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Desensitization of Nociceptive and Serotonergic Thermoregulatory Responses by Capsaicin in Chicken: Role of Capsaicin-Sensitive Subtype-1 Vanilloid Receptor-Independent Mechanisms

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Abstract: Background: It is well known that chicken is insensitive to pain burning sensation of capsaicin (main ingredient of hot chilli peppers) this may be due to insensitivity of transient receptors membrane potential of vanilloid subtype-1 (TRPV1) of chicken to capsaicin. So actions of capsaicin in chicken could be mediated through TRPV1-independent pathways. Serotonin (5-HT) acts as an excitatory transmitter in the heat loss effectors pathways producing hypothermia in mammals and birds. The objective of this study is to investigate the involvement of capsaicin sensitive TRPV1-independent mechanisms in nociceptive and thermoregulatory responses after exposure to mild heat stress and administration of 5-hydroxytryptamine (5-HT) in chicken. Methods: we induced desensitization to TRPV1-independent mechanisms by two times administration of capsaicin (10 mg/kg, i.v.) in newly hatched chicks. Thermoregulatory responses to administration of capsaicin, serotonin (5-HT) and exposure to heat and nociceptive responses to intraocular administration of ammonia were measured. Results revealed that a Administration of 5-HT produced a significant dose-dependent hypothermia in young chicks lasted up to 1 h. This hypothermic effect of 5-HT (10 mg/kg) was not observed in chicks that had been pretreated with capsaicin. Further, capsaicin pretreated-chicks showed impaired tolerance to heat as indicated by increased escape % from heat chamber (42 °C) and reduced nociceptive response to intraocular administration of 0.1%ammonia solution. In conclusion: These data may indicate that the thermoregulatory responses to 5-HT and heat and nociceptive response may be mediated through capsaicin-sensitive TRPV1-independent mechanisms.

Key words: Capsaicin · Hypothermia · 5-Ht · Trpv1 · Chicken

INTRODUCTION

Capsaicin (CAP: 8-methyl- N-vanillyl-6-noenamide) is an active component of chili peppers, belonging to the genus *Capsicum*. CAP and several related compounds, called capsaicinoids are produced as secondary metabolites by chili peppers. They are widely used in poultry rations to act as deterrents against certain rodents and fungi [1]. CAP has a wide range of physiological effects on mammals that include induction of pain sensation, thermoregulation and repeated application desensitizes animals against CAP and impairs nociception and thermoregulation against heat [2]. Further, repeated capsaicin treatment in neonates permanently reduces the number of sensory ganglia and destroys peripheral and dorsal root nerve fibers. These effects are attributed to its action on afferent neurons of nociceptive and central neurons involved in thermoregulation [3].

Recently, it has been demonstrated that a single CAP injection in chicken causes hypothermia that disappeared upon its repeated administration [4]. It is generally accepted that CAP acts on a ligand-gated nonselective cation channel termed TRPV1 (transient receptor potential ion channel of vanilloid subtype-1) which is mainly expressed by sensory neurons [5–7]. However, in chicken TRPV1 are insensitive to CAP [8]. Therefore the actions to CAP in chicken could be attributed to TRPV1-independent mechanisms. Serotonin (5-HT) has been implicated in the regulation of mammalian body temperature [9]. In this study we tried to investigate

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TRPV1 mechanisms in terms of serotonergic system, nociceptive response to irritants, hypothermic response of 5-HT and thermoregulatory response to heat stress.

It is well established that systemic administration of 5-HT leads to hypothermia in rats, mice, guinea pigs and rabbits perhaps via 5-HT 2 receptors [10–11]. The fact that both peripheral and central administration of 5-HT yields similar hypothermic responses indicates that the effect is mainly centrally mediated [11–13]. Such a mechanism is further supported by the finding that peripherally administered 5-HT can penetrate the blood–brain barrier [14]. On the other hand, 5-HT may induce hyperthermias effects in rabbits, rats and sheep [15]. The hypothermic effects of 5-HT in young chicks have been also reported [16, 17]. In addition, 5-HT in the hypothalamus is involved in the regulation of body temperature [18]. Stimulation of 5-HT2 receptors in hypothalamic neurons mediates hyperthermis effects, whereas stimulation of the 5-HT1A receptors in these neurons inhibits the endogenous release of hypothalamic 5-HT and leads to hypothermic effects [11, 19, 20]. In addition, there was evidence that treatment with CAP impairs the hypothermia induced by central administration of serotonin in proptic area or cisterna magna of rats [21].

