ris, pectoralis superficial, and masseter muscles),[12] but also in other organs and tissues (such as lung, spleen, and bone marrow),[14] indicating that PW may produce various effects on the whole body *via* the induction of HDC. However, in the present experiments, we observed no significant HDC elevation in brain after various time-points of PW in C57BL/6 mice (data not shown). In the present study, the peripheral H1 antagonist, fexofenadine, significantly reduced both PW-endurance and the muscle levels of NO metabolites and glycogen seen after 3 h walking. Thus, it may be the 'peripheral' effects of histamine (including effects in skeletal muscles) that are largely involved in favouring endurance and protecting against exhaustion.

Our present findings suggest that histamine is involved in protecting against exercise-induced fatigue or exhaustion. We previously reported that training decreases the magnitude of exercise-stimulated HDC induction,[14] and that starvation (which may promote fatigue) augments HDC induction in mice performing PW.[14] In the present study, the PW-endurance of H1-KO mice was less than that of WT C57BL/6 mice (Fig. 5B), while after PW the muscle HDC induction was larger in H1-KO mice than in WT mice (Fig. 5C). It is supposed that hypoxia might be an important factor to induce muscle fatigue. Indeed, a recent report has demonstrated that hypoxia-inducible factor-1 (HIF) has an HDC-inducing activity,[34] suggesting that the less PW-endurance of H1-KO mice might be due to a larger production of HIF due to hypoxia. Collectively, the results obtained by the present study may also support the idea that (i) histamine or HDC induction may be important to reduce exercise-induced fatigue as a protective mechanism, and (ii) mice with a lower PW-endurance may need more HDC induction against hypoxia due to PW.

We speculate upon the mechanism underlying the protective effect of histamine as follows. It is known that histamine dilates arterioles and enhances capillary permeability *via* stimulation of H1 receptors and the ensuing production of NO, and these effects may help to promote the supply of nutrients and O_2 and the removal of waste products, including CO_2. In addition, Li *et al.* have reported that the induction of eNOS by histamine in human vascular endothelial cells is mediated by stimulation of H1-receptors.[35] We previously showed that in mice PW also induces HDC in other peripheral organs with highly developed microcirculations (lung, spleen, and bone marrow).[14] Thus, HDC induction not only in skeletal muscles, but also in other peripheral organs, may help to prevent exercise-induced fatigue and/or aid the recovery from fatigue. Indeed, exercise-induced muscle NO production was significantly inhibited by fexofenadine, a peripheral H1 antagonist (Fig. 6). Therefore, HDC induction in skeletal muscles in response to exercise may be one of the defense mechanism that protects against exercise-induced fatigue or exhaustion. These ideas may be supported in part by the finding made in humans by Lockwood *et al.*[16] that H1 receptor-mediated vasodilation contributes to post-exercise hypotension and muscle hyperemia.

In conclusion, although the detailed mechanisms remain to be clarified, the results presented here support the ideas that in mice: (i) histamine is involved in protecting against exercise-induced fatigue or exhaustion, (ii) histamine exerts its protective effect *via* H1 receptors and the ensuing production of NO in skeletal muscle, and (iii) histamine is provided, at least in part, by HDC induction in skeletal muscles during prolonged exercise.

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ORIGINAL RESEARCH

Histamine Receptor Expression, Hippocampal Plasticity and Ammonia in Histidine Decarboxylase Knockout Mice

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Abstract Genetic ablation of the histamine producing enzyme histidine decarboxylase (HDC) leads to alteration in exploratory behaviour and hippocampus-dependent learning. We investigated how brain histamine deficiency in HDC knockout mice (HDC KO) affects hippocampal excitability, synaptic plasticity, and the expression of histamine receptors. No significant alterations in: basal synaptic transmission, long-term potentiation (LTP) in the Schaffer collateral synapses, histamine-induced transient changes in the CA1 pyramidal cell excitability, and the expression of H1 and H2 receptor mRNAs were found in hippocampal slices from HDC KO mice. However, when compared to WT mice, HDC KO mice demonstrated: 1. a stronger enhancement of LTP by histamine, 2. a stronger impairment of LTP by ammonia, 3. no long-lasting potentiation of population spikes by histamine, 4. a decreased expression of H3 receptor mRNA, and 5. less potentiation of population spikes by H3 receptor agonism. Parallel measurements in the hypothalamic tuberomamillary nucleus, the origin of neuronal histamine, demonstrated an increased expression of H3 receptors in HDC KO mice without any changes in the spontaneous firing of "histaminergic" neurons without histamine and their responses to the H3 receptor agonist (R)-α-methylhistamine. We conclude that the absence of neuronal histamine results in subtle changes in hippocampal synaptic transmission and plasticity associated with alteration in the expression of H3 receptors.

Keywords Long-term potentiation · Afterhyperpolarisation · Ammonia · Tuberomamillary nucleus · (R)-α-methylhistamine

Introduction

The brain histaminergic system originates from the tuberomamillary nucleus of the hypothalamus (TMN) projecting to virtually all brain regions (Panula et al. 1989). Histamine released in the target areas activates three types of G protein-coupled receptors: postsynaptic H1-receptors using mainly phospholipase C and IP3, postsynaptic H2 receptors using cyclic AMP for signaling and presynaptic H3 receptors negatively coupled to Ca^{2+} channels and adenylyl cyclase. In the TMN H3 receptors function as autoreceptors controlling firing, synthesis and release of histamine (Schwartz et al. 1990), while H3 heteroreceptors modulate the release of many other neurotransmitters (Haas et al. 2008). Histaminergic neurons display highest firing rates during attentive waking and are silent during sleep (Takahashi et al. 2006). Lesions and pharmacological manipulations in the TMN and its projection areas, as well as the knockout of genes encoding histamine receptors revealed a role of the histaminergic system in learning and memory processes (Dere et al. 2003; Passani et al. 2004).

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Mice lacking histidine decarboxylase (HDC), the histamine-producing enzyme, show subtle abnormalities in locomotion, a lower level of arousal and exploratory activity, impaired learning in some tests, and better learning in others (Dere et al. 2003; Liu et al. 2007; Parmentier et al. 2002). The hippocampus plays an essential role in associative learning. Histamine exerts prominent actions in this structure at both cellular and network levels. The CA1 and CA3 pyramidal cells in rat hippocampal slices are excited for long periods via postsynaptic H2 receptors (Brown et al. 1995; Yanovsky and Haas 1998) and the cAMP-PKA intracellular cascade (Selbach et al. 1997). Hippocampal network activity, involved in cognitive processes—fast 200 Hz oscillations (ripples), gamma-oscillations, and theta-rhythm are modulated via activation of H1 (Knoche et al. 2003; Ponomarenko et al. 2003) H2- (Atzori et al. 2000) and H3-receptors (Andersson et al. 2010; Hajos et al. 2008). Histamine can induce and facilitate long-term potentiation in the CA1 area (Brown et al. 1995; Haas et al. 2008; Luo and Leung 2010; Selbach et al. 1997), and the knockout of either H1 or H2 receptors reduces its magnitude (Dai et al. 2007).

We have now studied the expression and function of histamine receptors in the hippocampus in the absence of neuronal histamine by analysing the levels of mRNA for histamine receptors, basal synaptic transmission, its plasticity, and histamine effects on synaptic plasticity and excitability.

Methods

Animals

Histidine decarboxylase knock-out mice (HDC-KO) were generated and maintained as previously described (Ohtsu et al. 2001; Parmentier et al. 2002). All experiments were conducted according to German law and the local guidelines (Bezirksregierung Duesseldorf) and were in accordance with the European Communities Council directive regarding care and use of animals for experimental procedures. All efforts were made to minimize the number of animals and their suffering. Male HDC-KO and wild type (WT) littermates aged 6–10 weeks were used for electrophysiological experiments on hippocampal slices and RT-PCR analysis, younger (14–28 days) littermates were used for loose patch recordings of TMN neuronal activity.

Slice Preparation

After decapitation the brain was rapidly removed from the skull and immersed in ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 125 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 26 $NaHCO_3$, and 10 D-glucose (pH 7.4, 95% O_2/5% CO_2). Coronal hypothalamic slices (300 μm) containing the TMN or horizontal brain slices (400 μm) containing the hippocampus, were prepared with a vibratome. Slices were preincubated for at least 2 h at room temperature before the recording of spontaneous cell activity (TMN) or field and intracellular potentials (hippocampus).

Electrophysiology

Hippocampal field responses, excitatory postsynaptic potentials (fEPSPs), or population spikes (PS), were evoked by stimulation of the Schaffer collaterals and recorded in the CA1 stratum radiatum or stratum pyramidale, respectively, with ACSF-filled low-resistance micropipettes in a submersion-type recording chamber continuously perfused with ACSF at a flow rate of 1.5–2 ml/min at 32 °C. After the initial testing of stimulus—response relationships, the stimulus intensity was adjusted to induce the postsynaptic response of about 30–50% of its maximal value, and stimulation frequency was set to 0.033 Hz. Long-term potentiation (LTP) of synaptic responses was induced by either two 1-sec trains of high-frequency stimuli (HFS, 100 Hz) or theta-burst stimulation (TBS, 10 or 4 bursts of 5 stimuli at 100 Hz with 200 ms interburst intervals) at the test intensity. Signals were amplified, digitized at 10 kHz, and stored on a PC hard disk for further off-line analysis using pClamp8 software (Axon Instruments). Field EPSP slope was measured by straight line fitting, PS amplitude was measured as an average of its minimum (from the early positivity peak to the peak negativity) and maximum (from the peak negativity to the late positivity peak) values. All values were normalized to baseline, the mean value for 15–20 min period before tetanus or drug application. Every group of experiments included slices from at least three animals.

Loose patch recordings of spontaneous firing of TMN neurons were obtained using glass microelectrodes filled with ACSF (resistance 4–8 MΩ) and 0.1% biocytin. Neurons were recorded in the ventro-lateral, the most dense part of the TMN, which was visually identified under a dissecting microscope. Signals were recorded using an Axoclamp 2B amplifier and a Digidata 1200 interface board (Axon Instruments, USA), filtered between 0.5 and 10 kHz, sampled at 20 kHz and analyzed with pClamp8 software (Axon Instruments, USA). The frequency of extracellular action potentials was determined online in bins of 15 s duration.

