CD36 involvement in the olfactory perception of oleic aldehyde, an odour-active volatile compound, in mice

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ABSTRACT

Cluster of differentiation 36 (CD36) is a broadly expressed transmembrane receptor that has multiple ligands. It has been found to occur abundantly on the surface of the olfactory epithelium in mice and postulated to play a role in mammalian olfaction. However, there have been no ethological analyses of the mammalian behaviour showing CD36 involvement in the olfactory perception of a distinct odour-active volatile compound. In this study, we aimed to assess whether mammals perceive oleic aldehyde, an odour-active volatile that serves as a potential CD36 ligand, and if so, whether CD36 is involved in the sensing by following measurements using CD36-knockout mice and their wild-type littermates. In a two-bottle choice test, wild-type mice, but not CD36-knockout mice, discriminated a sucrose solution containing oleic aldehyde from the sucrose solution alone. To assess the importance of the olfactory system in the oleic aldehyde perception, we conducted an exploration test where the animals could rely primarily on the odour of test volatiles for recognition. We found that the wild-type, but not CD36-knockout mice, were aware of the compound. Our results provide behavioural evidence that CD36 plays a role in the perception of specific odour-active volatile compounds in the nasal cavity.

Chemosensory systems are responsible for the detection of soluble and volatile chemicals. The two chemosensory systems in mammals, gustatory and olfactory systems, distinguish nutritive compounds from harmful ones within the oral and nasal cavities (10). The sense of smell is particularly important for avoiding harmful chemicals in foods prior to ingestion (7). The olfactory perception starts from the

binding of odorants to specific G-protein-coupled receptors on olfactory sensory neurons located within the olfactory epithelium of the nasal cavity (5). However, for many odorants, the precise mechanisms of olfactory detection in mammals remain to be studied.

Cluster of differentiation 36 (CD36) is a membrane-bound receptor with two transmembrane spans that is produced and expressed by a variety of sites in the body of vertebrates (23). The receptor is involved in lipid binding and uptake within tissues, such as the small intestinal, adipose and muscle tissues (6). CD36 has also been postulated to play roles in the gustatory system. Indeed, CD36 has been shown to mediate the detection of long-chain fatty acids in the oral cavity, driving the consumption of the lipid species in rodents (12, 16). More

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recent studies demonstrated a novel role of CD36—the role in the olfactory system. This is exemplified by (i) the localisation of the receptor on the cilia of olfactory sensory neurons in the murine main olfactory epithelium (19), (ii) a weaker response to oleic acid in the olfactory sensory neurons of CD36-knockout mice compared with their wild-type littermates (21) and (iii) a reduction of preference for a lipid mixture odour through dysfunction of CD36 in mice (29). However, there have been no acoustic and ethological analyses of mammalian behaviour addressing the involvement of CD36 in the olfactory perception of specific odour-active volatile compounds.

In this study, *via* ethological tests using CD36-knockout mice and their wild-type littermates, we aimed to examine whether CD36 is involved in the olfactory perception of odour-active volatile compounds occurring in the environment. We attempted to use oleic aldehyde (or *cis*-9-octadecenal) as a model odorant (odour type; fatty, www.thegoodscentscompany.com/data/rw1128621.html), because it has been found not only to occur in the environment (28) but also to display a CD36 ligand activity (26). In this study, we show that (i) wild-type mice, but not CD36-deficient littermates, discriminate a sucrose solution with oleic aldehyde from the sucrose solution alone in a feeding test and (ii) wild-type mice perceive the fatty aldehyde as an odorant in an exploration test, whereas CD36-knockout mice do not. Our results support the notion that CD36 is involved in the olfactory perception of distinct odour-active volatile compounds in the environment.

