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EPISODIC-LIKE AND PROCEDURAL MEMORY IMPAIRMENTS IN HISTAMINE H1 RECEPTOR KNOCKOUT MICE COINCIDE WITH CHANGES IN ACETYLCHOLINE ESTERASE ACTIVITY IN THE HIPPOCAMPUS AND DOPAMINE TURNOVER IN THE CEREBELLUM

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Abstract—We investigated episodic-like (ELM) and procedural memory (PM) in histamine H1 receptor knockout (H1R-KO) mice. In order to relate possible behavioral deficits to neurobiological changes, we examined H1R-KO and wild-type (WT) mice in terms of acetylcholine esterase (AChE) activity in subregions of the hippocampus and AChE and tyrosine hydroxylase (TH) expression in the striatum. Furthermore, we analyzed acetylcholine (ACh), 5-HT and dopamine (DA) levels, including metabolites, in the cerebellum of H1R-KO and WT mice. The homozygous H1R-KO mice showed impaired ELM as compared with the heterozygous H1R-KO and WT mice. The performance of homozygous H1R-KO mice in the ELM task was primarily driven by familiarity-based memory processes. While the homozygous H1R-KO mice performed similar to the heterozygous H1R-KO and WT mice during the acquisition of a PM, as measured with an accelerating rotarod, after a retention interval of 7 days their performance was impaired relative to the heterozygous H1R-KO and WT mice. These findings suggest that, both, ELM and long-term PM are impaired in the homozygous H1R-KO mice. Neurochemical assays revealed that the H1R-KO mice had significantly lower levels of AChE activity in the dentate gyrus (DG) and CA1 subregions of the hippocampus as compared with the WT mice. The homozygous H1R-KO mice also displayed significantly reduced dihydroxyphenylacetic acid (DOPAC) levels and a reduced DOPAC/DA ratio in the cerebellum, suggesting that the DA turnover in the cerebellum is decelerated in homozygous H1R-KO mice. In conclusion, homozygous H1R-KO mice display severe long-term memory deficits in, both, ELM and PM, which coincide with changes in AChE activity in the hippocampus as well as DA turnover in the cerebellum. The importance of these findings for Alzheimer's (AD) and Parkinson's disease (PD) is discussed.

Key words: Alzheimer's disease, episodic memory, hippocampus, Parkinson's disease, rotarod.

The H1R is a metabotropic G-protein-coupled receptor, transcribed by an intronless gene and is expressed in the cortex, hippocampus, amygdala, hypothalamus, striatum and cerebellum (Lintunen et al., 1998). Histamine H1 receptor knockout (H1R-KO) mice (Inoue et al., 1996) exhibited a complex cognitive phenotype, including, both, impairments and improvements in a variety of learning and memory tasks (Dai et al., 2007; Zlomuzica et al., 2008).

The remembrance of unique personal experiences in terms of their details (what), their place (where) and temporal occurrence (when) is known as episodic memory (EM) (Dere et al., 2006) and critically depends on the hippocampus. On the other hand, procedural memory (PM) (the learning of motor skills and habits) involves more the striatum (caudate/putamen) and other brain structures, such as the cerebellum. Humans suffering from hippocampal damage (Hopkins et al., 2004) or early stages of Alzheimer's disease (AD) (Sabe et al., 1995; Small et al., 2003) exhibit impaired EM, while their PM remains intact. In contrast, early stage Parkinson's disease (PD) patients, with striatal dopamine (DA) dysfunctions, are impaired in PM, while their EM system seems preserved (Saint-Cyr et al., 1988). Interestingly, there is evidence that the cerebellum might be involved in both EM (Fliessbach et al., 2007) and PM (Molinari et al., 1997).

AD patients show changes in brain histamine levels (Panula et al., 1998) and a loss of histaminergic neurons in the nucleus tuberomammillaris, the only source of cerebral histamine (Saper and German, 1987; Airaksinen et al., 1991; Nakamura et al., 1993). Similar to AD, PD is also associated with changes in the histaminergic system. PD patients show increased brain histamine levels (Rinne et al., 2002) and changes in the activity of histidine decarboxylase (Garbarg et al., 1983). PD patients had also

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Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer's disease; DA, dopamine; DG, dentate gyrus; DOPAC, dihydroxyphenylacetic acid; ELM, episodic-like memory; EM, episodic memory; H1R-KO, histamine H1 receptor knockout; PD, Parkinson's disease; PM, procedural memory; PR, preference ratios; ROD, relative optical density; TH, tyrosine hydroxylase; WT, wild type; 5-HIAA, 5-hydroxyindole acetic acid.

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higher numbers of histaminergic fibers in the substantia nigra pars compacta. These results suggest an increase in histaminergic innervation of the substantia nigra in PD patients. We have previously demonstrated a close relationship between histaminergic and dopaminergic systems for motor performance of rats (Weiler et al., 1990; Maisonnette et al., 1998).

The hippocampus has long been implicated in EM in humans (Aggleton and Brown, 1999) and episodic-like memory (ELM) in animals (Ergorul and Eichenbaum, 2004). Recent human and animal studies suggest that hippocampal acetylcholine (ACh) and DA systems are involved in both the encoding of EM and PM (Gron et al., 2005; Hasselmo, 2006; Rammsayer et al., 2000; Schott et al., 2005). Given the involvement of the brain's histamine system in the brain pathology of AD and PD and their dissociative symptomatology in terms of EM and PM, we asked whether H1R-KO mice would show changes in ELM and/or PM. Furthermore, we examined H1R-KO mice in terms of acetylcholine esterase (AChE) histochemistry in subregions of the hippocampus, and AChE histochemistry and tyrosine hydroxylase (TH) immunohistochemistry in the striatum. Since the cerebellum has been implicated in different types of PM, such as eye-blink classical conditioning and motor-skill learning (Krakauer and Shadmehr, 2006), we analyzed ACh, 5-HT and DA levels in the cerebellum of H1R-KO and WT mice.