Real-Time RT-PCR Analysis of Histamine Receptor Expression

Histamine receptor-expression in HDC KO and WT mice was performed according to the previously described

protocols. In brief, total cellular mRNA was isolated from the slices of the hippocampus and posterior hypothalamic region prepared from the brain of KO ($n = 9$) and WT ($n = 7$) mice using an mRNA isolation kit (Pharmacia Biotech) in accordance with the manufacturer's protocol. The PCR was performed in a PE Biosystems GeneAmp 5700 sequence detection system using the SYBR green master mix kit. Semiquantitative analysis of histamine receptor expression relative to the β-actin endogenous control was performed according to the "$2^{-\Delta\Delta Ct}$" (ΔFold) method (Sergeeva et al. 2003). Primers for the histamine receptors were: H1up: 5′-CAA AGG AAA AGA GGT TCC TGG-3′; H1lo 5′-GTC ACC CTC TGT GGA CAG GT-3′. H2up 5′-AAC AGC AGA AAT GGG ACC AG-3′; H2lo: 5′-CCT AAG AGA GCC AGC CAT TG-3′, H3up: 5′-CCA AAG CAT CAC CCA GCG C-3′ and H3lo: 5′-CAC AGA AGC CAG AAG GAC GTC TCG-3′.

Data Analysis

All data are presented as mean ± standard error of mean (SEM). Statistical analysis was performed using *t*-tests provided by the GraphPad Prism software (San Diego, CA, USA).

Drugs

Histamine dichloride, thioperamide maleate, and ammonium chloride were purchased from Sigma, impromidine-from SKF (R)-(-)-α-methylhistamine dihydrobromide from Tocris.

Results

Histamine Deficiency Does Not Affect Basal Synaptic Transmission and LTP in the Schaffer Collateral Synapses

The analysis of fEPSPs recorded in the CA1 stratum radiatum in response to Schaffer collateral stimulation showed no significant difference between KO and WT mice in the basal synaptic transmission (Fig. 1a, b) and the magnitude or time course of LTP induced by standard tetanus protocols (Fig. 1c, d). After two HFS trains the magnitude of LTP measured at 50–60 min postHFS was 170 ± 5% of baseline in KO ($n = 13$) and 175 ± 5% ($n = 15$) in WT ($P = 0.44$). Moderate TBS (10 bursts) produced LTP with a magnitude of 153 ± 14%, $n = 5$ in KO and 147 ± 9%, $n = 5$ in WT ($P = 0.70$), and single weak TBS (4 bursts) 128 ± 4% and 124 ± 8%, $n = 5$ each, in KO and WT, respectively ($P = 0.65$).

Histamine-Deficient Mice Show Stronger Facilitation of Hippocampal LTP by Histamine

Histamine at 10 μM, given 10 min before and during weak TBS slightly enhanced LTP of field EPSPs in the CA1 area of hippocampal slices from WT mice (from 124 ± 8%, $n = 5$, to 138 ± 5%, $n = 6$, $P = 0.15$) and significantly enhanced LTP in HDC KO mice (from 128 ± 4%, $n = 5$, to 157 ± 8%, $n = 6$, $P = 0.003$) (Fig. 2a–c).

Brain Histamine Deficit Exacerbates the Impairment of Hippocampal LTP by Ammonia

We have found previously that exposure to ammonia, a major pathophysiological factor in hepatic encephalopathy (Butterworth 2002; Haussinger et al. 2002), impairs LTP in the CA1 area of murine hippocampus (Chepkova et al. 2006). Hepatic encephalopathy is associated with a significant increase in the brain level of histamine (Lozeva et al. 2003). Therefore, we have assessed the sensitivity of hippocampal LTP to ammonia in histamine-deficient mice. After 4 h preincubation with 1 mM ammonium chloride the hippocampal slices from both WT and KO mice showed a significant decrease in the amount of potentiation at 80–90 min postHFS (Fig. 3a, b). In WT slices the potentiation decreased to 134 ± 6%, $n = 7$ vs. 165 ± 4%, $n = 12$ in control and in KO slices to 110 ± 8%, $n = 11$ vs. 153 ± 5% in control. Thus, although the magnitude and time course of HFS-induced LTP in the slices from KO and WT mice were similar under standard conditions, exposure to ammonia resulted in a stronger impairment of LTP in KO (Fig. 3c).

Histamine Effects on the Excitability of Hippocampal Pyramidal Neurons in Histamine-Deficient Mice

Histamine (10 μM, 10 min) potentiated synaptically evoked population spikes to a similar extent in hippocampal slices from HDC KO ($n = 5$) and WT ($n = 7$) mice, to 122 ± 8% and 136 ± 8% of baseline, respectively. However, in the KO preparations histamine-induced potentiation was easily reversible and followed by a depression to 80 ± 3% of baseline, whereas in the WT slices, potentiation persisted for a long time (123 ± 9% of baseline after 40–50 min washout) (Fig. 4). The difference between the two genotypes in the long-lasting histamine effects was highly significant ($P = 0.0007$).

An increased excitability of the CA1 pyramidal neurons after activation of H2 receptors is associated with a transient depolarization and suppression of the slow afterhyperpolarization (sAHP) (Haas and Konnerth 1983; Selbach et al. 1997). Intracellular recordings of the CA1 pyramidal neurons in hippocampal slices from HDC KO and WT

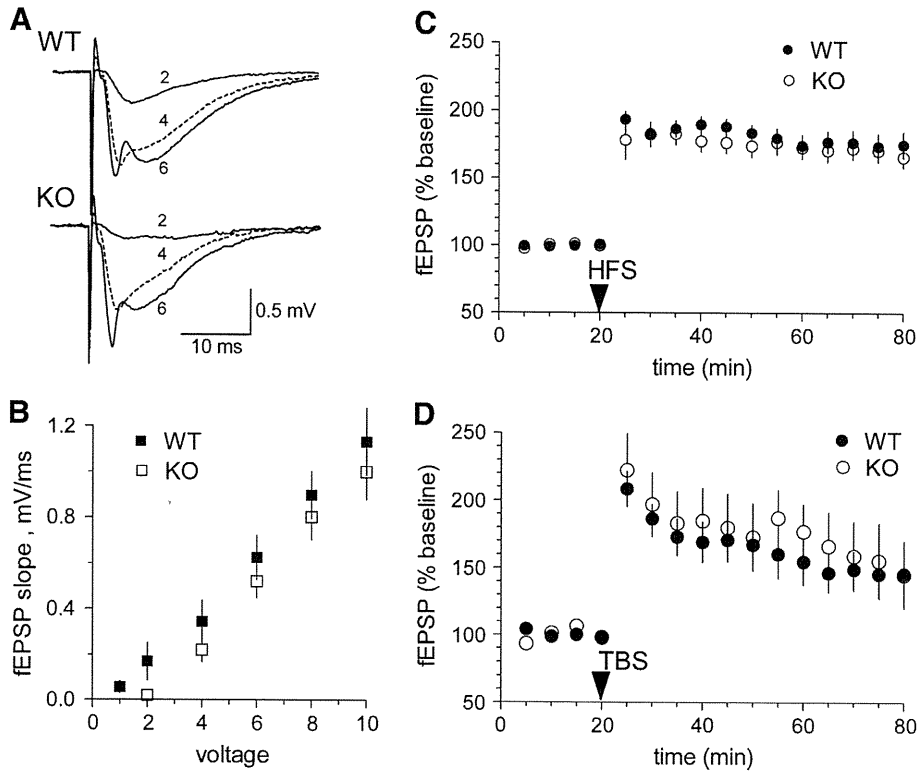

Fig. 1 Histamine-deficient mice show no abnormalities in basal synaptic transmission and synaptic plasticity in the Schaffer collaterals–CA1 pathway. **a** representative examples of stimulus—response relationships in hippocampal slices from WT and KO mice; each trace represents the average of three responses to the stimulus at 2, 4, and 6 V, respectively. **b** Summary of averaged Schaffer collateral–CA1 field EPSPs evoked at increasing intensities as shown in (**a**). **c** time course of LTP induced by high frequency stimulation (HFS, 2 trains of 100 stimuli at 100 Hz). **d** Time course of LTP induced by moderate theta-burst stimulation (TBS, ten bursts of five stimuli at 100 Hz and test intensity separated by 200 ms intervals)

mice revealed no difference between them in the transient suppression of sAHP by histamine (5 μM) or the H2 agonist impromidine (3 μM) (Fig. 5).

Expression of Histamine Receptors in The Hippocampus and Hypothalamic Tuberomamillary Nucleus

Small and non-significant increases were seen in the expression of H1 (3.0 ± 0.8, $n = 9$ vs. 2.0 ± 0.31 $n = 6$) and H2 (2.8 ± 0.48, $n = 10$ vs. 2.07 ± 0.47, $n = 5$) receptor transcripts in the hippocampus of KO versus WT mice, whereas the expression of H3 receptors in the KO hippocampus was significantly ($P < 0.05$) decreased—to 60% of the WT level (2.9 ± 0.35, $n = 10$ in KO vs. 4.88 ± 0.7, $n = 6$ in WT). Since a facilitating effect of histamine on LTP is likely mediated by allosteric modulation of the NMDA receptor (Brown et al. 1995), we also measured the hippocampal expression of GluN2B (formerly NR2B) transcripts, a histamine-sensitive NMDA receptor subunit (Vorobjev et al. 1993; Williams 1994). No difference in the expression of this transcript was found between HDC KO (1.8 ± 0.3, $n = 10$) and WT (1.9 ± 0.4, $n = 6$) mice.

Next we determined the levels of H1, H2, and H3 receptor mRNA in the TMN. The expression levels of H1- and H2-receptor transcripts in this structure did not differ significantly between HDC KO and WT mice (Fig. 6a), whereas the level of H3 receptor mRNA in KO was nearly two times higher than in WT ($P < 0.05$). Thus, the expression of H3 receptors underwent opposite changes in hippocampus and TMN.

