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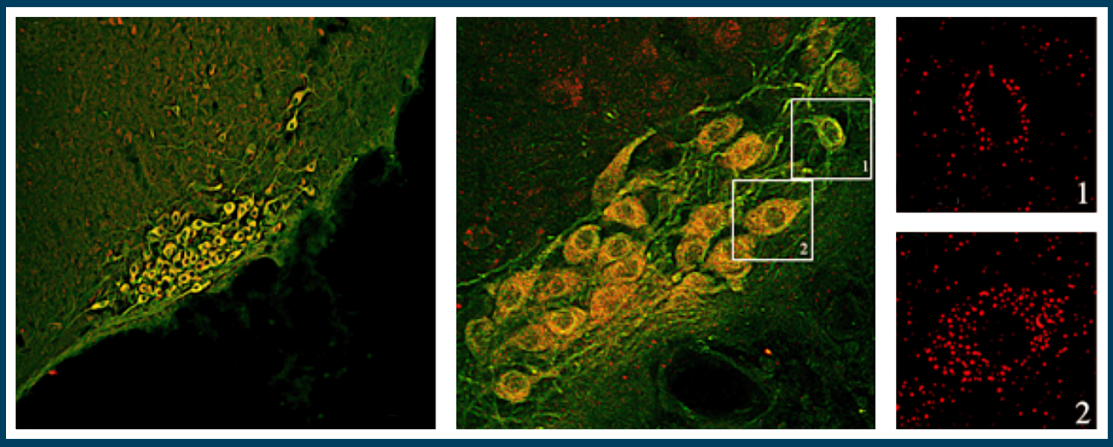

HISTAMINE IN THE BRAIN

Topic Editors
Jian-Sheng Lin, Pertti Panula and
Maria Beatrice Passani

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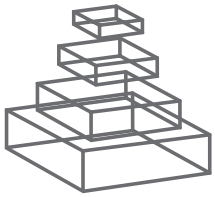

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HISTAMINE IN THE BRAIN

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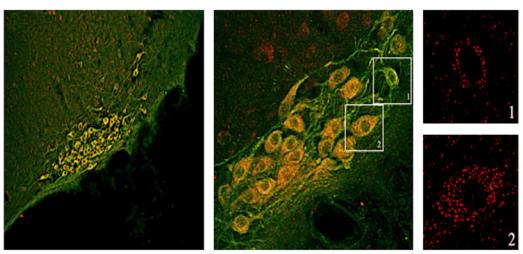

Histidinedecarboxylase (HDC) immunopositive neurons in the E2–E3 subdivision of the tuberomamillary nucleus in the posterior hypothalamus showing H3-R immunofluorescence (left panels). High resolution z-projections of H3-R expression in the HDC-positive neurons (right panels)

Brain aminergic pathways are organized in parallel and interacting systems, which support a range of functions, from homoeostatic regulations to cognitive, and motivational processes. Despite overlapping functional influences, dopamine, serotonin, noradrenaline and histamine systems provide different contributions to these processes. The histaminergic system, long ignored as a major regulator of the sleep-wake cycle, has now been fully acknowledged also as a major coordinator of attention, learning and memory, decision making. Although histaminergic neurons project widely to the whole brain, they are functionally heterogeneous, a feature which may provide the substrate for differential regulation, in a region-specific manner, of other neurotransmitter systems. Neurochemical preclinical studies have clearly shown that histamine interacts and modulates the release of neurotransmitters that are recognized as major modulators of cognitive processing and motivated behaviours. As a consequence, the histamine system has been proposed as a therapeutic target to treat sleep-wake disorders and cognitive dysfunctions that accompany neurodegenerative and neuroinflammatory pathologies. Last decades have witnessed an unexpected explosion of interest in brain histamine system, as new receptors have been discovered and selective ligands synthesised. Nevertheless, the complete picture of the histamine systems fine-tuning and its orchestration with other pathways remains rather elusive.

This Research Topic is intended to offer an inter-disciplinary forum that will improve our current understanding of the role of brain histamine and provide the fundamentals necessary to drive innovation in clinical practice and to improve the management and treatment of neurological disorders.

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EDITORIAL
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Histamine in the brain

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Keywords: histamine receptors, cognition, wakefulness, heterogeneity, anxiety

Brain histamine promotes wakefulness and orchestrates disparate behaviors and homeostatic functions. Recent evidence suggests that aberrant histamine signaling in the brain may also be a key factor in addictive behaviors and degenerative disease such as Parkinson's diseases and multiple sclerosis. The intent of this research Topic is to provide an overview of the recent advances in the understanding of the many functions of brain histamine and to propose neurobiological substrates and mechanisms of action that might explain the reasons why the histaminergic system is a potential target for therapeutic interventions. This may justify the search for new histaminergic compounds.

The authors that contributed to this e-book offered several approaches to the study of brain histamine function. Tomasch et al. (2012) synthesized a novel fluorescent ligand of the human histamine H3 receptor with potential to be used as pharmacological tools for visualization in different tissues. Shibuya et al. (2012) by using positron emission tomography (PET) in the human brain examined whether the levels of neuronal release of histamine might change binding of [(11)C]doxepin to the H1 receptors (a standard method for measuring H1 distribution) under the influence of physiological stimuli.

Histamine acts as a modulator of several neurotransmitters in the brain and its role in promoting wakefulness has for long overshadowed other important functions. In fact, histamine signaling controls feeding behavior in a complex fashion and it has been considered for long a satiety system as brain histamine decreases the drive to consume food. In their paper, Ishizuka and Yamatodani (2012) demonstrated the fine regulation of histamine release during feeding and in taste perception. Furthermore, they showed that histamine neurons respond to both mechanical and chemical sensory input from the oral cavity, as may be expected for a danger detection system.

Brain histamine is crucial for motivation and goal-directed behaviors as reviewed by Torrealba et al. (2012). The authors evaluated recent works demonstrating that histamine is differentially involved in the appetitive, food anticipatory responses, and in food consumption, suggesting that it may have an important role in abnormal appetites not only for food but also for substances of abuse. Indeed, preclinical studies on both rats and mice are hinting at a possible role of the histaminergic system in alcohol consumption, as blockade of the H_3 receptor (which regulates histamine and other neurotransmitters' release), decreases alcohol drinking in several behavioral tasks, like operant alcohol administration and "drinking in the dark" paradigm (Nuutinen et al., 2012). However, the authors caution that despite the evidence that the H_3 receptor is a key element in alcohol drinking and place preference, the role of histamine in these behaviors is poorly understood and deserves further investigation.

The importance of H_3 receptor signaling in the brain to acquire and store short- and long- term memories has been documented extensively. However a limited number of studies have investigated the role of the H_3 receptor in anxiety. By using novel behavioral test, Abuhamdah et al. (2012) present their results with selective agonist and antagonist for the H_3 receptor providing new evidence that the H_3R may have a role in fear-induced avoidance responses, but not in anxiety. In addition, Vohora and Bhowmik (2012) provided comprehensive neurobiological/neurochemical evidence of the role of histaminergic H3 receptor antagonists in the physiopathology of cognitive dysfunction and motor impairments.

Dysfunctions of the histaminergic system may also contribute to the pathogenesis of multiple sclerosis and its murine model of experimental autoimmune encephalomyelitis, although the role of the different histamine receptors is complex and still controversial (Passani and Ballerini, 2012).

Histaminergic neurons are sensitive to CO_2, Yanovsky et al. (2012) showed the complex mechanism of histaminergic neuron activation by acidification in murine brain slices. Their results contribute to understand the neuronal mechanisms controling acid/CO_2-induced arousal in hepatic encephalopathy and obstructive sleep apnoea.

Recent evidence summarized by Blandina et al. (2012) suggest that such a complexity of the brain histamine system may be served by different neuronal subpopulations that are recruited at different times during the unfolding of a specific behavior. Histamine neurons send broad projections within the CNS that are organized in functionally distinct circuits impinging on different brain regions. This implies independent functions of subsets of histamine neurons according to their terminal projections and their selective participation in different aspects of behavioral responses.

In conclusion, we believe that this Research Topic offered an inter-disciplinary forum that improved our current knowledge of the role of brain histamine. It also provided the necessary drive to stimulate innovation in clinical practice to manage and treat neurological disorders.

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Novel chalcone-based fluorescent human histamine H_3 receptor ligands as pharmacological tools

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Novel fluorescent chalcone-based ligands at human histamine H_3 receptors (hH_3R) have been designed, synthesized, and characterized. Compounds described are non-imidazole analogs of ciproxifan with a tetralone motif. Tetralones as chemical precursors and related fluorescent chalcones exhibit affinities at hH_3R in the same concentration range like the reference antagonist ciproxifan (hH_3R pK_i value of 7.2). Fluorescence characterization of our novel ligands shows emission maxima about 570 nm for yellow fluorescent chalcones and ≥600 nm for the red fluorescent derivatives. Interferences to cellular autofluorescence could be excluded. All synthesized chalcone compounds could be used to visualize hH_3R proteins in stably transfected HEK-293 cells using confocal laser scanning fluorescence microscopy. These novel fluorescent ligands possess high potential to be used as pharmacological tools for hH_3R visualization in different tissues.

Keywords: human histamine H_3 receptor, fluorescent ligand, fluorescence confocal laser scanning microscopy, pharmacological tool

INTRODUCTION

Histaminergic receptors belong to class A of membrane bound G-protein-coupled receptors (GPCRs). They consist of four subtypes, the histamine H_1, H_2, H_3, and H_4 receptors (Walter and Stark, 2012). The H_3 receptor has a neurotransmitter function. High receptor densities could be found in different areas of the central nervous system (Martinez-Mir et al., 1990; Sander et al., 2008). Possible indications of H_3 receptor antagonists/inverse agonists could be the treatment of cognitive and sleep disorders as well as schizophrenia, epilepsy, adipositas, and neuropathic pain (Girard et al., 2004). To get information on etiopathology and accumulation or depletion of human histamine H_3 receptors (hH_3R) and to accelerate the clinical development of pharmaceuticals in the screening of drugs it is interesting to design labeled H_3 receptor ligands. To date several different techniques have been used to measure receptor occurrence in tissues. Radioactive competition is often used for instance in brain slices with [^{3}H](*R*)-alpha-methylhistamine (Martinez-Mir et al., 1990), [^{3}H]N^{α}-methylhistamine (Le et al., 2009), [^{3}H]-A-349821 (Miller et al., 2009), [^{125}I]iodoproxyfan (Ligneau et al., 1994; Stark et al., 1996), and [^{125}I]iodophenpropit (Jansen et al., 2000). Adversely, analysis of *ex vivo* autoradioactivity often takes longer time (Le et al., 2009) and synthesis and storage of radioligands causes high costs and special equipment/rooms. Radioactive exposure as generally known is very harmful. Fluorescent ligands are preferred over radioligands in terms of safety precautions and often applicability. Fluorescent ligands and their use for the localization and detection of GPCRs is still a topical area of investigation (Kuder and Kiec-Kononowicz, 2008). Attempts to design fluorescent human hH_3R were established with motif structure elements of Sangers reagent, dansyl, NBD, cyanoisoindol, and tetramethylrhodamine groups (Amon et al., 2006, 2007; Cowart et al., 2006; Kuder et al., 2010). Most of these compounds showed high affinity at histamine H_3 receptors (hH_3R K_i: 0.1–10 nM), but their fluorescence absorption and emission wavelengths were mainly between 300 and 500 nm. In this wavelength range interactions with cellular autofluorescence occur and in addition to this problem, most of these ligands possess low fluorescence intensities. A PhD thesis from the working group of Prof. Buschauer (University Regensburg, Erdmann, 2010) presented further fluorescent human histamine H_3 ligands. Applied fluorophores possess good fluorescent features but are expensive or difficult to synthesize. The aim of this study was the optimization of the fluorescent properties taking advantage of already published fluorescent ligands as lead structures. A literature survey indicated bio-active fluorescent benzylidine tetralones (Kamakshi et al., 2010) possessing different antibacterial activity and useful physicochemical data (Al-Ansari, 1998). Tomecková et al. (2004) reported on related cyclic chalcone analogs demonstrating biological effects on mitochondrial outer membrane via fluorescence microscopy. These results motivated us to use chalcones as fluorescent element for labeling of histamine H_3 receptor ligands to generate novel fluorescent pharmaceutical tools (**Figure 1**).

