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Behavioural phenotype of histamine H4 receptor knockout mice: Focus on central neuronal functions

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17 April 2024

Graphical Abstract

unaltered

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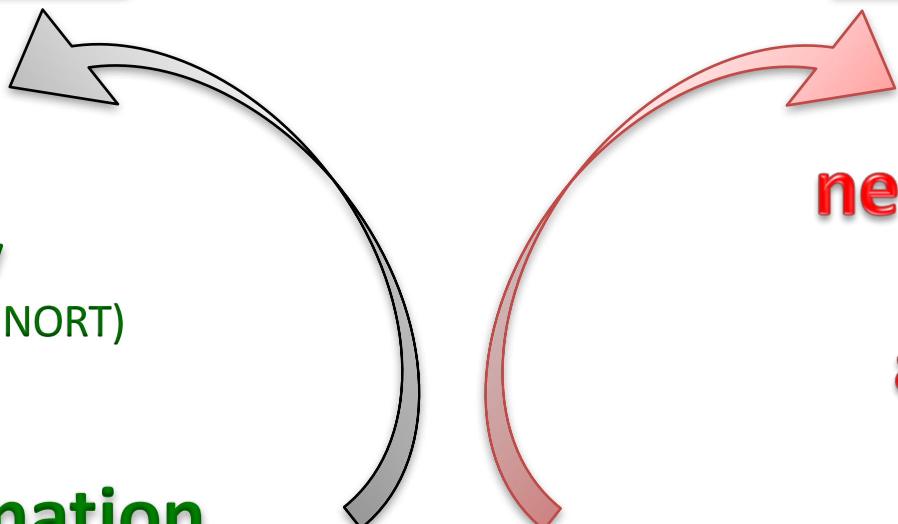

memory

(passive avoidance, NORT)

motor coordination

(rotarod)

acute thermal pain

(hot plate, Hargreaves' plantar test)

acute mechanical pain

(von Frey)

H_4R-KO

neuropathic pain

(SNI)

anxiety (light dark box)

feeding behaviour

exploratory activity

(hole board)

depressive behaviour

(TST)

spontaneous mobility

(hole board)

*Title page

Behavioural phenotype of histamine H_4 receptor knockout mice: focus on central neuronal functions

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Running head: behavioural phenotype of H_4R-KO

Conflicts of interest: none

Author contribution: Dr. Sanna conducted the behavioural experiments, acquired and analysed the data. Prof. Galeotti designed and directed the protocol, and drafted the article. Prof. Masini participated in the protocol design and critically revised the article. Prof Ghelardini was involved in critically revising the article. Dr. Thurmond genotyped and provided H_4R knockout mice.

Abbreviations: DRG = dorsal root ganglia; H_4R = histamine H_4 receptors; H_4R-KO = Histamine H_4 receptor knockout; i.c.v. = intracerebroventricular; i.p. = intraperitoneal; NORT = novel object recognition test; SNI = spared nerve injury; TST = tail suspension test; Wt = wild type

*Abstract

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The functional expression of H_4 receptors (H_4R) within neurons of the central nervous system has been recently reported, but their role is poorly understood. The present study aims to elucidate the role of neuronal H_4R by providing the first description of the behavioural phenotype of H_4R-deficient (H_4R knockout, H_4R-KO) mice. Mice lacking H_4R underwent behavioural studies to evaluate locomotor activity, pain perception, anxiety, depression, memory and feeding behaviour. H_4R-KO mice showed a significant increase in ambulation in an open field as well as in exploratory activity in the absence of any modification of motor coordination. The sensitivity of mutant mice to a thermal or a mechanical stimulus was identical to that of the wild type mice, but H_4R-KO showed sensory hypersensitivity toward a condition of neuropathic pain. The lack of H_4R is associated with the promotion of anxiety in the light-dark box test. H_4R-KO mice showed an increased immobility time in the tail suspension test, experimental procedure used to evaluate the response of H_4R deficient mice to a behavioural despair paradigm. Cognitive function parameters of H_4R deficient mice, examined using the passive avoidance and the novel object recognition tests, were unaltered showing the lack of influence of H_4R on working and recognition memory. Finally, H_4R-deficient mice showed an orectic phenotype. These results illustrate that H_4R modulates various neurophysiological functions such as locomotor activity, anxiety, nociception and feeding behaviour, confirming the importance of the integrity and functionality of neuronal H_4R in the histaminergic regulation of neuronal functions.

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Behavioural phenotype of histamine H_4 receptor knockout mice: focus on central neuronal functions

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Key words: histamine H_4 receptors; central nervous system; pain; anxiety; memory; feeding

1. Introduction

Histamine activates four G protein-coupled receptors designated H_1R, H_2R, H_3R and H_4R (Bongers et al., 2010; Walter and Stark, 2012; Seifert et al., 2013; Strasser et al., 2013). Peripheral histamine is produced by mast cells, basophils and gastric enterochromaffine-like cells with a key role in immunological processes, allergy and inflammation that involves all histamine receptor subtypes. In addition to its peripheral functions, histamine also acts as a neurotransmitter. The H_1R, H_2R and H_3R as well as the histamine-synthesizing enzyme histidine decarboxylase (HDC) are neuronally expressed (Haas et al., 2008; Schneider et al., 2014a). Neuronal histamine activates postsynaptic H_1R and H_2R and regulates a multitude of behaviours and metabolic functions, e.g. food intake, energy consumption, respiration, susceptibility to seizures, locomotor activity, cognition, pain perception, circadian rhythm, sleep and wakefulness, arousal or emotional states (Schneider et al., 2014a). The release of neuronal histamine is controlled by a negative feedback, which is mediated by the presynaptic H_3R (Haas et al., 2008; Schneider et al., 2014b).

The physiological role of the histamine H_4R, the most recently discovered histamine receptor subtype (Nakamura et al., 2000; Oda et al., 2000; Liu et al., 2001), has not yet been fully elucidated. The immunological function of the peripheral H_4R has been clearly identified (Seifert et al., 2013; Neumann et al., 2014). However, much less is known about expression and function of H_4R in the central nervous system and since the discovery of H_4R, its functional presence in the peripheral and central nervous system has been controversially discussed (Schneider and Seifert, 2016).

The H_4R mRNA was detected in human dorsal root ganglia (DRG), spinal cord and brain regions including hippocampus, cortex, thalamus and amygdala. In the rat H_4R mRNA was found in the DRG, spinal cord, cortex, cerebellum, brainstem, amygdala, thalamus and striatum (Strakhova et al., 2009). In addition, significantly increased H_4R mRNA expression was observed in the putamen and caudate nucleus of Parkinson’s patients (Shan et al., 2012). Moreover, on the protein level, H_4R was immunohistochemically identified in the DRG and spinal cord (Strakhova et al., 2009; Lethbridge and Chazot, 2010) as well as in several regions of human and mouse brain, such as

thalamus, hippocampus and cerebral cortex (Connelly et al., 2009). The functional expression of H_4R on human and rodent neurons highlights their implication in neuronal functions, but the difficulty to generate H_4R antibodies with high specificity (Beermann et al., 2012) generates results that should be interpreted with some caution.