Functional Consequences of Altered Expression of H3 Receptors

Looking for possible functional correlates of altered expression of H3 receptors in the hippocampus, we tested the effects of the H3 receptor agonist (R)-(-)-α-methylhistamine ((R)-α-MeHA) on synaptically evoked population spikes in the CA1 area. In 10 of 14 hippocampal slices from WT mice 10 min perfusion with 2 μM (R)-α-MeHA caused slowly developing and persistent changes in response amplitudes with long-lasting potentiation ($149 \pm 8\%$ of baseline after 50–60 min washout, $n = 6$) prevailing over long-lasting depression ($82 \pm 3\%$ of baseline, $n = 4$). In hippocampal slices from KO mice ($n = 7$) (R)-α-MeHA -induced depression was more frequent ($n = 3$ of 7) and stronger (to $67 \pm 3\%$ of baseline, $n = 3$, $P < 0.05$ with WT value) while potentiation was less frequent and slightly smaller ($130 \pm 8\%$ of baseline, $n = 2$). The difference in responsiveness to (R)-α-MeHA between KO and WT slices is clearly seen in the pooled data presented in Fig. 6a. The hippocampal slices from WT and KO mice did not significantly differ in their responses to 20 min perfusion with the H3 receptor inverse agonist/antagonist thioperamide (TPA, 10 μM, Fig. 6b), although KO slices were slightly less responsive (4 of 6, 67% vs. 10 of 12, 83%). In both

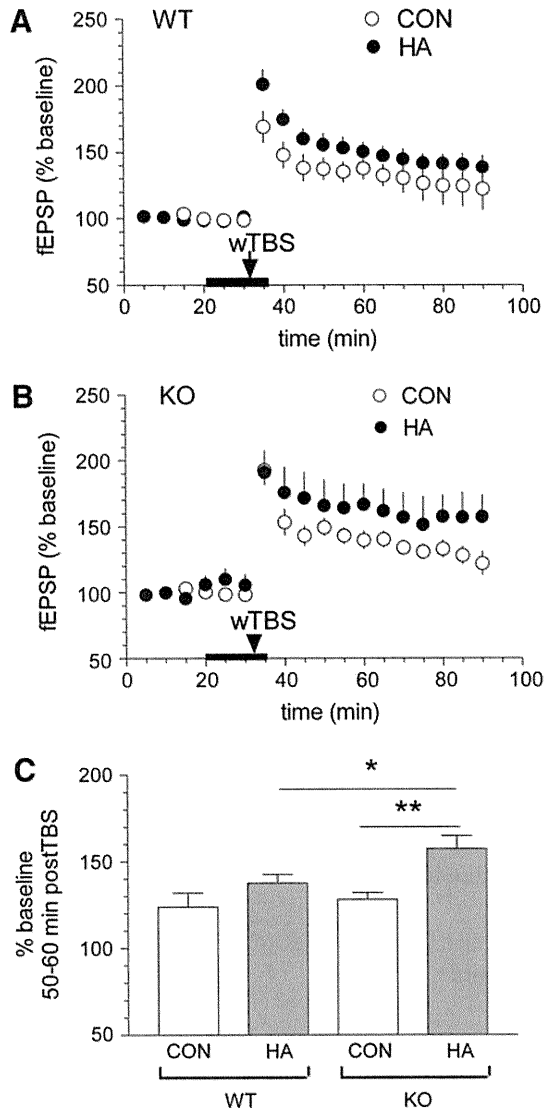

Fig. 2 Histamine significantly enhances LTP induced by weak TBS in HDC KO, but not WT mice. **a** The time course of LTP in control and histamine-treated slices in WT. **b** The time course of LTP in control and histamine-treated slices in KO mice. Histamine (10 μM) was applied 10 min before and washed out 5 min after weak TBS as indicated by black bars. $*P < 0.05$, $**P < 0.01$ Student's t test

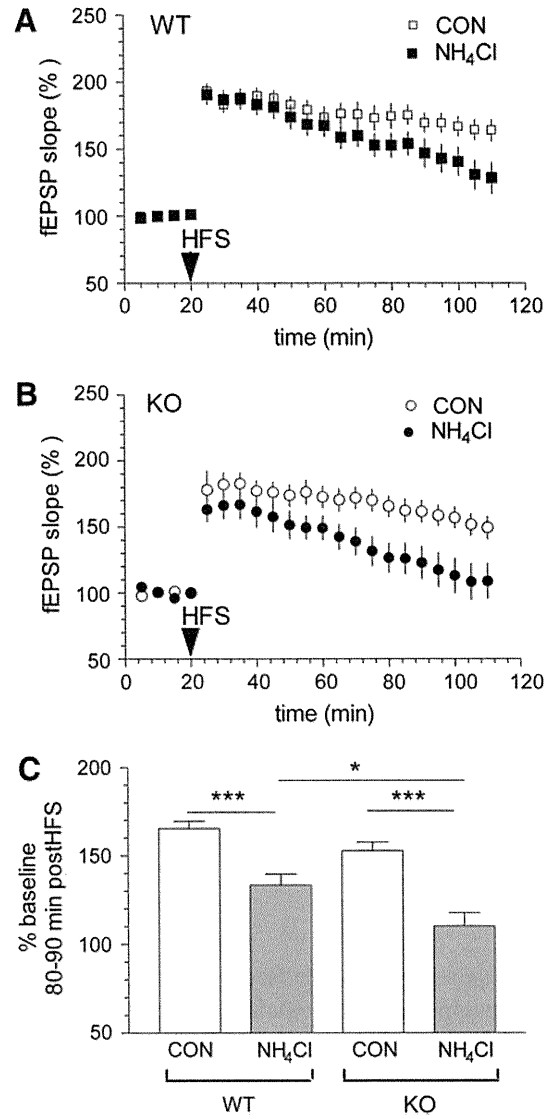

Fig. 3 Ammonia causes more severe impairment of hippocampal LTP in histamine deficient mice (HDC KO). **a** The time course of changes in fEPSP amplitude in the CA1 area after HFS of Schaffer collaterals in control and ammonia-treated hippocampal slices from WT. **b** The time course of changes in fEPSP amplitude in the CA1 area after HFS of Schaffer collaterals in control and ammonia-treated hippocampal slices from HDC KO mice. **c** the mean magnitude of potentiation at 80–90 min postHFS in control and ammonia-treated slices from WT and KO mice. $*P = 0.033$, $***P < 0.0001$

WT and KO slices, TPA induced largely potentiation (to 148 ± 6%, $n = 7$ and 137 ± 6%, $n = 3$ in WT and KO, respectively) and less frequently depression (to 73 ± 2%, $n = 3$ and 53%, $n = 1$ in WT and KO, respectively) of responses. Although the mechanisms of long-lasting changes in field responses after both, activation and blockade of H3 receptors remain to be determined, attenuation of (R)-α-MeHA-induced potentiation of SC–CA1 population spike in KO may be related to the decreased expression of hippocampal H3 receptors.

We also recorded the activity of the TMN neurons with the idea that the increased expression of H3 autoreceptors in HDC KO may affect spontaneous firing of the TMN neurons and their responsiveness to the H3 receptor agonist (R)-α-MeHA. Histaminergic neurons were recorded in the most compact ventrolateral cellular group of the TMN (Fig. 7b); they displayed their typical regular firing at 0.5–3 Hz and wide biphasic action potentials and were identified *post hoc* by the co-localization of immunoreactivities for biocytin and peripherin, a novel marker of TMN histaminergic neurons (Eriksson et al. 2008). The spontaneous firing rate of the TMN neurons in slices from KO mice (2.2 ± 0.2 Hz, $n = 14$) as well as its suppression by 2 μM (R)-α-MeHA (to

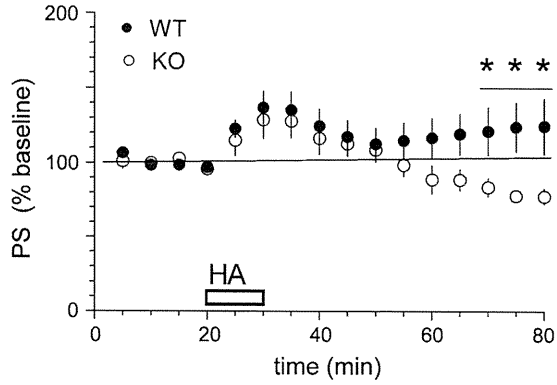

Fig. 4 Histamine effects on population spikes (PS) in HDC KO and WT mice. The time course of changes in the amplitude of PS during and after perfusion with 10 μM histamine (HA, marked by open bar). A long lasting enhancement is observed in WT, whereas the potentiation gives way to a long lasting depression of PS about 20 min after washout in KO. The mean amplitudes of responses at minutes 40–50 after histamine washout are significantly different (***$P = 0.0007$)

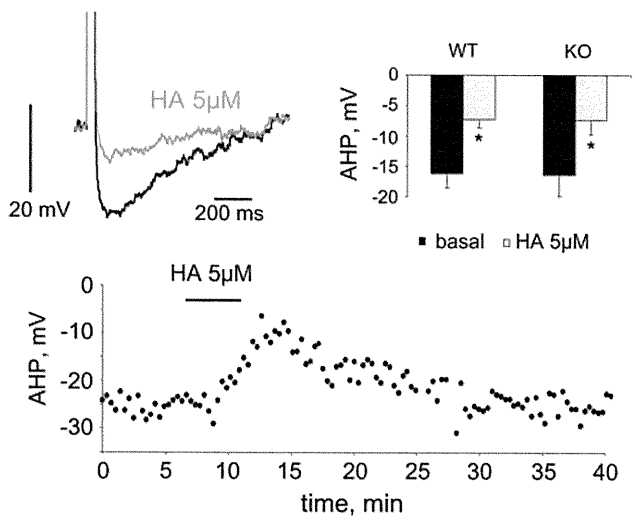

Fig. 5 Histamine equally blocks slow afterhyperpolarizations in HDC KO and WT mice. Representative traces (*left*) and the mean amplitude of AHPs in control and in the presence of histamine (5 μM) or the H2 receptor agonist impromidine (3 μM) (*right*). *Bottom* the time course of changes in the AHP amplitude in one representative cell

30 ± 7%, $n = 15$) did not differ from these indices in WT neurons (2.1 ± 0.3 Hz, $n = 18$ with the suppression to 26 ± 8%, $n = 16$ of the baseline) (Fig. 7a, c).