MATERIALS AND METHODS

CHEMISTRY

All reagents and solvents were purchased from VWR (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Ward Hill, MA, USA), Perkin Elmer Life and Analytical Sciences (Rodgau, Germany), and Acros Organics (Geel, Belgium), and were used without further purification (unless otherwise stated). ^{1}H and ^{13}C NMR spectra were recorded on a AV 250 Spektrometer (5.9 T; ^{1}H: 250 MHz; ^{13}C: 63 MHz), AV 300 Spektrometer (7.1 T; ^{1}H: 300 MHz; ^{13}C: 75 MHz), or AV 400 Spektrometer (0.4 T; ^{1}H: 400 MHz, ^{13}C: 100 MHz): Bruker (Rheinstetten, Germany). Electro-spray-ionization MS (ESI MS) was performed on a: VG

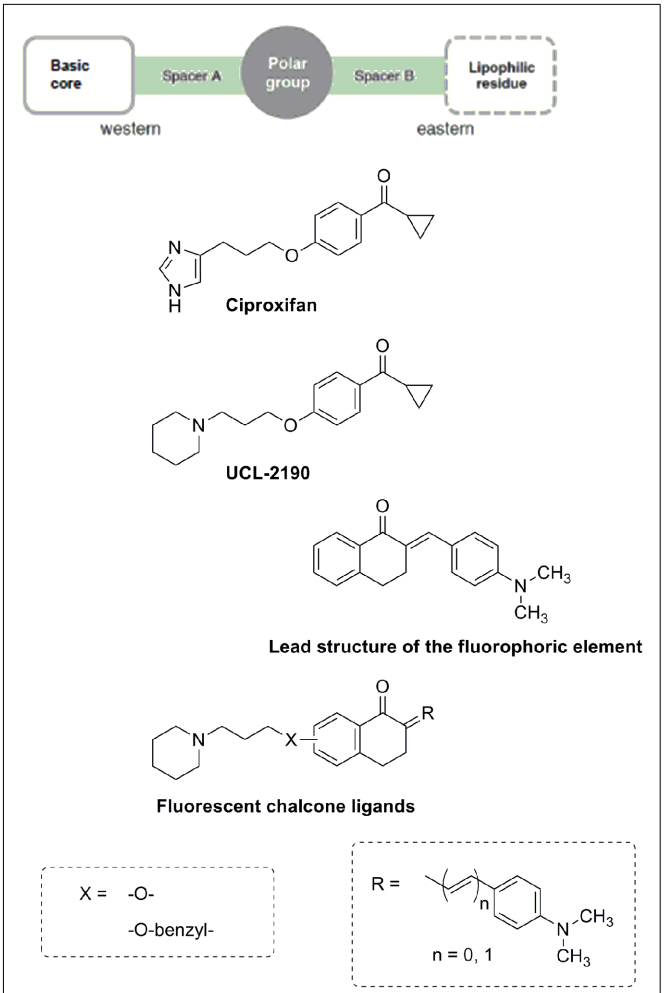


FIGURE 1 | Design strategy for novel fluorescent human histamine H_3 receptor ligands.

Platform II: Fisons Instruments (Manchester, GB) and nano-ESI (nESI): Mariner Workstation TOF: Applied Biosystems (Carlsbad, CA, USA). High resolution MS (HRMS) was recorded on a LTQ Orbitrap XL: Thermo Fisher Scientific (Waltham, MA, USA). Elemental analyses (C, H, N) were measured on a Vario MicroCube: Elementar Heraeus (Hanau, Deutschland) and were within ±0.4% of the theoretical values for all final compounds. Preparative column chromatography was performed on silica gel 60 F254, coat thickness: 0.2 mm (VWR, Darmstadt, Germany). The microwave oven used was a Biotage Initiator 2.0 (400 W): Biotage (Uppsala, Sweden). For detailed synthesis procedures and analytical data see Section "Appendix."

The initializing precursor 3-(piperidin-1-yl)propan-1-ol hydrochloride was synthesized by alkylation of piperidine with 3-chloropropan-1-ol as described in the literature (Apelt et al., 2005) and chlorinated (Sander et al., 2010). 6-Hydroxy-1-tetralone was commercially available whereas its regioisomer 7-hydroxy-1-tetralone was prepared from the corresponding methoxy derivative (**Scheme 1**). Ether cleavage has been carried out with *para*-toluenesulfonic acid and 1-butyl-3-methyl-1*H*-imidazolium bromide as ionic liquid under microwave condition with yields greater than 90% as described before (Boovanahalli et al., 2003). Synthesis of compound **1** and **2** started with the alkylation of 6- and 7-hydroxy-1-tetralone with 1-(3-chloropropyl)piperidine hydrochlorid (**Scheme 1**) by Williamson ether reaction, respectively (Williamson, 1851). Compound **1** and **2** were converted into fluorescent chalcones (compound **3**, **4**, **5,** and **6**) via aldol condensation with appropriate aldehyde.

Alkylation of 4-(hydroxymethyl)phenol with 1-(3-chloropropyl)piperidine hydrochlorid was the initializing step in the synthesis of compounds with elongated spacer B (**Scheme 2**). Resulting (4-(3-(piperidin-1-yl)propoxy)phenyl)methanol was chlorinated with thionylchloride. After alkylation with 6- and 7-hydroxy-1-tetralone fluorescent chalcones were synthesized with corresponding aldehydes (compounds **9–12**), respectively. All tetralone and chalcone derivatives were purified by column chromatography. Oily products were crystallized as salts of oxalic acid in ethanol.

For fluorescence characterization all corresponding histamine H_3 receptor ligands were dissolved in buffer (12.5 mM $MgCl_2$, 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4) at a concentration of 10 mM. Fluorescence absorption and emission spectra were recorded on a Fluorolog HORIBA JOBIN YVON fluorometer (HORIBA scientific, Kyoto, Japan) at room temperature. Spectra were analyzed with FluorEssence™(HORIBA scientific, Kyoto, Japan) for Windows®.

BINDING STUDIES

Determination of human histamine H_3 receptor affinity

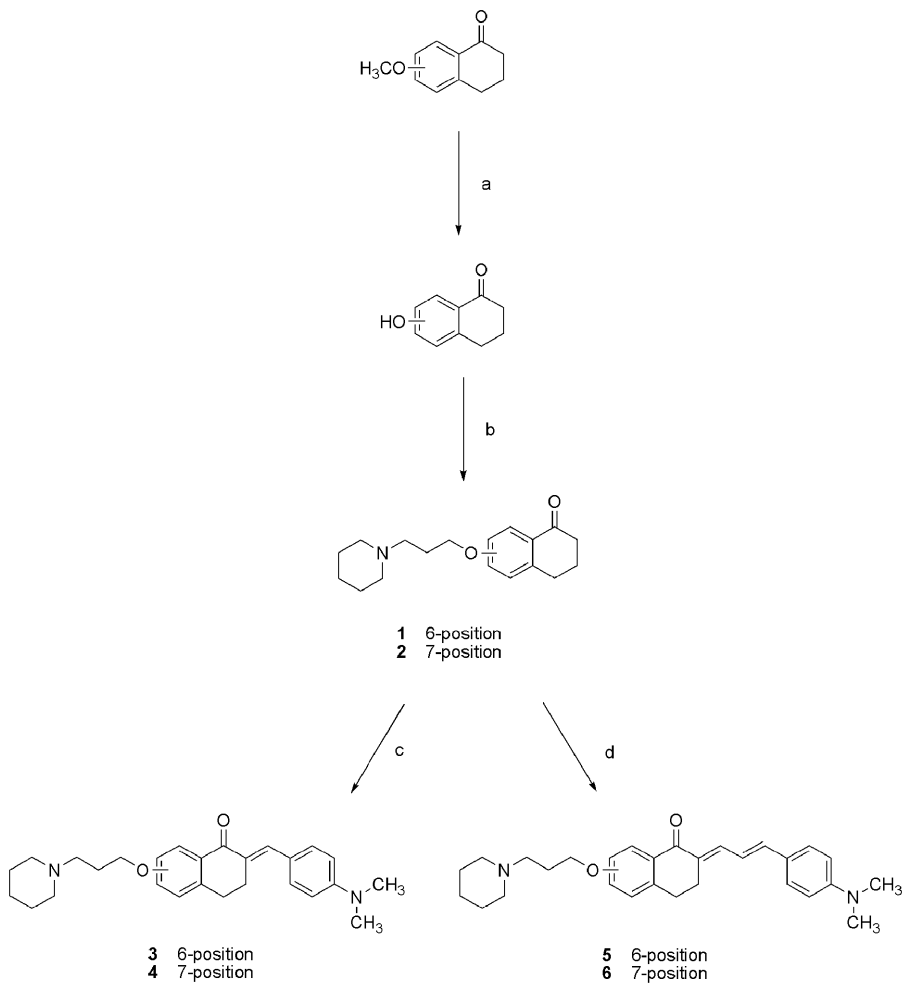
HEK-293 cells stably expressing the recombinant human histamine H_3 receptor were used for membrane extraction. In brief, membrane protein concentration was determined by the method of Bradford (1976). Competition binding experiments were carried out as described before (Kottke et al., 2011). Membranes were incubated with [^{3}H]N^{α}-methylhistamine (2 nM) and test ligand in a concentration range between 0.01 nM and 100 μM. For determination of non-specific binding 10 μM of pitolisant was used. All values are means of at least three independent measurements, each in triplicates and on seven different concentrations. Binding data were analyzed by the software GraphPad Prism™(2000, version 3.02, San Diego, CA, USA).

Determination of human histamine H_1 receptor affinity

CHO-K1 cells expressing the human histamine H_1 receptor were used for membrane extraction. In brief, human histamine H_1 receptor radioligand competition binding assay was performed as described before (Rossbach et al., 2011). Membranes were incubated with 1 nM of [^{3}H]pyrilamine and test ligand in a concentration range from 100 nM to 100 μM. For determination of non-specific binding 10 μM of chlorpheniramine was used. All values are means of at least two independent measurements, each in triplicates and four different concentrations. Binding data were analyzed by the software GraphPad Prism™(2000, version 3.02, San Diego, CA, USA).

Determination of human histamine H_4 receptor affinity

Sf9 cells transiently expressing the human histamine H_4 receptor and co-expressed with G-protein $G_{\alpha i/o}$ and $G_{\beta 1\gamma 2}$ subunits were



SCHEME 1 | Synthesis of compounds 1–6. (a) *para*-Toluenesulfonic acid, 1-butyl-3-methyl-1*H*-imidazolium bromide, microwave irradiation, 160°C, 1 h, $\eta = 97\%$; (b), 1-(3-chloropropyl)piperidine hydrochlorid, K_2CO_3, KI, acetone, 60°C, 48 h, $\eta = 71$–94%; (c) 4-(dimethylamino)benzaldehyde, ethanol/water, NaOH, RT, over night, $\eta = 63$–85%; (d) 4-(dimethylamino)cinnamic aldehyde, ethanol/water, NaOH, RT, over night, $\eta = 69$–89%.

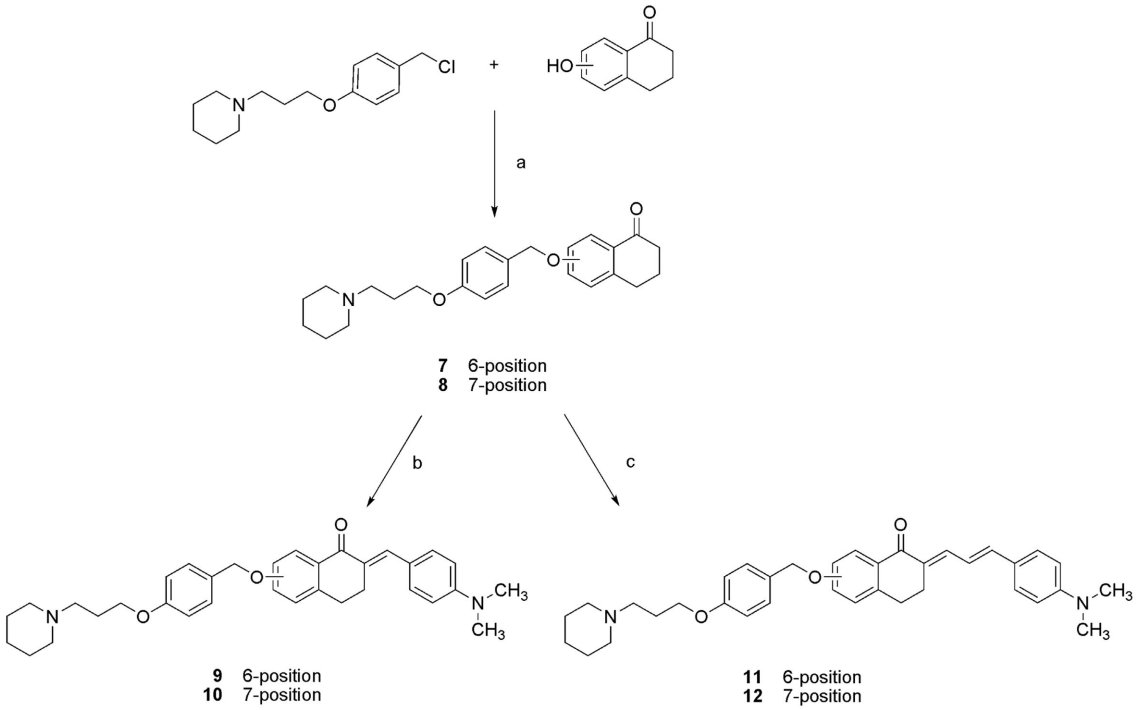
used for membrane extraction. Competition binding experiments were carried out as described before (Kottke et al., 2011). Membranes were incubated with 10 nM [^{3}H]histamine and test ligand in a concentration range from 0.01 nM to 100 μM. Non-specific binding was determined by using 10 μM JNJ-7777120. All values are means of at least two independent measurements, each in triplicates and four different concentrations. Binding data were analyzed by the software GraphPad Prism^{TM} (2000, version 3.02, San Diego, CA, USA).