EXPERIMENTAL PROCEDURES

Animals

Homozygous H1R-KO mice were delivered from the Riken Research Center for Allergy and Immunology in Yokohama, Japan and were maintained at the animal breeding facilities of the University of Düsseldorf. The generation of H1R-KO mice and the absence of specific [^{3}H]pyrilamine binding (an H1R selective antagonist) in brain sections of homozygous H1R-KO mice have been described elsewhere (Inoue et al., 1996). The H1R-KO mice founder animals were backcrossed 10 times to C57BL/6J/Jcl mice. The C57BL/6J/BomTac founder animals, used as controls, were initially obtained from Taconic/Artemis, Cologne, Germany. We examined the impact of possible genetic background diversity between homozygous H1R-KO mice and C57BL/6J/BomTac control mice on behavior by comparing their performance in the episodic-like and PM tasks to that of heterozygous H1R-KO mice with a mixed genetic background. For this purpose, both male and female C57BL/6JBomTac mice were mated with homozygous H1R-KO mice to obtain heterozygous H1R-KO mice with a mixed 50% C57BL/6J/Jcl and 50% C57BL/6J/BomTac genetic background. These heterozygous H1R-KO mice were shown to express functional H1R throughout the brain, as measured by [^{3}H]-pyrilamine autoradiography in brain sections from the hypothalamus, cerebral cortex, amygdala, thalamus, hippocampus and cerebellum (Inoue et al., 1996). Consequently, the heterozygous H1R-KO mice should not exhibit a behavioral phenotype that is related to H1R-deficiency. However, since the heterozygous H1R-KO mice do express functional H1Rs (Inoue et al., 1996) and have a mixed 50% C57BL/6J/Jcl and 50% C57BL/6J/BomTac genetic background, they can be used to probe whether a possible genetic diversity between C57BL/6J mice originally obtained from Clea/Japan and those originally obtained from Taconic/Artemis would impact on the performance of mice in the behavioral tasks used (Fig. 1).

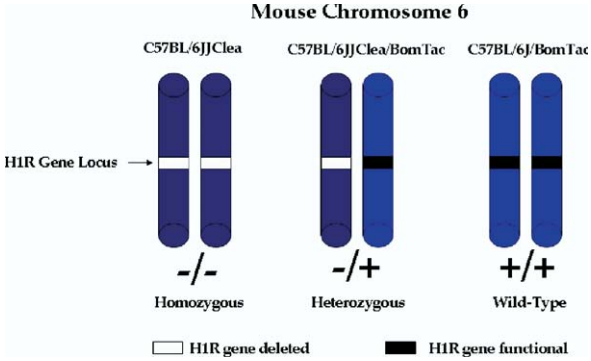

Fig. 1. Scheme of the H1R-gene locus on mouse chromosome 6 and genetic background of WT, heterozygous and homozygous knockout mice.

Our approach to control for genetic background effects still does not discard the unlikely possibility that a recessive gene other than H1R from the C57BL/6J/Jel background would be causing the phenotype, but giving the combination of our behavioral and neurochemical data, we think that possibility is improbable.

Three-months-old male homozygous H1R-KO (n=43) and C57BL/6J/BomTac mice (wild type, WT) (n=46) were used for behavioral or neurobiological experiments. Eighteen homozygous H1R-KO and 20 WT mice were tested in the ELM experiment. Thereafter, after a test pause of 2 weeks, half of these animals (nine homozygous H1R-KO and 10 WT mice) were used for the rotarod test. Other batches of eight homozygous H1R-KO and eight WT mice were subjected to neurochemical tests for changes in postmortem ACh, DA and 5-HT levels in the cerebellum. Finally, another batch of eight homozygous H1R-KO and 8 WT mice was used for AChE and TH measurements in subregions of the hippocampus and striatum (caudate/putamen).

In separate experiments the episodic-like and PM tests were repeated with other batches of male 3-month-old homozygous H1R-KO (n=15), WT (n=15) and heterozygous H1R-KO mice (n=15).

Animals were held in standard Makrolon cages (type 2: 22×16×13 cm) with metal covers and rodent chow (Ssniff, Spezialdiäten GMBH, Soest, Germany) and liquid available *ad libitum*. They were maintained on a 12-h light/dark cycle and were tested during the light phase between 9 a.m. and 6 p.m. All experiments were performed according to the guidelines of the German Animal Protection Law and were approved by the North Rhine Westphalia State Authority. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

ELM: memory for what, where and when

The test apparatus was a square open-field arena (30×30 cm) with 40-cm-high walls made of gray polyvinyl chloride, a metal floor and an open roof. Two visual cues were fixed on the east and north wall of the maze, which were intended to be used for spatial orientation. The open field was placed in a sound-attenuating cubicle. A white light bulb (60 W) was located on the ceiling of the cubicle, adjusted to provide an equal light intensity inside the open-field. The experimenter observed the animal's behavior on a screen monitor, which was connected to an overhead camera suspended above the open-field arena.

All objects were available in triplicate copies and varied in terms of surface texture, color and shape, but were of similar height (18 cm). Objects used in the sample trials were substituted by identical copies for the test trial, thus precluding unwanted

olfactory cues. The objects were made of plastic and were of sufficient weight so that they could not be displaced by the mice. None of the objects had previously been paired with a reinforcing stimulus. The objects were always placed in the corners of the open-field at a distance of 0.5 cm from the side walls. Previous work ensured that the mice were able to discriminate the different objects, and there was no, per se, preference for one of these objects. For each animal two of these three objects were randomly chosen, and the order of presentation during the sample trials was randomized.

The mice were first subjected to three daily 10-min open-field habituation trials. On the fourth day, animals received two 10-min sample trials, followed by a test trial of 5-min duration. The mice were always placed in the central part of open-field. The inter-trial interval between the three trials was 50 min. After each trial, the objects and the open-field were thoroughly cleaned with 0.1% acetic acid solution in order to remove odor cues. The open-field was virtually divided into nine squares by 2×2 parallel lines. The central square was not used for object placement. For each animal, four out of eight squares were randomly chosen to position the four copies of the "old familiar" object in the first sample trial. The second sample trial was identical to the first, except that four copies of another "recent familiar" object was present. Two copies of the "recent familiar" object were randomly placed onto positions that had been occupied in the first sample trial, and two copies were positioned in new positions, that were randomly chosen from the remaining four peripheral positions. In the test trial (trial 3), two copies of both objects were present in either stationary or displaced positions, i.e. one of the copies of each object was presented in a position encountered in the respective sample trial, i.e. sample trial 1 ("old familiar-stationary" object) or sample trial 2 ("recent familiar-stationary" object). The remaining objects were presented in new positions ("old familiar-displaced," and "recent familiar-displaced"). All four objects were placed onto positions previously encountered in the sample trials (Kart-Teke et al., 2006, 2007). Fig. 2 gives an example for the arrangement of objects during the three trials of the ELM task.