CD36-knockout mice (C57BL/6J background) (20) were provided as previously described (11). Wild-type and CD36-knockout littermates were obtained from the same breeding pair, thus having a genetically identical background. For genotyping the mice lines, we used the primer set described in a previous study (11). All mice used were females from 8 to 12 weeks of age. Two weeks prior to the experiments, each mouse was individually housed with *ad libitum* access to water and standard mouse chow (MF; Oriental Yeast, Japan). Animal experiments were performed in accordance with the ethics guidelines of the Kyoto University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Oleic aldehyde was prepared by the chemical conversion of oleic acid as described in a previous study (26). The synthesised oleic aldehyde (more than 98% of purity) was dissolved in ethanol ($\geq 99.5\%$;

Nacalai Tesque, Kyoto, Japan) at a concentration of 1% (w/v) (*i.e.* 38 mM) and stored at -70°C until use.

Mice used in the two-bottle choice test were bred under a 12 : 12 hour light-dark cycle (dark phase at 18 : 00–6 : 00). All the tests including the training sessions were performed at 18 : 00 under food and water deprivation for 1 hour prior to the presentation of test fluids. In the first training session, each mouse was given 0.3 M sucrose (Nacalai Tesque) solution and distilled water for 10 min. This was repeated for 3 days. In the second training session, the mice were given access to 0.15 M sucrose solution and distilled water for 10 min. After mice learned to select the 0.15 M sucrose solution for 2 consecutive days, they were regarded to be eligible for the tests. The test fluids with and without oleic aldehyde were prepared by 1000-fold dilutions of 1% (w/v) oleic aldehyde in ethanol and ethanol alone, respectively, into 0.15 M sucrose solution (the final concentration of oleic aldehyde; 38 μM). The test fluids were simultaneously presented to the mice for 10 min. This was repeated for 3 days. The fluid consumption in each cage was measured as a weight and was expressed as grams consumed *per* cage. To avoid developing a preference for a particular side (left or right), the positions of fluids were randomly altered every day throughout all training and test sessions.

Mice used in the exploration test (4, 9, 22) were bred under a 12 : 12 hour light-dark cycle (dark phase at 6 : 00–18 : 00). All the tests including the training sessions were performed at 10 : 00. Food was deprived for 18 hours and provided only after a test session for 6 hours. For the experiments, each mouse was moved from the home cage to the exploration cage, a 20 $\times$ 25 $\times$ 30 cm Plexiglas box with solid transparent walls and a hole of 1 cm of diameter in one of the sides, located 8 cm above the cage ground. The cage ground was covered with a layer of bedding material (Alpha-Dry bedding; Lab Supply, Fort Worth, TX, USA). A stainless cover with a square transparent window was set during the tests to avoid odour dissipation. The video recording system was fixed above the exploration cage at a distance of 30 cm from the cage ground. After each test, the mouse was returned to the home cage and the exploration cage was thoroughly wiped clean with hypochlorous acid solution in order not to influence the following tests. Prior to the training and test sessions, the mice were placed in the exploration cage for 15 min (cage habituation). This was repeated for 3 days. During a session, the mice were allowed a 3-min free exploration for stabilising their behaviour. Subsequently, a vessel containing a sheet of filter

Fig. 1 Assessment of the perception of oleic aldehyde and CD36 involvement in feeding *via* two-bottle choice test. Wild-type and CD36-knockout mice were given 0.15 M sucrose solution with and without oleic aldehyde (38 μ M) at the same time for 10 min. The test was repeated over 3 days. Each column and bar represents the intake $\pm$ S.E. (wild-type = 8; CD36-knockout = 10). * $P < 0.05$ *versus* the intake of sucrose solution alone.

paper soaked in 40 μ L of 1/100 (v/v) ethanol-distilled water (vehicle control) was placed in front of the hole for 1 min. The first exposure to the vehicle control was followed by two more successive exposures to newly prepared vehicle controls. After the three trials with the vehicle control, we placed another vessel containing a sheet of filter paper soaked with either 40 μ L of vehicle control containing acetic acid (Nacalai tesque) (for training sessions) or oleic aldehyde (for test session) at a concentration of 83 mM or 38 μ M, respectively. The exposure to the test vessels lasted for 1 min. A 2-min inter-trial interval without stimulus was given between trials. The total time of exploratory behaviour was assessed in every trial through video recordings. Exploratory behaviours considered to be related to olfactory perception include smelling, sniffing or directing the nose towards the hole at a close distance.