The use of selective ligands for H_4R can greatly help understand the physiological role of H_4R. However, in the absence of knockout controls, off-target effects of the H_4R ligands cannot be fully excluded. Although H_4R-deficient mice were generated more than a decade ago (Hofstra et al., 2003), they have not yet been used for systematic experiments to elucidate the physiological and pathological role of this receptor subtype within the central nervous system. The aim of the present study is to clarify the functional role of neuronal H_4R and its involvement in neuronal processes through the phenotypic characterization of H_4R-deficient mice.

2. Materials and methods

2.1. Animals and reagents

Histamine H_4 receptor knockout (H4R$^{-/-}$) mice were generated by Lexicon Genetics (Woodlands Park, TX, USA) as previously described (Hofstra et al., 2003) provided by Janssen Research & Development, LLC La Jolla, CA, USA and back crossed to CB57 background. Corresponding wild-type (Wt) mice were obtained from Harlan Laboratories (Bresso, Italy).

Male mice were randomly assigned to standard cages, with four to five animals per cage. The cages were placed in the experimental room 24 h before behavioural test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence, Italy, under license from the Italian Department of Health (54/2014-B) and in compliance with international laws and policies (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals (McGrath & Lilley, 2015). All effort was taken to minimize the number of animals used and their suffering.

All behavioural experiments were performed during the light phase with a blind procedure. The number of animals per experiment was based on a power analysis (Charan & Kantharia, 2013) and ten animals per group were used to have the probability of 86% that the study detects a difference between groups at a two-sided 0.05 significance level. Sample size was calculated by G power software.

VUF 8430 (40 μg per mouse), diazepam (1 mg/kg) (Sigma, Milan, Italy), D-amphetamine (1 mg/kg; De Angeli, Rome, Italy), amitriptyline (10 mg/kg; Sigma, Milan, Italy) were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared in such a

way that the necessary dose could be administered in a volume of 10 ml/kg by intraperitoneal (i.p.) or in a volume of 5 μl per mouse by intracerebroventricular (i.c.v.) injection, as previously described (Galeotti et al., 2003). Doses and administration schedule were chosen on the basis of time-course and dose-response curves performed in our laboratory (Galeotti et al., 2013).

2.2. Locomotor activity

2.2.1. Rotarod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus up to 5 mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s.

2.2.2. Hole-board test

The spontaneous locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous mobility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

2.3. Nociceptive behaviour

2.3.1. Mechanical threshold (von Frey's test)

Mechanical allodynia was measured by using Dynamic Plantar Aesthesiometer (Ugo Basile, Bologna, Italy). The mice were placed in individual Plexiglas cubicles (8.5 x 3.4 x 3.4 (h) cm) on a wire mesh platform and allowed to acclimate for approximately 1 h, during which exploratory and grooming activity ended. After that, the mechanical stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic ascending force (0-5 g in 35 s). When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. Nociceptive response for mechanical sensitivity was expressed as mechanical paw withdrawal threshold (PWT) in grams. The mean PWT was calculated from six consecutive trials (each performed every 30 min) and averaged for each group of mice.

2.3.2. Hargreaves' plantar test

Thermal nociceptive threshold was measured using Hargreaves' device as described (Hargreaves et al., 1988). Paw withdrawal latency in response to radiant heat (infrared) was assessed using the plantar test apparatus (Ugo Basile, Comerio, Italy). Each mouse was placed under a transparent Plexiglas box (7.0 (d) × 12.5 (w) x 17.0 (h) cm) on a 0.6-cm-thick glass plate and allowed to acclimatize for 1–2 h before recording. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W) that was positioned 0.5 cm under the glass plate directly beneath the hind paw. The time elapsed between switching on the infrared radiant heat stimulus and manifestation of the paw withdrawal response was measured automatically. The intensity of the infrared light beam was chosen to give baseline latencies of 10 s in control mice. A cut-off of 20 s was used to prevent tissue damage. Each hindpaw was tested 2–3 times, alternating between paws with an interval of at least 1 min between tests. The interval between two trials on the

same paw was of at least 5 min. Nociceptive response for thermal sensitivity was expressed as thermal paw withdrawal latency in seconds. All determinations were averaged for each animal.

2.3.3. Hot-plate test

The hot plate test was performed as previously described (Galeotti et al. 2003). Mice were placed inside an hot plate apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy), which was set thermostatically at 52.5 ± 0.1°C. Reaction times (s) were measured with a stopwatch. The endpoint used was the licking of the fore or hind paws. Mice were removed from the hot plate immediately after the first response. Data were recorded as raw latencies and an arbitrary cut-off time of 45 s was adopted.

2.3.4. Spared nerve injury (SNI)

Mono-neuropathy was induced according to the method of Bourquin et al. (2006). Mice were anaesthetized with sodium pentobarbital (60 mg/kg i.p.). The right hind limb was immobilized in a lateral position and slightly elevated. Incision was made at mid-thigh level using the femur as a landmark. The sciatic nerve was exposed at mid-thigh level distal to the trifurcation and freed of connective tissue; the three peripheral branches (sural, common peroneal, and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures. Both tibial and common peroneal nerves were ligated and transacted together. A microsurgical forceps with curved tips was delicately placed below the tibial and common peroneal nerves to slide the thread (5.0 silk, Ethicon; Johnson & Johnson Intl, Brussels, Belgium) around the nerves. A tight ligation of both nerves was performed. The sural nerve was carefully preserved by avoiding any nerve stretch or nerve contact with surgical tools. Muscle and skin were closed in two distinct layers with silk 5.0 suture. Intense, reproducible and long-lasting thermal hyperalgesia and mechanical allodynia-like behaviours are measurable in the non-injured sural nerve skin territory. The SNI model offers the advantage of a

distinct anatomical distribution with an absence of co-mingling of injured and non-injured nerve fibers distal to the lesion such as the injured and not injured nerves and territories can be readily identified and manipulated for further analysis (i.e. behavioural assessment). The sham procedure consisted of the same surgery without ligation and transection of the nerves.

Animals were habituated to the testing environment daily for at least 2 days before baseline testing. Nociceptive responses to mechanical and thermal stimulus were measured before surgery, to establish a baseline for comparison with post-surgical values, and 7 days after nerve injury.

2.4. Antidepressant-like activity

2.4.1. Tail suspension test

A piece of tape was adhered to the upper middle of the tail of each animal, creating a flap with the overlap of tape. Mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. The duration of the test was 6 minutes and immobility was measured in the first 2 min, when animals react to the inescapable stress, and in the last 4 min of the test, when the behavioural despair is established. Immobility was defined as the absence of any limb or body movements, except those caused by respiration.

2.5. Evaluation of food consumption

Mice did not have access to food for 4 or 12 h but water was available ad libitum. A weighed amount of food (standard laboratory pellets) was given and the weight consumed (evaluated as the difference between the original amount and the food left in the cage, including spillage), was measured 15, 30, 45 and 60 min after the beginning of the test, to an accuracy of 0.1 g. An arbitrary cut-off time of 60 min was used.