Birds are generally indifferent to the burning pain sensation induced by capsaicin [22, 23]. CAP as a severe trigeminal irritant when treated to neonates it impairs trigeminal chemoreception in mammals [24, 25] and therefore CAP-desensitized animals has been used as a tool to study the perception of olfactory stimuli. Unlike mammals, birds lack a vomero-nasal system [26] and thus they represent a natural model for study of olfactory-trigeminal interaction. Further, some avian species like pigeon and grey partridges are insensitive to strong ammonia solutions and parrots normally eat *Capsicum* peppers and they prefer pungent than non-pungent hot peppers [22, 23]. Moreover, preferences for capsaicin or other severe trigeminal irritants cannot be induced in these species [24, 25].

The aim of the present study was to investigate the involvement of CAP-sensitive, TRPV1-independent mechanisms in nociceptive response and thermoregulatory responses to heat and administration of 5-HT as regards to its ability to induce a hyperthermia.

MATERIALS AND METHODS

Experimental Birds: A day old, newly hatched male chicks (White Leghorn strain, body weight of 45±5 g), were permitted to free access for standard drinking bottles and feed trough. Chicks were maintained on 12:12 light-dark cycle and kept in thermostatically controlled cages to match chick requirements. Starting ambient temperature was 35°C and decreased 1°C each other day.

Measurement of Temperature: Colonic temperature (Tc) was measured by using a lubricated thermistor probe as described previously [4]. In Brief: chicks were allowed 1h to acclimatize to the laboratory conditions before commencing any temperature measurement and had free access to food and water. The experiments were performed in electrostatically controlled thermal cages. The colonic temperature was recorded with the aid of a lubricated thermistor probe (model XN-64, Technol Seven, Yokohama, Japan) was inserted gently 2.5 cm beyond the vent and the body temperature was monitored using a peripheral processor connected to a computerized medical system. Baseline temperature recordings were made for one hour before any injection and chicks exhibiting no stress fever were used for the experiment [26]. Each chick was used only once. To avoid circadian variations in body temperature recordings, all measurements being made between 09.00 and 15:00. Each animal received ten habituating exposures to the rectal probe.

Drug Treatments: CAP was purchased from Sigma (St Louis, MO, U.S.A.). CAP was dissolved in ethanol (90.0%) and then mixed with Tween 80 (10%). 5-Hydroxytryptamine (5-HT, Sigma), was given as a single i.p. injection by using the doses freshly prepared by dissolving in phosphate buffered saline (PBS, 0.4 ml/chick) and PBS was injected to the control group.

Induction of CAP-desensitization in Chicken: CAP (10 mg/kg, i.v.) or vehicle was injected in the same chicks once a day for three successive days as previously described [4]. Various doses of 5-HT (0.1, 1.0 and 10 mg/kg) were injected into chicks to determine the hypothermic effect of the lowest dose. Next, 5-HT (10 mg/kg) was administered to CAP-pretreated chicks at 5th day old. Rectal temperature was recorded prior to and after treatment with CAP, 5-HT or vehicle with 30 min intervals.

Drinking Bottles Preference Test: The preference to CAP was measured in two bottles preference test and standardized by body weights in universal units (1m/gram

body weight) as previously described [27]. Briefly, the preference to CAP (10 ppm) or, vehicle in tap water was measured at 5th day of age in chicks previously treated with CAP (10 mg/kg, BWT) or injected with vehicle at 3rd and 4th days of age. Since there were no differences between vehicle (10 ppm) and water consumption, water consumption was not analyzed *per se*. Counter balanced drinking bottles of the same size and color were used. The bottles were orientated every 12 h to preclude the location preference [28].