The following behaviors were scored during the sample trials and the test trial: The cumulative time spent (s) exploring the individual objects. Exploration of an object was assumed when the mouse approached an object and had physical contact with it, with its snout and/or forepaws. Sitting next to the object or leaning against the object while exploring the wall of the open-field was not considered as object exploration. The behavioral parameters were scored by an experienced observer, who was blind with respect to the mice's genotype. Object exploration was scored semi-automatically using the EthoVision tracking system (Noldus, The Netherlands), run under the "manually record behaviors" option.

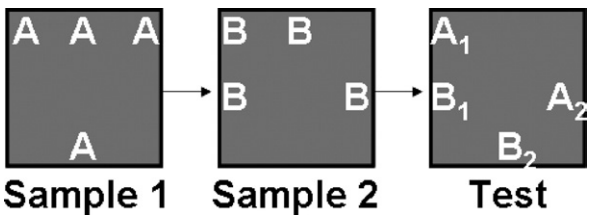

Fig. 2. Experimental design of the ELM task. This schematic drawing shows one example of a possible object arrangement for the ELM task. The mice received three 10-min trials with a 50 min inter-trial interval. During the test trial, two "old familiar" and two "recent familiar" objects, known from the sample trials, were presented at familiar and novel locations relative to the respective sample trials. A1: "old familiar-stationary"; A2: "old familiar-displaced"; B1: "recent familiar-stationary" and B2: "recent familiar-displaced."

PM: motor learning and long-term memory

Motor coordination was tested with an accelerating rotarod (TSE systems; Bad Homburg, Germany; model no.: 7650). The rotating rod was elevated 10 cm off the floor, had an axis diameter of 3.5 cm and a striated surface made of black rubber. During the acquisition phase, each mouse was given three trials (with an inter-trial interval of 10–25 min to control for possible effects of physical exhaustion) per day for three consecutive days. After a retention-delay of 7 days, the animals were given another three trials with the same inter-trial-interval as during the acquisition phase. The mouse was placed on the inactive drum, which thereafter was accelerated to a speed of 40 rpm over a period of 5 min. The mouse had to move forward on the drum, which was rotating with increasing speed along its vertical axis, in order to avoid falling off. As some mice tend to passively ride around the rod, especially at higher velocities, the duration (s) of active performance until the mouse fell off the drum was registered with a cutoff after 300 s.

ACh, DA and 5-HT levels in the cerebellum

ACh and monoamine concentrations, including metabolites and turnover ratios in the cerebellum, were determined for H1R-KO and WT mice. The animals were killed by cervical dislocation followed by decapitation. Their cerebellum was removed and weighed. Thereafter, the probe was homogenized with an ultrasonic device in ice cold 0.05 N perchloric acid containing ethylhomocholine and deoxyepinephrine as internal standards, centrifuged, filtered and kept at −80° until being analyzed. Samples were analyzed for ACh, DA, dihydroxyphenylacetic acid (DOPAC), 5-HT, and 5-hydroxyindole acetic acid (5-HIAA) using high-performance liquid chromatography with electrochemical detection (for technical details, see De Souza-Silva et al., 1997, 2000). Furthermore, 5-HIAA/5-HT and DOPAC/DA ratios were calculated as a measure for the rate of neurotransmitter turnover.

Hippocampal and striatal AChE histochemistry and TH immunohistochemistry

Tissue preparation. The mice were anesthetized with a mixture of ketamine hydrochloride (90.0 mg/kg, Ketavet, Pharmacia & Upjohn GmbH, Erlangen, Germany) and xylazine hydrochloride (8.0 mg/kg, Rompun, Bayer, Leverkusen, Germany). Their heart was exposed and the left ventricle was cannulated and the right auricle was cut. The brain was first perfused with 50 ml saline and, thereafter, with 100 ml ice-cold 4% paraformaldehyde in PBS pH 7.4 (room temperature) under constant pressure. Then the brain was extracted and postfixed in the same fixative for 3 h at 4 °C. Thereafter, the brain was washed for 1 h in PBS at 4 °C, overnight, cryoprotected with sucrose 25% in PBS, frozen by immersion in isopentane, cooled in dry ice, and stored at −80 °C. Brain coronal sections (25 μm thick) were cut with a cryostat and collected on Superfrost Plus glass slides. Adjacent sections were mounted on different slides, in order to perform different immunoreactions on alternate sections. Slides were stored at −80 °C until staining. The following brain regions were analyzed for TH and/or AChE staining: Subregions of the hippocampus (included the hilus of the dentate gyrus (DG), DG, CA1 and CA3) and the striatum (caudate/putamen).

AChE histochemistry. AChE cleaves released ACh from the synaptic cleft and, thus, limits or terminates its effects on postsynaptic cholinergic receptors. Quantitative histochemistry for AChE activity was performed as previously described (Tien et al., 2004). Briefly, sections were washed in PBS and then incubated for 15 h at 4 °C in the following solution: S-acetylthiocholine iodide (Sigma) 4 mM, ethopropazine 0.086 mM, copper sulfate 4 mM, glycine 16 mM in acetate buffer 50 mM pH5. Slides were then

rinsed in PBS and immersed in 1% sodium sulfide pH 7.5. Finally, slides were dehydrated in alcohol and coverslipped with Permount. All brains were stained at the same time with the same solutions to minimize experimental variations.

