

Research Note: Behavioral preference and conditioned taste aversion to oleic acid solution in chickens

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ABSTRACT A functional fatty acid taste receptor, GPR120, is present in chicken oral tissues, and chickens show a preference for lipid in feed. However, it remains unclear whether chickens can detect fatty acids. To address this issue, we adopted 2 behavioral paradigms: a one-bowl drinking test to evaluate the preference for oleic acid solution and a conditioned taste aversion test to investigate the role of gustation in chickens' ability to detect oleic acid. In the one-bowl drinking test, chickens did not show any preference for solution containing

0.001, 0.01, 0.03, 0.1, or 30 mmol/L oleic acid although 30 mmol/L oleic acid was enough to fully activate GPR120, confirmed by Ca²⁺ imaging. On the other hand, chickens conditioned to avoid 30 mmol/L oleic acid solution also learned to avoid the solution. These results suggested that chickens have a gustatory perception of oleic acid solution but do not have a preference for it. The present results support the idea that chickens prefer lipid in feed, not only by a post-ingestive effect but also by sensing the taste of fatty acid.

Key words: chicken, conditioned taste aversion, oleic acid

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INTRODUCTION

Lipids constitute one of the 3 main nutrients in food and feed, along with carbohydrates and proteins. Lipids play an important role in feed preference in chickens because chickens prefer feed containing long-chain triacylglycerol (Furuse et al., 1996). Their preference for long-chain triacylglycerol is affected by oral anesthesia, suggesting that the gustation is involved in chickens' ability to detect lipid in feed (Furuse et al., 1996). Earlier, the perception of the fatty acid taste was thought to be attributed mainly to texture and smell in humans (Andersen et al., 2020). However, recent studies have revealed a molecular basis for fatty acid detection in the gustatory system and the evidence for cortical response to fatty acid taste in humans, providing one of the evidences to include fatty acid

taste in the repertoire of basic taste qualities (Andersen et al., 2020).

Rodent taste bud cells include a fatty acid transporter called cluster of differentiation 36 (CD36) and 2 fatty acid receptors: G-protein-coupled receptor 40 (GPR40) and G-protein-coupled receptor 120 (GPR120) (Shanmugamprema et al., 2020). More recently, F-type fiber, which responds to fatty acids best among the various tastants, was identified in the mouse gustatory nerves (Yasumatsu et al., 2019). These findings suggest that fatty acid has a unique position in the gustatory system and is an important tastant in foods.

Previously, we cloned the fatty acid receptor *GPR120* from chicken oral tissue and found that heterologous cells, which transiently express chicken GPR120 (cGPR120), were activated by oleic acid and linoleic acid, the main fatty acids in chicken feed (Sawamura et al., 2015). We also reported that *GPR120* and *CD36*, along with several lipase genes that digest triacylglycerol to fatty acids, were widely expressed in the chicken oral tissues in addition to the gastrointestinal tissues (Kawabata et al., 2018). However, it remains unknown whether chickens can sense fatty acids behaviorally, using their gustatory system, and whether chickens can detect fatty acids by sensing physical stimuli (texture).

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MATERIALS AND METHODS

Chemicals

Oleic acid was obtained from Sigma-Aldrich (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan) and stored at -20°C .

Animals

Rhode Island Red strain chicks were obtained from the National Livestock Breeding Center's Okazaki station (Okazaki, Japan), and their offspring (60 birds) were used for the present experiments (0–2 wk old, males and females). The chicks were maintained in poultry housing lit on a 12–12-h dark–light cycle with the temperature and humidity maintained at around 30°C and 55%, respectively. This study was carried out according to the Guide for Animal Experiments issued by Kyushu University, the Law Concerning the Human Care and Control of Animals (Law No. 105; October 1, 1973), and the Japanese Government Notification on the Feeding and Safekeeping of Animals (Notification No. 6; March 27, 1980). This study was approved by the committee for Laboratory Animal Care and Use at Kyushu University, Japan (approval no. A28-183-0 and A28-151-1).