Heat Escape Test: Chicks were either treated with CAP (10 mg/kg, ip) or vehicle. Chicks were placed in thermal cages adjusted at 42 °C for 50 min. The front and back doors of thermal cages were left opened. The number of chicks escaping from the cage was counted at 10 min intervals from the total number of 10 chicks /group [29].

Nociceptive Response to Ammonia: Ammonia solution (0.1%) or distilled water were dropped into the eyes and number of eye wipes/30 sec, duration (sec) of sustained eye blinks or the reaction time (head shaking and leg scratching) were recorded at 5 days-old in the CAP-treated and control (vehicle at 3rd and 4 th day) groups according to Dogan *et al.* [6].

Statistical Analyses: GraphPad Prism 5 software (GraphPad Software Inc.) was used. Data were presented as mean ± SD. Statistical analyses were performed using Student's *t*-test and two-way ANOVA repeated measures for colonic temperature data. The levels of statistical significance were presented as asterisks. A P value of $P < 0.05$ was considered statistically significant.

RESULTS

Thermoregulatory Response to Capsaicin: The changes in colonic temperature in response to repeating capsaicin treatment in same chicks at 3, 4 and 5 days of age are shown in Fig. (1A). One-way ANOVA showed that CAP (10mg / kg, iv) injection in chicks for the first time caused a significant fall in colonic temperature reached down to 40.0 ± 0.51 °C at 1.5 hours post-injection ($F_{(1,119)} = 5.65$, $p < 0.01$). On the next day (Fig.1B) at 4 days age when the same chicks injected with CAP (10 mg/kg, iv) a non-significant hypothermia was produced. While third CAP (10 mg/kg, iv) application on the 5th day (Fig. 1 C) did not cause any obvious changes in T_c. Together these results suggest that twice treatment with CAP can induce desensitization that was enough to prevent hypothermia induced by 3rd treatment with CAP.

Thermoregulatory Response to Serotonin in CAP-pretreated Chicks: There was no difference in basal temperature between 5-HT injected chicks (0.1, 1.0 and 10 mg/kg) at the beginning of each experiment. Saline injections had no effect on body temperature in any chicks group. We observed a strong but transient dose-dependent hypothermic response that persisted until 90–120 min after the administration of 5-HT (Fig. 1D). Intraperitoneal administration of a higher dose of 5-HT (10 mg/kg) reduced the temperature significantly at 60 min and then continued to potentiate this decrease during the 2-h recording period. 5-HT (10 mg/kg) in 4-day-old chicks induced a significant decrease in body temperature (down to 40.31 ± 0.33 °C from the control value of 41.41 ± 0.13 °C ($P < 0.05$) (Fig.1D). When the curve profile analysis was carried out, it showed a maximum drop in body temperature between 60 and 120 min after 5-HT (10 mg/kg) administration. The lower doses of 5-HT had no effect on body temperature in chicks. The decrease in body temperature lasted for 2 hours and after 150 min the body temperature had returned to base level (Fig.1D). The results suggest that 5-HT induced significant hypothermia in chicks with a peak effect at 90 min after the injection (Fig. 1).

To this end, we have studied the hypothermic response induced by 5-HT in CAP-pretreated chicks at 5th-day old. Data in Figure (1E) showed that 5-HT had no significant effect on body temperature in the CAP-pretreated chicks. In other words, the 5-HT-induced decrease in body temperature was abolished by CAP pretreatment.

Impaired Heat Loss Mechanisms in CAP-pretreated Chicks: Escape from thermal cages was performed in 5th day-old chicks that have been treated with CAP (10 mg/kg, iv) or injected with vehicle at 3rd and 4th days post hatching. The % of chicks escape from heat chamber was measured every 10 min during a 50-min test (Fig. 2A). CAP-pretreated chicks (10 mg/kg, iv) showed impaired adaptation within 50 min to hot environment as indicated by the higher escape percent (40.0 ± 12 %) than the control (78 ± 6.0 %). The results may refer to impaired heat loss mechanisms in CAP-pretreated chick.