TH immunohistochemistry. TH is a synthesizing enzyme which converts tyrosine into L-DOPA, the precursor of DA. TH-expression in the striatum was measured as follows: sections were washed in TBS and incubated with a mouse monoclonal antibody against TH (Diasorin, Stillwater, USA) at a 1:5000 dilution in 10% normal bovine serum, 0.2% Triton X-100 in PBS overnight at +4 °C. After overnight incubation, sections were washed three times in PBS and then incubated with anti-mouse-biotin, conjugated (Sigma, USA) at a 1:200 dilution in 10% normal bovine serum, 0.2% Triton X-100 in PBS for 1 h at room temperature. Sections were then washed again three times in TBS and incubated in ABC (Vector Laboratory, USA) for 1 h. After three washes in TBS, the reaction was visualized with 0.1% diaminobenzidine, 0.02% hydrogen peroxide in TB 0.05 M at pH 7.4 for 10 min in the dark. The reaction was then stopped with cold TBS. Finally, slides were dehydrated in alcohol and coverslipped with Permount.

Morphometric data acquisition

All morphological analyses and staining were conducted blind. Slides were analyzed with a Zeiss Axioskop 20, equipped with a CCD high-resolution camera (Hamamatsu Photonics, Italy, C5405) and motorized XYZ stage (Proscan II, Prior). The images were captured with a 5× objective and converted by a microcomputer-assisted image analyzer (MCID Elite; Imaging Res. Inc., Canada). Tiled images over the entire filed of interest had a final resolution of 1824×1440 pixels.

AChE and TH expression levels were quantified over the entire sampled field according to the guidelines of Capowski (1989), and measured as relative optical density (ROD) units (ROD=log (256/gray level)). ROD units are correlated with the enzyme activity and antigen concentration (Burke et al., 1990).

Statistics

Behavioral and neurochemical data are expressed as mean±SEM. The rotarod data were analyzed with ANOVA procedures with repeated measures on blocks of three daily trials. Student's *t*-test for unpaired data was performed on the rotarod retention test, and the neurotransmitter and histochemical data. The total time spent exploring the four objects during the sample and test trials of the ELM memory experiment were analyzed by means of one-way ANOVA. The test-trial object exploration data were used to calculate the following preference ratios (PR) for each mouse:

PR1=Temporal order memory

Time spent exploring the old familiar stationary object/Time spent exploring the old familiar stationary+recent familiar stationary object

PR2=Object-place memory for the recent familiar objects

Time spent exploring the recent familiar displaced object/Time spent exploring the recent familiar displaced+recent familiar stationary object

PR3=Object-place memory for the old familiar objects

Time spent exploring the old familiar displaced object/Time spent exploring the old familiar displaced+old familiar stationary object

Single group *t*-tests (each against a comparison value of 0.5 ~chance level) were performed on these PRs for each group separately. Between-group differences in the above indicated PRs were analyzed by means of Student's *t*-test for unpaired data. The *P*-values given are considered to be significant when lower than 0.05.

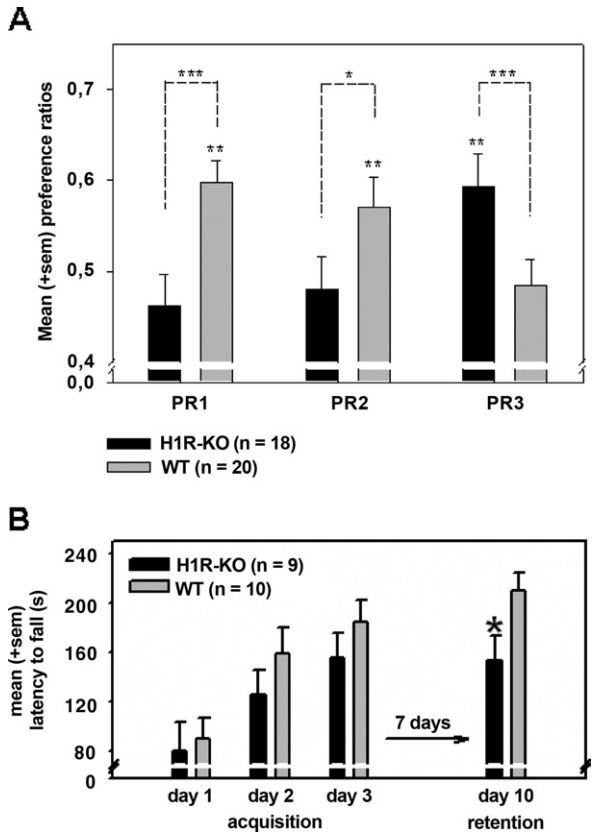

Fig. 3. (A) ELM deficits of homozygous H1R-KO mice. Bars represent mean (and SEM) PRs of H1R-KO and WT mice. ** $P<0.05$, single-group *t*-test; *** $P<0.05$, Student's *t*-test for unpaired data; * $P<0.1$, Student's *t*-test for unpaired data. (B) Long-term PM deficits of homozygous H1R-KO mice. Bars represent mean (and SEM) latency to fall on indicated days for H1R-KO and WT mice. * $P<0.05$, Student's *t*-test for unpaired data.

RESULTS

ELM in homozygous H1R-KO mice

The total time spent exploring the four objects during the sample and test trials was not significantly different between the H1R-KO and WT mice ($P>0.05$; repeated measures ANOVA; data not shown), suggesting that H1R-KO and WT mice were equally motivated to explore the objects during the sample trials and showed comparable levels of object exploration during the sample and test trials.