Statistical analysis for the two-bottle choice test was conducted by a Bonferroni-corrected paired *t*-test *per* genotype. The data for exploration test was analysed by repeated measures analysis of variance (ANOVA) with genotype as the between-subjects factor and trial as the within-subjects factor *per* session. To discern fundamental differences in the responsiveness to test odorants (*i.e.*, the magnitude of the change in exploration time upon their presentation) between wild-type and CD36-knockout mice, this ANOVA was conducted after the conversion of original measured values in such a way that the mean exploration time during the first trial was to be 1.0 s/min in both lines of mice. Differences in mean values were assessed with Bonferroni multiple comparison procedure. We also examined to determine the statistical differences in the sum of exploration time during the first three trials (trials with vehicle

control) across session *per* genotype using the same procedure as described above. These analyses were performed by means of the aid of Prism 5 software package (GraphPad, San Diego, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

We initially assessed whether animals could recognise oleic aldehyde in feeding, and if so, whether CD36 is involved in the sensing. Therefore, we examined the choice of the two fluids with or without oleic aldehyde in wild-type and CD36-knockout mice. It has been shown that (i) mice can consciously perceive 1-(palmitoyl)-2-(5-keto-6-octene-diyl) phosphatidylcholine (KODiA-PC), a CD36 ligand, included in a sucrose solution at a concentration of 7.5 μ M (18) and (ii) the CD36 ligand activity is 3.5 times lower in oleic aldehyde than in KODiA-PC (27). On the basis of these data, we set the concentration of oleic aldehyde at 38 μ M, expecting that animals certainly perceive it. In addition, we used a 0.15 M sucrose solution as a vehicle fluid to ensure minimum intake in animals (18).

On the first day, the wild-type mice ingested both fluids in a comparable manner (Fig. 1, left panel). We repeated the test for an additional 2 days. On the second day, the intake of the sucrose solution with oleic aldehyde was significantly less than that of the sucrose solution alone (Fig. 1, left panel). This result suggests that the mice could consciously perceive oleic aldehyde in the fluid and avoid ingesting it. Fatty aldehydes are known to occur through the oxidation of lipids (1, 3). These are believed to be carcinogenic and teratogenic when introduced to and accumulated in the bodies (15). Considering the potentially harmful effects of fatty aldehydes, we infer that oleic aldehyde served as an unpleasant stimulus

for the animals. It is unclear why the mice did not avoid oleic aldehyde on the first day, but they did on the second day (Fig. 1, left panel). One possible explanation is that a negative metabolic reflex through the ingestion of oleic aldehyde on the first day (15) may have led to the aversive response to the aldehyde on the next day. On the third day of tests, the intake did not differ between the test fluids with and without oleic aldehyde (Fig. 1, left panel). We assume that this was due to a reduced sensitivity of mice to oleic aldehyde, which might be caused by desensitisation and/or down regulation of receptors/binding proteins that participate in the perception of the odour-active compound, including CD36 (13, 25). We conducted the two-bottle choice test using CD36-knockout mice. The mice ingested the two fluids in a similar pattern during 3 consecutive days (Fig. 1, right panel). These findings obtained using the two lines of mice suggested CD36 involvement in the perception of oleic aldehyde in feeding in mice.