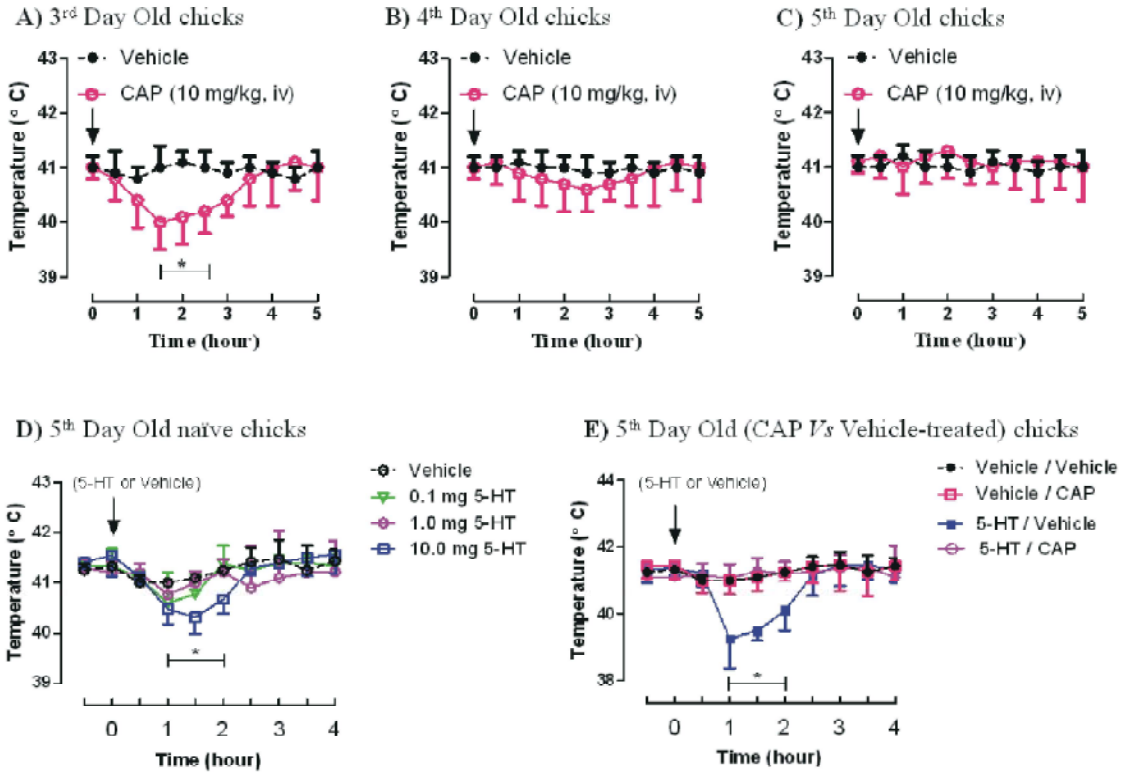

Fig. 1: Thermoregulatory responses to capsaicin and serotonin. The changes in colonic temperature in response to repeated capsaicin (CAP) treatment in same chicks at 3, 4 and 5 days of age (A, B and C). Serotonin (5-HT; 0.1, 1.0 and 10 mg/kg, or vehicle (saline, 1ml/Kg) were injected ip and body temperature was recorded at 30 min-intervals. 5-HT at 10 mg/kg caused significant decrease in body temperature of chicken. (E) Effect of 5-HT (10 mg/kg) was abolished in CAP-desensitized chicken. (n = 5 per group). Data are presented as Mean ± SD.

Increased Consumption of CAP in CAP-pretreated Chicks: In this study, the preference to capsaicin was compared between two groups, CAP (10 mg/kg, iv) pretreated and vehicle injected chicks. All chicks were treated once a day at the first three days post hatching (Fig. 2B, right). The consumption of CAP and vehicle was not different in vehicle treated groups. The results of this experiment showed a tendency for more consumption of capsaicin in the CAP-treated group compared to vehicle injected control group (Fig.2B). These results refer to increased consumption of CAP in CAP-desensitized chicken.