2.6. Evaluation of mnemonic functions

2.6.1. Passive-avoidance test

The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. As soon as the mouse entered the dark compartment, it received a punishing electrical shock (0.5 mA, 1 sec). The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. The maximum entry latency allowed in the training and retention sessions was, respectively, 60 and 180 s.

2.6.2. Novel object recognition test

The novel object recognition, based on spontaneous exploratory activity, is a test to measure a form of recognition memory (Okamura et al., 2011). The novel object recognition test (NORT) was performed in the open field device (cylinder diameter: 78 cm, height walls: 60 cm). Mice were habituated to the open field without objects for 10 min. Then, in the training phase, animals were placed in the center of the apparatus with two identical objects placed 16 cm away from the walls and allowed to explore both objects for 5 min. Object exploration was recorded by an experienced observer only when the animal's nose or mouth was in contact with the object. In the test phase, the animals were placed back in the open field with one object familiar (the same as the previous phase) and one novel object, 3 h or 24 h after the training phase in order to measure short-term memory or long-term memory. The time spent exploring the objects was recorded for 5 min. The test phase reflects the preference for the novel object. Recognition index for the novel object was calculated as the ratio between time spent exploring the novel object with respect to overall exploring time, expressed by (TN-TF/TN + TF) x100 [TF = time spent exploring familiar object; TN = time spent exploring the novel object]. During training session both objects are novel and the time spent on both objects should be similar. The objects were always placed in the same location, 16 cm from the

wall and 37 cm apart. Velcro into the base of the objects was used to secure the objects in place. In addition, the objects to be explored were made of plastic in three different shapes that had no significance for animals and had never been associated with reinforcement. The objects and the apparatus were cleaned with ethanol solution between trials.

2.7. Anxiolytic-like activity

2.7.1. Light dark box

The light–dark box was made of white and black opaque apparatus (length 50 cm, width 20.5 cm, and height 19 cm) consisted of two equal acrylic compartments, one dark and one white, illuminated by a 60-W bulb lamp and separated by a divider with a 10 x 3.2 cm opening at floor level. Each mouse was placed in the middle of the light chamber facing a side away from the door and then released. Animals' behaviours were scored for 5 min and included the latency to the first step into the dark compartment, the duration of time spent in the light chamber, the number of full-body transitions between chambers. These behaviours have previously been measured as a reflection of anxiety in this apparatus (Bourin and Hascöet, 2003). After testing, subjects were removed from the light–dark box and returned to their home cage in colony room. The apparatus was cleaned with 70% ethanol after each use and allowed to dry before the next subject was tested.
This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light.

2.8. Statistical analysis

All experimental results are given as the mean ± s.e.mean. Data were analysed using Student's t-test, one-way or two-way ANOVA. Tukey's test was used for post hoc analysis following a significant one-way ANOVA. Multiple comparisons following two-way ANOVA were conducted

with Bonferroni post hoc comparison. A P-value of <0.05 was considered statistically significant. The computer programme GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used in all statistical analyses.

3. Results

3.1. Influence of H_4R on locomotor behaviour

H_4R-KO mice did not show any visible sign of altered gross behaviour or poor health. The body weight was comparable to that of wild type mice (Fig. 1A). In addition, specific tests were performed to unmask alterations of locomotor behaviour that were not visible to the operator. Mice were evaluated for motor coordination by use of the rotarod test and for spontaneous mobility and exploratory activity by use of the hole board test.

H_4R-KO mice did not show any impairment in the motor coordination and the number of falls from the rotating rod was identical to that of wild type mice (Fig. 1B). The spontaneous mobility (Fig. 1C) and exploratory activity (Fig. 1D) of H_4R-KO mice were significantly increased in comparison with the control group.

3.2. Role of H_4R on nociception

The involvement of histamine H_4 receptors in the modulation of the pain threshold was evaluated by applying a thermal (Hargraves's test, hot plate test) or a mechanical (von Frey's test) stimulus. H_4R-KO mice showed a thermal (Fig. 2A,B) and mechanical (Fig. 2C) threshold comparable to that of wild type (Wt) mice, indicating the lack of any spontaneous hypernociceptive or antinociceptive phenotype. The role of H_4R on nociceptive behaviour was also investigated in the presence of a condition of neuropathic pain (SNI model). 7 days after surgery, Wt mice showed a marked thermal (Fig. 2D) and mechanical (Fig. 2E) hyperalgesia on the ipsilateral side. H_4R-KO mice that underwent SNI showed a thermal and mechanical hypersensitivity on the ipsilateral side which was significantly more severe than control mice, while the nociceptive response in the contralateral side was comparable to that detected in Wt mice. A specific contribution of neuronal H_4R in the control of chronic pain of neuropathic origin was illustrated.

3.3. Role of H_4R in a depressant-like paradigm

The role of H_4R in the modulation of a depressant-like behaviour was investigated in the tail suspension test (TST), a widely used test to evaluate antidepressant-like activities. In the TST animals are subjected to the short term, inescapable stress of being suspended by their tail. Animals then develop an immobile posture considered as a depressive-like behaviour. The presence of a depressant/antidepressant-like phenotype of H_4R-KO mice was detected in the first 2 min when animals react to the inescapable stress (Fig. 3A), and in the last 4 min (Fig. 3B) of the test, when the behavioural despair is established. Experiments showed an increased immobility time in the first 2 min of the test that was still significant in the last 4 min. The immobility time detected in the 6 min duration of the test (Fig. 3C) confirmed that the immobility time values were significantly higher than that showed by Wt control animals.

To confirm the trend towards a depressant-like phenotype in mice lacking the H_4R, the TST was performed in control mice following neuronal H_4R activation. However, the intracerebroventricular (i.c.v.) administration of the H_4R agonist VUF 8430 (20 μg) was unable to modify the immobility time in the TST (Fig. 3D), showing the lack of any antidepressant-like activity and suggesting the lack of a prominent role of H_4R in the modulation of mood. The administration of amitriptyline, used as reference drug, decreased the immobility time values in the mouse TST, thus validating our experimental results (Fig. 3D).

3.4. Anxiogenic-like phenotype in H_4R-KO mice

The role of H_4R in anxiety-related behaviours was investigated in the light dark box paradigm. H_4R-KO mice spent a shorter time in the light chamber than the Wt mice (Fig. 4A) without modifying the latency to the first step in the dark chamber (Fig. 4B). A second behavioural parameter detected to evaluate the presence of an anxiolytic-like behaviour in mice lacking H_4R

was the number of transitions from the two chambers, that was markedly reduced in comparison with Wt mice. The anxiolytic reference drug diazepam markedly increased the transitions in comparison with the control group (Fig. 4C). All these data indicate an anxiogenic-like phenotype of H_4R-deficient mice.

In order to confirm the role of H_4R in the response to an anxiety-inducing environment, the anxiolytic-like properties of the H_4R agonist VUF 8430 were tested. The compound, after i.c.v. administration (20 μg), prolonged the time spent in the light chamber producing an anxiolytic-like effect (Fig. 4D). Diazepam, used as a reference molecule, prolonged the time spent in the lighted compartment, thus evidencing its anxiolytic-like properties and validating our experimental approach. The intensity of the anxiolytic-like effect of VUF 8430 was comparable to that induced by diazepam (Fig. 4D).