CELL CULTURE

Slides were coated with poly-D-lysine (50 μg/mL, Sigma-Aldrich, Steinheim, Germany) and cooled for 2 h. HEK-293 cells stably expressing the recombinant human histamine receptor (mean protein content: 10 μg/mL; B_{max}: 300–900 fmol/mg) were seeded (100,000 cells/mL) and grown in 2 mL of Dulbecco´s modified Eagle´s medium (without phenol red) with 2 mM glutamine, 10 mM HEPES, 10% fetal bovine serum, and 10 μL/mL penicillin/streptomycin in an atmosphere of 5% CO_2 at 37°C for 48 h in six-well plates with poly-D-lysine coated slides. The medium was removed, and the slides were incubated with 3% BSA in buffer (12.5 mM $MgCl_2$, 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4) for 30 min at 37°C. Afterward the slides were washed with PBS buffer and incubated with 2 mL 200 nM of appropriate fluorescent H_3 ligand in buffer (12.5 mM $MgCl_2$, 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4) for 1 h at 37°C. The ligand solution was removed, washed with PBS buffer, and the cells were fixed in methanol for 20 min at a temperature of −20°C. After equilibration with PBS buffer the nucleus was stained with 100 nM blue fluorescent DAPI (4′,6-diamidino-2-phenylindole; Kapuscinski, 1995). The slides were mounted on cover slips with 30 μL Mowiol. For reasons of comparison HEK-293 cells were cultured as blind reference and treated with respective ligand in a similar manner.

FLUORESCENCE MICROSCOPY

Cell preparations were fluorescently visualized with a confocal laser scanning microscope (Leica TCS SP5, Wetzlar, Germany). Fluorescence intensities were adjusted minimizing



SCHEME 2 | Synthesis of compound 7–12. (a) K_2CO_3, KI, acetone, 60°C, 48 h, η = 65–71%; (b) 4-(dimethylamino)benzaldehyde, ethanol/water, NaOH, RT, over night, η = 85–91%; (c) 4-(dimethylamino)cinnamic aldehyde, ethanol/water, NaOH, RT, over night, η = 93–95%.

autofluorescence and all slices were measured with the same intensity to generate comparable images. All images were recorded with a 60× oil immersions objective. Fluorescence colors were adapted to appropriate fluorescence emission wavelengths. Fluorescent ligands were excited with a 488 nm multiline argon laser and emission was detected between 520 and 790 nm. DAPI-stained nuclei of the HEK-293 cells were excited with a 405 nm diode laser and emission was measured between 420 and 500 nm. Images were recorded in sequential mode to avoid interferences.

RESULTS AND DISCUSSION

DESIGN OF NOVEL HUMAN HISTAMINE H_3 LIGANDS

Historically the first potent histamine H_3 receptor ligands were derivatized from the endogenous ligand histamine. Ciproxifan as parent compound for these lead structures possesses a basic imidazole ring as core moiety (**Figure 1**). Spacer A is a propyl group, followed by a polar ether functionality linking the phenyl group which is then connected to structurally different affinity enhancing elements. The imidazole-containing ciproxifan exerts an affinity at the human histamine H_3 receptor in the medium nanomolar concentration range (**Table 1**; Ligneau et al., 2000). Since the imidazole group may potentially be associated with interaction to the cytochrome P_{450} enzyme system, the change from imidazole to more robust piperidine systems in the western part of the molecule has been performed resulting in an improved pharmacological profile (Lazewska et al., 2006, 2008; Sander et al., 2008). In 2001 the piperidine analog of ciproxifan, UCL-2190 (**Figure 1**), was published (Meier et al., 2001). Likewise we introduced the piperidine core element in our newly designed compounds, kept spacer A constant with three methylene groups as well as the benzyl ether spacer. For compound **7–12** spacer B was elongated by the benzyl ether. The keto group in the lipophilic residue of ciproxifan was incorporated into a tetrahydronaphthalene system (**1**, **2**, **7**, and **8**) which were converted to fluorescent chalcones (**3**, **4**, **5**, **6**, **9**, **10**, **11**, and **12**).

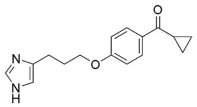
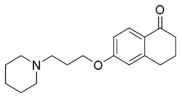
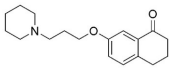
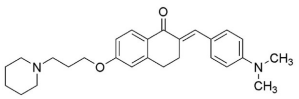
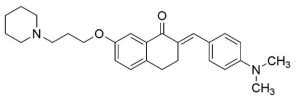
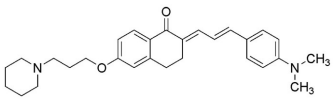
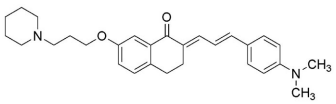
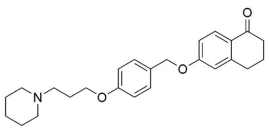
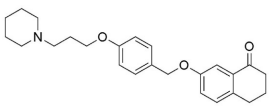
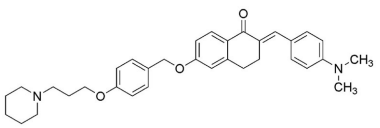
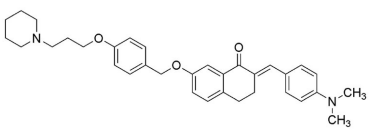
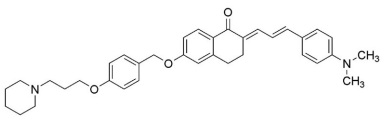
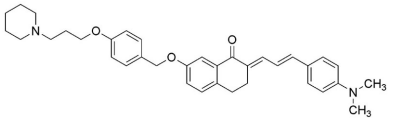
During design of novel fluorescent pharmacological tools it is important to generate compounds with high affinity at the target receptor, high fluorescence intensities, and emission wavelengths near the infrared wavelength range. It is difficult to retain binding affinity and low molecular weight with structurally larger fluorophoric elements in small molecule GPCR ligands. Fluorophores emitting with high wavelengths frequently consist of bulky structures. Al-Ansari (1998) and Kamakshi et al. (2010) described the synthesis of unbulky, fluorescent benzylidene tetralones which have been used as fluorophore lead element (**Figure 1**).

DETERMINATION OF HUMAN HISTAMINE H_3 RECEPTOR AFFINITY

Affinities of the newly designed fluorescent chalcones (**3**, **4**, **5**, **6**, **9**, **10**, **11**, and **12**) at the human histamine H_3 receptor are in a comparable nanomolar concentration range like that of ciproxifan (**Table 1**). Tetralones with elongated aromatic spacer B exhibit slightly lower K_i values than those of the shorter tetralones (**1** → **7**; **2** → **8**) most probably due to increased lipophilic interactions. Elongation of the tetralones with appropriate aldehydes gives no further improvement in binding properties.

The variation from 6- to 7-hydroxy-1-tetralone derivatives causes no remarkable difference in binding and does not result in higher affinities at the human histamine H_3 receptor.

Table 1 | Human histamine H_1, H_3, and H_4 receptor affinities and fluorescence absorption and emission maximum wavelengths of novel non-imidazole ligands.

Compound	Structure	hH_1R K_i [nM][a]	hH_3R K_i [nM][b]	hH_4R K_i [nM][c]	λ_{max} Abs. [nm][d]	λ_{max} Em. [nm][d]	Stokes shift [nm]
Ciproxifan		>10,000[e]	46 ± 4[f]	612 ± 32[g]			
1		3,473 ± 475	68 ± 12	100,535 ± 21,305			
2		3,717 ± 873	89 ± 8	169,895 ± 118,942			
3		791 ± 192	70 ± 21	12,370 ± 3,055	405	584	179
4		2,142 ± 886	149 ± 29	13,725 ± 1,110	397	583	186
5		881 ± 203	94 ± 26	5,634 ± 2,100	468	670	202
6		1,974 ± 89	101 ± 13	8,704 ± 204	467	674	207
7		1,968 ± 416	19 ± 5	37,260 ± 13,209			
8		3,686 ± 950	17 ± 5	43,875 ± 9,581			
9		1,768 ± 472	41 ± 4	12,598 ± 6,608	405	567	162
10		4,228 ± 1,121	97 ± 34	15,610 ± 5,841	405	583	178
11		1,374 ± 10	68 ± 3	6,400 ± 2,189	467	600	133
12		1,837 ± 837	87 ± 20	10,984 ± 3,403	467	607	140

[a][^{3}H]Pyrilamine competition binding assay with membrane preparation of CHO-K1 cells expressing human histamine H_1 receptor. [b][^{3}H]N^{α}-Methylhistamine competition binding assay with membrane preparation of HEK-293 cells over-expressing human histamine H_3 receptor. [c][^{3}H]Histamine displacement assay with membrane preparation of Sf9 cells transiently expressing human histamine H_4 receptor and co-expressed with supporting G-Protein $G_{\alpha i/o}$ and $G_{\beta 1\gamma 2}$. K_i values are means ± SEM. [d]Measured in buffer (12.5 mM $MgCl_2$, 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4) at a concentration of 10 mM. [e]Zhao et al. (2008). [f]Ligneau et al. (2000). [g]Gbahou et al. (2006).

DETERMINATION OF HUMAN HISTAMINE RECEPTOR SELECTIVITY

For selectivity validation the new compounds have been screened on affinity at related human histamine H_1 and H_4 receptors. All ligands possess highest affinity at the human histamine H_3 receptor demonstrating their receptor preference (**Table 1**). Affinity at the human H_1 receptor is about one log unit and at the human histamine H_4 receptor about two log units higher than that at the human histamine H_3 receptor.

Compound **8** demonstrated one of the highest selectivity over all 12 compounds. Compound **9** is the most selective compound in the fluorescent chalcone series whereas compound **5** the less selective chalcone. These data further prove the beneficial effects of elongation of spacer B via benzyl ether concerning selectivity and affinity.

FLUORESCENCE CHARACTERIZATION

Kamakshi et al. (2010) described the fluorophore lead structure (**Figure 1**) as compound with intense emission signals. Absorption maximum was found at 406 nm. The fluorophore lead is described as charge transfer compound where the *N*,*N*-dimethyl group behaves as the electron donor moiety and the carbonyl group as the electron acceptor moiety. These two groups form a donor–acceptor complex upon excitation. Fluorescence measurement confirmed the absorption maxima (compounds **3**, **9,** and **10**: 405 nm; cf. Appendix).

Introduction of an additional vinyl group resulted in an increased maximum in excitation and emission wavelength. (cf. **3** → **5**: $\Delta\lambda_{max}$ Abs.: 63 nm, $\Delta\lambda_{max}$ Em.: 102 nm). Compounds with propenone element (**3**, **4**, **9**, **10**) emit in the yellow area of the fluorescence spectrum and compounds with pentadienone elements (**5**, **6**, **11**, **12**) in the red one. It is important to design fluorophores which emit fluorescence higher than 500 nm. In the blue spectroscopic area (300–450 nm) high autofluorescence occurs and causes difficulties in detection of the actual target. In fluorescence visualization it is the aim to achieve emission in the near infrared wavelength area to improve the signal-to-noise ratio. Therefore it is a need to use fluorophores with high quantum yields and low quenching properties. To avoid self-quenching it is important to synthesize fluorophores with large Stokes shifts. All compounds fulfill this requirement (**Table 1**).

Elongation of spacer B via benzyl ether group causes decrease of emission wavelengths of red emitting chalcones. Introduction of a phenylether group can influence the donor–acceptor complex of the fluorophore. Elongated compounds show higher fluorescence emission intensities (**Figure 2**; cf. Appendix).

For the measurement of fluorescence absorption and emission buffer (12.5 mM $MgCl_2$, 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4) has been used to simulate assay conditions and simultaneously find the ideal settings for confocal laser microscope studies.

VISUALIZATION OF HUMAN HISTAMINE H_3 RECEPTORS

To minimize unspecific binding cells were incubated for 30 min with 3% BSA solution. Fluorescent histamine H_3 receptor ligands were added in a concentration range of two up to three times of K_i-value at the human histamine H_3 receptor (200 nM)

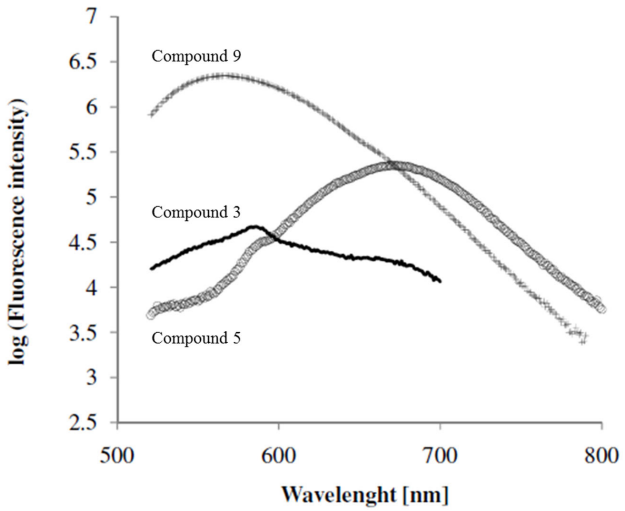


FIGURE 2 | Fluorescence emission spectra of selected chalcone derivatives.

on hH_3-HEK-293 and HEK-293 cells. After washing steps and staining of the nucleus with blue fluorescent DAPI as reported in literature (Kapuscinski, 1995), slides were fixed with Mowiol and analyzed with a confocal laser scanning microscope (**Figure 3**).