The WT mice were able to discriminate the temporal order in which the objects were presented and, accordingly, spent significantly more time exploring the "old familiar stationary" object as compared with the "recent familiar stationary" object (WT: PR1: $P=0.001$; single group *t*-test; Fig. 3A). In contrast, the H1R-KO mice failed to discriminate the temporal order in which the two objects had been presented (H1R-KO: PR1: $P>0.05$; single group *t*-test). Compared with the WT mice, the H1R-KO mice showed significantly lower temporal order memory scores (H1R-KO vs. WT: PR1: $P=0.003$; Student's *t*-test for unpaired data). In the group of WT mice, the mean time spent exploring the displaced copy of the recent familiar object

was significantly higher than the time spent exploring the stationary copy of the recent familiar object (WT: PR2: $P=0.046$; single group *t*-test), suggesting that the WT mice were able to remember where the recent familiar object had been placed during sample trial 2. Unlike the WT mice, the H1R-KOs failed to detect that one "recent familiar" object had been displaced to a novel position (H1R-KO: PR2: $P>0.05$, single group *t*-test). However, there was no significant difference between the WT and H1R-KO mice for the PR2 scores (H1R-KO vs. WT: $P=0.074$; Student's *t*-test for unpaired data). While there was no significant difference in the exploration times of the stationary compared with the displaced copy of the old familiar object in the WT mice (WT: PR3: $P>0.05$; single group *t*-test), the H1R-KO mice preferred the "old familiar" object in the novel position compared with the one placed in its former location known from sample trial one (H1R-KO: PR3: $P>0.026$; single group *t*-test). Consequently, the PR3 ratios of the H1R-KO mice were significantly increased as compared with the WT mice (H1R-KO vs. WT: $P=0.028$; Student's *t*-test for unpaired data). The H1R-KO spent most of the time exploring the old familiar displaced object, which obviously appeared least familiar to them, as it was not seen most recently, and, additionally, was placed in a novel location. It seems that the object-preference of the H1R-KO mice was merely guided by familiarity-based memory.

While the WT mice were able to remember the temporal order in which the objects were presented and the spatial position in which the objects had been encountered during the second sample trial, the H1R-KO mice failed to do so and, instead, showed a preference for the old familiar displaced object as compared with the other three objects. The object exploration pattern of the H1R-KO is in accord with merely familiarity-based memory performance, suggesting that the absence of the histamine H1R in C57BL/6 mice impairs their object memory for what, where and when.

ELM in heterozygous H1R-KO mice

In order to know whether the behavioral differences between the homozygous H1R-KO and the WT mice are due to the complete absence of the H1R, or due to possible differences in the genetic background, we repeated the experiment with heterozygous H1R-KOs. The heterozygous H1R-KOs, generated by the mating of homozygous H1R-KO and WT mice, express functional histamine H1Rs (Inoue et al., 1996), but have a mixed 50% C57BL/6J/Jcl and 50% C57BL/6J/BomTac genetic background. If the heterozygous H1R-KOs would perform similar to the homozygous H1R-KOs, then the differences between homozygous H1R-KO and WT mice are likely to be due to the differences in the genes of the general genetic background. On the contrary, if the heterozygous H1R-KOs would perform similar to the WT mice with a pure C57BL/6J/BomTac genetic background, then it would be more reasonable to assume that the differences between homozygous H1R-KO and WT mice are due to the complete absence of functional H1Rs in the brains of homozygous

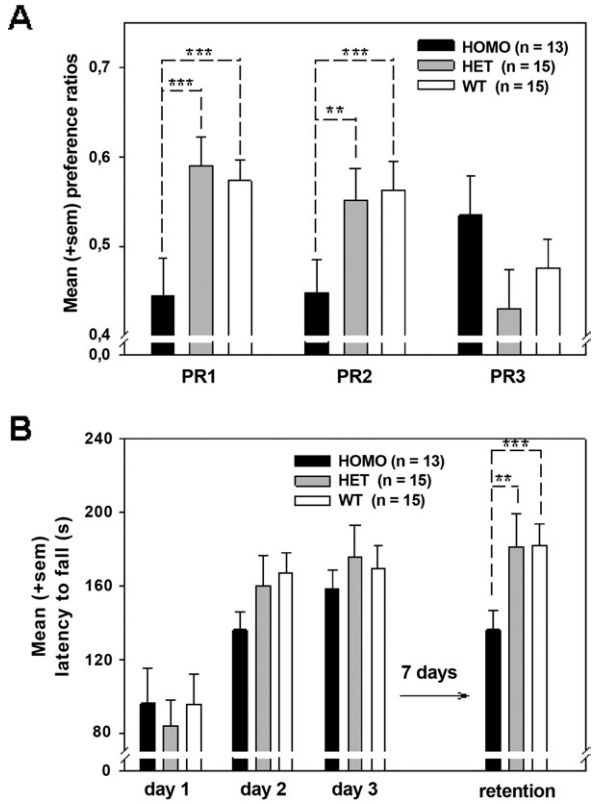

Fig. 4. (A) ELM in heterozygous H1R-KO mice. Bars represent mean (and SEM) PRs of homozygous, heterozygous H1R-KO and WT mice. *** $P<0.05$, Student's *t*-test for unpaired data; ** $P<0.1$, Student's *t*-test for unpaired data. (B) Long-term PM of heterozygous H1R-KO mice. Bars represent mean (and SEM) latency to fall on indicated days for homozygous, heterozygous H1R-KO and WT mice. *** $P<0.05$, ** $P<0.1$, Student's *t*-test for unpaired data.

H1R-KO mice and not due to differences in the general genetic background.

Two mice from the homozygous H1R-KO group showed very low levels of total test-trial object-exploration (<10 s) and were excluded from data analysis. There were no significant differences between the heterozygous H1R-KO and WT mice in the three PRs (all *P*s>0.05; Student's *t*-test for unpaired data; Fig. 4A), suggesting that ELM performance is not affected by the general genetic background of the mice. Compared with the homozygous H1R-KOs, both, the WT and the heterozygous H1R-KO mice showed significantly better temporal order memory (WT vs. homozygous H1R-KO: $P=0.013$; Heterozygous vs. homozygous H1R-KOs: $P=0.012$) and object-place memory for the recent objects (WT vs. homozygous H1R-KO: $P=0.030$; Heterozygous vs. homozygous H1R-KOs: $P=0.060$). There were no significant differences between the homozygous H1R-KO and heterozygous H1R-KO or WT mice in the PR three (both *P*s>0.05; Student's *t*-test for unpaired data). These results suggest that the general genetic background of the mice had no significant effect on the performance in the ELM task and that only the complete absence of functional H1Rs significantly impairs ELM performance.