Discussion

Histamine deficiency has no impact on basal transmission and LTP in the Schaffer collateral (SC)–CA1 synapses, but modifies histamine-induced facilitation of synaptic plasticity and excitability and aggravates LTP impairment by

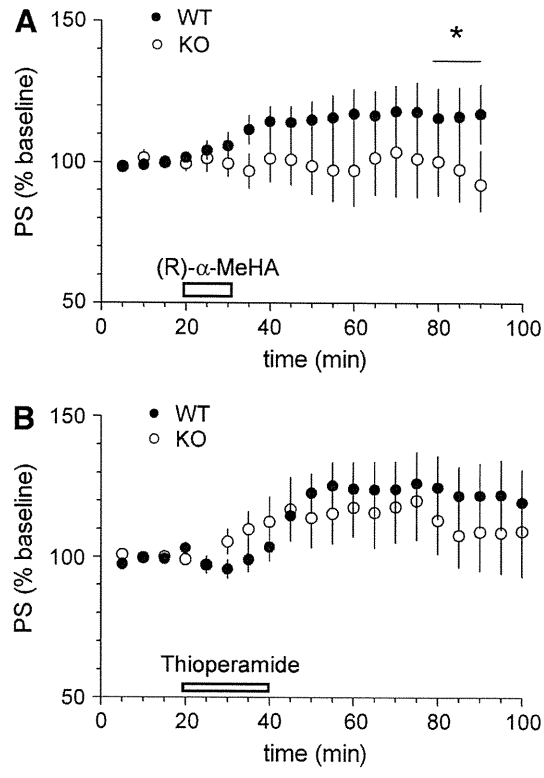

Fig. 6 Effects of pharmacological activation and blockade of H3 histamine receptors on population spike (PS) amplitude in hippocampal slices from HDC KO and WT mice. **a**, **b** Time course of changes in the amplitude of PS during and after 10 min perfusion with the H3 receptor agonist (R)-α-methylhistamine ((R)-α-MeHA, 2 μM) and 20 min perfusion with the H3 receptor antagonist thioperamide (TPA, 10 μM), respectively. The periods of applications are marked by *open bars*. The mean amplitudes of population spike responses at minutes 50–60 after (R)-α-MeHA washout are significantly different (*$P = 0.040$), indicating less potentiation in the KO hippocampus

hyperammonemia. These functional modifications were accompanied by the pronounced down-regulation of H3 receptors without significant alterations in the expression of H1 and H2 receptors. In contrast, H3 receptor expression in the TMN was increased, in the absence of changes in the other two receptors, the spontaneous firing rate, and its response to H3 receptor antagonism.

The unchanged activity-dependent hippocampal synaptic plasticity in HDC KO mice reported here is at variance with a previous study reporting an enhanced magnitude of SC–CA1 LTP and its correlation with enhanced performance in contextual fear conditioning (Liu et al. 2007). This may be accounted for by different parameters chosen for the analysis: population spikes (Liu et al. 2007) versus field EPSPs (our study). The former, reflecting largely the neuronal excitability, is sensitive to external histamine (Brown et al. 1995, and this study) and shows more pronounced activity-dependent modifications than would be

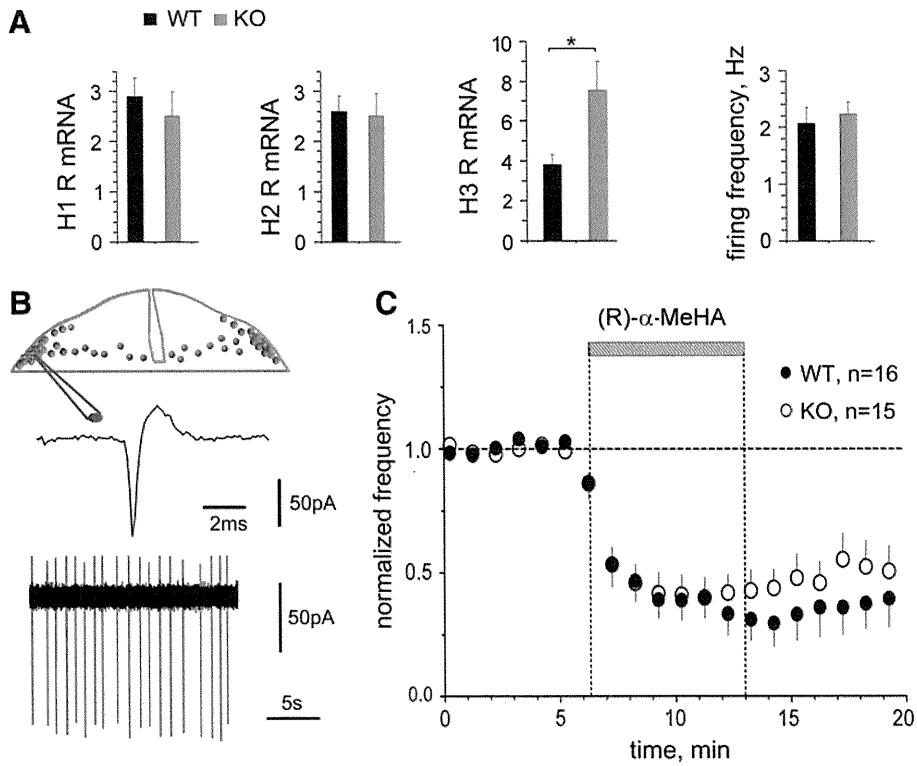

Fig. 7 Expression and function of histamine receptors in the tuberomamillary nucleus of HDC knockout and wild type mice. **a** Expression of H1, H2, and H3 histamine receptor transcripts in the TMN as well as comparison of spontaneous firing frequencies of TMN histaminergic neurons from HDC WT and KO mice. **b** Schematic drawing of coronal slice illustrating the location of histaminergic cells (marked by *dots*) and recording site in the ventrolateral TMN. *Below* action potential, recorded in cell-attached mode (average of 12 individual traces) of the WT histaminergic neuron and example of its typical regular firing. **c** Inhibition of firing of TMN neurons by the H3R agonist (R)-α-methylhistamine at 2 μM (period of application is marked by *bar*)

expected from fEPSP LTP due to the parallel increase in intrinsic excitability (Abraham et al. 1987; Daoudal and Debanne 2003; Xu et al. 2005).

Histamine modulates the properties of LTP in the Schaffer collateral synapses via activation of both H1 and H2 receptors (Dai et al. 2007) and direct interaction with NMDA receptors (Brown et al. 1995). Although the histamine-deficient hippocampus showed only non-significant increases in the expression of H1 and H2 receptors, they may be responsible for a slightly stronger facilitation of the weak tetanus-induced LTP by external histamine observed in the HDC KO mice, and the absence of histamine may have made these receptors more sensitive in KO mice (Haas et al. 1978). Histamine H1 and H2 receptors are also involved in the long-lasting enhancement of neuronal excitability by histamine through the H2 receptor-activated adenylyl cyclase/PKA signal transduction cascade (Selbach et al. 1997). The absence of long-lasting population spike potentiation by histamine in HDC KO hippocampus may indicate alterations in H2 receptor coupling to this intracellular cascade. On the other hand, it may be related to the decreased expression of hippocampal H3 receptors, which modulate several signal transductions, including stimulation of the ERK/MAPK pathway (Bongers et al. 2007), which is critical for both synaptic plasticity and the long-term enhancement of neuronal excitability (Cohen-Matsliah et al. 2007). This suggestion is supported by our data on the decreased incidence and amplitude of long-lasting potentiation of the SC–CA1 population spike in HDC KO hippocampus after exposure to the specific H3 receptor agonist (R)-(α)-MeHA.

Our study shows that histamine deficiency in HDC KO brain exacerbates the impairment of hippocampal synaptic plasticity by ammonia, the main pathogenic factor in hepatic encephalopathy (Butterworth 2002; Haussinger et al. 2002) indicating that the increased histamine level in the brain counteracts the deleterious effects of ammonia. These data are in line with findings in other models of brain pathology: irreversible inhibition of its synthesis aggravates the postischemic death of the CA1 pyramidal cells (Adachi 2005) and kainate-induced damage of hippocampal pyramidal neurons (Kukko-Lukjanov et al. 2006). Neuroprotection by histamine was mediated largely by activation of either H1 or H2 receptors, but some in vitro models were also sensitive to H3 receptor antagonists (Kukko-Lukjanov et al. 2006; Dai et al. 2006). The potentiation of GABA-mediated inhibition by histamine (Saras et al. 2008) may contribute to its neuroprotective action. H3 receptor inverse agonists/antagonists activate various signaling pathways, and are developed as novel therapeutics for the treatment of various brain pathologies, including cognitive disorders (Esbenshade et al. 2008; Lin et al. 2011; Parmentier et al. 2007; Passani et al. 2004; Schwartz et al. 2003).

The hippocampus of HDC KO mice showed a considerably reduced amount of H3 histamine receptor transcript

indicating significant downregulation of H3 receptors. We show here that both (R)-α-MeHA (an H3R-agonist) and thioperamide (an inverse agonist/antagonist at the H3R) can induce a long-lasting increase in the CA1 population spike. Although similar in appearance, these increases must be mediated by different mechanisms which remain to be determined. The thioperamide-induced potentiation cannot be caused by histamine following H3 autoreceptor blockade, since it was equally expressed in the histamine-deficient HDC-KO mice. However, it may be mediated by noradrenaline, whose release in the hippocampus is controlled by H3 heteroreceptors (Alves-Rodrigues et al. 1998; Di Carlo et al. 2000) and can induce long-lasting enhancement of the SC–CA1 population spike (Kostopoulos et al. 1988). It remains obscure how activation of hippocampal H3 receptors can trigger potentiation of CA1 population spikes, but the attenuation of this effect in hippocampal slices from HDC KO mice is associated with decreased expression of hippocampal H3 receptors. Taking into account that H3 receptor antagonists/inverse agonists improve the performance in a variety of rodent cognition paradigms (Esbenshade et al. 2008; Passani et al. 2004) we can suggest that the reduced expression of H3 receptors may be responsible for the reported superior performance of some hippocampus-dependent tasks by HDC KO mice (Dere et al. 2003; Liu et al. 2007).