Chalcones synthesized from 4-(dimethylamino)benzaldehyde result in yellow fluorescent ligands like compound **3** and **9**. Chalcones synthesized from 4-(dimethylamino)cinnamic aldehyde in red emitting ligands like compound **5**. Due to their maximum emission wavelengths ligand color on the confocal laser scanning microscope was adjusted. Fluorescence images were recorded in sequential mode to avoid interfering of emission wavelengths of ligand and DAPI fluorescence. In the first step DAPI-stained nuclei were recorded (**Figures 3B,E,H,K**) between 420 and 500 nm, then fluorescent ligand emission was measured between 520 and 790 nm (**Figures 3A,D,G,J**). Appropriate images were over-laid to co-localize fluorescent ligand and fluorescent nucleus (**Figures 3C,F,I,L**). **Figure 3D** shows the enrichment of the yellow emitting compound **3** in the outer membrane of hH_3-HEK-293 cells. **Figure 3G** shows the enrichment in same regions in hH_3-HEK-293 cells of the red emitting compound **5** which emits more intensive (**Figure 2**). The most human histamine H_3 receptor selective and most intense compound (**9**) is shown in **Figures 3J–L**. Fluorescent human histamine H_3 ligands stain the outer membrane of hH_3-HEK-293 cells where human hH_3R are mainly expressed. In HEK-293 cells which do not have human hH_3R, no enrichment of ligand in the outer cell membrane can be identified (**Figures 3A,C**; cf. Appendix) indicating selectivity to the aimed receptor (**Table 1**). The fluorescent ligands on HEK-293 cells are diffusely distributed and emit in a low manner if compared to images **3D**, **3G,** and **3J** where outer membranes emit strong fluorescence (cf. Appendix). Differences between hH_3- and HEK-293 cells are facile to detect. Fluorescence emission of chalcone derivatives on HEK-293 cells is in the same range as autofluorescence (cf. Appendix) and therefore negligible.

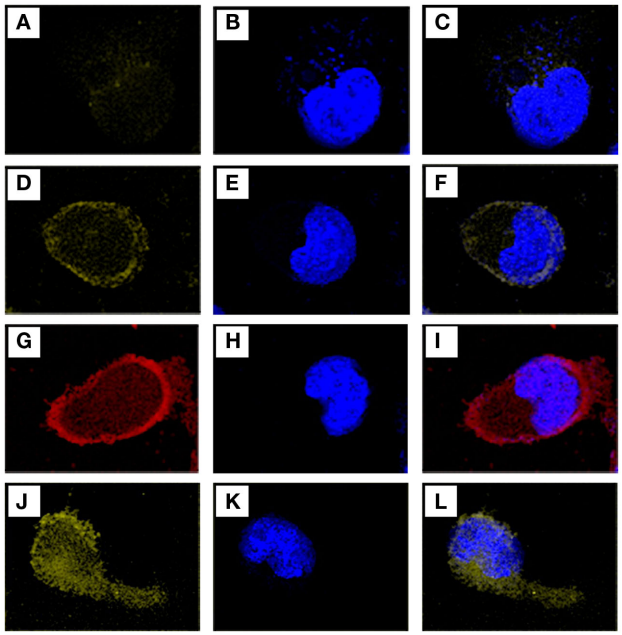


FIGURE 3 | Confocal laser scanning microscope images of fluorescent human histamine H_3 ligands on HEK-293 cells and hH_3-HEK-293. All images were taken on a Leica TCS SP5 microscope. Fluorescent chalcones were incubated for 1 h and after washing steps nuclei were stained with blue fluorescent DAPI. **(A)** Shows a HEK-293 cell labeled with compound **3** and **(B)** shows the DAPI-stained nucleus of the same cell. **(C)** Is an overlay of image **(A,B)**. **(D)** Shows compound **3** on an hH_3-HEK-293 cell, **(E)** the DAPI-stained nucleus of the same cell and **(F)** an overlay of **(D)** and **(E)**. Red fluorescent compound **5** on an hH_3-HEK-293 cell is shown in **(G)**, cell nucleus in **(H)**, and the overlay of **(G)** and **(H)** in **(I)**. **(J)** Shows an hH_3-HEK-293 cell stained with compound **9**, **(K)** its nucleus, and **(L)** the overlay of **(J,K)**.

CONCLUSION

In our work we achieved the design of selective and fluorescent human histamine H_3 receptor ligands. Affinities at the human histamine H_3 receptor of all newly synthesized compounds are in the nanomolar concentration range. All 12 compounds showed high H_3 receptor preference in comparison to their binding properties at H_1 and H_4 receptors.

The fluorescent chalcone ligands facilitate visualization of human hH_3R on hH_3–HEK cells. Their fluorescence characteristics (fluorescence emission maxima higher than 500 nm) avoid appearance of interference with autofluorescence. Intense light signals label the localization of targeted receptor. Reference HEK-293 cells which do not over-express human hH_3R show negligible binding of fluorescent ligands demonstrating low unspecific binding.

Compounds **3**, **5,** and **9** showed excellent, but slightly diverse properties concerning absorption, emission, Stokes shift, intensity and H_3 receptor affinity and selectivity. Best compound concerning wavelength represents compound **5** (λ_{max} Abs./Em.: 468/670 nm) but it is not as selective as compound **9** which emits with highest intensity of all synthesized compounds in the yellow area of spectrum. Compound **9** has the highest affinity at the human histamine H_3 receptor compared to the other chalcone derivatives in this series.

Our new fluorescent ligands open new possibilities for non-radioactive alternatives for novel binding assays and novel visualization tools. They are precious tools for detailed pharmacological analyses on detection of receptors in cells and tissues. Studies on different tissues with these novel ligands are envisaged to provide further evidence of their convenience as pharmacological tools.

More detailed information is available in the Appendix.

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APPENDIX

CHEMICAL ANALYSIS OF SYNTHESIZED COMPOUNDS

NMR chemical shifts (δ) are reported in ppm downfield from tetramethylsilane as internal reference. Multiplicities of peaks have following abbreviations: br, broad; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; q, quintet. Coupling constants are given in Hertz (Hz). Numbers and assignments of protons or carbon atoms are named: ox, oxalate; pip, piperidine; ph, phenyl; prop, propyl; tetr, tetralone; dmab, 4-(dimethylamino)benzyl; dmap, 4-(dimethylamino)phenyl; benzylid, benzylidene; allylid, allyllidene.

3-(Piperidin-1-yl)propan-1-ol hydrochloride

Piperidine (47.4 g, 0.56 mol), 3-chloropropan-1-ol (35.1 g, 0.37 mol), potassium carbonate (77.0 g, 0.56 mol) and potassium iodide (61.6 g, 0.37 mol) were refluxed in absolute acetone (300 mL) for 72 h under inert atmosphere. The mixture was allowed to cool to room temperature, inorganic components were removed by filtration and the filtrate was concentrated to dryness. The crude oil was further purified by distillation (10 mbar, 88°C). The oily product was crystallized as hydrochloride from isopropanolic HCl. White solid, 54.1 g, 81%.

$C_8H_{17}NO \times HCl$

Molecular weight: 179.7

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.59 (br s, 1H, NH^+), 4.39 (s, 1H, OH), 3.46 (t, J = 5.9, 2H, prop-1H_2), 3.36–3.31 (m, 2H, pip-2,6H_{eq}), 3.01–2.94 (m, 2H, prop-3H_2), 2.84–2.80 (m, 2H, pip-2,6H_{ax}), 1.86–1.66 (m, 7H, prop-2H_2, pip-3,5H_2, pip-4H_{eq}), 1.42–1.28 (m, 1H, pip-4H_{ax}).

^{13}C NMR (75 MHz, DMSO-d_6) δ 57.99 (prop-1C), 53.49 (prop-3C), 52.99 (pip-2,6C), 26.36 (prop-2C), 22.18 (pip-3,5C), 21.33 (pip-4C).

ESI MS: 143.8 [M + H$^+$] (100).

1-(3-Chloropropyl)piperidine hydrochloride

3-(Piperidin-1-yl)propan-1-ol hydrochloride (35.3 g, 0.2 mol) was suspended in toluene (100 mL) an excess of thionyl chloride (28.5 mL, 0.4 mol) was added dropwise at a temperature of 0°C under inert atmosphere. After beginning of the exothermic reaction the mixture was stirred for 3 h at 60°C. Thionyl chloride and toluene were distilled off. The beige product crystallized during the reaction and could be filtered off. Beige solid, 38.1 g, 98%.

$C_8H_{16}ClN \times HCl$

Molecular weight: 198.1

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.69 (br s, 1H, NH^+), 3.68 (t, J = 6.4, 2H, prop-3H_2), 3.41–3.36 (m, 2H, pip-2,6H_{eq}), 3.11–3.04 (m, 2H, prop-1H_2), 2.94–2.81 (m, 2H, pip-2,6H_{ax}), 2.31–2.22 (m, 2H, prop-2H_2), 1.77–1.60 (m, 5H, pip-3,5H_2, pip-4H_{eq}), 1.39–1.28 (m, 1H, pip-4H_{ax}).

^{13}C NMR (75 MHz, DMSO-d_6) δ 54.14 (prop-3C), 52.01 (pip-2,6C), 41.44 (prop-1C), 25.19 (prop-2C), 23.08 (pip-3,5C), 22.31 (pip-4C).

ESI MS: 161.6 [M + H$^+$] (100).

Compound 1

6-(3-(Piperidin-1-yl)propoxy)-1-tetralone. 1-(3-Chloropropyl) piperidine hydrochloride (1 g, 5.1 mmol), 6-hydroxy-1-tetralone (0.9 g, 5.6 mmol), potassium carbonate (2.3 g, 16.7 mmol) and potassium iodide (0.9 g, 5.6 mmol) were refluxed in absolute acetone (100 mL) for 72 h under inert atmosphere. After cooling to room temperature inorganic salts were filtered off. The filtrate was concentrated to dryness and resuspended in dichloromethane. The organic layer was washed three times with 2 M NaOH solution and brine, dried with $MgSO_4$, and concentrated to dryness. Resulting brown oil was crystallized with waterfree oxalic acid in absolute ethanol. Beige crystals were filtered off. Beige solid, 1.4 g, 71%.

$C_{18}H_{25}NO_2 \times (COOH)_2 \times 0.25\ H_2O$

Molecular weight: 381.9

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83 (d, J = 8, 1H, tetr-8H), 6.90 (m, J = 12, 2H, tetr-5,7H), 4.09 (t, J = 8, 2H, prop-3H_2), 2.91 (t, J = 8, 2H, tetr-2H_2), 2.52 (t, J = 9, 2H, tetr-4H_2), 2.38 (t, 2H, J = 8, prop-1H_2), 2.36–2.34 (m, 4H, pip-2,6H_2), 2.01 (q, 2H, J = 8, tetr-3H_2), 1.88 (q, 2H, J = 7, prop-2H_2), 1.54–1.46 (m, 4H, pip-3,5H_2), 1.43–1.35 (m, 2H, pip-4H_2).

^{13}C NMR (100 MHz, DMSO-d_6) δ 196.04 (tetr-1C), 164.40 (ox-COOH), 162.53 (tetr-7C), 147.18 (tetr-8aC), 128.67 (tetr-8C), 125.63 (tetr-4aC), 113.58 (tetr-5C), 113.08 (tetr-7C), 66.24 (prop-3C), 54.96 (prop-1C), 54.08 (pip-2,6C), 38.38 (tetr-2C), 29.27(tetr-4C), 26.13 (prop-2C), 25.57 (pip-3,5C), 24.10 (pip-4C), 22.93 (tetr-3C).

ESI MS: 288.52 [M + H$^+$] (100).

Anal. calc.: C, 62.89; H, 7.26; N, 3.67. Found: C, 62.91; H, 7.41; N, 3.58.

7-Hydroxy-1-tetralone. 7-Methoxy-1-tetralone (1 g, 5.7 mmol), *para*-toluenesulfonic acid (3.2 g, 17.0 mmol), and 1-butyl-3-methyl-1H-imidazol-3-ium bromide (12.2 g, 34.1 mmol) were mixed in a 20 mL microwave vial. Mixture was heated for 1 h for 160°C under microwave condition. After cooling down to room temperature the reaction mixture was diluted with water and extracted three times with ethyl acetate. The organic phase was washed with brine, dried with $MgSO_4$, and concentrated to dryness. Resulting yellowish crystals were used without further purification. Yellowish solid, 0.9 g, 97%.

$C_{10}H_{10}O_2$

Molecular Weight: 162.1

^{1}H NMR (250 MHz, DMSO-d_6) δ 9.58 (s, 1H, OH), 7.25 (s, 1H, tetr-8H), 7.19 (d, J = 10, 1H, tetr-5H), 6.98 (d, J = 10, 1H, tetr-6H), 2.83 (t, J = 7.5, 2H, tetr-4H_2), 2.55 (t, J = 7.5, 2H, tetr-2H_2), 2.00 (q, J = 6.3, 2H, tetr-3H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 197.48 (tetr-1C), 155.86 (tetr-7C), 135.27 (tetr-4aC), 133.06 (tetr-8aC), 130.18 (tetr-5C), 121.10 (tetr-6C), 111.80 (tetr-8C), 38.30 (tetr-2C), 28.12 (tetr-4C), 23.25 (tetr-3C).

ESI MS: 160.5 [M − H$^+$] (100).

Compound 2

7-(3-(Piperidin-1-yl)propoxy)-1-tetralone. 1-(3-Chloropropyl) piperidine hydrochloride (1.55 g, 7.9 mmol), 7-hydroxy-1-tetralone (1.4 g, 8.6 mmol), potassium carbonate (3.6 g, 25.9 mmol), and potassium iodide (1.4 g, 8.6 mmol) were refluxed in absolute acetone (50 mL) for 72 h under inert atmosphere. After cooling to room temperature inorganic salts were filtered off. The filtrate was concentrated to dryness and resuspended in dichloromethane.

The organic layer was washed three times with 2 M NaOH solution and brine, dried with $MgSO_4$, and concentrated to dryness. Beige crystals, 2.1 g, 94%.