3.5. Orectic phenotype of H_4R-KO mice

The role of H_4R in feeding behaviour was evaluated in H_4R-KO mice that were previously deprived of food. The cumulated amount of food eaten by mice which had no access to food for 4 h before the test, experimental condition suitable to highlight an increase in food intake, is reported in Fig. 5A. Wt mice showed a modest but constant increase in the amount of food consumed in the 60 min test. Similarly, H_4R-KO mice showed a constant increase in the food intake, but the cumulative amount of food eaten was significantly higher than that consumed by the Wt, showing an orectic phenotype (Fig. 5A). To further delineate the role of H_4R in the feeding behaviour, the same experiment was performed in mice which were not provided with food for 12 h, experimental condition able to highlight an anorectic behaviour. In a condition of more prolonged food deprivation, the cumulated amount of food eaten by H_4R-KO was comparable to that eaten by Wt mice at any time point (Fig. 5B), ruling out the presence of an anorectic phenotype. Amphetamine, used as a reference molecule, greatly reduced the food consumption at any time point (Fig. 5B), thus evidencing its anorectic properties and validating our experimental approach.

To confirm these results we evaluated the feeding behaviour in control mice exposed to the H_4R agonist VUF 8430. I.c.v. treatment with VUF 8430 did not modify the food consumption within the first 30 min, whereas an anorectic effect was produced 45 and 60 min after the beginning of the test (Fig. 5C). VUF 8430-treated mice showed an anorectic behaviour which was of smaller intensity than that produced by the reference drug amphetamine, indicating that H_4R, while able to influence food consumption, are not endowed with a prominent role in the eating behaviour.

3.6. Role of H_4R on memory functions

To clarify the role of H_4R on memory processes, we determined the behavioural response of H_4R-KO mice in a passive avoidance task. Mice lacking H_4R showed the absence of any impairment in working memory since the latency values recorded in both the retention and training sessions were identical to those detected in the control group (Fig. 6A).

To evaluate whether H_4R might produce a detrimental or ameliorative effect on recognition memory, the novel object recognition test (NORT) was performed. In the NORT the total time spent exploring both objects in the training session (internal control) was similar in Wt mice (Fig. 6B). Also H_4R-KO mice showed similar exploration times in the training session, but time values were higher than those of control mice (Fig. 6C), in confirmation of the higher exploratory activity of H_4R-KO mice observed in the hole board test (Fig. 1D). The evaluation of the training object exploration index confirmed the lack of any difference on training exploration activity between Wt and H_4R-KO (Fig. 6B). The evaluation of the exploration times between training object and novel object in the retention session illustrated that in H_4R-KO there was an increased novel object exploration time of intensity comparable to that detected in Wt mice (Fig. 6C). The novel object exploration index (Fig. 6E) and the discrimination index (Fig. 6F) were comparable to those of Wt mice, indicative of a lack of any recognition memory impairment.

4. Discussion

Even though the immunological function of H_4R is well established, little is known about expression and function of H_4R in the central nervous system. The present study investigated the role of H_4R in the histaminergic regulation of neuronal functions by characterizing the behavioural phenotype of H_4R-deficient mice.

H_4R-KO mice did not show any visible sign of altered gross behaviour, weight loss or poor health. However, the histaminergic system is known to be involved in the regulation of locomotor activity. $HDC^{(-/-)}$ mice show reduced locomotor and exploratory activity (Acevedo et al., 2006), that likely reflects a lack of histaminergic stimulation of H_1R, H_2R and H_3R since knockout mice for each histamine receptor subtype demonstrated reduced locomotor activity (Yanai et al., 1998; Shiba et al., 2001; Toyota et al., 2002). Investigating into the locomotor behaviour of H_4R-deficient mice, we observed that the lack of H_4R increased spontaneous mobility and exploratory activity, conversely to the locomotor reduction produced by the deficiency of the other histamine receptor subtypes. However, similarly to H_1 deficiency (Yanai et al., 1998), H_4 deficiency did not impair the rotarod performance indicating the lack of influence on motor coordination.

In human and rodents, expression of H_4R mRNA appears to be the highest in the spinal cord. Immmunohistochemical detection revealed that H_4R are strongly expressed in sensory neurons of dorsal root ganglia (DRG) with more intense staining of small and medium diameter cells, with a H_4 signal which is neuronal and not of glial origin, and in the lumbar spinal cord, especially laminae I and II (Strakova et al., 2009; Sanna et al., 2015). Detection of H_4R in the spinal cord and on cell bodies of DRG neurons raises the possibility that these histamine receptors might have a role in nociception. Behaviour experiments performed with mice after intracerebroventricular (i.c.v.) administration of H_4R agonists, revealed pain-reducing effects of neuronal H_4R activation in acute thermal nociception (Galeotti et al., 2013) and neuropathic pain (Smith et al., 2007; Sanna et al., 2015), further supporting this hypothesis. H_4R-deficient mice showed a thermal and mechanical threshold comparable to that of Wt animals, but showed a significantly increased pain

hypersensitivity after spared nerve injury, a surgical procedure to produce a peripheral mononeuropathy characterized by a long lasting thermal and mechanical hyperalgesia. This hypernociceptive phenotype, that selectively emerges in a neuropathic pain condition in the absence of any influence on normal pain threshold, indicates that modulation of neuronal H_4R appeared not to be involved in the regulation of the physiological maintenance of the pain threshold, while it is highly involved in the response to a condition of pathological chronic pain hypersensitivity of neuronal origin. These results also importantly highlight an opposite involvement of peripheral and neuronal H_4R on pain processes, with peripheral H_4R involved in promoting inflammatory pain, as widely reported, and neuronal H_4R involved in the relief from neuropathic pain.

The role of the histaminergic system in the modulation of anxiety-like behaviours in animals has been suggested, but with contradictory results. Increased anxiety has been reported in mice after peripheral administration of the histamine precursor l-histidine (Kumar et al., 2007). However, these results are at odds with more recent findings demonstrating increased anxiety in several behavioural tests in histidine decarboxylase (HDC)-deficient mice (Dere et al., 2004; Acevedo et al., 2006). Opposite roles for histamine receptor subtypes in the modulation of anxiety behaviour has also been reported. H_1R-deficient mice showed reduced anxiety (Yanai et al., 1998; Zlomuzica et al., 2008) whereas H_2R-deficient mice were more anxious than wild-type controls (Shiba et al., 2001). H_3R-deficient mice and treatment with H_3R antagonists showed increased or decreased anxiety depending on the experimental paradigm used (Rizk et al., 2004; Bongers et al., 2004). The role of H_4R in the modulation of anxiety by the histaminergic system has not been elucidated. Present results obtained in the light-dark box test, indicate a clear involvement of H_4R in anxiety behaviour since H_4R-KO mice spent a shorter time in the light chamber than the Wt mice, showing an anxyogenic-like phenotype. H_4R-KO mice also showed a reduced number of transitions between the two compartments, a parameter usually considered to evaluate the anxiolytic-like efficacy of compounds along with the light/dark performance, further supporting the increased response to anxiety of H_4R-deficient mice. This hypothesis is supported by previous data in which i.c.v. administration of the H_4R agonist VUF 8430 caused anxiolytic effects (Galeotti et al., 2013).