Cell Culture

Human embryonic kidney (HEK)-derived 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK), and Penicillin-Streptomycin Solution ($\times 100$) (FUJIFILM Wako Pure Chemical Corporation) at 37°C in 5% CO_2 .

Ca^{2+} Imaging

For the Ca^{2+} imaging experiments, HEK293T cells were transfected with either empty vector pcDNA3.1(+) or cGPR120/pcDNA3.1(+) by using ScreenFectA (FUJIFILM Wako Pure Chemical Corporation) on a 96-well clear bottom black plate (Thermo Fisher Scientific, Waltham, MA) coated by poly-D-lysine (0.1 mg/mL) like our previous report (Sawamura et al., 2015). After transfection, the cells were incubated for 48 h at 37°C and 5% CO_2 . Then, we loaded Fura 2-AM solution per well in accordance with the manufacturer's manual for the Calcium Kit II–Fura 2 (Dojindo Laboratories, Kumamoto, Japan). After incubation for 1 h in the dark at 37°C , calcium imaging was performed using a multimode microplate reader (FlexStation 3, Molecular Devices, San Jose, CA). The assay was carried out at about 37°C , and 0.005 to 50 mmol/L oleic acid solution diluted by the standard bath solution (containing 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L MgCl_2 , 2 mmol/L CaCl_2 , 10 mmol/L HEPES, and 10 mmol/L

glucose at pH 7.4, adjusted with NaOH just before each experiment) was applied. Final concentrations of oleic acid in the well were 0.001 to 10 mmol/L after injections. Cell activity was analyzed by the value of the ratio of fluorescence intensity excited at 340 nm and 380 nm before and after injection. Cell viability was confirmed by responses to 5 $\mu\text{mol/L}$ ATP (Sigma-Aldrich).

One-Bowl Drinking Test

Eight birds of almost same age (3–10 d old) were used in the one-bowl drinking test of each oleic acid concentration (5 treatments). Chicks were raised in a box brooder (length 154.7 cm \times width 56.2 cm \times height 30.3 cm, Showa Furanki, Saitama, Japan) before and during experimental period. Briefly, the behavioral test was performed for 5 consecutive days. Throughout the 5 d, commercial layer feed was fed to the chicks *ad libitum* (PowerLayer 17Y; JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan). For 6 h before drinking test, the chicks' access to water was restricted. On days 1 and 2, the chicks were supplied the bowl containing normal tap water for 5 min. From day 3, chicks entered into the individual space divided to 8 spaces by transparent acrylic board in the same box brooder. The isolation stress was inhibited by seeing other chicks. On day 3, the chicks were presented the bowl containing fatty acid solution for 5 min to avoid neophobia. On days 4 and 5, the chicks were supplied the bowl containing fatty acid solution or control solution randomly for 5 min (the chicks that were presented the fatty acid solution on day 4 were presented the control solution on day 5, and vice versa), and their solution intakes were measured. We used 0.001, 0.01, 0.03, 0.1, and 30 mmol/L oleic acid as the test solutions, and 0.2% xanthan gum, which mimics the texture of fatty acid, as the control solution. These experiments were one-time replicate.

Conditioned Taste Aversion Test

The conditioned taste aversion (CTA) tests were conducted based on our previous report with some modifications (Yoshida et al., 2018). Briefly, they were performed for 6 consecutive days. Throughout the 6 d, commercial layer feed was fed to the almost same age (3–10 d old) chicks (20 birds) *ad libitum*. The chicks' water intake was restricted for 6 h before each test. On days 1 and 2, the chicks were presented the bowl containing normal tap water for 5 min. On day 3, the chicks were presented the bowl containing test solution and then were immediately injected intraperitoneally with 230 mg/kg body weight of lithium chloride (LiCl) by using 0.24 mol/L LiCl solution (4 birds per treatment and 2 treatments) or the same volume of saline (6 birds per treatment and 2 treatments). On day 4, the chicks were presented the bowl containing normal tap water. On days 5 and 6, the chicks were randomly presented the bowl containing a test solution or the control solution (the chicks that were presented the test solution on day 5 were presented the control solution on day 6, and vice versa), and their