Histamine-containing neurons represent an important component of neuronal network promoting wakefulness (Brown et al. 2001; Haas et al. 2008; Parmentier et al. 2007; Passani et al. 2004). The "ex-histaminergic" neurons in the TMN maintain the level of spontaneous firing and H3 autoreceptor inhibitory function in the absence of their natural transmitter histamine. Sakai et al. (2010) recording TMN neuronal activity in HDC KO mice during the sleep-waking cycle also reported recently that the histamine-deprived TMN neurons exhibit the same firing properties as seen in control mice. Since theoretically the constitutive activity characteristic of H3 receptors (Arrang et al. 2007) should increase with the increase in receptor concentration (Kenakin 2007), the increased expression of the TMN H3 receptors may provide the level of constitutive activity sufficient for the proper control of firing of histamine-deprived neurons.

In conclusion, the absence of brain histamine leads to significant alteration in the expression of H3 histamine receptors, modifies histamine-induced modulation of hippocampal synaptic plasticity and excitability, and exacerbates the damage caused by hyperammonemia. These findings are relevant for the possible clinical use of H3 receptor modulators as cognitive enhancers.

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MolecularMedicine

Arrhythmogenic Effect of Sympathetic Histamine in Mouse Hearts Subjected to Acute Ischemia

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The role of histamine as a newly recognized sympathetic neurotransmitter has been presented previously, and its postsynaptic effects greatly depended on the activities of sympathetic nerves. Cardiac sympathetic nerves become overactivated under acute myocardial ischemic conditions and release neurotransmitters in large amounts, inducing ventricular arrhythmia. Therefore, it is proposed that cardiac sympathetic histamine, in addition to norepinephrine, may have a significant arrhythmogenic effect. To test this hypothesis, we observed the release of cardiac sympathetic histamine and associated ventricular arrhythmogenesis that was induced by acute ischemia in isolated mouse hearts. Mast cell–deficient mice (MCDM) and *histidine decarboxylase* knockout (*HDC*$^{-/-}$) mice were used to exclude the potential involvement of mast cells. Electrical field stimulation and acute ischemia-reperfusion evoked chemical sympathectomy–sensitive histamine release from the hearts of both MCDM and wild-type (WT) mice but not from *HDC*$^{-/-}$ mice. The release of histamine from the hearts of MCDM and WT mice was associated with the development of acute ischemia-induced ventricular tachycardia and ventricular fibrillation. The incidence and duration of induced ventricular arrhythmias were found to decrease in the presence of the selective histamine H_2 receptor antagonist famotidine. Additionally, the released histamine facilitated the arrhythmogenic effect of simultaneously released norepinephrine. We conclude that, under acute ischemic conditions, cardiac sympathetic histamine released by overactive sympathetic nerve terminals plays a certain arrhythmogenic role via H_2 receptors. These findings provided novel insight into the pathophysiological roles of sympathetic histamine, which may be a new therapeutic target for acute ischemia-induced arrhythmias.

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INTRODUCTION

Previous investigations have shown that endogenous histamine (HA) and HA receptors play highly significant roles in many cardiovascular diseases, such as hypertension (1,2), artherosclerosis (3–5) and chronic heart failure (6,7). Additionally, early reports found that cardiac HA and its receptors were important in the development of abnormal cardiac rhythms, especially those induced by ischemia reperfusion (8). More recently, it was reported that increased histaminergic tone could induce tachycardia via HA H_2 receptors (9) and that sympathetic presynaptic H_3 receptors are key modulating elements in ventricular arrhythmogenesis induced by ischemia reperfusion (10–12). Following the results of both fundamental and clinical investigations (6,7,13), corresponding HA receptors have therefore been proposed as new therapeutic targets for curing ischemic cardiovascular diseases.

However, despite the consensus view that HA exerts an important influence on ischemic hearts, the origin of endogenous HA still remains unclear. Several previous reports have suggested that the cardiac endogenous HA originates from mast cells. In an ischemic state, cardiac mast cells are degranulated and release HA to induce arrhythmogenesis (8). However, mast cells are not the sole source of cardiac HA (14). Our previous findings indicated that cardiac sympathetic nerves also express HA and *histidine decarboxylase* (*HDC*), the rate-limiting enzyme in the synthesis of HA (15–17),

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and that sympathetic nerve terminals release HA upon appropriate stimulation (16,18–20). On the basis of these findings, we proposed HA to be a newly recognized sympathetic neurotransmitter. Furthermore, the postsynaptic effects of sympathetic HA have been found to be positively related to the firing activities of sympathetic nerves (19). The overactivation of cardiac sympathetic nerves in ischemic hearts, as reported in previous studies, further correlates with ischemia-induced ventricular arrhythmias (11–13,21,22). Therefore, it is rational to speculate that sympathetic HA, in addition to norepinephrine (NE), is also released and plays certain pathophysiological roles in ischemia-induced ventricular arrhythmias.

In the present study, mast cell–deficient mice (MCDM) and HDC knockout ($HDC^{-/-}$) mice were used to test the aforementioned hypothesis and to further clarify the pathophysiological roles of cardiac sympathetic HA.

MATERIALS AND METHODS

Animals

MCDM (Wads c-kit mutant mice) were provided by Dr. Xiang Gao of Model Animal Research Center of Nanjing University (Nanjing, China). $HDC^{-/-}$ mice used in the present study were provided by H Ohtsu. Both strains had a pure C57BL/6 genetic background. C57BL/6 mice were used as the wild-type (WT) controls. All experiments and animal care procedures were reviewed and approved by the Animal Resource Center of the Fourth Military Medical University, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication number 8023, revised 1978).

Morphological Identification

Hearts dissected from WT, homozygotes of MCDM and $HDC^{-/-}$ mice were fixed using Bouin fixative. The hearts were dehydrated in rising concentrations of ethanol, embedded in paraffin and sectioned (4 μm). Mouse anti-tryptase monoclonal antibody (1:200 dilution; Abcam), biotinylated goat anti-mouse antibody (1:20 dilution; Sigma, St. Louis, MO, USA) and ExtrAvidin Peroxidase (1:20 dilution; Sigma) were used for mast cell identification. Positive staining was detected with 0.05% diaminobenzidine/0.01% H_2O_2 in 0.05 mol/L Tris-HCl buffer. Superior cervical ganglions (SCGs) obtained from the same mice were also sectioned, and double immunofluorescence staining was performed. Briefly, SCG sections were incubated with a mixture of rabbit anti-HA polyclonal antibody (1:1,000 dilution; Sigma) and mouse anti-dopamine β hydroxylase (DβH) monoclonal antibody (1:1,000 dilution; Incstar, Stillwater, MN, USA) for 24 h at 4°C. The incubation medium was prepared using 0.05 mol/L phosphate-buffered saline (PBS) containing 0.5% (v/v) Triton X-100, 0.25% (w/v) L-carrageenan, 0.05% (w/v) NaN_3 and 0.5% (v/v) normal goat serum. After rinsing three times in PBS, the slides were incubated overnight at room temperature in the secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse IgG and Texas Red–conjugated goat anti-rabbit IgG), in a 1:200 dilution with 0.05 mol/L PBS containing 0.3% (v/v) Triton X-100 (PBS-T). Slides were then rinsed in PBS and cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (antifading agent) in 0.05 mol/L PBS and examined with a fluorescence microscope (Olympus FV300; Olympus, Tokyo, Japan) under excitation wavelengths of 543 and 488 nm. In the control experiments, one of the primary antibodies was omitted and replaced with normal IgG or the corresponding preneutralized antibody. No immunoreactivity for the replaced or neutralized antibody was found.

Perfusion of Isolated Mouse Hearts

Mice weighing 30–35 g were sacrificed by cervical dislocation under deep CO_2 anesthesia. Hearts were quickly dissected and placed in ice-cold Krebs-Henseleit (KH) solution with the following composition (mmol/L): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11 (pH 7.4) and quickly cleared of surrounding excess tissues. The aorta of each heart was cannulated with a flanged stainless-steel needle, and the heart was perfused with KH solution at 37°C and constant pressure (100 cm H_2O). The solution contained 1 μmol/L atropine and 0.1 μmol/L desipramine and was equilibrated with 95% O_2 and 5% CO_2. Only hearts with an initially stable sinus rhythm were considered for further experiments. The coronary effluents were collected every 5 min, and coronary HA and NE overflows were measured simultaneously by high-performance liquid chromatography with electrochemical detection, as previously described (23). Hearts were weighed after the experiments for result standardization, quickly frozen with liquid nitrogen and stored at –80°C before cyclic adenosine 3′,5′-cyclic monophosphate (cAMP) assaying.

Electrical Field Stimulation

Electrical field stimulation (EFS) experiments were performed according to previous studies (21). After perfusion with KH solution and equilibration for 30 min, two stainless steel paddles were apposed to the heart with their stimulating surface parallel to the interventricular septum. EFS was performed for 60 s at 2, 4 or 8 Hz with pulse duration of 1 ms and voltage of 5 V, generated by PowerLab/8SP (ADInstruments). Each tissue was stimulated at one of the selected frequencies only.

Ischemia/Reperfusion and Electrocardiogram Recording

After 30-min stabilization, hearts were subjected to 10-, 20-, or 30-min stop-flow normothermic global ischemia followed by a 30-min reperfusion. Surface electrocardiograms were obtained using two electrodes with one attached to the aortic cannula and the other to the apex of the left ventricle (recorded online with recording frequency of 4 kHz, analyzed

with PowerLab/8SP). According to previous studies (24), the electrode was purpose-built with a small (approximately 1 mm) bare silver ball to achieve steady contact between the electrode and the heart, ensuring minimal movement occurred. The coronary HA overflow was measured and the incidence and duration of reperfusion arrhythmias were calculated according to the Lambeth Conventions (25).

In experiments to exhaust endogenous HA, 50 mg/kg α-fluoromethylhistidine (αFMH) was injected intraperitoneally 2 h before the study (26). To explore the effects of the antagonists, the reagents were applied 10 min before ischemia and continuously perfused until the end of the 30-min reperfusion. Vehicles were used as controls. To explore the effects of exogenous HA and/or NE, the corresponding reagents were applied for 5 min. The surface ECGs were recorded soon after the application of HA and/or NE for a duration of 30 min.

