPY-Y4 receptor and thus the Y4 receptor is considered a PP-preferring receptor (Bard et al., 1995; Lundell et al., 1995; Larsen and Kristensen, 1997). PP is mainly expressed in endocrine cells of the pancreas and is involved in satiety (Kojima et al., 2007; Larhammar, 1996; Michel et al., 1998; Simpson et al., 2012). In the current study, neither 1 nmol nor 47 or 188 pmol central NPY induced splenic NPY-Y4 receptor expression. Therefore, further research is needed to examine the involvement of PYY and PP besides NPY in the regulation of NPY-Y4 receptor expression the chick spleen under HT.

It has previously been shown that NPY is stored in large dense-core vesicles within sympathetic nerve terminals (Fried et al., 1985), is co-localized with NE, and is co-released during nerve stimulation (Allen et al., 1984). Moreover, NE is known as the first trigger for regulation of inflammatory cytokine production when receiving signals from the brain and transmitting them to the mouse spleen (Rosas-Ballina et al., 2011; Zhang et al., 2020). In the current study, splenic NE concentrations did not change in response to 1 nmol NPY injection or temperature treatment; however, concentrations of MHPG, which is a NE metabolite, were significantly increased under HT and the change in MHPG was further enhanced by 1 nmol NPY central injection. Therefore, it could be suggested that catecholamine metabolism in the spleen may be enhanced by central NPY under HT.

It was reported that chicken plasma GLU increased, and plasma TG and UA decreased after acute exposure to HT (Chowdhury et al., 2012b; Han et al., 2018; Deyhim et al., 1995). In the present study, plasma GLU was increased by acute and chronic HT. Plasma UA has an antioxidative effect in chickens (Sevanian et al., 1991). In the present study, decreased plasma UA may indicate that acute and chronic HT both reduced antioxidative activity. Plasma TG and TCHO were decreased to a greater degree by chronic exposure than acute exposure. Although chronic exposure to CT did not cause a reduction in food intake in the current study, chronic exposure may affect the metabolism in chicks.

In conclusion, the spleen expressed NPY-Y4 receptor that were localized in the splenic pulps, with no expression of NPY-Y4 receptor in other immune tissues. An HT increased splenic NPY-Y4 receptor expression, and central injection of NPY under HT enhanced splenic catecholamine metabolism. The current results, therefore, suggest that the NPY-Y4 receptor of male chicks plays an important role in the spleen to regulate splenic functions under heat stress.

Disclosure summary

The authors of this manuscript have nothing to declare.

CRediT authorship contribution statement

Haruka Nishimura: Writing – original draft, Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Mohamed Z. Elhussiny:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Yoshimitsu Ouchi:** Writing – review & editing. **Shogo Haraguchi:** Writing – review & editing, Funding acquisition. **Taichi Q. Itoh:** Writing – review & editing. **Elizabeth R. Gilbert:** Writing – review & editing. **Mark A. Cline:** Writing – review & editing. **Shotaro Nishimura:** Writing – review & editing. **Yoshinao Z. Hosaka:** Writing – review & editing. **Eiki Takahashi:** Writing – review & editing. **John F. Cockrem:** Writing – review & editing. **Takashi Bungo:** Writing – review & editing, Funding acquisition. **Vishwajit S. Chowdhury:** Writing – review & editing, Resources, Supervision, Funding acquisition, Conceptualization, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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