$C_{18}H_{25}NO_2$

Molecular Weight: 287.4

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.34 (s, 1H, tetr-8H), 7.29 (d, $J = 7.5$, 1H, tetr-5H), 7.16 (d, $J = 7.5$, 1H, tetr-6H), 4.02 (t, $J = 5$, 2H, prop-1H_2), 2.87 (t, $J = 6.25$, 2H, tetr-4H_2), 2.59 (t, $J = 6.25$, 2H, tetr-2H_2), 2.38 (t, 2H, $J = 5$, prop-3H_2), 2.35–2.31 (m, 4H, pip-2,6H_2), 2.02 (q, 2H, $J = 5$, tetr-3H_2), 1.86 (q, 2H, $J = 7$, prop-2H_2), 1.54–1.48 (m, 4H, pip-3,5H_2), 1.41–1.34 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 197.38 (tetr-1C), 157.35 (tetr-7C), 137.14 (tetr-4aC), 133.18 (tetr-8aC), 130.60 (tetr-5C), 122.08 (tetr-6C), 109.99 (tetr-8C), 66.39 (prop-1C), 55.09 (prop-3C), 54.10 (pip-2,6C), 38.45 (tetr-2C), 27.94 (tetr-4C), 26.56 (prop-2C), 25.60 (pip-3,5C), 24.10 (pip-4C), 23.09 (tetr-3C).

ESI MS: 288.6 [M + H$^+$] (100).

HRMS: calc.: 288.19581; found: 288.19633.

Compound 3

(E)-2-(4-(Dimethylamino)benzylidene)-6-(3-(piperidin-1-yl) propoxy)-1-tetralone. Compound 1 (500 mg, 1.7 mmol) and 4-(dimethylamino)benzaldehyde (260 mg, 1.7 mmol) were dissolved in 5 mL ethanol. 15 M NaOH (0.25 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). The oily product was crystallized with waterfree oxalic acid in absolute ethanol. Crystals were filtered off. Yellow solid, 582 mg, 63%.

$C_{27}H_{34}N_2O_2 \times (COOH)_2 \times 1.25\ H_2O$

Molecular weight: 530.78

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, $J = 8$, 1H, tetr-8H), 7.63 (s, 1H, benzylid-2H), 7.45 (d, $J = 8$, 2H, dmab-2,6H), 6.95 (d, $J = 8$, 1H, tetr-7H), 6.90 (s, 1H, tetr-5H), 6.79 (d, $J = 8$, 2H, dmab-3,5H), 4.16 (t, $J = 12$, 2H, prop-3H_2), 3.12–3.08 (m, 6H, prop-1H_2, pip-2,6H_2), 3.00 (s, 6H, dmab-N(CH_3)), 2.96 (t, $J = 9$, 2H, tetr-4H_2), 2.92 (q, $J = 8$, 2H, tetr-3H_2), 2.15 (q, $J = 7$, 2H, prop-2H_2), 1.80–1.69 (m, 4H, pip-3,5H_2), 1.61–1.41 (m, 2H, pip-4H_2).

^{13}C NMR (100 MHz, DMSO-d_6) δ 196.12 (tetr-1C), 164.32 (ox-COOH), 161.87 (tetr-7C), 150.52 (dmab-4C), 145.45 (tetr-8aC), 136.37 (benzylid-2C), 132.05 (tetr-2C), 131.84 (dmab-2,6C), 129.67 (tetr-8C), 126.64 (tetr-4aC), 122.64 (dmab-1C), 113.77 (tetr-5C), 112.72 (tetr-7C), 111.68 (dmab-3,5C), 65.30 (prop-3C), 53.40 (prop-1C), 52.28 (pip-2,6C), 40.12 (dmab-N(CH$_3$)$_2$), 28.18(tetr-4C), 26.82 (prop-2C), 23.54 (pip-4C), 22.78 (pip-3,5C), 21.53 (tetr-3C).

ESI MS: 419.6 [M + H$^+$] (100).

Anal. calc.: C, 65.58; H, 7.31; N, 5.27. Found: C, 65.63; H, 6.92; N, 5.27.

Compound 4

(E)-2-(4-(Dimethylamino)benzylidene)-7-(3-(piperidin-1-yl) propoxy)-1-tetralone. Compound 2 (500 mg, 1.7 mmol) and 4-(dimethylamino)benzaldehyde (260 mg, 1.7 mmol) were dissolved in 7 mL ethanol. 15 M NaOH (0.25 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Yellow solid, 619 mg, 85%.

$C_{27}H_{34}N_2O_2$

Molecular Weight: 418.6

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.67 (s, 1H, benzylid-2H), 7.47 (d, $J = 10$, 2H, dmab-3,5H), 7.42 (s, 1H, tetr-8H), 7.30 (d, $J = 7.5$, 1H, tetr-5H), 7.16 (d, $J = 8.75$, 1H, tetr-6H), 6.79 (d, $J = 7.5$, 2H, dmab-2,6H), 4.06 (t, $J = 7.5$, 2H, prop-3H_2), 3.09 (t, $J = 6.25$, 2H, tetr-3H_2), 2.99 (s, 6H, dmab-N(CH_3)), 2.86 (t, $J = 7.5$, 2H, tetr-4H_2), 2.62–2.52 (m, 6H, prop-3H_2, pip-2,6H_2), 1.99 (q, 2H, $J = 9$, prop-2H_2), 1.65–1.56 (m, 4H, pip-3,5H_2), 1.48–1.41 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 186.24 (tetr-1C),157.44 (tetr-7C), 150.68 (dmab-4C), 137.24 (benzylid-2C), 134.30 (tetr-8aC), 131.91 (dmab-2,6C), 130.96 (tetr-4aC), 130.34 (tetr-5C), 122.63 (dmab-1C), 117.11 (tetr-6C), 111.93 (dmab-3,5C), 110.60 (tetr-8C), 65.94 (prop-1C), 54.54 (prop-3C), 53.21 (pip-2,6C), 39.53 (dmab-N(CH$_3$)$_2$), 27.37 (pip-3,5C), 26.12 (tetr-4C), 25.47 (prop-2C), 24.32 (pip-4C), 23.18 (tetr-3C).

ESI MS: 419.7 [M + H$^+$] (100).

HRMS: calc.: 419.26930; found: 419.26971.

Compound 5

(E)-2-((E)-3-(4-(Dimethylamino)phenyl)allylidene)-6-(3-(piperidin-1-yl)propoxy)-1-tetralone. Compound 1 (500 mg, 1.7 mmol) and 4-(dimethylamino)cinnamic aldehyde (305 mg, 1.7 mmol) were dissolved in 3 mL ethanol. 15 M NaOH (0.5 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). The oily product was crystallized with ethanol. Crystals were filtered off. Red solid, 545 mg, 69%.

$C_{29}H_{36}N_2O_2 \times 0.5\ H_2O$

Molecular weight: 453.6

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, $J = 8$, 1H, tetr-8H), 7.52 (d, $J = 16$, 1H, allylid-2H), 7.31 (d, $J = 8$, 2H, dmap-2,6H), 6.95 (d, $J = 8$, 1H, tetr-7H), 6.94 (d, J = 16, 1H, allylid-4H), 6.91 (s, 1H, tetr-5H), 6.80 (d, $J = 8$, 2H, dmap-3,5H), 6.50 (t, $J = 16$, 1H, allylid-3H), 4.13 (t, $J = 12$, 2H, prop-3H_2), 2.99 (s, 6H, dmap-N(CH_3)), 2.97–2.93 (m, 6H, prop-1H_2, pip-2,6H_2), 2.47–2.31 (m, 4H, tetr-3H_2, tetr-4H_2), 1.96 (q, 2H, $J = 7$, prop-2H_2), 1.56–1.48 (m, 4H, pip-3,5H_2), 1.46–1.36 (m, 2H, pip-4H_2).

^{13}C NMR (100 MHz, DMSO-d_6) δ 196.08 (tetr-1C), 162.37 (tetr-7C), 149.33 (dmap-4C), 144.58 (tetr-8aC), 141.31 (allylid-2C), 138.02 (allylid-4C), 135.99 (tetr-2C), 133.04 (dmap-2,6C), 129.06 (tetr-8C), 127.02 (tetr-4aC), 125.56 (allylid-3C), 121.97 (dmap-1C), 112.57 (tetr-5C), 112.21 (tetr-7C), 111.08 (dmap-3,5C), 64.98 (prop-3C), 52.27 (prop-1C), 51.98 (pip-2,6C), 40.32 (dmab-N(CH$_3$)$_2$), 28.88 (tetr-4C), 26.31 (prop-2C), 23.40 (pip-4C), 22.18 (pip-3,5C), 21.55 (tetr-3C).

ESI MS: 445.74 [M + H$^+$] (100).

Anal. calc.: C, 76.79; H, 8.22; N, 6.18. Found: C, 76.71; H, 8.47; N, 5.90.

Compound 6

(E)-2-((E)-3-(4-(Dimethylamino)phenyl)allylidene)-7-(3-(piperidin-1-yl)propoxy)-1-tetralone. Compound 2 (500 mg, 1.74 mmol) and 4-(dimethylamino)cinnamic aldehyde (305 mg,

1.74 mmol) were dissolved in 3 mL ethanol. 15 M NaOH (0.5 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Red solid, 689 mg, 89%.

$C_{29}H_{36}N_2O_2$

Molecular Weight: 444.6

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.53 (d, $J = 7.5$, 1H, allyl-2H), 7.43–7.36 (m, 3H, dmap-2,6H, tetr-8H), 7.28 (d, $J = 7.5$, 1H, tetr-5H), 7.18–7.12 (m, 2H, tetr-6H, allylid-4H), 6.80 (t, $J = 16$, 1H, allyl-3H), 6.74 (d, $J = 10$, 2H, dmap-3,5H), 4.07 (t, $J = 6.25$, 2H, prop-1H_2), 3.09 (t, $J = 6.25$, 2H, tetr-3H_2), 2.98 (s, 6H, dmap-N(CH_3)), 2.87 (t, $J = 7.5$, 2H, tetr-4H_2), 2.74–2.60 (m, 6H, prop-3H_2, pip-2,6H_2), 2.01 (q, $J = 9$, 2H, prop-2H_2), 1.68–1.54 (m, 4H, pip-3,5H_2), 1.52–1.40 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 185.72 (tetr-1C), 157.42 (tetr-7C), 151.24 (dmap-4C), 142.62 (allylid-2C), 137.31 (allylid-4C), 134.41 (tetr-8aC), 130.92 (tetr-4aC), 129.71 (tetr-5C), 128.98 (dmap-2,6C), 124.22 (allylid-3C), 120.54 (dmap-1C), 118.97 (tetr-6C), 112.03 (dmap-3,5C), 110.64 (tetr-8C), 65.97 (prop-1C), 54.54 (prop-3C), 53.41 (pip-2,6C), 40.29 (dmap-N(CH$_3$)$_2$), 28.86 (tetr-4C), 27.17 (prop-2C), 25.86 (pip-3,5C), 24.74 (pip-4C), 23.24 (tetr-3C).

ESI MS: 445.7 [M + H$^+$] (100).

HRMS: calc.: 445.28495; found: 445.28500.

(4-(3-(Piperidin-1-yl)propoxy)phenyl)methanol. 1-(3-Chloropropyl)piperidine hydrochloride (4 g, 20.2 mmol), 4-hydroxybenzyl alcohol (2.5 g, 22.2 mmol), potassium carbonate (8.3 g, 60.6 mmol), and potassium iodide (1.7 g, 20.2 mmol) were refluxed in absolute acetone (100 mL) for 72 h under inert atmosphere. After cooling to room temperature inorganic salts were filtered off. The filtrate was concentrated to dryness and resuspended in dichloromethane. The organic layer was washed three times with 2 M NaOH solution and brine, dried with $MgSO_4$, and concentrated to dryness. Yellowish solid, 3.6 g, 72%.

$C_{15}H_{23}NO_2$

Molecular weight: 249.4

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.29 (d, $J = 10$, 2H, ph-2,6H), 6.96 (d, $J = 10$, 2H, ph-3,5H), 5.12 (t, $J = 7$, 1H, OH), 4.49 (d, $J = 10$, 2H, CH_2-OH), 4.03 (t, $J = 7.5$, 2H, prop-1H_2), 2.42 (t, $J = 7.5$, 2H, prop-3H_2), 2.37–2.28 (m, 4H, pip-2,6H_2), 1.93 (q, $J = 6.26$, 2H, prop-2H_2), 1.60–1.49 (m, 4H, pip-3,5H_2), 1.49–1.38 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 157.45 (ph-4C), 134.30 (ph-1C), 127.95 (ph-2,6C), 113.93 (ph-3,5C), 66.01 (prop-1C), 62.75 (CH$_2$-OH), 55.25 (prop-3C), 54.11 (pip-2,6C), 26.57 (prop-2C), 25.26 (pip-3,5C), 23.80 (pip-4C).

ESI MS: 250.46 [M + H$^+$] (100).

1-(3-(4-(Chloromethyl)phenoxy)propyl)piperidine hydrochloride. (4-(3-(Piperidin-1-yl)propoxy)phenyl)methanol (3.6 g, 14.5 mmol) was dissolved in 100 mL toluene and an excess of thionyl chloride (3.2 mL, 43.6 mmol) was added dropwise at 0°C under inert atmosphere. Once the exothermic reaction had decayed the mixture was stirred for 3 h at 60°C. Afterward toluene and thionylchloride was distilled off. Crude product was re-crystallized in ethanol. Beige solid, 4.1 g, 93%.