Sympathetic Denervation

To denervate sympathetic nerves, 6-hydroxydopamine (6-OHDA) was dissolved in saline containing 0.1% ascorbic acid, and mice were treated as previously mentioned (16). Briefly, 6-OHDA was injected through the caudal vein with 50 mg/kg on d 1 and 100 mg/kg 6-OHDA on d 7. For the control experiments, solutions containing only 0.1% ascorbic acid in saline were used. Mice were sacrificed for study 7 d after the final injection.

cAMP Assay

To measure myocardial cAMP levels, each sample was homogenized mechanically in frozen PBS (pH = 7.4) with a mechanical homogenizer. The homogenate was thawed and centrifuged at 5,000*g* at room temperature for 15 min, and the 100-mL aliquots of supernatant were subjected to the assay. Levels of cAMP were determined by enzyme-linked immunosorbent assay (Biotrak EIA, Amersham Pharmacia Biotech, Bucks, UK).

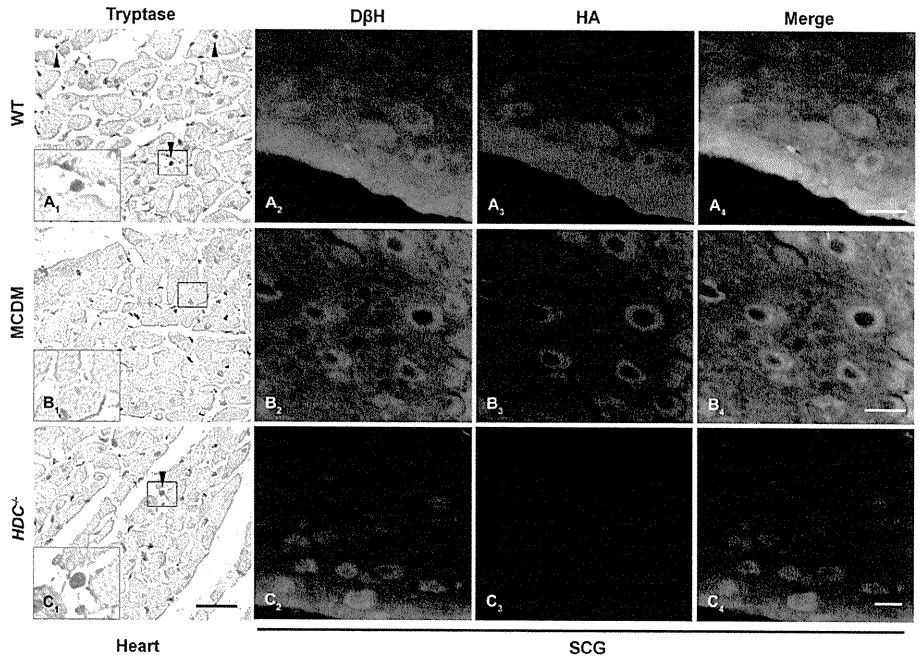

Figure 1. Morphological evaluation of hearts and SCG neurons from WT (A), MCDM (B) and *HDC*$^{-/-}$ mice (C). Heart and SCGs were obtained from the same mouse. Black arrowheads identify mast cells. Magnified images of the boxed areas are shown in the bottom left of the corresponding panels (A_1, B_1 and C_1). No positive staining of tryptase was found in MCDM sections (B_1). SCG sections were double stained with antibody to DβH (green) and HA (red). All mice strains were positive for DβH (A_2, B_2 and C_2). Majority of WT mice and MCDM SCG neurons were simultaneously positive for HA (A_{3-4} and B_{3-4}), whereas *HDC*$^{-/-}$ SCG sections were negative for HA (C_3). Experiments were repeated in three mice from each strain, and five sections of heart or SCG from each one mouse were observed. Scale bar, 50 μm.

Chemicals

Atenolol, biotinylated secondary antibody, famotidine, FITC-conjugated secondary antibody, HA, HA antibody, 6-hydroxydopamine and NE were from Sigma. DβH antibody was from Incstar. Texas Red–conjugated antibody was from Molecular Probes (Eugene, OR, USA). Tryptase antibody was from Abcam (Cambridge, UK). α-Fluoromethylhistidine was provided by C Kamei (Okayama University, Japan).

Data Analysis

Values were expressed as mean ± standard deviation (SD), and statistical differences between groups were determined by the χ^2 test, Student *t* test or analysis of variance (ANOVA) followed by Student-Newman-Keuls *t* test where necessary. A value of $P < 0.05$ was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Morphological Identification

The coat color of heterozygous MCDM is black with white spots, whereas that of homozygous MCDM is pure white with eyes remaining black (27). The staining of tryptase, a specific marker of mast cells, showed that heart sections from WT and *HDC*$^{-/-}$ mice expressed a number of mast cells (Figures $1A_1$, C_1), but none were found in the hearts of the homozygote of MCDM (Figure $1B_1$). HA-like immunopositive signals were found to be present in SCG neurons of the same WT mice and homozygote of MCDM (Figures $1A_3$, B_3), but were absent in the *HDC*$^{-/-}$ mice (Figure $1C_3$).

Table 1. Basic parameters of perfused isolated hearts of WT mice, MCDM and *HDC*$^{-/-}$ mice.

	WT	MCDM	*HDC*$^{-/-}$
n	12	12	15
Heart weight (g)	0.18 ± 0.03	0.17 ± 0.03	0.18 ± 0.01
Basal heart rate (bpm)	315 ± 24	313 ± 25	305 ± 19
Coronary flow (mL/min)			
Preischemia	1.54 ± 0.21	1.66 ± 0.16	1.53 ± 0.18
5 min after reperfusion	1.60 ± 0.22	1.68 ± 0.11	1.56 ± 0.17
10 min after reperfusion	1.56 ± 0.20	1.66 ± 0.15	1.54 ± 0.19

Effect of EFS or Acute Ischemia on Coronary HA and NE Overflows

Table 1 shows the basic parameters of isolated hearts from the three phenotypes of mice and indicates that there were no significant differences among the groups. As shown in Figure 2A$_1$, EFS frequency-dependently evoked HA release from Langendorff-perfused hearts of WT mice and MCDM. No significant differences in HA overflow were observed between the two groups. A 10-min period of acute global stop-flow ischemia induced a similar amount of HA release from isolated hearts of WT mice and MCDM after reperfusion (Figure 2B$_1$). The HA concentration in coronary effluents peaked in the first 5 min of reperfusion and decreased thereafter. Neither EFS nor ischemia evoked significant HA release in *HDC*$^{-/-}$ mice. However, coronary NE overflow induced by EFS or acute ischemia exhibited a similar pattern among the three mouse strains, being slightly higher in *HDC*$^{-/-}$ mice (Figures 2A$_2$, B$_2$).

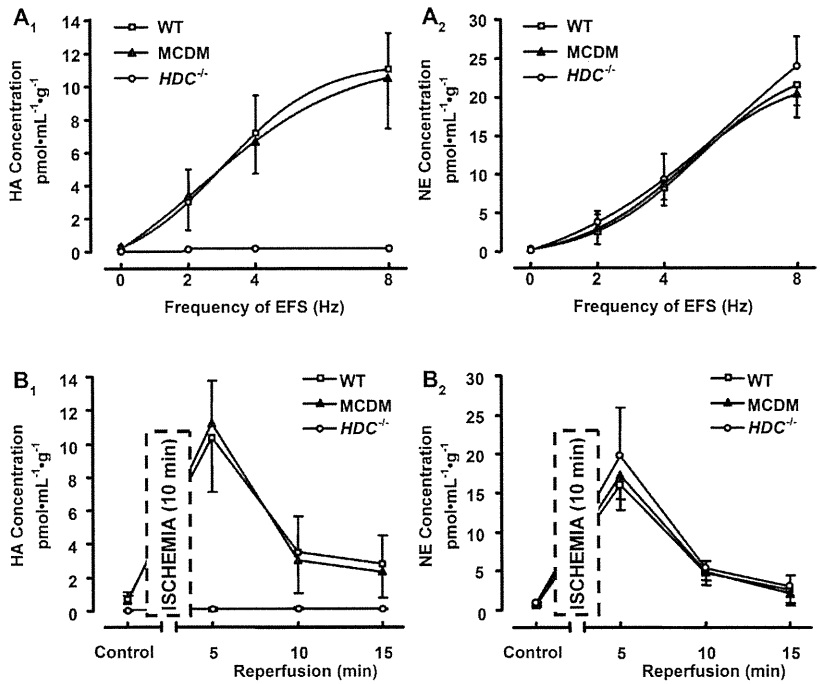

Figure 2. Coronary HA and NE overflows from perfused mice isolated hearts. (A) Effect of EFS on coronary HA (A$_1$) and NE overflows (A$_2$) in WT, MCDM and *HDC*$^{-/-}$ mouse hearts. Each point represents the mean ± SD value of HA or NE concentration in the first 5 min of coronary effluent after EFS (n = 5). (B) Effect of global stop-flow ischemia followed with reperfusion on coronary HA (B$_1$) and NE overflows (B$_2$) in WT, MCDM and *HDC*$^{-/-}$ mouse hearts. Ischemia was applied for 10 min after an initial stabilization period of 30 min. Each point represents the mean ± SD value of HA concentration in the coronary effluent after reperfusion (n = 12–15).

Effect of Ischemia Time on Coronary HA and NE Overflows

To explore coronary HA and NE overflows upon acute or prolonged global ischemia, different time courses of stop-

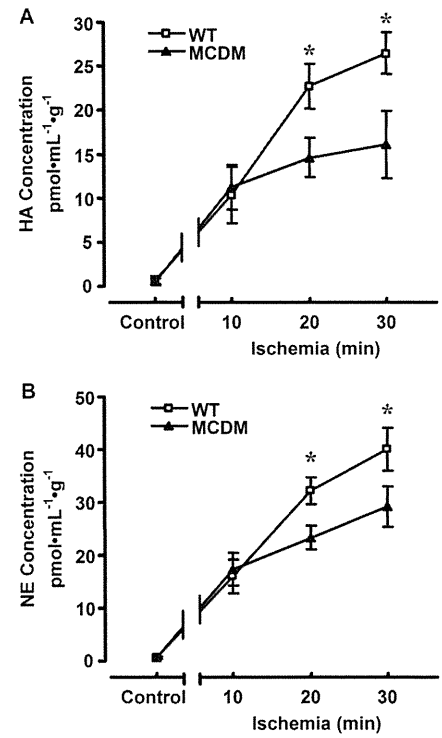

Figure 3. Effect of different durations of global stop-flow ischemia on coronary HA (A) and NE overflows (B) from WT and MCDM hearts after reperfusion. Ischemia was applied for 10, 20 or 30 min after an initial stabilization period of 30 min. Each point represents the mean ± SD value of HA concentration in the coronary effluent in the first 5 min of reperfusion. *$P < 0.05$ between hearts of different phenotypes subjected to the same ischemia time by ANOVA followed by a Student-Newman-Keuls t test (n = 5).