Table 1. Mean±SEM levels of neurotransmitters and their metabolites, as well as metabolite/transmitter turnover ratios in the cerebellum of H1R-KO and WT mice

Mice	ACh	DA	DOPAC	DOPAC/DA	5-HT	5-HIAA	5-HIAA/5-HT
H1R-KO	5.75±0.27	26.34±4.87	11.86±2.45*	0.52±0.10*	310.7±28.3	220.2±14.1	0.73±0.03
WT	5.38±0.53	34.46±7.73	28.21±3.59	1.05±0.17	349.8±23.2	226.8±18.7	0.65±0.04

Abbreviations: ACh, pmol/mg; DA, pg/mg; DOPAC, pg/mg; 5-HT, pg/mg; 5-HIAA, pg/mg.
* $P<0.05$ (Student *t*-test for unpaired data).

PM in homozygous knockout mice

A repeated measures ANOVA revealed that the rotarod performance of both H1R-KO (H1R-KO: main effect of trials: $F(2, 10)=14.3$, $P<0.001$, Fig. 3B) and WT mice (WT: $F(2, 18)=23.1$, $P<0.001$) improved significantly across the 3 days of acquisition. However, there were no significant differences between H1R-KO and WT mice (H1R-KO vs. WT: main effect of genotype: $P>0.05$, genotype×trials interaction: $P>0.05$). These data suggest that, similar to a previous report (Inoue et al., 1996), neither initial motor coordination performance nor motor learning is affected in H1R-KO mice and that both groups reached a similar final performance level (*P*s>0.05; Student's *t*-test for unpaired data). Next, we asked whether the H1R-KO and WT animals might differ in terms of motor long-term memory, and subjected them to three more trials on the rotarod after a retention interval of 7 days. Here, the H1R-KOs were significantly impaired relative to the WT mice (H1R-KO vs. WT: $P=0.029$; Student's *t*-test for unpaired data).

PM in heterozygous H1R-KO mice

In order to evaluate the impact of genetic background on PM, the experiment was repeated with an additional group of heterozygous H1R-KOs. There were no significant differences between the three groups during the 3 days of rotarod acquisition (main effect of genotype: $P>0.05$, ANOVA with repeated measures; genotype×trials interaction: $P>0.05$; Fig. 4B). On the retention day, the homozygous H1R-KOs showed significantly impaired performance relative to the control groups (Homozygous H1R-KO vs. WT: $P=0.008$; Heterozygous vs. homozygous H1R-KOs: $P=0.054$; Student's *t*-test for unpaired data; Fig. 4B). There was no significant difference between the retention performance of the heterozygous H1R-KO and the WT mice ($P>0.05$). In conclusion, it seems that the general genetic background of the mice had no significant effect on acquisition and retention on the rotarod task. Furthermore, the complete absence of functional H1Rs in the homozygous H1R-KOs significantly impairs long term retention in the rotarod task.

ACh, DA and 5-HT levels in the cerebellum

We also analyzed whether the motor memory impairment of the H1R-KO mice might be associated with changes in ACh, DA and/or 5-HT levels in the cerebellum. Compared with the controls, the H1R-KO mice had significantly lower DOPAC concentrations (H1R-KO vs. WT: $P=0.003$; Student's *t*-test for unpaired data; Table 1) and a lower DOPAC/DA ratio (H1R-KO vs. WT: $P=0.024$) in the cerebellum. These results suggest that the DA turnover in the cerebellum is decelerated in H1R-KO mice. No significant differences between H1R-KO and WT were found for the other neurochemical parameters considered (all *P*s>0.05).

Hippocampal and striatal AChE histochemistry and TH immunohistochemistry

Compared with the WT, the H1R-KO mice had significantly lower levels of AChE activity in the DG (H1R-KO vs. WT: $P=0.026$; Student's *t*-test for unpaired groups; Table 2; Fig. 5A) and CA1 subregions of the hippocampus ($P=0.031$). Interestingly, compared with the WT mice, the H1R-KO mice had a thicker oriens layer in the CA1 region (H1R-KO: 103.71±6.32 μm vs. WT: 81.29±3.07 μm; $P<0.05$; Fig. 5B), whereas no significant differences were found for the pyramidal layer, stratum radiatum or lacunosum moleculare in the CA1 region (*P*s<0.01). The AChE and TH expression levels in the striatum were not statistically different between groups (*P*s>0.05).

DISCUSSION

ELM deficits in the H1R-KO mice

The homozygous H1R-KO mice showed impaired ELM as compared with the heterozygous H1R-KO and WT mice. Unlike the heterozygous H1R-KO and WT mice, the H1R-

Table 2. Mean±SEM AChE and TH expression as measured by histochemistry and immunohistochemistry and expressed as ROD units in subregions of the hippocampus and the striatum of H1R-KO and WT mice

Mice	AChE histochemistry in subregions of the hippocampus				Striatum	
	Hilus	DG	CA1	CA3	AChE	TH
H1R-KO	0.028±0.004	0.092±0.004*	0.081±0.004*	0.158±0.008	0.738±0.018	0.056±0.005
WT	0.035±0.005	0.111±0.006	0.102±0.007	0.174±0.006	0.749±0.022	0.048±0.004

* $P<0.05$, *t*-test for independent groups.