Impaired Nociceptive Response to Ammonia in CAP-pretreated Chicks: Effect of CAP (10 mg /kg, iv) pretreatment on the reaction time (s) and number of eye wipes/30 s to topical administration of 0.1% ammonia (NH4OH) solution was recorded at 5 days old chicks (Fig.2C). The results of *t*-Test for comparing the differences between means showed that CAP pretreated chicks was affected as the control to 1% ammonia solution (data not shown), while the effect of capsaicin was prominent at 0.1 % ammonia solution ($t_{(18)} = 11.47, p < 0.1\times10^{-2}$, Fig. 2C, left). At 5th day of age, CAP (10 mg /kg, iv) pretreatment at first three days reduced significantly the number of eye wipes to 3.20 ± 1.03 compared with control chicks 8.60 ± 1.51 ($t_{(18)} = 9.35$, $p < 0.1\times10^{-2}$, Fig. 2C right). The reaction time was also markedly reduced (14.10 ± 6.78 s) in CAP-pretreated chicks compared to vehicle injected control (43.10 ± 5.36 s, $t_{(18)} = 10.61$, $p < 0.1\times10^{-2}$, Fig. 2C). These data suggested a reduced nociceptive response in terms of reduced number of eye wipes and reaction time to intraocular instillation of ammonia solution in CAP-desensitized chicks.