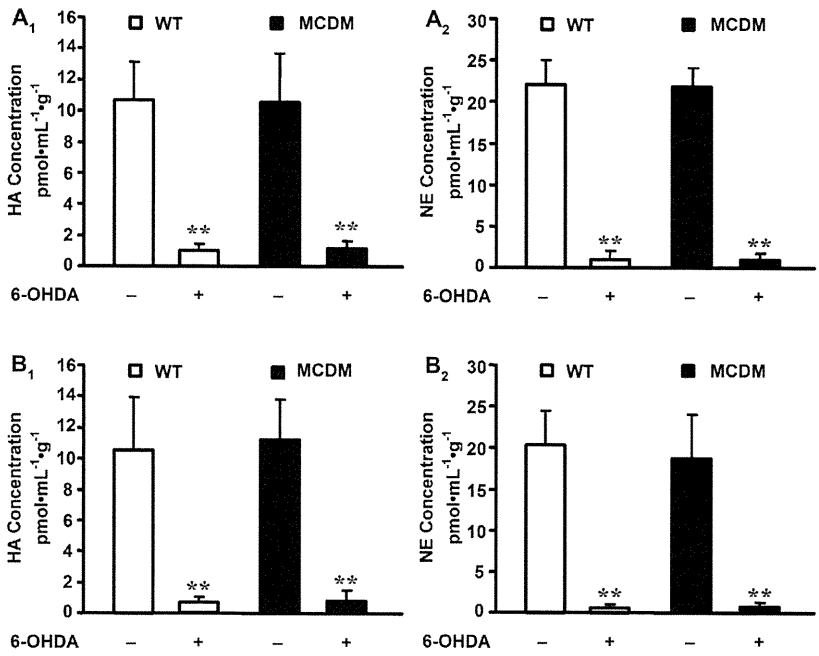

Figure 4. Effect of 6-OHDA on EFS (A) and global stop-flow ischemia (B) induced coronary HA and NE overflows in WT mice and MCDM. (A) The frequency of EFS applied was 8 Hz for 60 s with pulse duration of 1 ms. Bars represent the mean ± SD values of HA (A_1) and NE (A_2) concentrations in the first 5 min of coronary effluent after EFS. (B) Ischemia was applied for 10 min after an initial stabilization period of 30 min. Bars represent the mean ± SD values of HA (B_1) and NE (B_2) concentrations in the coronary effluent in the first 5 min of reperfusion. $^{**}P < 0.01$ versus the 6-OHDA untreated group of corresponding phenotype by Student *t* test (n = 5).

flow (10, 20 or 30 min) were applied. As shown in Figure 3, no significant differences were observed in the coronary HA (A) and NE (B) overflows in the first 5 min of reperfusion between WT mice and MCDM after 10 min of ischemia. However, when the time courses of stop-flow were increased to over 20 min, the coronary HA and NE overflows of WT mice group were significantly greater than those of the MCDM group.

Effect of 6-Hydroxydopamine on EFS or Acute Ischemia Evoked Coronary HA and NE Overflows

The coronary HA and NE overflows induced by EFS in WT mice and MCDM were almost entirely eliminated when mice were pretreated with 6-OHDA (Figures $4A_1$, A_2). Similarly, pretreatment with 6-OHDA also eliminated acute ischemia-induced coronary HA and NE overflows (Figures $4B_1$, B_2).

Effect of Acute Ischemia on Ventricular Arrhythmogenesis

Representative ECG recordings from one WT mouse, MCDM or $HDC^{-/-}$ mouse after reperfusion are shown in Figure 5A. Ischemia for 10 min resulted in the occurrence of ventricular tachycardia (VT) and/or ventricular fibrillation (VF) in WT mice and MCDM groups after reperfusion. The rhythm became irregular during the first minute of reperfusion and gradually returned to regular sinus rhythm thereafter. In the $HDC^{-/-}$ mice group, the incidence and duration of VT and/or VF induced by ischemia was markedly reduced (Figures 5B, C). Furthermore, after pretreatment with αFMH, the coronary HA overflow in MCDM after ischemia was markedly decreased (see Supplementary Figure S1) with the incidence and duration of VT and/or VF reduced to the level of the $HDC^{-/-}$ mice group (Figures 5B, C).

Effects of Atenolol and Famotidine on Acute Ischemia-Induced Ventricular Arrhythmogenesis

To explore the effects of atenolol and famotidine on the incidence and duration of VT and/or VF, selective β_1 adrenoceptor antagonist atenolol (1 μmol/L) and selective H_2 receptor antagonist famotidine (100 nmol/L) were used either alone or in combination. When used alone, each of these two drugs only marginally (not significantly) reduced the incidence of VT and/or VF but markedly shortened the VT and/or VF duration (72.5% decrease in WT mice, 63.2% decrease in MCDM; $P < 0.05$). However, when used in combination, both the incidence and duration of VT and/or VF were significantly reduced (Figures 6A, B). Furthermore, these two drugs affected neither the coronary HA overflow (Figure 6C) nor the other basic parameters of perfused isolated hearts, including the initial normal sinus rhythm and the coronary NE overflow (data not shown).

Effects of Exogenous HA and NE on Ventricular Arrhythmogenesis

Exogenous application of either HA (1 μmol/L) or NE (1 μmol/L) independently induced ventricular arrhythmogenesis in WT mice and MCDM isolated hearts with a respective incidence of 100% (n = 5). When used in combination, synergism was observed between the arrhythmogenic effect of HA and NE. Specifically, the durations of VT and/or VF induced by NE together with HA were markedly prolonged (219% increase in WT mice, 234% increase in MCDM), which was longer than the sum of VT and/or VF durations induced by HA and NE independently (Figures 7A, B).

Effect of Famotidine on the Cardiac cAMP Level in Isolated Mouse Hearts After Reperfusion

At the end of the 30-min reperfusion, the cardiac cAMP levels in hearts of the three strains of mice were all significantly increased compared with the cor-

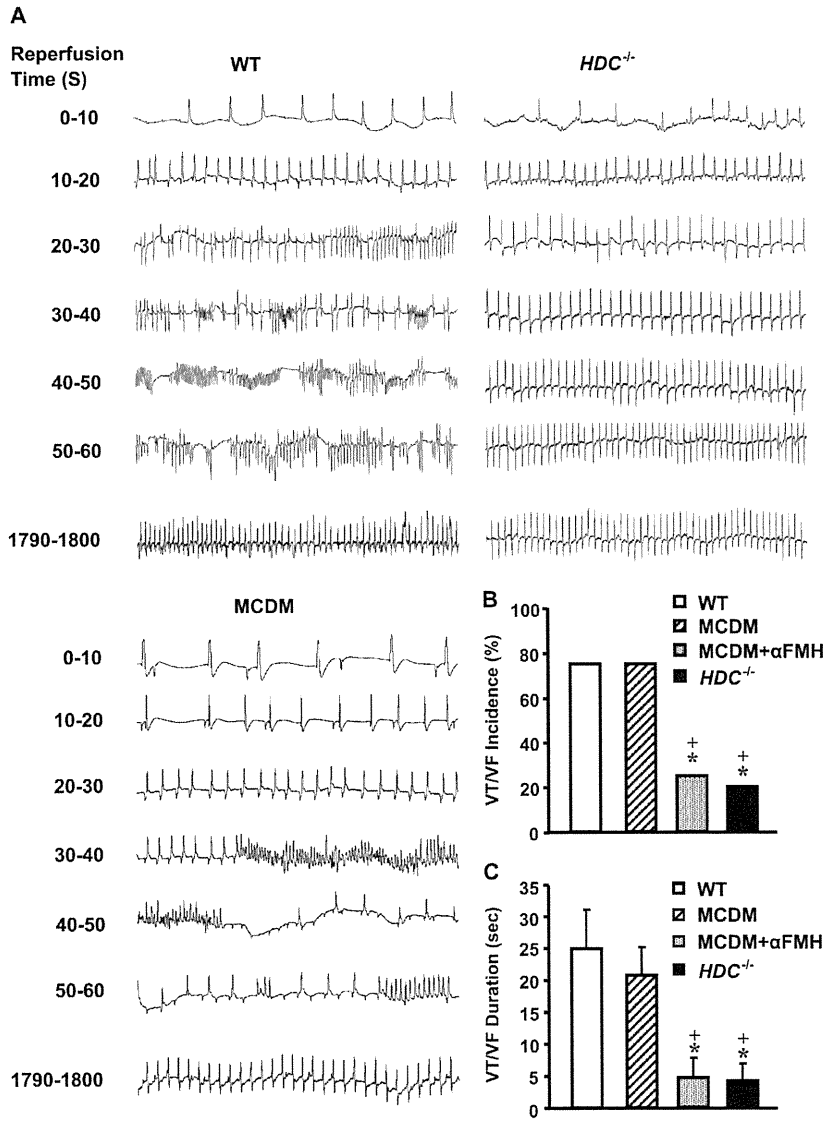

Figure 5. Effect of global stop-flow ischemia on ventricular arrhythmias in mice isolated hearts during a 30-min reperfusion. Ischemia was applied for 10 min after an initial stabilization period of 30 min. (A) ECG tracings from one heart of WT, MCDM and $HDC^{-/-}$ mouse, respectively, showing the first 60 s and the last 10 s of the 30-min reperfusion. (B) The incidence of VT and VF is expressed as percentages of the total number of hearts used in each group. Bars represent the percentage values; $^{*}P < 0.05$ versus WT group and $^{+}P < 0.05$ versus the MCDM group by χ^2 test. (C) Duration of VT and VF represents the cumulative duration of arrhythmia during the 30-min reperfusion. Bars represent the mean ± SD values of duration time. $^{*}P < 0.05$ versus WT group, $^{+}P < 0.05$ versus MCDM group by ANOVA followed by Student-Newman-Keuls *t* test (n = 12–15).