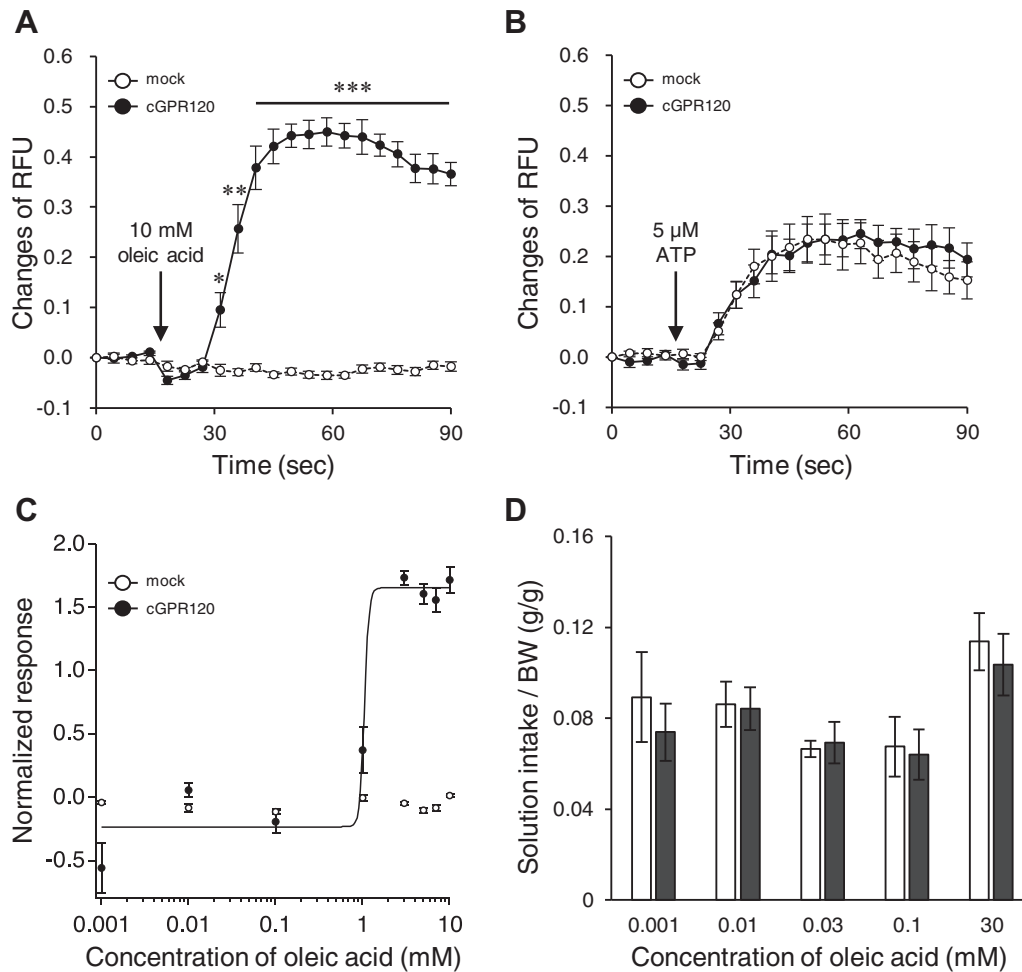


Figure 1. Responses of cGPR120-expressing cells or mock cells for oleic acid solutions and intakes of oleic acid solutions in one-bowl drinking test. (A) Ten mmol/L oleic acid did not increase the relative fluorescein unit (RFU), which is an index of intracellular Ca^{2+} , in HEK293T cells transfected with empty vector (mock, $n = 4$ wells) but 10 mmol/L oleic acid increased RFU in cGPR120-expressing HEK293T cells ($n = 4$ wells). (B) Five $\mu\text{mol/L}$ ATP activated both cells ($n = 4$ wells). (B, C) The arrows indicate the injection timing. Data were analyzed using 2-way repeated ANOVA, followed by unpaired t -test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) RFU changes of mock cells and cGPR120-expressing cells for each concentration of oleic acid were normalized by each 5 $\mu\text{mol/L}$ ATP response in same Ca^{2+} imaging plate. Normalized data were plotted and fitted with the Hill equation ($n = 4$ wells for each concentration). (A–C) Values are the means \pm SE. (D) Mean solution intakes/body weight (BW) of 0.001, 0.01, 0.03, 0.1, and 30 mmol/L oleic acid solutions (black bar) compared to those of 0.2% xanthan gum (white bar) in the one-bowl drinking test for 5 min. There were no significant differences in solution intakes/BW between the fatty acid solutions and 0.2% xanthan gum on days 4 and 5 by the paired t -test at any concentration. Values are the mean solution intakes/BW \pm SE. ($n = 8$ birds in each bar).