The light/dark box test is limited by its ability to yield false-positive results if a drug increases general motor function. H_4R-deficient mice showed increased spontaneous mobility and exploratory activity. However, the latency to the first step into the dark compartment is identical to that of the Wt animals and the number of transitions, reported to be an index of exploration activity because of habituation over time (Bourin and Hascoët, 2003), is reduced. It appears that the response to an anxiety-inducing environment predominates over the locomotor and exploratory behaviour, making the results reliable.

Histaminergic neurons project to regions especially important for cognitive functions such as the frontal cortex, basal forebrain, hippocampus and amygdala (Panula and Nuutinen, 2013). The role of histamine H_1R, H_2R and H_3R has been intensively investigated, but these studies have used many behavioural tasks and both improvements and impairments in cognitive behaviour have been obtained (Dere et al., 2010). To investigate the role of the neuronal H_4R in the modulation of memory processes, we evaluated the memory performance of H_4R-deficient mice on a passive avoidance task and on a novel object recognition paradigm. H_4R-deficient mice did not show any alteration of both working and recognition memory. The capability of a H_4R agonist to counteract scopolamine-induced amnesia has been demonstrated in a passive avoidance paradigm (Galeotti et al., 2013). However, the authors did not detect any procognitive activity following neuronal H_4R stimulation in mice devoid of pharmacologically-induced memory deficits. These data, along with the lack of any memory impairment in H_4R-KO mice might suggest that the H_4R subtype did not play a prominent role in the histaminergic modulation of memory processes.

Brain histamine plays a fundamental role in eating behaviour as it induces loss of appetite and it has long been considered a satiety signal that is released during food intake (Sakata et al., 1997; Provensi et al., 2014). Brain histamine appears to suppress food intake mainly via H_1R. Centrally administered H_1R agonists suppressed food consumption in rats (Lecklin et al., 1998), whereas injection of H_1R-antagonists elicited food intake (Sakata et al., 1998; Ookuma et al., 1993). Experiments with H_1R-KO mice further confirmed that histamine inhibits food intake via H_1R (Masaki et al., 2004). H_2R was not involved in feeding since both H_2-agonists (Lecklin et al., 1998)

and antagonists (Sakata et al., 1998) centrally injected had no effect on food consumption. More recently a role of H_3R in the eating behaviour has been postulated. H_3R agonists increase feeding (Chiba et al., 2009) whereas H_3R antagonists have been reported to induce weight loss (Malmlöf et al., 2005). To date, little is known on the role of neuronal H_4R in the modulation of eating behaviour and no data from knockout mice are available. The evaluation of cumulative food intake over a 60 min period in mice deprived of food for 4 h, experimental condition suitable to highlight an increase in food intake, showed a higher amount of food eaten by H_4R-deficient mice in comparison with Wt animals. The orectic phenotype showed by H_4R-KO mice is in agreement with previous results in which the i.c.v administration of the H_4R agonist VUF 8430 significantly reduced food consumption, further supporting the involvement of H_4R in the regulation of food consumption by the histaminergic system.

Pharmacological or genetic loss of histamine or histamine receptor function in animals produces phenotypes that mimic human depression (Haas et al., 2008). Activation of H_1R reduces the time of immobility in the forced swimming test, suggesting an antidepressant-like effect (Lamberti et al., 1998). More recently, the involvement of the H_3R has been postulated since blockade of H_3R produced an antidepressant-like activity in rats that was prevented by H_1R and H_2R antagonists (Femenia et al., 2015). Investigating into the role of neuronal H_4R, an increased immobility time in the TST was showed by H_4R-deficient mice. This effect was particularly evident in the first 2 min of the test, when animals react to the inescapable stress, more than what showed in the last 4 min of the test, when the behavioural despair is established. Since H_4R-edficient mice showed an anxiogenic-like behaviour, we cannot exclude that the increased immobility time was secondary to a response to an anxiety-inducing environment. The lack of any antidepressant-like effect by i.c.v. administration of an H_4R agonist, along with a lower expression of H_4R in the CA1 hippocampal area (Connelly et al., 2009) in comparison with other histamine receptor subtypes (Vizuete et al., 1997; Pillot et al., 2002), further supports the hypothesis of a secondary role of H_4R in depression.

The H_4R-KO model used is constitutive and it cannot be excluded that the altered neuronal functions detected on H_4R-deficient mice are a consequence of developmental effects. However,

behavioural experiments performed with mice after pharmacological H_4R stimulation within the central nervous system revealed pain-reducing, anxiolytic and orectic behaviour (Galeotti et al., 2013; Smith et al., 2007; Sanna et al., 2015), effects that were opposite to those showed by H_4R deficiency. These data, taken as a whole, let hypothesize a role for central H_4R in the modulation of neuronal functions and that the behavioural phenotype of H_4R-deficient mice is likely to be related to a role of H_4R in adult mice rather than to a consequence of developmental processes.

5. Conclusions

Present results represent the first description of the behavioural phenotype of H_4R-deficient mice. These data illustrate that H_4R modulates various neurophysiological functions such as locomotor activity, nociception, anxiety and feeding behaviour, confirming the functional role of neuronal H_4R and the importance of their integrity in the histaminergic regulation of neuronal functions. Selective stimulation of neuronal H_4R might have important clinical relevance as an innovative approach for neuropathic pain relief and for the treatment of anxiety and anxiety-related disorders.

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Conflicts of interest: none

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Fig. 1. Locomotor behaviour of H_4R-deficient mice. (A) H_4R-KO mice showed a body weight similar to wild type (WT) mice. (B) Lack of impairment of motor coordination by H_4R mutant mice. Increased of spontaneous mobility (C) and exploratory activity (D) in mice lacking H_4R. *P<0.05 in comparison with wild type mice; n=10 per group.

Fig. 2. Hypernociceptive phenotype of H_4R-deficient mice. **(A)** H_4R-KO mice showed similar pain threshold to wild type (WT) mice again thermal (plantar test, A; hot plate test, B) and mechanical (C) stimuli. Spared nerve injury (SNI) produced, 7 days after surgery, thermal (D) and mechanical (E) allodynia in the ipsilateral side of control mice that was significantly more prominent in H_4R-KO mice. *P<0.05 vs contralateral side; °P<0.05 vs ipsilateral side of wild type mice; n=10 per group.

Fig. 3. Depressant-like phenotype of H4R-deficient mice in the tail suspension test. H_4R-KO mice showed increased immobility time in comparison with wild type (WT) animals detected in the first 2 min (A), in the last 4 min (B) and in the whole 6 min duration of the test (C). Treatment with the H_4R agonist VUF 8430 (20 µg per mouse i.c.v.) did not modify the immobility time that was reduced by the antidepressant drug amitriptyline (AMI), used as reference drug (D). *P<0.05 in comparison with control mice; n=10 per group.