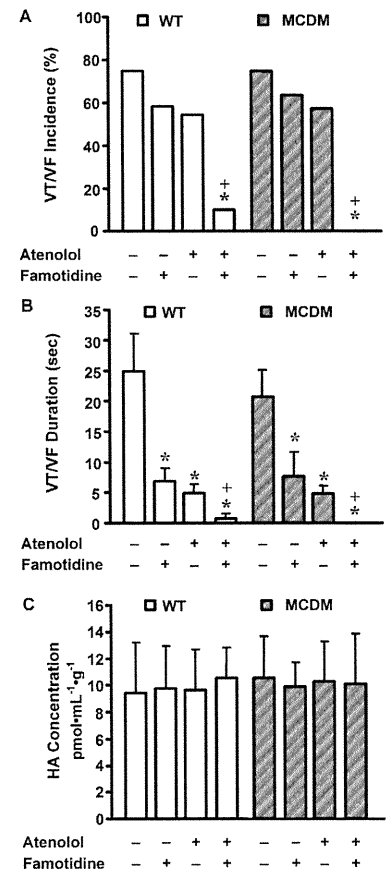

Figure 6. Effect of atenolol and famotidine, alone and in combination, on ventricular arrhythmias induced by global stop-flow ischemia in isolated hearts of WT mice and MCDM mice during a 30-min reperfusion. Ischemia was applied for 10 min after an initial stabilization period of 30 min. (A) The incidence of VT and VF is expressed as percentages of the total number of hearts used in each group. Bars represent the percentage values. $^{*}P < 0.05$ versus untreated group; $^{+}P < 0.05$ versus either atenolol alone or famotidine alone group by χ^2 test. (B) The duration of VT and VF represents the cumulative duration of arrhythmia during the 30-min reperfusion. Bars represent the mean ± SD values of duration time. $^{*}P < 0.05$ versus untreated group; $^{+}P < 0.05$ versus either atenolol alone or famotidine alone group by ANOVA followed by Student-Newman-Keuls *t* test. (C) Effect of atenolol and famotidine, alone or in combination, on coronary HA overflow obtained from the same isolated hearts as in (A) and (B). Bars represent the mean ± SD values of HA concentrations in the coronary effluent in the first 5 min of reperfusion (n = 10–12).

responding preischemia control groups. Pretreatment with famotidine markedly reduced the increased cardiac cAMP levels in WT mice and MCDM groups, but did not significantly affect the increased cAMP level in the $HDC^{-/-}$ mice group (Figure 8).

DISCUSSION

Cardiac mast cells are a well-acknowledged source of cardiac HA (28). However, the morphological results from the present study consistently show that HA is also present in the SCGs of MCDM and absent in $HDC^{-/-}$ mice. This strongly

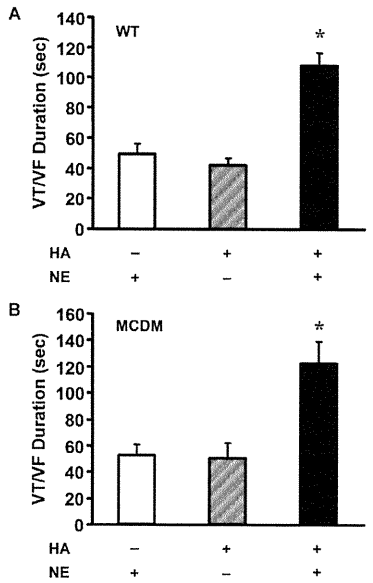

Figure 7. Effect of HA and NE, alone and in combination, on ventricular arrhythmogenesis in isolated hearts of WT mice (A) and MCDM mice (B). Exogenous reagents were applied for 5 min after an initial stabilization period of 30 min. Surface ECG recording began when exogenous reagents were applied and lasted for 30 min. Bars represent the mean ± SD values of duration time. $^{*}P < 0.05$ versus respective HA or NE treatment group by ANOVA followed by Student-Newman-Keuls *t* test (n = 5).

indicates that sympathetic HA is synthesized and stored independently of mast cells and is in support of previous work, which found HA present in cardiac sympathetic axons and varicosities projected from SCGs (16). Therefore, the MCDM model is ideal for studying the functions of cardiac sympathetic HA, and the $HDC^{-/-}$ mice, which do not express HA, are also appropriate as negative controls.

The results of previous studies have shown that with appropriate stimulation, such as EFS (21) and acute ischemia (11,13,21), cardiac sympathetic nerve terminals are activated, triggering neurotransmitter release. In the present study, such stimulations also evoked mast cell–independent HA overflow. To clarify the origin of HA, we performed chemical sympathectomy with 6-OHDA and achieved cardiac sympathectomy, as indicated by diminished NE release. HA release was found to be sensitive to 6-OHDA pretreatment, firmly suggesting a sympathetic neural origin.

The NE release induced by EFS or ischemia in the present study is in accordance with previous reports (11–13,21) and remained high in $HDC^{-/-}$ mice, which confirmed the activation of cardiac sympathetic nerve terminals. The slightly (but not significantly) increased coronary NE overflow in $HDC^{-/-}$ mice may be due to the lack of endogenous HA required to activate sympathetic presynaptic H_3 receptors, which in turn exert an inhibitory effect on NE release (11–13,16). However, since H_3 receptors have been reported to show constitutive activity in absence of an agonist (29), the NE overflow from $HDC^{-/-}$ mice is unlikely to increase as significantly as that in H_3 receptor knockout mice (11).

The choice of a 10-min global stop-flow period instead of the more frequently used 30 min (including the cardiac creatine phosphate kinase [CPK]) release data; see Supplementary Figure S2) was because prolonged ischemia (>10 min) caused significant mast cell activation and severe adverse cellular injury. This step not only releases HA but also triggers NE, one of the most important arrhythmogenic factors, released via the local renin system (30). Mast cell activation and severe cellular injury would significantly complicate interpretation of the present results. Therefore, to simplify the experimental model and focus on the pathophysiological roles of cardiac sympathetic HA, we investigated short-lived acute myocardial ischemia using a 10-min ischemia period, during which time barely any biochemical and cellular changes were observed. Further research is recommended to clarify the nature of cardiac HA release in protracted myocardial ischemia.

Cardiac H_2 receptors mediate positive chronotropic and inotropic effects (9,31–33). In the present study, the selective H_2 receptor antagonist famotidine was found to slightly decrease the incidence of ischemia-induced ventricular arrhythmia and markedly reduced the duration. This result was evidently a postsynaptic effect, since famotidine did not affect sympathetic HA release. This result is in accordance with previous studies, in which certain H_2 receptor antagonists have been found to exhibit a cardiac protecting effect in ischemic heart diseases, including antiarrhythmia (6,7,9,34). Moreover, results in the present study confirm the mediating role of the H_2 receptor (35,36) and indicate the therapeutic potential of its antagonist in treatment of ischemia-induced ventricular arrhythmias. Furthermore, our experiments show that famotidine did not fully reduce cardiac cAMP to the preischemia level. One possible reason for this is that NE, which is released simultaneously, plays an important role in triggering the rise of cardiac cAMP levels, via postsynaptic β_1 adrenoreceptors and in inducing positive chronotropic and in-

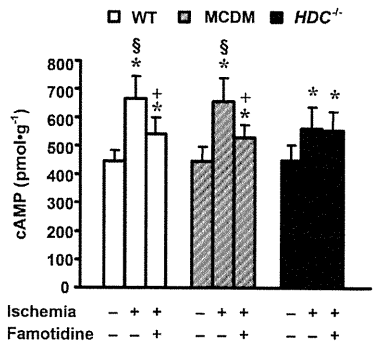

Figure 8. Myocardial cAMP levels in isolated hearts of WT, MCDM and $HDC^{-/-}$ mice. The basal groups of myocardial cAMP levels were obtained from hearts without being subjected to global stop-flow ischemia. The ischemia-induced myocardial cAMP levels were obtained from hearts subjected to a 10-min global stop-flow ischemia followed by 30-min reperfusion with or without famotidine treatment. Bars represent the mean ± SD values of cAMP levels. $^{*}P < 0.05$ versus corresponding basal group; $^{+}P < 0.05$ versus corresponding famotidine untreated group; $^{\S}P < 0.05$ versus famotidine untreated group of $HDC^{-/-}$ mice by ANOVA followed by Student-Newman-Keuls *t* test (n = 8–12).

otropic effects. This is the basis for the clinical use of β adrenoreceptor antagonists in the treatment of ischemia-induced arrhythmias (37).

The finding that the selective β_1 adrenoreceptor antagonist atenolol used independently exhibited a similar antiarrhythmic effect to famotidine but when used in combination, a stronger antiarrhythmic effect was observed, confirms the hypothesis that cardiac sympathetic HA facilitates the arrhythmogenic effect of NE. This finding is consistent with the results of previous research, which showed that the postsynaptic effect of NE is facilitated by sympathetic HA in guinea pig vas deferens (19) and is because both the β_1 adrenoreceptor (38) and the H_2 receptor (35) are Gs protein coupled receptors, sharing the same second messenger.

While the present study has investigated the relationship between HA and ischemia-induced arrhythmogenesis, HA is only one of the arrhythmogenic factors, and the arrhythmogenic roles of many other factors (such as renin, angiotensin, endothelins and so on) still require clarification. Furthermore, although the cardiac protection effect of H_2 receptor antagonists is reasonably well established from previous investigations (28,39) and the present study, the origin of cardiac HA after prolonged ischemia is complicated, and the relationship between the effects of HA from different origins remains uncertain. This issue is likely to prove a valuable area of research for future investigations in this field.

CONCLUSION

In summary, the present study has demonstrated for the first time that cardiac sympathetic HA plays a certain arrhythmogenic role in acute myocardial ischemia, via postsynaptic H_2 receptors. These findings provide considerable insight into the pathophysiological function of cardiac sympathetic HA and H_2 receptors and offer novel experimental evidence in the search for therapeutic targets to cure acute ischemia-induced arrhythmias.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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