$C_{15}H_{22}ClNO \times HCl$

Molecular weight: 304.3

^{1}H NMR (250 MHz, DMSO-d_6) δ 10.62 (br s, 1H, NH^+), 7.42 (d, $J = 15$, 2H, ph-2,6H), 6.99 (d, $J = 15$, 2H, ph-3,5H), 4.73 (s, 2H, CH_2-Cl), 4.07 (t, $J = 10$, 2H, prop-1H_2), 3.45 (t, $J = 10$, 2H, prop-3H_2), 3.21–3.11 (m, 2H, pip-2,6H_{eq}), 2.94–2.81 (m, 2H, pip-2,6H_{ax}), 1.91 (q, $J = 6.26$, 2H, prop-3H_2), 1.69–1.50 (m, 5H, pip-3,5H_2, pip-4H_{eq}), 1.49–1.38 (m, 1H, pip-4H_{ax}).

^{13}C NMR (63 MHz, DMSO-d_6) δ 158.38 (ph-4C), 130.35 (ph-2,6C), 130.33 (ph-1C), 114.58 (ph-3,5C), 65.35 (prop-1C), 53.44 (prop-3C), 51.86 (pip-2,6C), 46.08 (CH$_2$-Cl), 23.30 (prop-2C), 22.43 (pip-3,5C), 21.55 (pip-4C).

ESI MS: 268.42 [M + H$^+$] (100), 571.78 [2M + HCl] (19).

Compound 7

6-(4-(3-(Piperidin-1-yl)propoxy)benzyloxy)-1-tetralone. 1-(3-(4-(Chloromethyl)phenoxy)propyl)piperidine hydrochloride (1.7 g, 5.6 mmol), 6-hydroxy-1-tetralone (1.0 g, 6.2 mmol), potassium carbonate (2.6 g, 18.5 mmol) and potassium iodide (1.0 g, 6.2 mmol) were refluxed in absolute acetone (70 mL) for 72 h under inert atmosphere. After cooling to room temperature inorganic salts were filtered off. The filtrate was concentrated to dryness and resuspended in dichloromethane. The organic layer was washed three times with 2 M NaOH solution and brine, dried with $MgSO_4$, and concentrated to dryness. Beige solid, 1.4 g, 65%.

$C_{25}H_{31}NO_3$

Molecular weight: 393.5

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.84 (d, $J = 7.5$, 1H, tetr-8H), 7.42 (d, $J = 7.5$, 2H, ph-2,6H), 6.99–6.93 (m, 4H, ph-3,5H, tetr-5H, tetr-7H), 5.11 (s, 2H, ph-CH_2), 4.06 (t, $J = 6.3$, 2H, prop-1H_2), 3.11–3.04 (m, 6H, prop-3H_2, pip-2,6H_2), 2.90 (t, $J = 6.3$, 2H, tetr-2H_2), 2.53 (t, $J = 6.3$, 2H, tetr-4H_2), 2.15–1.96 (m, 4H, tetr-3H_2, prop-2H_2), 1.73–1.69 (m, 4H, pip-3,5H_2), 1.55–1.53 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 196.37 (tetr-1C), 162.30 (tetr-6C), 158.39 (ph-4C), 147.23 (tetr-8aC), 130.00 (ph-2,6C), 128.63 (tetr-8C, ph-1C), 126.08 (tetr-4aC), 114.93 (ph-3,5C), 113.56 (tetr-5C), 113.54 (tetr-7C), 69.50 (prop-1C), 65.39 (ph-CH$_2$), 53.84 (prop-3C), 52.67 (pip-2,6C), 38.93 (tetr-2C), 29.17 (tetr-4C), 24.28 (prop-2C), 23.11 (pip-3,5C), 23.09 (pip-4C), 21.73 (tetr-3C).

ESI MS: 394.7 [M + H$^+$] (100).

HRMS: calc.: 394.23767; found: 394.23800.

Compound 8

7-(4-(3-(Piperidin-1-yl)propoxy)benzyloxy)-1-tetralone. 1-(3-(4-(Chloromethyl)phenoxy)propyl)piperidine hydrochloride (2.4 g, 7.9 mmol), 7-hydroxy-1-tetralone (1.4 g, 8.6 mmol), potassium carbonate (3.6 g, 25.9 mmol) and potassium iodide (1.4 g, 8.6 mmol) were refluxed in absolute acetone (300 mL) for 72 h under inert atmosphere. After cooling to room temperature inorganic salts were filtered off. The filtrate was concentrated to dryness and resuspended in dichloromethane. The organic layer was washed three times with 2 M NaOH solution and brine, dried with $MgSO_4$, and concentrated to dryness. Beige solid, 2.2 g, 71%.

$C_{25}H_{31}NO_3$

Molecular Weight: 393.5

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.34 (s, 1H, tetr-8H), 7.33 (d, $J = 10$, 2H, ph-2,6H), 7.23 (d, $J = 7.5$, 1H, tetr-5H), 7.15 (d, $J = 7.5$, 1H, tetr-6H), 6.90 (d, $J = 10$, 2H, ph-3,5H), 5.00 (s, 2H, ph-CH_2), 3.97 (t, $J = 5$, 2H, prop-1H_2), 2.80 (t, $J = 7.5$, 2H, tetr-4H_2), 2.50 (t, $J = 7.5$, 2H, tetr-2H_2), 2.46–2.40 (m, 6H, prop-3H_2, pip-2,6H_2), 2.02 (q, 2H, $J = 5$, tetr-3H_2), 1.92 (q, 2H, $J = 7$, prop-2H_2), 1.68–1.61 (m, 4H, pip-3,5H_2), 1.52–1.43 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 197.29 (tetr-1C), 157.88 (ph-4C), 156.80 (tetr-7C), 137.10 (tetr-4aC), 133.03 (tetr-8aC), 130.88 (ph-1C), 129.17 (ph-2,6C), 129.10 (tetr-5C), 121.67 (tetr-6C), 114.60 (ph-3,5C), 110.11 (tetr-8C), 69.41 (prop-1C), 67.55 (ph-CH$_2$), 57.84 (prop-3C), 56.07 (pip-2,6C), 38.34 (tetr-2C), 30.42 (tetr-4C), 27.88 (prop-2C), 26.09 (pip-3,5C), 24.95 (pip-4C), 23.14 (tetr-3C).

ESI MS: 394.7 [M + H$^+$] (100).

HRMS: calc.: 394.23767; found: 394.23767.

Compound 9

(E)-2-(4-(Dimethylamino)benzylidene)-6-(4-(3-(piperidin-1-yl) propoxy)benzyloxy)-1-tetralone. Compound 7 (300 mg, 0.8 mmol) and 4-(dimethylamino)benzaldehyde (113, 0.8 mmol) were dissolved in 3 mL ethanol. 15 M NaOH (0.2 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Yellow solid, 357 mg, 85%.

$C_{34}H_{40}N_2O_3$

Molecular Weight: 524.7

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.89 (d, $J = 7.5$, 1H, tetr-8H), 7.61 (s, 1H, benzylid-2H), 7.43–7.37 (m, 4H, dmab-2,6H, ph-2,6H), 7.04–6.96 (m, 4H, tetr-7H, ph-3,5H, tetr-5H), 6.74 (d, $J = 7.5$, 2H, dmab-3,5H), 5.10 (s, 2H, ph-CH_2), 4.02 (t, $J = 5$, 2H, prop-1H_2), 3.07 (t, $J = 7.5$, 2H, tetr-3H_2), 2.97 (s, 6H, dmab-N(CH_3)$_2$), 2.85 (t, $J = 7.5$, 2H, tetr-4H_2), 2.68–2.61(m, 6H, prop-3H_2, pip-2,6H_2), 1.99 (q, $J = 5$, 2H, pip-2H_2), 1.62–1.58 (m, 4H, pip-3,5H_2), 1.48–1.38 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 185.13 (tetr-1C), 162.03 (tetr-6C), 158.31 (ph-4C), 150.38 (dmab-4C), 145.37 (tetr-2C), 136.27 (benzylid-2C), 132.01 (tetr-8aC), 131.79 (dmab-2,6C), 130.70 (tetr-8C), 130.55 (ph-2,5C), 129.61 (ph-1C), 126.79 (tetr-4aC), 122.64 (dmab-1C), 114.40 (ph-3,5C), 113.06 (tetr-5C), 111.65 (dmab-3,5C), 110.94 (tetr-7C), 69.22 (prop-1C), 65.50 (ph-CH$_2$), 54.35 (prop-3C), 53.21 (pip-2,6C), 40.13 (N(CH$_3$)$_2$), 28.17 (tetr-4C), 26.79 (prop-2C), 24.97 (pip-4C), 24.22 (pip-3,5C), 22.86 (tetr-3C).

ESI MS: 525.5 [M + H$^+$] (100).

HRMS: calc.: 525.31117; found: 525.31034.

Compound 10

(E)-2-(4-(Dimethylamino)benzylidene)-7-(4-(3-(piperidin-1-yl) propoxy)benzyloxy)-1-tetralone. Compound 8 (60 mg, 0.2 mmol) and 4-(dimethylamino)benzaldehyde (23 mg, 0.2 mmol) were dissolved in 1.5 mL ethanol. 15 M NaOH (0.04 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Yellow solid, 72 mg, 91%.

$C_{34}H_{40}N_2O_3$

Molecular weight: 524.7

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.58 (s, 1H, benzylid-2H), 7.41 (d, $J = 5$, 2H, dmab-3,5H), 7.36 (d, $J = 5$, 2H, ph-2,6H), 7.31 (s, 1H, tetr-8H), 7.23 (d, $J = 7.5$, 1H, tetr-5H), 7.14 (d, $J = 7.5$, 1H, tetr-6H), 6.90 (d, $J = 7.5$, 2H, ph-3,5H), 6.72 (d, $J = 10$, 2H, dmab-3,5H), 5.01 (s, 2H, ph-CH_2), 3.97 (t, $J = 5$, 2H, prop-1H_2), 3.02 (t, $J = 5$, 2H, tetr-3H_2), 2.92 (s, 6H, dmab-N(CH_3)$_2$), 2.89 (t, $J = 7.5$, 2H, tetr-4H_2), 2.84–2.78 (m, 6H, prop-3H_2, pip-2,6H_2), 1.99 (q, $J = 5$, 2H, prop-2H_2), 1.66–1.60 (m, 4H, pip-3,5H_2), 1.42–1.33 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 185.93 (tetr-1C), 158.30 (ph-4C), 157.28 (tetr-7C), 150.98 (dmab-4C), 137.16 (benzylid-2C), 135.54 (tetr-2C), 134.52 (tetr-8aC), 132.01 (dmab-2,6C), 130.43 (tetr-4aC), 129.75 (tetr-5C), 129.48 (ph-2,6C), 129.13 (ph-1C), 122.73 (dmab-1C), 120.70 (tetr-6C), 114.81 (ph-3,5C), 111.69 (dmab-3,5C), 110.80 (tetr-8C), 69.50 (prop-1C), 65.43 (ph-CH$_2$), 55.73 (prop-3C), 53.04 (pip-2,6C), 39.62 (dmab-N(CH$_3$)$_3$), 28.24 (tetr-4C), 27.78 (prop-2C), 27.03 (pip-3,5C), 24.58 (pip-4C), 23.94 (tetr-3C).

ESI MS: 525.8 [M + H$^+$] (100).

HRMS: calc.: 525.31117; found: 525.31129.

Compound 11

(E)-2-((E)-3-(4-(Dimethylamino)phenyl)allylidene)-6-(4-(3-(piperidin-1-yl)propoxy)benzyloxy)-1-tetralone. Compound 7 (300 mg, 0.8 mmol) and 4-(dimethylamino)cinnamic aldehyde (133 mg, 0.8 mmol) were dissolved in 5 mL ethanol. 15 M NaOH (0.22 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Yellow solid, 398 mg, 95%.

$C_{36}H_{42}N_2O_3$

Molecular Weight: 550.7

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.96 (d, $J = 10$, 1H, tetr-8H), 7.57 (d, $J = 7.5$, 2H, ph-2,6H), 7.48 (d, $J = 7.5$, 2H, dmap-2,6H), 7.40 (d, $J = 16$, 1H, allylid-2H), 7.14–7.01 (m, 6H, ph-3,5H, dmap-3,5H, tetr-5H, tetr-7H), 6.84 (t, $J = 16$, 1H, allylid-3H), 6.79 (d, $J = 16$, 1H, allylid-4H), 5.19 (s, 2H, ph-CH_2), 4.11 (t, $J = 6.3$, 2H, prop-1H_2), 3.56–3.42 (m, 6H, prop-3H_2, pip-2,6H_2), 3.12–3.05 (m, 2H, tetr-3H_2), 3.03 (s, 6H, N(CH_3)$_2$), 2.18 (q, $J = 4.7$, 2H, pip-2H_2), 1.81–1.76 (m, 4H, pip-3,5H_2), 1.65–1.59 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 184.91 (tetr-1C), 162.19 (tetr-6C), 158.09 (ph-4C), 151.02 (dmap-4C), 145.99 (tetr-8aC), 141.89 (allylid-2C), 136.49 (allylid-4C), 131.56 (tetr-2C), 129.79 (ph-2,6C), 128.89 (ph-1C),128.86 (tetr-8C), 127.37 (tetr-4aC), 124.39 (dmap-1,2,6C), 119.17 (allylid-3C), 114.89 (ph-3,5C), 114.15 (dmap-3,5C), 113.40 (tetr-5C), 111.91 (tetr-7C), 69.46 (prop-1C), 65.36 (ph-CH$_2$), 53.81 (prop-3C), 52.70 (pip-2,6C), 40.22 (N(CH$_3$)$_2$), 28.30 (tetr-4C), 25.70 (pip-3,5C), 24.21 (prop-2C), 23.09 (pip-4C), 21.60 (tetr-3C).