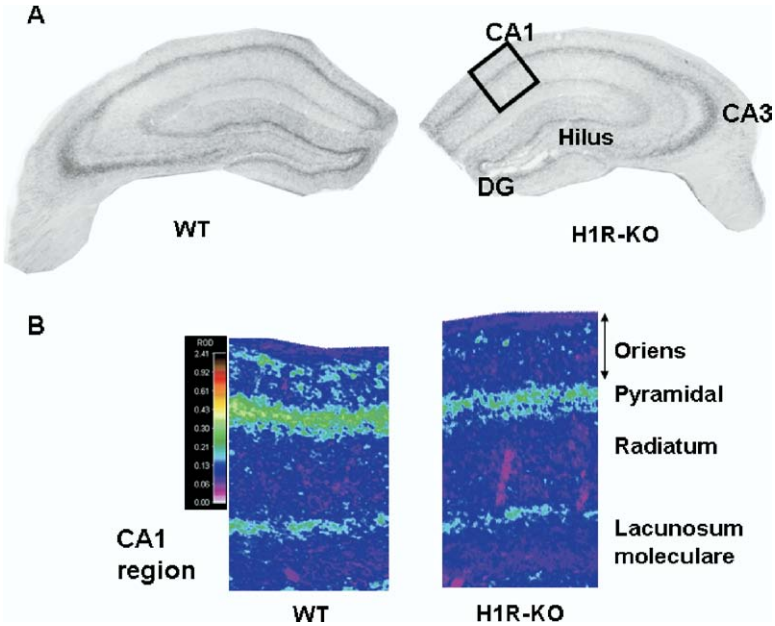

Fig. 5. (A) AChE histochemistry. Representative pictures of AChE histochemistry (upper panels) in H1R-KO mice (right panels) and WT mice (left panels). (B) Morphometric analysis of the CA1 region. Representative pictures of the CA1 region in H1R-KO mice (right panels) and WT mice (left panels). The arrow indicates an increase in thickness of the oriens layer in the H1R-KO mice.

KOs were not able to remember the temporal order in which two different objects had been encountered during the sample trials. Compared with the heterozygous H1R-KO and WT mice, the H1R-KOs also showed impaired spatial memory for the locations in which the recent familiar objects were placed during the second sample trial. In the heterozygous H1R-KO and WT mice the spatial displacement effect of the old familiar objects was blocked, suggesting an interaction between spatial and temporal factors in their ELM. In contrast, the homozygous H1R-KOs preferred the old familiar displaced object in relation to the old familiar stationary object. In fact, the homozygous H1R-KOs spent most of the time exploring the old familiar displaced object, which was not seen most recently, and, additionally, was placed in a novel location. This exploratory profile of the homozygous H1R-KOs is not compatible with ELM, and, instead, indicates familiarity-based memory.

ELM deficits of homozygous H1R-KOs coincide with changes in AChE in the DG and CA1 subregions of the hippocampus

Neuropsychological evidence suggests that the hippocampus is of utmost importance for EM (Moscovitch et al., 2005). Within the hippocampus, the information relayed from the DG to the CA3 subregion has been proposed to be used to establish configural representations, which provide the spatiotemporal context of EM and support spatial pattern separation during the retrieval of EM (Eldridge et al., 2005; Knierim et al., 2006; Rolls and Kesner, 2006). In line with these assumptions, it was shown that lesions to the CA3 region of the hippocampus impair ELM in rats (Li and Chao, 2008). CA3-lesioned rats failed to show the interaction between the temporal and spatial factors of ELM, whereas object recognition, spatial memory, or temporal order memory was similar to controls (Li and Chao, 2008). This finding suggests that the information relayed from the DG to the CA3 subregion of the hippocampus is especially important for the integration of object, spatial, and temporal information and, thus, ELM. Alternatively, it has been proposed that temporal vs. spatial processing is based on different hippocampal subregions, which could provide the foundation of EM within the hippocampus (Kesner et al., 2004). The DG and CA3 subregions of the hippocampus are thought to provide a metric spatial representation and allow spatial pattern separation, whereas the CA1 subregion is involved in temporal pattern association and temporal pattern completion. In the present study, we observed that the homozygous H1R-KOs had a thicker oriens layer in the CA1 region of the hippocampus. It remains to be determined whether the ELM deficits of homozygous H1R-KOs are related to this morphological alteration, possibly leading to specific impairments in temporal order memory.

The pharmacological manipulation of cholinergic neurotransmission affects EM in humans (Hasselmo et al., 1996) and modulates hippocampal synaptic plasticity (Ovsepian et al., 2004), which might subserve the encoding of new episodic memories (Hasselmo, 2006). Furthermore, it is known that AChE inhibitors improve cognitive symptoms in AD patients (Riepe, 2005) and patients with amnestic mild cognitive impairment (Gron et al., 2006). We therefore examined whether the ELM deficits of H1R-KO mice are correlated with changes in levels of AChE in the DG, CA3 and CA1 subregions of the hippocampus. The homozygous H1R-KOs had significantly lower levels of AChE in the DG and CA1 region as compared with the WT

mice. It is known that reduced AChE activity is an indicator of cholinergic impairment in AD (Eggers et al., 2006; Herholz et al., 2005). In contrast, hippocampal ACh levels, in the H1R-KOs and WT mice were similar (Zlomuzica et al., 2008). It is possible that the reduction in AChE expression reflects a compensatory response, which masks a cholinergic deficit in the homozygous H1R-KOs. Given that H1R-KOs exhibited deficits in ELM, such a compensatory response, similar to the one seen in AD patients, who also exhibit decreased AChE levels, would seem not to be effective in terms of preventing memory impairments. It is, therefore, possible that the ELM deficits of the homozygous H1R-KOs might be related to a dysfunctional cholinergic innervation of the hippocampus, as reflected by decreased AChE activity in the DG and CA1 subregions of the hippocampus. Future studies are needed to specify how exactly the changes in cholinergic metabolism in the DG and CA1 regions of the homozygous H1R-KOs affect information processing in the hippocampus, and how changes in hippocampal functioning translate into deficits in ELM. Furthermore, it remains to be determined whether the administration of cholinergic drugs, which are effective in ameliorating cognitive symptoms in AD, to homozygous H1R-KO mice would ameliorate their ELM deficit.

The stratum oriens is composed of the basal dendrites of pyramidal cells, few interneurons, and fibers from (i) CA3 region (ii) contralateral hippocampus (via commissure) and (iii) septum. The latter are mainly cholinergic fibers. Therefore, the change in the thickness of the stratum oriens might be related to a quantitative change in the density of the cholinergic innervation of the CA1 region.

Long-term PM deficits of homozygous H1R-KO mice

We also investigated whether homozygous H1R-KOs would show changes in PM in terms of motor coordination learning and motor long-term memory using the accelerating rotarod task. The homozygous H1R-KOs performed similar to the heterozygous H1R-KO and WT mice during the acquisition of the rotarod task. Their performance improved significantly across the 3 days of acquisition and their final performance level was similar to the heterozygous H1R-KO and WT mice. After a retention interval of 7 days, however the homozygous H1R-KOs showed lower performance scores relative to the heterozygous H1R-KO and WT mice. These findings suggest that, besides ELM, also motoric long-term memory is impaired in the homozygous H1R-KO mice.