The ability of mice to perceive oleic aldehyde, coupled with the volatile nature of the compound, led us to assume that the olfactory system plays a major role in odour-sensing in mice. To provide evidence for this, we conducted an exploration test where animals might have to rely primarily on the odour of test substances for perception. In such a test, animals were placed in a cage with a hole and were offered presentation of a vessel containing a sheet of filter paper soaked with test fluids outside the cage. In our setting, mice were presented with a vehicle control (1/100 [v/v] ethanol-water) on the first three trials to habituate to the test environment and to sustain the exploratory interest. Subsequently, mice were presented with the vehicle control containing test odorants on the fourth trial. Mice were regarded to have perceived the odours in the vessel when they stayed a longer (or shorter) time around the hole while exhibiting exploratory behaviours, including sniffing, upon presenting test compounds.

Prior to the test with oleic aldehyde, we executed the training session using acetic acid, which was reportedly recognised by rodents through sniffing (24). Moreover, there have been no reports showing that acetic acid serves as a CD36 ligand. This prior training was mandatory to train mice on the presentation of test compounds. Indeed, we could not evaluate the response of the animals to oleic aldehyde without the prior training (data not shown). We conducted the training session twice on consecutive days. In the first session, wild-type mice exhibited an exploratory behaviour toward the vehicle control over 2.5

± 0.6 , 2.4 ± 1.2 and 1.9 ± 0.7 s/min during the first, second and third trials, respectively (Fig. 2, upper left panel). Upon the presentation of acetic acid (fourth trial), the exploration time was extended to 5.4 ± 2.0 s/min (Fig. 2, upper left panel). CD36-knockout mice also displayed an increased exploratory behaviour upon acetic acid presentation (Fig. 2, upper right panel). The exploration time during the fourth trial was 6.5 ± 2.0 s/min. Repeated measures ANOVA regarding the first training session revealed a significant effect of trial ($P = 0.0009$). On the other hand, we found no significant effects of CD36 gene disruption ($P = 0.13$) and CD36 gene disruption $\times$ trial interaction ($P = 0.28$). Based on these data, we judged that both the wild-type and CD36-knockout mice raised awareness of acetic acid. The odour of acetic acid is likely perceived to be unpleasant by rodents (17). It is also known that certain of the aversive compounds induce a strong exploratory behaviour in rodents (8). It is thus tempting to speculate that the increased exploratory behaviour toward acetic acid reflects a need for caution in mice. We detected statistically significant differences in the exploration time between the fourth and any of the other trials with vehicle control in CD36-knockout mice (Fig. 2, upper right panel). On the other hand, this was not the case in the wild-type littermates (Fig. 2, upper left panel). A previous report has shown that, in anxiety tests, the locomotive activity was apparently higher in CD36-knockout mice than in wild-type mice (30). It is thus likely that the knockout mice are more cautious upon the presentation of acetic acid than the wild-type mice. In any event, results obtained in the first session suggest that (i) both lines of mice perceive acetic acid possibly *via* the olfactory system and (ii) the olfactory perception of acetic acid does not rely on CD36 in mice.

On the second round of training session with acetic acid, the wild-type and CD36-knockout mice displayed an exploratory behaviour upon presentation of acetic acid with the time of 3.7 ± 1.4 and 8.6 ± 3.1 s/min, respectively (Fig. 2, middle panels). Repeated measures ANOVA revealed only a nearly statistical significance for the effect of trial ($P = 0.064$). Like in the first session, no significant effects were observed in CD36 gene disruption ($P = 0.95$) and CD36 gene disruption $\times$ trial interaction ($P = 0.84$). Furthermore, we only detected a tendency toward significant difference between the fourth and third trials in the data for CD36-knockout mice ($P = 0.095$) (Fig. 2, middle right panel). Altogether, we suggest that both mouse lines exhibit a reduced sensitivity