ESI MS: 551.4 [M + H$^+$] (100).

HRMS: calc.: 551.32682; found: 551.32618.

Compound 12

(E)-2-((E)-3-(4-(Dimethylamino)phenyl)allylidene)-7-(4-(3-(piperidin-1-yl)propoxy)benzyloxy)-1-tetralone. Compound 8

(60 mg, 0.2 mmol) and 4-(dimethylamino)cinnamic aldehyde (27 mg, 0.2 mmol) were dissolved in 1.5 mL ethanol. 15 M NaOH (0.04 mL) was added. The mixture was stirred over night at room temperature, concentrated to dryness, and purified by column chromatography (CH_2Cl_2/MeOH, 9/1). Yellow solid, 77 mg, 93%.

$C_{36}H_{42}N_2O_3$

Molecular Weight: 550.7

^{1}H NMR (250 MHz, DMSO-d_6) δ 7.59 (d, $J = 16$, 1H, allylid-2H), 7.54 (d, $J = 7.5$, 2H, dmap-2,6H), 7.47 (d, $J = 5$, 2H, ph-2,6H), 7.38 (s, 1H, tetr-8H), 7.35 (d, $J = 5$, 1H, tetr-5H), 7.29 (d, $J = 10$, 1H, tetr-6H), 7.17 (d, $J = 16$, 1H, allylid-3H), 7.04 (d, $J = 10$, 2H, ph-3,5H), 6.86 (t, $J = 16$, 1H, allylid-3H), 6.80 (d, $J = 10$, 2H, dmap-3,5H), 5.14 (s, 2H, ph-CH_2), 4.11 (t, $J = 6.25$, 2H, prop-1H_2), 3.18 (t, $J = 5$, 2H, tetr-3H_2), 3.03 (s, 6H, dmap-N(CH_3)$_2$), 2.97 (t, $J = 7.5$, 2H, tetr-4H_2), 2.95–2.81 (m, 6H, prop-3H_2, pip-2,6H_2), 2.15 (q, $J = 5$, 2H, prop-2H_2), 1.85–1.77 (m, 4H, pip-3,5H_2), 1.60–1.45 (m, 2H, pip-4H_2).

^{13}C NMR (63 MHz, DMSO-d_6) δ 183.03 (tetr-1C), 158.34 (ph-4C), 157.83 (tetr-7C), 150.38 (dmap-4C), 141.18 (allylid-2C), 138.52 (allylid-4C), 135.46 (tetr-2C), 133.62 (tetr-8aC), 131.08 (dmap-2,6C), 129.63 (tetr-4aC), 129.88 (tetr-5C), 129.84 (ph-2,6C), 128.31 (ph-1C), 125.34 (allylid-3C), 124.71 (dmap-1C), 120.63 (tetr-6C), 114.62 (ph-3,5C), 114.92 (tetr-8C), 111.76 (dmap-3,5C), 73.58 (prop-1C), 70.44 (ph-CH_2), 56.35 (prop-3C), 54.47 (pip-2,6C), 39.89 (dmap-N(CH_3)$_3$), 28.56 (tetr-4C), 27.85 (prop-2C), 25.83 (pip-3,5C), 24.83 (pip-4C), 23.45 (tetr-3C).

ESI MS: 551.8 [M + H$^+$] (100).

HRMS: calc.: 551.32682; found: 551.32705.

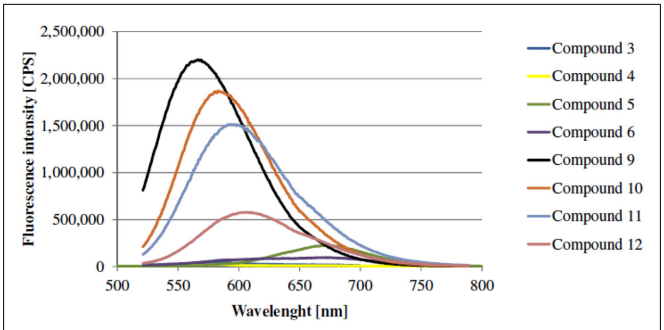


FIGURE A2 | Fluorescence emission spectra of chalcone derivatives (Excitation wavelength: 405 nm).

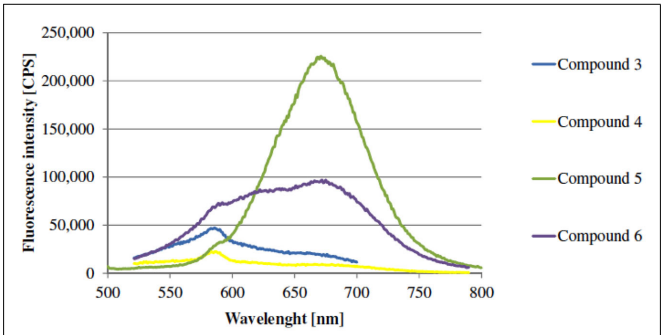


FIGURE A3 | Fluorescence emission spectra of chalcone derivatives with *O*-alkyl-ether as spacer B (Excitation wavelength: 405 nm).

FLUORESCENCE CHARACTERIZATION

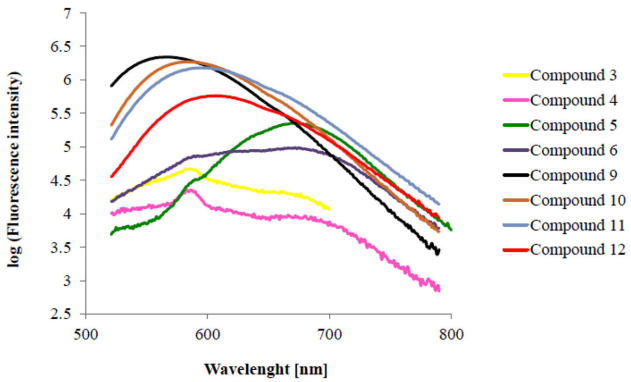


FIGURE A1 | Fluorescence emission spectra of chalcone derivatives in logarithmic delineation (Excitation wavelength: 405 nm).

SELECTIVITY DATA

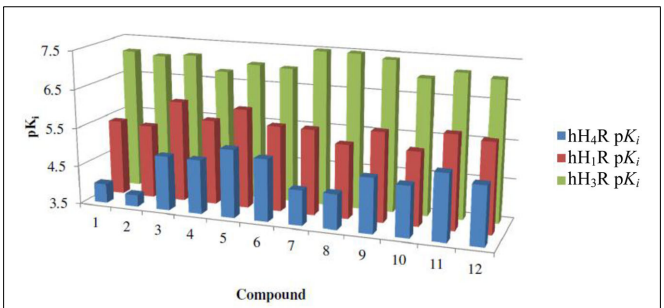
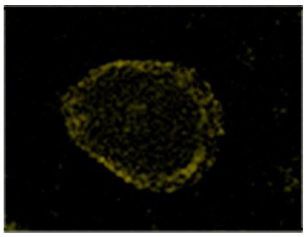
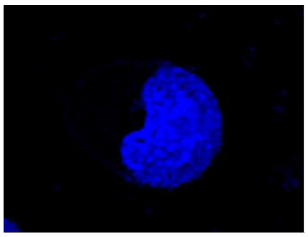
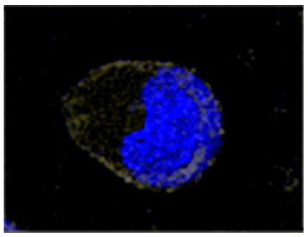
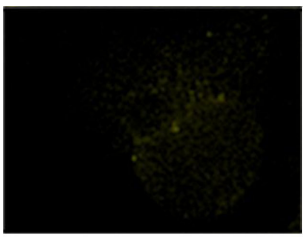
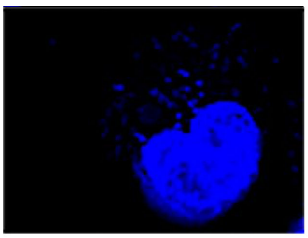
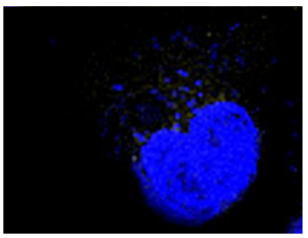
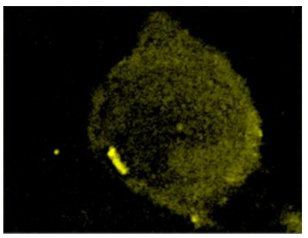
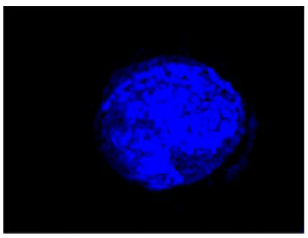
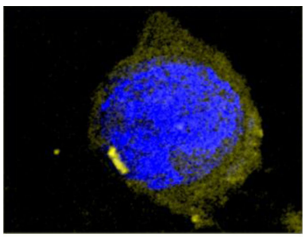
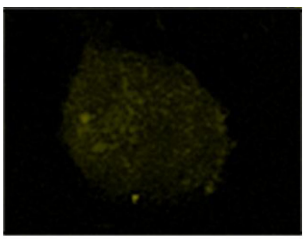
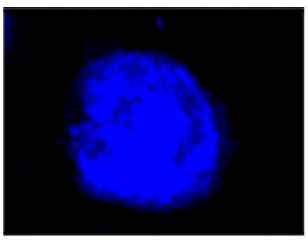
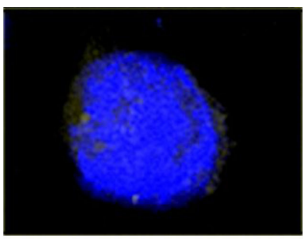
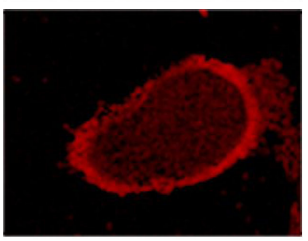
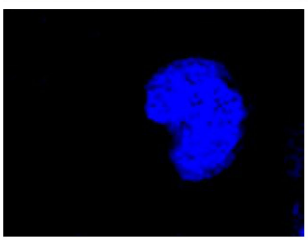
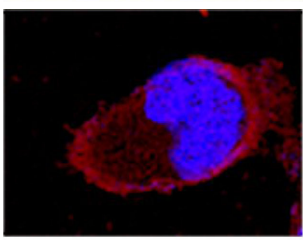
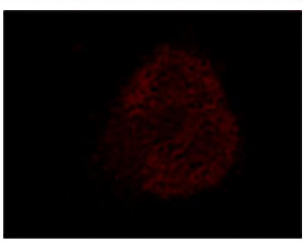
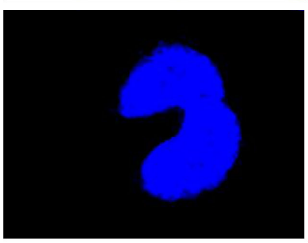
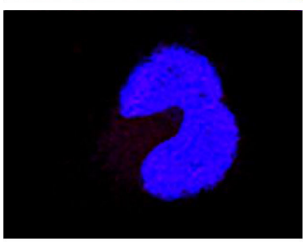