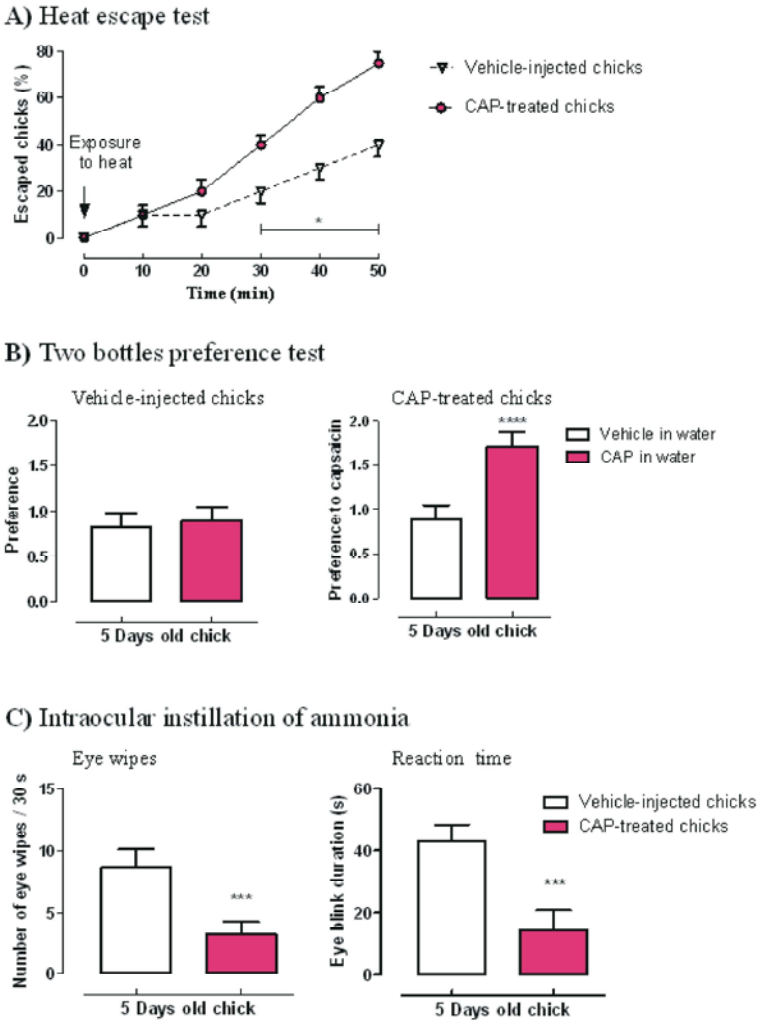

Fig. 2: Examination of thermoregulatory, gustatory and nociceptive responses in capsaicin- desensitized chicken. (A) The preference of chicks to capsaicin (CAP; 10 ppm) or vehicle was assessed in two bottles preference test at 5 days old naïve chicks (left) or chicks previously de sensitized with CAP (10 mg/kg, iv at 3rd, 4th and 5th days of age, *n* = 10 chicks/group) (right). Increased the preference to CAP (10 ppm) was observed in CAP-desensitized chicken (B). The reaction time and the number of eye wipes to intra-ocular administration of 0.1% ammonia solution (*n* = 10 chicks/group). (C) Effect of CAP (10 mg /kg iv) or vehicle pretreatment on escape response to mild heat stress (42 °C for 50 min) in thermal cages at 5 days old. Each point represents the % of chicks escaped at each 10 min intervals. Data represent mean ± SD. (*n* = 10 chicks/group). Asterisks indicated the level of significance, $P < 0.05$.

DISCUSSION

CAP transiently decreased the body temperature of young chicks at 4th day post-hatching [4]. CAP-induced hypothermia has been observed in mammals [30–32]. Although effects of CAP on body temperature are apparently similar, the initiating mechanisms may be completely different in mammals and birds. In mammals, the hypothermic effect of CAP is mediated entirely by TRPV1 [33]. The mechanism by which CAP induces hypothermia in birds has not been elucidated well. It has been suggested that CAP induces hypothermia through autonomic responses such as vasodilatation and salivation or panting [34]. Unlike mammals, the hypothermia induced by CAP in chickens may not be related to TRPV1 [4, 33].

First, the present findings indicate a dose-dependent hypothermic effect of 5-HT in young chicks. It was thought worthwhile to plot the thermal response index at various dose levels (Fig.1D). 5-HT induced hypothermia in a dose-dependent manner and with magnitudes and time course similar to that found in previous studies [11, 35]. Thus, 5-HT-induced hypothermia may be at least primarily mediated by the 5-HT7 receptor [36]. Additionally, an activation of 5-HT1A receptors in the hypothalamus leads to hypothermia [37]. Due to the immaturity of the blood-brain barrier in young chicks [38] a central involvement is possible. However, an additional involvement of peripheral mechanisms (e.g. vasodilation) cannot be ruled out. The results may suggest that 5-HT-induced hypothermia may be mediated by the 5-HT7 receptor.

Serotonin (5-HT) has been implicated in energy homeostasis. 5-HT acts through the 5-HT1B receptor subtype in the paraventricular nucleus of the hypothalamus, leading to potential modification of neuronal transcriptional and secretory machinery [39]. It has been described that local perfusion of the hypothalamus with 5-hydroxytryptophan (a 5-HT precursor) increases body temperature by an increase in extracellular concentrations of 5-HT, which activate central 5-HT2A receptors [40, 41] although activation of these receptors in the peripheral system leads to hypothermia [42, 43]. In the present study, 5-HT induced hypothermia in young chickens was not observed after pretreatment with CAP. Our results (Fig. 2) showing that CAP pretreatment in chicks abolished the decrease in body temperature after 5-HT injection clearly demonstrate that the CAP-sensitive TRPV1-independent mechanism plays a pivotal role in 5-HT-induced hypothermia.

TRPV1-independent effects of CAP in mammals have been demonstrated by *in vitro* experiments [44]. Transient decrease in body temperature in 5-HT, 5-day-old chicks totally disappeared when chicks were pretreated with CAP on days 4 (Fig. 2). These results are consistent with an earlier. The hypothermic effect observed may be the result of an activation of post-synaptic 5-HT receptors in the brain caused by 5-HT [42]. According to Weber and Angell [43], the infusion of 5-HT increased the formation of 5-HT in the hypothalamus, pons, medulla and thalamus in rats. It is possible that the metabolism of 5-HT in the avian species is similar to that in rodents. These findings agree with the view that most of the pharmacological effects obtained after injection of 5-HT are attributable to 5-HT formed *in vivo* from the injected 5-HT [44].Thus in this study, the hypothermic effects observed after 5-HT injection might be attributed to 5-HT formed *in vivo*.