1st training session

2nd training session

Test session

Fig. 2 Assessment of the olfactory detection of oleic aldehyde and CD36 involvement in mice *via* an exploration test. In all of the sessions, wild-type and CD36-knockout mice were given a vessel containing a sheet of filter paper soaked in vehicle control (1/100 [v/v] ethanol-water) during the first three trials. During the fourth trial of the training session, both lines of mice were presented with a sheet of paper soaked in vehicle control containing acetic acid at a concentration of 83 mM (upper and middle panels). During the fourth trial of the test session, we used a sheet of filter paper soaked in vehicle control containing oleic aldehyde at a concentration of 38 μ M (lower panels). Each column and bar represents the exploration activity $\pm$ S.E. of 10 individuals expressed by the exploration time (s/min). * $0.05 < P < 0.1$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, between indicated bars.

to acetic acid in the second session. Also, we assume that the reduced response may be caused by the desensitisation and/or down-regulation of olfac-

tory receptor(s) involved in the acid recognition (5, 13, 25). It is noted that the sum of exploration time during the first three trials using vehicle control was

similar between the first session (6.8 ± 1.6 s/min) and the second session (6.4 ± 1.8 s/min) in wild-type mice (Fig. 2, upper and middle left panels), whereas that was significantly longer during the second session (13 ± 2.5 s/min) compared with the first session (5.8 ± 0.7 s/min) in CD36-knockout mice ($P < 0.05$) (Fig. 2, upper and middle right panels). We suggest that CD36-knockout mice were anxious about the exploration test *per se* during the second session, which might be caused by the first exposure to acetic acid.

In the test session, wild-type mice exhibited consistent exploratory behaviours during the exposure to vehicle control (Fig. 2, lower left panel). On the fourth trial, during the presentation of oleic aldehyde, the mice exhibited an increased exploratory behaviour (Fig. 2, lower left panel). There were statistically significant differences in the exploration time between the fourth and any of the other trials. These results indicate that the wild-type mice responded to oleic aldehyde. We also assume that the mice might have taken precautions against the volatile compound. In CD36-knockout mice, the sum of exploration time during the first three trials with vehicle control was similar between the second training session (13 ± 2.5 s/min) and this test session (13 ± 2.9 s/min) (Fig. 2, middle and lower right panels), suggesting that the knockout mice still exhibited anxiety due to the test. More importantly, CD36-knockout mice did not display increased exploratory behaviours upon the presentation of oleic aldehyde (Fig. 2, lower right panel). Indeed, the exploration time during the fourth trial did not differ significantly from any of the other trials. This result led us to suggest that the CD36-knockout mice were unaware of oleic aldehyde. Repeated measures ANOVA detecting a significant effect of CD36 gene disruption $\times$ trial interaction ($P = 0.025$) further supports this conclusion (P values: 0.33 for the effect of CD36 gene disruption; 0.0083 for the effect of trial). Altogether, we suggest that (i) mice (wild-type mice) perceive oleic aldehyde possibly *via* the olfactory system and (ii) the volatile compound is perceived by a mechanism dependent on CD36 in the mammals.

In this study, we provided behavioural evidence that CD36 is involved in the olfactory perception of oleic aldehyde. This reinforces the idea that CD36 plays a part in the mammalian olfactory system through its ability to recognise specific odorants. However, it is still unclear what role, if any, CD36 plays in the olfactory perception of specific volatiles. Unlike G-protein-coupled olfactory receptors (5), CD36 might not function as a signal transduction

receptor in the conscious perception of volatiles. It has been shown that (i) the *Drosophila melanogaster* CD36 homologue, a sensory neuron membrane protein, is expressed in a population of olfactory sensory neurons implicated in pheromone detection and (ii) this protein acts in concert with a distinct G-protein coupled odorant receptor to capture distinct pheromone molecules (2, 14). By analogy, we hypothesise that CD36 is co-expressed with distinct types of olfactory receptors and serves as the obligate transmembrane partner for such olfactory receptors for facilitating the detection of specific odorants. Further studies are required to prove this hypothesis. Nevertheless, our present findings contribute to expanding the knowledge of the diverse functions of CD36.

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