PM deficits of homozygous H1R-KOs are not related to cholinergic or dopaminergic parameters in the striatum

Since the striatum has been implicated in motor coordination on the rotarod (Lindgren et al., 2007), we asked whether the homozygous H1R-KOs would exhibit changes in AChE and TH levels in the striatum. However, striatal AChE and TH levels were similar between homozygous H1R-KO and WT mice. Previously, we showed that neither ACh nor DA levels, including metabolites, are altered in the striatum of homozygous H1R-KO mice (Zlomuzica et al., 2008). Therefore, we asked whether the present results are related to neurochemical changes in the cerebellum of homozygous H1R-KOs.

Both ELM and PM deficits of homozygous H1R-KOs might be due to changes in DA turnover in the cerebellum

There is evidence that H1R-related agents modulate the neurotransmission within the dopaminergic systems. Recently it has been reported that H1R activation results in the modulation of dopaminergic transmission associated with stereotyped behavioral patterns induced by methamphetamine (Kitanaka et al., 2007). Furthermore, various H1-receptor antagonists inhibit neuronal uptake of DA after alpha-methyl-p-tyrosine-induced depletion of DA in the mouse brain (Oishi et al., 1994). We have shown that systemic application of H1R-antagonists increases the extracellular levels of DA and decreases the levels of its metabolites DOPAC and HVA in the neostriatum and nucleus accumbens of anesthetized rats (Dringenberg et al., 1998).

Besides the striatum, the cerebellum has also been implicated in motor learning (Evans, 2007; Molinari et al., 1997; Schlett et al., 2004) and motor memory consolidation (Krakauer and Shadmehr, 2006).

In order to know whether the long-term motor-memory deficit of homozygous H1R-KOs is due to neurochemical alternations in the cerebellum, we analyzed the levels of ACh, DA and 5-HT, as well as their metabolites, in homozygous H1R-KO and WT mice. ACh and 5-HT levels were similar in homozygous H1R-KO and WT mice. In contrast, DOPAC levels and the DOPAC/DA ratio were significantly reduced in the cerebellum of homozygous H1R-KOs, suggesting that the DA turnover in the cerebellum is decelerated in homozygous H1R-KOs. It is possible that the long-term memory deficit of the homozygous H1R-KOs is related to these changes in cerebellar DA parameters.

There is evidence that the cerebellum might also be involved in EM (Fliessbach et al., 2007). The cerebellum might contribute to the temporal component of EM. It has been proposed that the cerebellum, together with the basal ganglia and the prefrontal cortex, is involved in temporal order memory (Lalonde and Hannequin, 1999) and that DA is an important modulator of this function (Hotte et al., 2005). Previously, we showed that ACh and monoamine concentrations in the hippocampus and striatum of homozygous H1R-KO mice and WT mice are not significantly different (Zlomuzica et al., 2008). Therefore, it remains possible that, both, ELM and PM deficits of homozygous H1R-KOs are related to changes in DA turnover in the cerebellum.

Are cognitive deficits in Alzheimer's and Parkinson's disease due to pathological changes in the histamine system?

Beside the well-known brain pathologies in AD, such as degeneration of cholinergic systems, extracellular amyloid plaques, and intracellular neurofibrillary tangles, AD patients also show dysfunctions in the histaminergic system (Mazurkiewicz-Kwilecki and Prell, 1984; Mazurkiewicz-Kwilecki and Nsonwah, 1987). Compared with age-

matched non-demented controls, AD patients show changes in brain histamine levels (Cacabelos et al., 1989; Mazurkiewicz-Kwilecki and Nsonwah, 1989; Panula et al., 1998). AD is also associated with a loss of histaminergic neurons in the nucleus tuberomammillaris, the only source of cerebral histamine (Saper and German, 1987; Airaksinen et al., 1991; Nakamura et al., 1993). Most importantly, tacrine (an inhibitor of cholinesterase), which is prescribed to AD patients during early stages of the disease, inhibits the catabolic activity of histamine-N-methyltransferase, which normally degrades histamine to tele-methylhistamine, and, thereby, increases hippocampal histamine levels (Nishibori et al., 1991; Morisset et al., 1996). In contrast to tacrine, physostigmine is less effective in ameliorating AD symptoms and has a lower affinity to histamine-N-methyltransferase (Nishibori et al., 1991).

Similar to AD, PD is also associated with changes in the histaminergic system. PD patients show increased brain histamine levels (Rinne et al., 2002) and changes in the activity of histidine decarboxylase, the enzyme which synthesizes histamine from L-histidine (Garbarg et al., 1983). Examination of the distribution of histaminergic fibers in the substantia nigra in postmortem brain samples PD patients revealed increased density of histaminergic fibers in the middle portion of substantia nigra, pars compacta and reticulata. The morphology of histaminergic fibers was also altered in PD patients. They had thinner fibers and enlarged varicosities at histaminergic terminals (Anichtchik et al., 2000). These results suggest an increase of histaminergic innervation of the substantia nigra in PD patients.

Conclusions

In the present study, we demonstrated that homozygous H1R-KO mice display severe long-term memory deficits in both ELM and PM. These memory deficits coincide with changes in AChE activity in the DG and CA1 subregions of the hippocampus as well as DA turnover in the cerebellum. Given that AD and PD patients show cognitive deficits along with changes in parameters of the histaminergic system, it is tempting to speculate that some of their cognitive symptoms might be related to changes in H1R expression, their sensitivity or function. Indeed, it has been shown that H1R levels in the frontal and temporal areas in brains of AD patients are decreased as compared with the healthy aged subjects. Moreover, the H1R binding was correlated with the severity of cognitive symptoms in AD patients within several brain areas (Higuchi et al., 2000). It would, therefore, be interesting to know whether H1R-agonists can ameliorate cognitive deficits in animal models of AD or PD.

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