Fig. 4. Anxiogenic-like effect of H_4R-KO mice in the mouse light/dark box test. **(A)** H4R-KO mice showed a reduced permanence in the light compartment (A) in comparison with wild type (WT) mice. (B) The latency to the first step into the dark compartment of mutant mice was similar to that of WT mice. **(C)** The number of transitions was reduced in H_4R-KO mice. Diazepam (1 mg/kg i.p.), used as reference anxiolytic drug, increased the number of transitions. **(D)** Treatment of WT mice with the H_4R agonist VUF 8439 (20 µg i.c.v.) increased the time spent in the light chamber producing an anxiolytic-like effect comparable to that showed by diazepam. *P<0.05 in comparison with the control group; n=10 per group.

Fig. 5. Orectic phenotype of H_4R-deficient mice. The food intake values were evaluated as the cumulated amount of food eaten 60 min after the beginning of the test. (A) After 4h-food deprivation H_4R-KO mice showed a significant increase in the food consumption. (B) No difference between H_4R-KO and wild type (WT) mice was detected after 12 h-food deprivation. D-amphetamine (amph,1 mg/kg i.p.) was used as reference drug. (C) Decrease of food intake by i.c.v. administration of the H_4R agonist VUF 8430 (40 μg). **P<0.01, ***<0.001 in comparison with the control group; n=10 per group.

Fig. 6. Lack of effect of H_4R deficiency on memory processes. (A) H_4R-KO mice showed no impairment of working memory in the passive avoidance test. (B) In the novel object recognition test wild type mice reduce exploration of the familiar object in the retention test. No difference between exploration times was detected in the training session. (C) H_4R-KO mice showed an exploratory activity similar to wild type mice. No difference in the training object exploration index (D), the novel object exploration index (E) and discrimination index (F) between wild type and H_4R-KO mice. °P<0.05 in comparison with training values; *P<0.05 vs TA1; n=10 per group.

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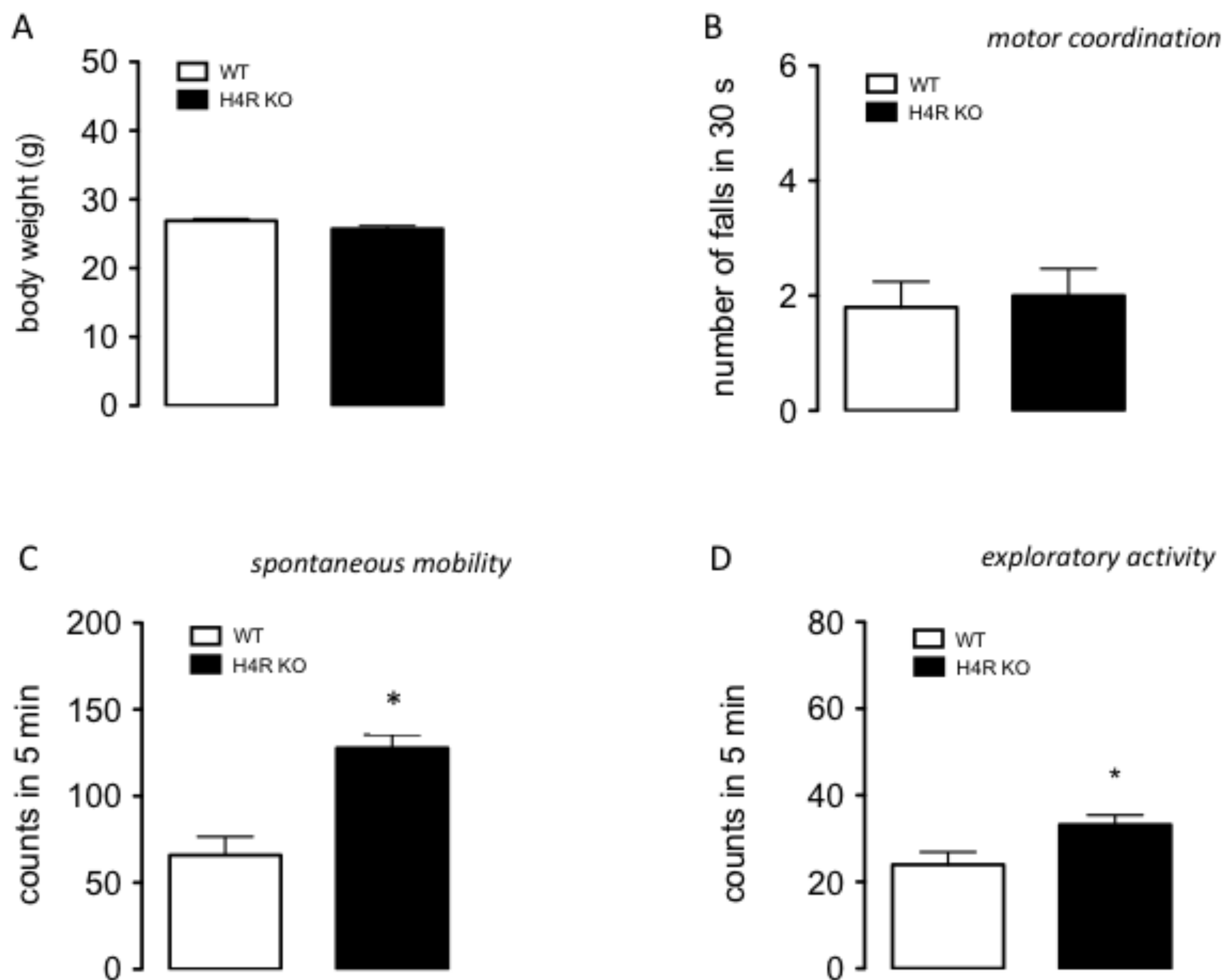