solution intakes were measured. To confirm the establishment of CTA by the test solutions, we compared the intake of the same test solutions between saline group and LiCl group. In the CTA tests, there were 2 treatments. First, we used 0.2% xanthan gum solution as the test solution and normal tap water as the control solution to address whether the chicks could perceive the texture of the fatty acid itself. Then, we used 30 mmol/L oleic acid dissolved in 0.1% DMSO solution as the test solution and 0.1% DMSO solution as the control solution to test the chicks' gustatory perception to oleic acid. These experiments were one-time replicate.

Statistical Analysis

The data are expressed as means \pm SE. Statistical analyses were performed by Student paired t -test, unpaired t -test, or 2-way repeated ANOVA. The analyses, fitting

with the Hill equation, and the calculation of EC_{50} value were conducted using the IGOR Pro software package (Version 6.34J, WaveMetrics, Portland, OR), and differences with P -values < 0.05 were considered significant in all experiments.

RESULTS AND DISCUSSION

First, we examined the activity range of cGPR120 for oleic acid by Ca^{2+} imaging. In cGPR120-expressing cells, relative fluorescein unit, which is an index of intracellular Ca^{2+} , was increased by 10 mmol/L oleic acid, and there were significant differences between cGPR120 cells and mock cells (Figure 1A). Although mock cells were not activated by oleic acid, we confirmed the mock cell's activity by 5 $\mu\text{mol/L}$ ATP as same as cGPR120 cells (Figure 1B). The maximum responses by oleic acid solutions normalized by the average of

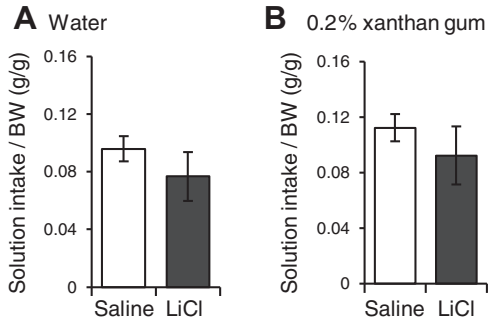
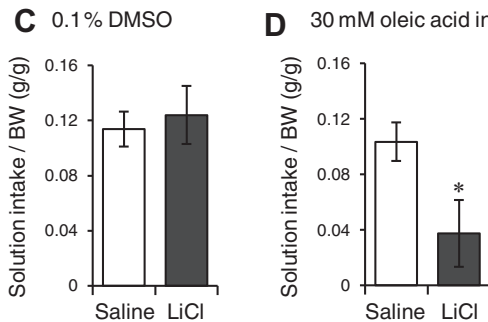
Conditioned stimulus: 0.2% xanthan gum**Conditioned stimulus: 30 mM oleic acid in 0.1% DMSO**