Also repeated high doses of CAP cause generalized desensitization (both splanchnic and peripheral nerves) and reduced or no response to nociceptive stimuli such as strong alkalies (eye irritants such as ammonium hydroxide [45–47]. Hence, a single injection of CAP to chicken causes hypothermia and repeated injection induces impaired thermoregulation to high ambient temperature together with loss of ability to discriminate between warm and cool drinking water but not permanent desensitization [24, 46]. There may be species differences among the sensitivity to CAP; from a relatively insensitive species like parrots and pigeons to a relatively sensitive one like chicken [25, 34, 48]. A good example of these differences is pigeons; insensitive to relatively high strong ammonia concentrations and about 10 folds more resistant to capsaicin–induced hypothermia than chicken [37].

From the present TRPV1-independent findings in chicken, it appears that CAP acts as 5-HT antagonist. These results may be due to a complex modification of yet undefined processes at the receptor sites. CAP has been reported to be a peripheral 5-HT receptor blocker so the hypothermic effect of 5-HT (10 mg/kg) was significantly antagonized. Due to lack of a functional blood-brain barrier in young chicks, penetration of CAP into the brain is possible. In addition, blockade of the peripheral actions of 5-HT by CAP pretreatement might also contribute to the antagonism of 5-HT-induced hypothermia in this study. In our experiments, 5-HT induced hypothermia was abolished by CAP lead us to conclude that hypothermia induced by 5-HT can be the consequence of 5-HT release that activates postsynaptic 5-HT1A receptors. Even in mammals, TRPV1-independent mechanisms has been suggested controlling temperatures, possibly mediated by additional thermosensitive TRP channels in primary afferent terminals [46]. Further, the activation of the sensory nerves was still observed in TRPV1 knockout mice indicating that the neurogenic inflammatory component occurs via a TRPV1 receptor independent process [47], where via capsaicin-sensitive afferents and prostanoids are involved in mediating inflammation in such mice [48]. In addition, the anti-tumor effects of CAP (prototypic TRPV1 agonist) were not revered by capsazepine (TRPV1 antagonist), were independent of TRPV1 activation and are most likely due to ROS induction and subsequent apoptosis [49]. In parallel,

CAP-sensitive TRPV1-indepent mechanisms have been involved in fever and hypothermia induced by lipopolysaccharide and in thermoregulatory response to cold in chicken [4, 50]. Therefore, this study supports in part the role of TRPV1-independent pathways in serotonergic, thermoregulatory, gustatory and nociceptive responses.

As described by Fig. 2A, the results obtained after repeated treatment with CAP (10 mg/kg, iv) was similar to those observed for disruption of thermoregulation to high ambient temperature in rodents [48] and confirmatory to those in chicken [25, 37] where these chicks seemed to be less able to defend basal body temperature against increases in the ambient environmental temperature as indicated by percent of escaped chicks (Fig. 2A). This result in chicks was in agreement with studies of Sann *et al*. [34] they found that capsaicin pretreatment increase heat loss by causing vasodilatation. They interpret the results based on the fact that capsaicin excites nociceptive fibers that are sensitive to mechano-heat and mechano-cold in the rat skin [50]. Unlike mammals, complete desensitization were obtained after repeating CAP (10 mg/kg, i.p. or s.c.) treatment, in chicken repeating the same dose of CAP within the first three days did not affect the nociceptive response to 1.0 % ammonia solution (data not shown) but affect 0.1 % of ammonia solution (NH4 OH) at 5 days of age (Fig. 2C). These finding would be parallel to the results obtained by Meredith [25]. Where, the reaction time and number of eye wipes to 0.1% ammonia solution were reduced significantly (Fig. 1B). Response threshold was about 0.23 % of ammonia vapour saturation for adult chicken [51]. Our data refer to reduced nociceptive response to eye irritants that could be beneficial under intensive rearing conditions.

CONCLUSIONS

To sum up, thermoregulatory responses to 5-HT and heat and nociceptive responses are likely to be mediated by capsaicin-sensitive TRPV1-independent mechanisms in chicken.

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