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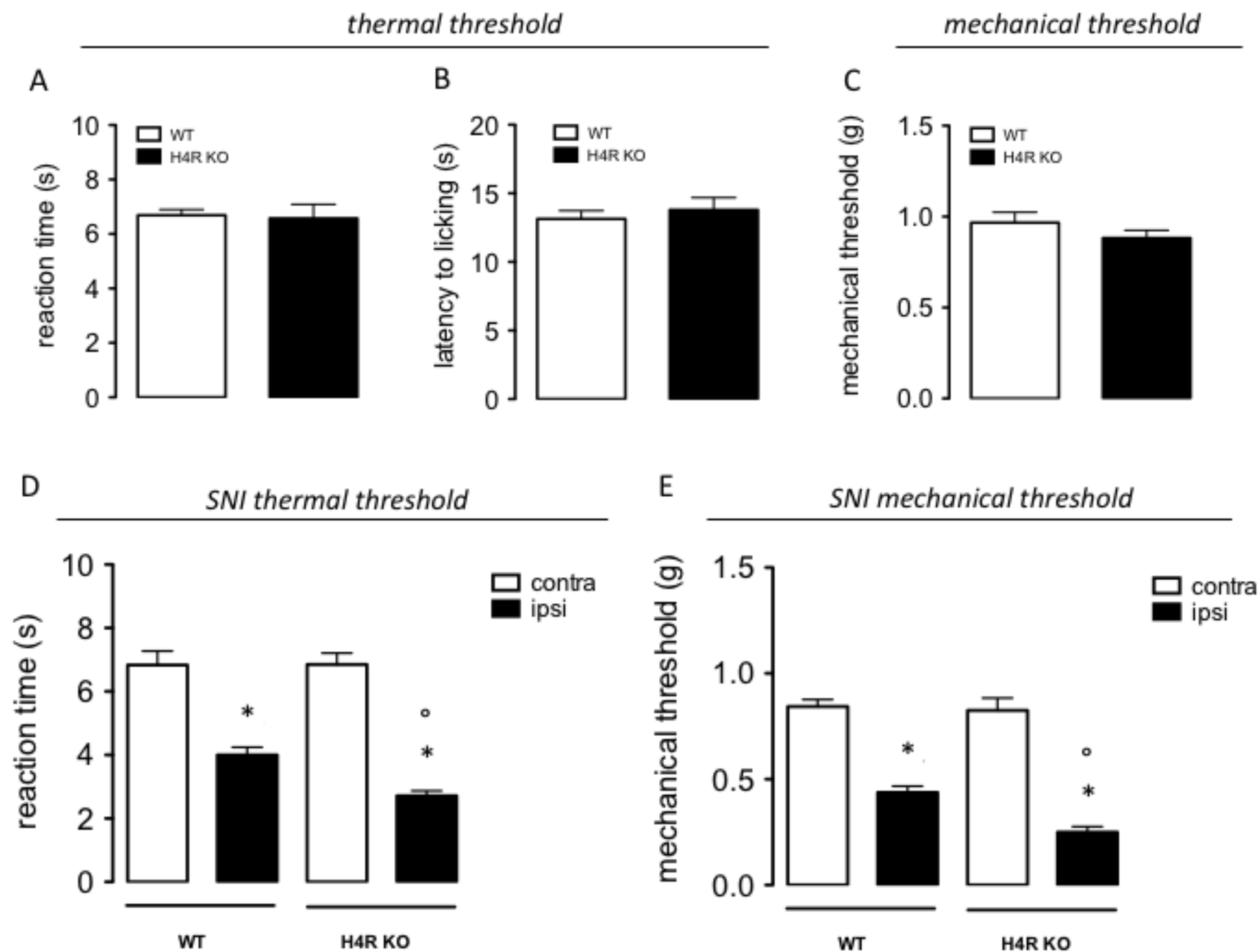

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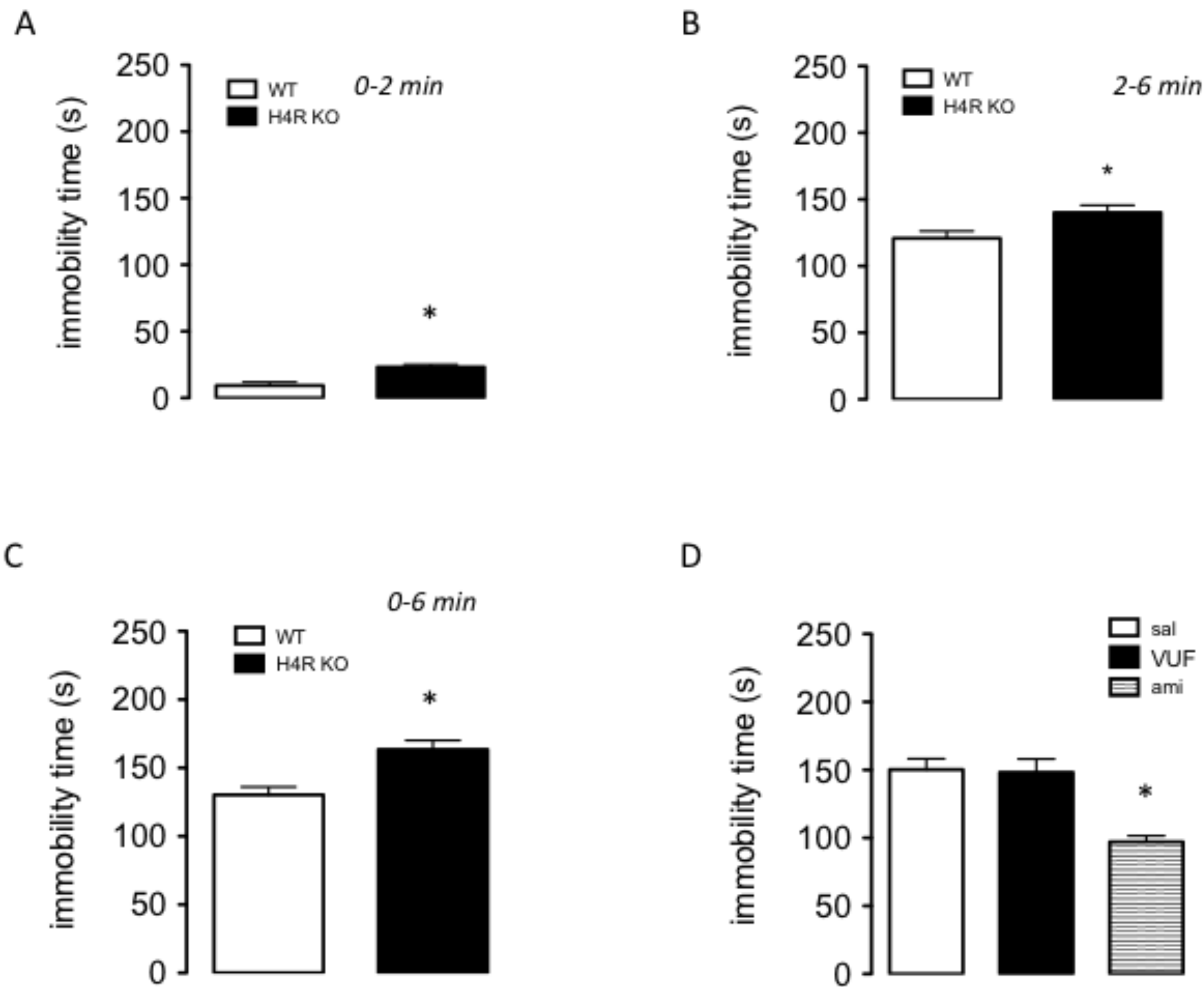

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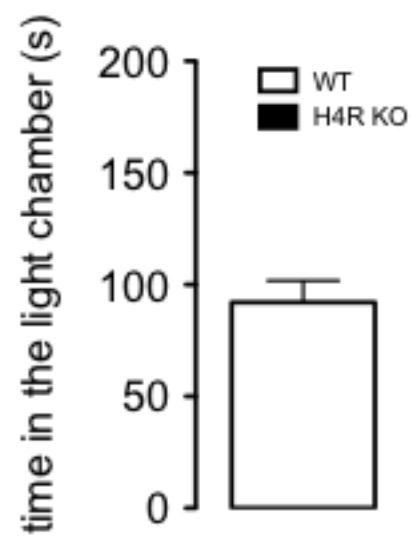