Figure 2. (A, B) Mean solution intakes/BW of water (A) or 0.2% xanthan gum (B) in chickens conditioned to avoid 0.2% xanthan gum by injection of LiCl ($n = 4$ birds), or control chickens injected with saline ($n = 6$ birds). There were no significant differences in intake of water and 0.2% xanthan gum solution between the conditioned chickens and the control chickens by the unpaired t -test. (C, D) Mean solution intakes/BW of 0.1% DMSO (C) or 30 mmol/L oleic acid dissolved in 0.1% DMSO (D) in the chickens conditioned to avoid 30 mmol/L oleic acid dissolved in 0.1% DMSO by injection of LiCl ($n = 4$ birds) or the control chickens injected with saline ($n = 6$ birds). There were significant differences in 30 mmol/L oleic acid dissolved in 0.1% DMSO solution intakes/BW between the conditioned chickens and the control chickens on days 5 and 6 by the unpaired t -test. * $P < 0.05$. Values are the mean solution intakes/BW \pm SE.

maximum responses of 5 μ mol/L ATP in the same 96-well plate were used for analyzing dose-dependency (Figure 1C). cGPR120 cells were dose-dependently activated by oleic acid but not in mock cells. We also found that EC_{50} of oleic acid to cGPR120 is 1.047 mmol/L by calculating from Hill equation and above 3 mmol/L oleic acid solutions can maximally activate cGPR120 (Figure 1C).

Second, we investigated whether chickens preferred pure oleic acid solution. In the present one-bowl drinking tests, the chicks did not consume significantly more oleic acid solution than control solution at any of the concentrations tested (0.001, 0.01, 0.03, 0.1, and 30 mmol/L) although 30 mmol/L was enough concentration to activate cGPR120, suggesting that the chicks had no preference for oleic acid solution (Figure 1D).

Next, we performed the CTA tests to clarify whether chicks have a gustatory perception of fatty acid. We used 0.2% xanthan gum solution, which mimics the texture of fatty acid, as a test solution to confirm whether chicks learn to avoid fatty acid by texture rather than by taste. The chicks conditioned to avoid 0.2% xanthan gum

solution did not avoid the control solution (normal tap water) (Figure 2A) or the test solution (0.2% xanthan gum) (Figure 2B). We then tested the chicks' gustatory perception of 30 mmol/L oleic acid solution. The chicks conditioned to avoid 30 mmol/L oleic acid solution did not avoid the control solution (0.1% DMSO) (Figure 2C) but did avoid the test solution (30 mmol/L oleic acid solution dissolved in 0.1% DMSO) (Figure 2D). These results suggested that the chicks have a gustatory perception of oleic acid solution. Because the CTA paradigm is a well-established learning behavior elicited by a pairing of taste and illness in animals specifically, the present results strongly suggest that chicks have a gustatory perception of fatty acid taste. Thus, these results suggested that chicks can sense oleic acid using gustatory perception, although they do not prefer oleic acid itself.

When mice are presented solutions containing fatty acids such as oleic and linoleic acids, the number of their licks is upregulated (Cartoni et al., 2010), suggesting that mice have a preference for fatty acid solutions. However, the present results demonstrated that chicks totally lacked a behavioral preference for oleic acid solution. Because chickens have fatty acid sensors and lipase in their oral tissues (Sawamura et al., 2015; Kawabata et al., 2018), the difference between these species in their preference for oleic acid solution may be due to the central taste processing systems or to the lack of *GPR40* in the chicken genome.

Chickens show a preference for lipid-containing feeds such as corn oil (Furuse et al., 1996; Sawamura et al., 2015). However, the present results showed that chickens did not prefer pure oleic acid itself, whereas they indicated a gustatory perception of oleic acid solution in the CTA test. It is possible that this discrepancy is due to a postingestive effect. In fact, in a long-term test, mice acquired a behavioral preference for fatty acid solution without taste perception (Sclafani et al., 2013). The present one-bowl drinking test was performed for only 5 min to minimize the postingestive effect. Thus, it is possible that the postingestive effect may contribute to the oleic acid preference in chickens while gustatory perception contributes to their ability to detect oleic acid.

In conclusion, the present study demonstrated that chickens lacked a behavioral preference for oleic acid solution but perceived it gustatorily.

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DISCLOSURES

The authors declare no conflicts of interest.

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