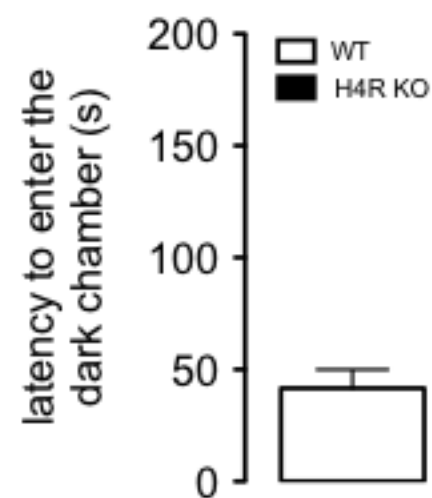

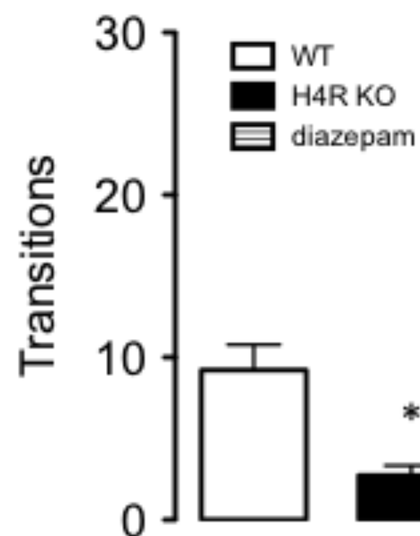

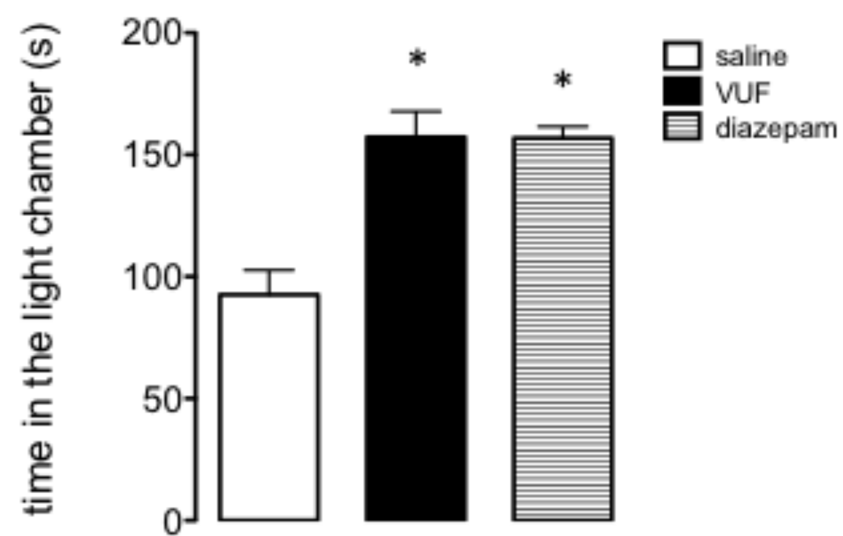

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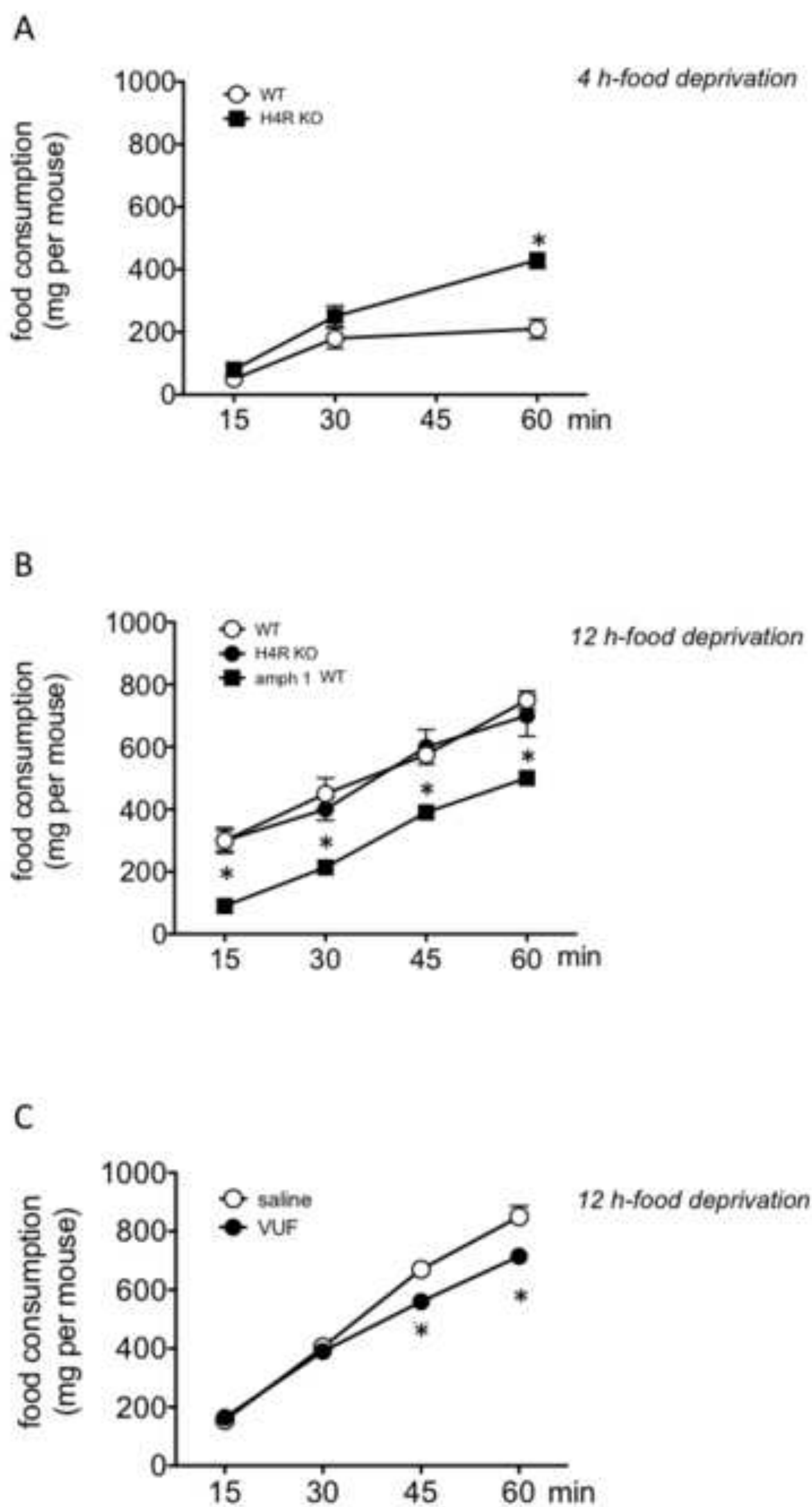

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