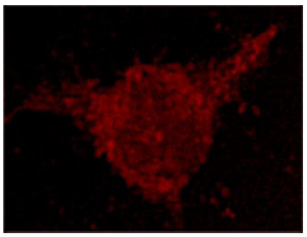
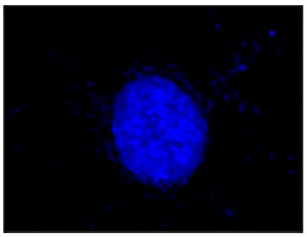
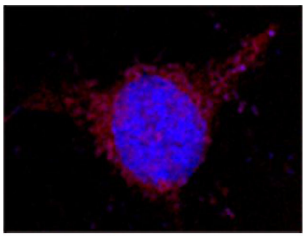
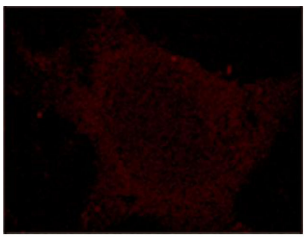
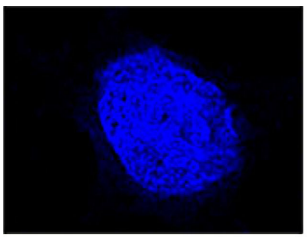
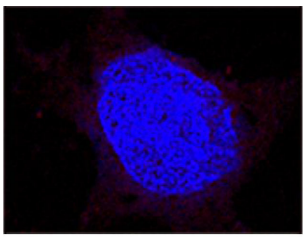
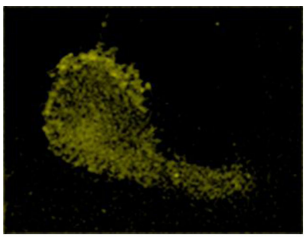
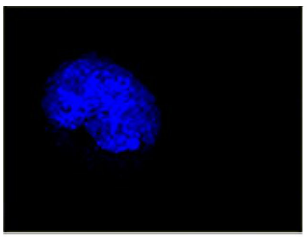
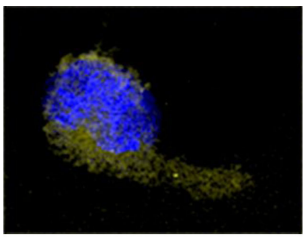
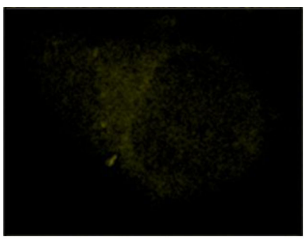
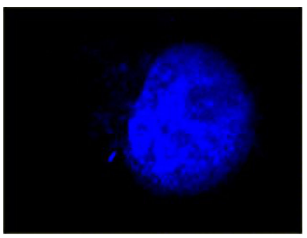
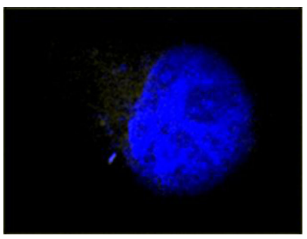
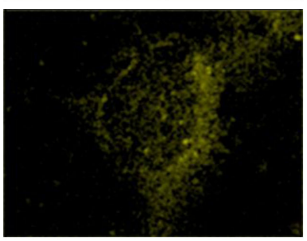
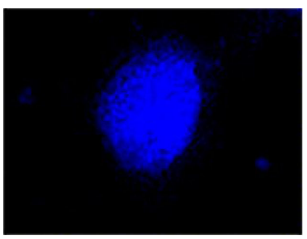
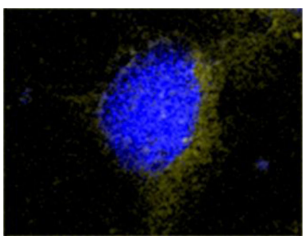
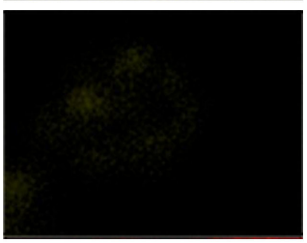
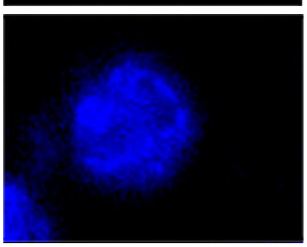
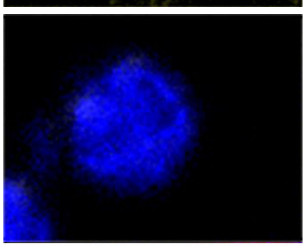
FIGURE A4 | Graphic chart of human histamine H$_1$, H$_3$, and H$_4$ receptor pK_i values.

Table A1 | Fluorescence images of fluorescent compounds.

Compound	Cell line	Ligand image	DAPI image	Overlay of ligand and DAPI image
3	hH_3-HEK-293			
3	HEK-293			
4	hH_3-HEK-293			
4	HEK-293			
5	hH_3-HEK-293			
5	HEK-293			

(Continued)

Table A1 | Continued

Compound	Cell line	Ligand image	DAPI image	Overlay of ligand and DAPI image
6	hH_3-HEK-293			
6	HEK-293			
9	hH_3-HEK-293			
9	HEK-293			
10	hH_3-HEK-293			
10	HEK-293			

(Continued)

Table A1 | Continued

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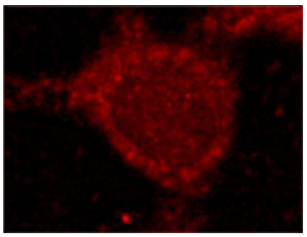
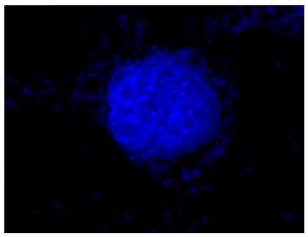
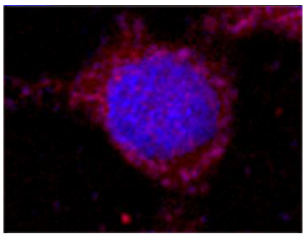
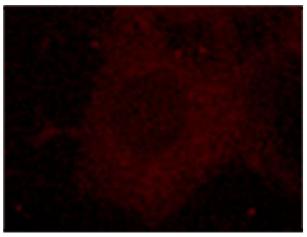
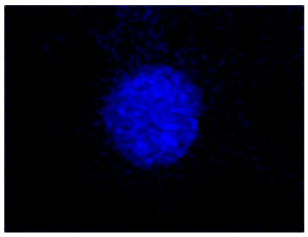
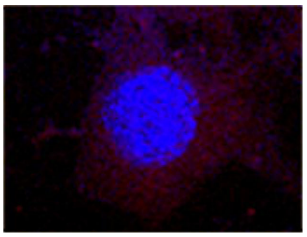
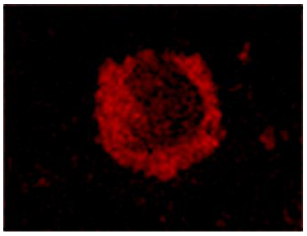
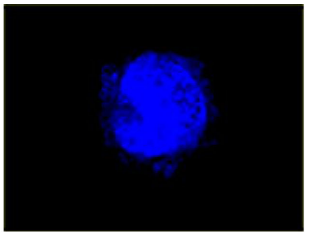
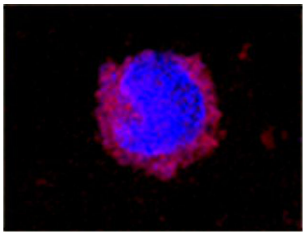
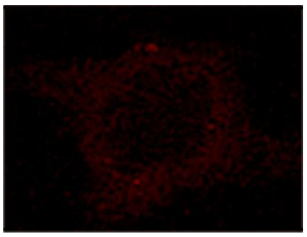
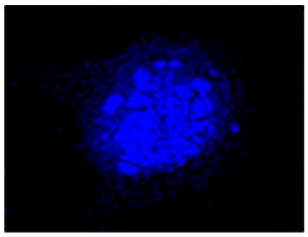
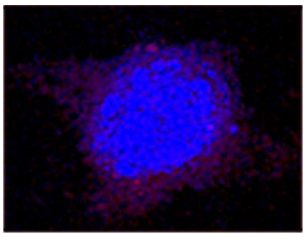
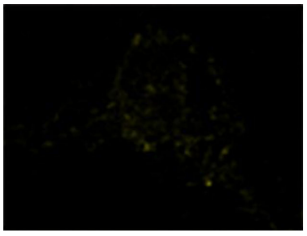
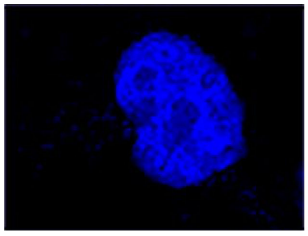
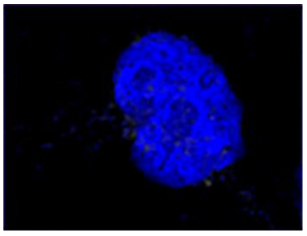
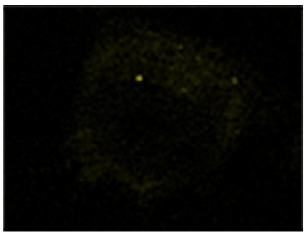
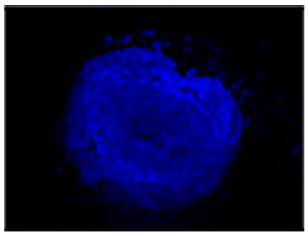
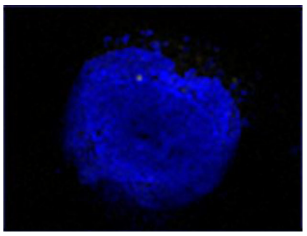
11	hH_3-HEK-293			
11	HEK-293			
12	hH_3-HEK-293			
12	HEK-293			
–	H_3-HEK-293			
–	HEK-293			

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[11C]Doxepin binding to histamine H1 receptors in living human brain: reproducibility during attentive waking and circadian rhythm

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Molecular imaging in neuroscience is a new research field that enables visualization of the impact of molecular events on brain structure and function in humans. While magnetic resonance-based imaging techniques can provide complex information at the level of system, positron emission tomography (PET) enables determination of the distribution and density of receptor and enzyme in the human brain. Previous studies using [11C]raclopride and [11C]FLB457 revealed that the release of neuronal dopamine was increased in human brain by psychostimulants or reward stimuli. Following on from these previous [11C]raclopride studies, we examined whether the levels of neuronal release of histamine might change [11C]doxepin binding to the H1 receptors under the influence of physiological stimuli. The purpose of the present study was to evaluate the test–retest reliability of quantitative measurement of [11C]doxepin binding between morning and afternoon and between resting and attentive waking conditions in healthy human subjects. There was a trend for a decrease in [11C]doxepin binding during attentive calculation tasks compared with that in resting conditions, but the difference (less than 10%) was not significant. Similarly, the binding potential of [11C]doxepin in the cerebral cortex was slightly higher in the morning than that in the afternoon, but it was also insignificant. These data suggest that higher histamine release during wakefulness could not decrease the [11C]doxepin binding in the brain. This study confirmed the reproducibility and reliability of [11C]doxepin in the previous imaging studies to measure the H1 receptor.

Keywords: H1 receptor, attentive waking, circadian rhythm, histamine release, positron emission tomography (PET), test–retest reliability, human brain

INTRODUCTION

Histamine is a transmitter in the nervous system and a signaling molecule in the gut, the skin, and the immune system. Histamine neurons in mammalian brain are located exclusively in the tuberomammillary nucleus of the posterior hypothalamus and their axons extend throughout the central nervous system (CNS). Four known histamine receptors and histamine binding to glutamate NMDA receptors serve multiple functions in the brain, particularly control of excitability and plasticity (Haas and Panula, 2003; Haas et al., 2008). The H1 and H2 receptor-mediated actions are mostly excitatory, while H3 receptors act as inhibitory auto- and heteroreceptors. Histamine neurons are proposed to have a dual effect on the CNS, with both stimulatory and suppressive actions (Watanabe and Yanai, 2001). As a stimulator, neuronal histamine is one of the most important systems that stimulate and maintain attentive wakefulness. Brain histamine also functions in bioprotection as a suppressor of various noxious and unfavorable stimuli of convulsion, drug sensitization, denervation supersensitivity, ischemic lesions, and stress susceptibility. We have examined the functions of histamine neurons using various approaches, such as histamine-related gene knockout mice and human positron emission tomography (PET) (Yanai and Tashiro, 2007).

Histamine neurons play an important role in the forebrain waking systems (Lin, 2000; Eriksson et al., 2001; Huang et al., 2001). Their neuronal activity is specific for a high-vigilance waking state and histamine neurons might play a role not in the initiation of wakefulness, but in maintenance of the high level of vigilance necessary for cognitive processes (Takahashi et al., 2008; Sakai et al., 2010). An increase in histaminergic transmission promotes wakefulness, whereas its blockade by sedating antihistamines causes somnolence and impaired performance in humans (Yanai et al., 2011). Several lines of evidence suggest that histamine modulates circadian rhythms (Tuomisto et al., 2001; Abe et al., 2004) and it has even been suggested to play a pivotal role in circadian entrainment (Jacobs et al., 2000). Accordingly, a clear circadian rhythm of histamine release was demonstrated in the anterior hypothalamus by *in vivo* microdialysis studies

(Mochizuki et al., 1992). Histamine release increased before the active phase and was maintained at an elevated level during the active phase.

Stress in our daily lives has been associated with various psychiatric disorders including depression, schizophrenia, cognitive disorders, and psychosomatic diseases such as anorexia nervosa and irritable bowel syndrome. To date, we have conducted several PET studies to elucidate the pathophysiological mechanism behind the above-mentioned disorders, focusing on alteration in neural transmission of the histaminergic neuron systems (Yanai and Tashiro, 2007). For PET studies, [11C]doxepin, a potent antagonist of histamine H1 receptors, has been utilized as an effective PET imaging tracer. Using [11C]doxepin, increasing evidence has been accumulated regarding the role of the histaminergic neuron system in the pathophysiology of stress-related neuropsychiatric disorders. For example, histamine H1 receptor binding was measured using [11C]doxepin in patients with schizophrenia (Iwabuchi et al., 2005), major depression (Kano et al., 2004) and anorexia nervosa (Yoshizawa et al., 2009). Another application of [11C]doxepin-PET is the measurement of histamine H1 receptor occupancy by antihistamines. The inhibition of histaminergic activity at the brain H1 receptor level may have some favorable anxiolytic effects, however, more often this is accompanied by unfavorable effects like increased daytime somnolence, impaired memory and learning, decreased attention, and weight gain. The brain H1 receptor occupancy measured by [11C]doxepin-PET is one of the most reliable and objective methods to estimate the sedating properties of antihistamines (Yanai et al., 2011, 2012). A critical step in validating [11C]doxepin to measure the brain H1 receptor occupancy is to evaluate the reproducibility of its binding potential (Bmax/KD) in the different attentive conditions. Clinically-used PET probes should be examined on the test–retest reproducibility for the *in vivo* binding (Kim et al., 2006; Yasuno et al., 2007; Edison et al., 2009; Narendran et al., 2011), although [11C]doxepin binding to brain has not been examined on this aspect until now.

Previous studies have shown that imaging with PET radiotracers that are specific for brain receptors can be used to visualize changes in the release of neurotransmitters indirectly. Most successful studies have focused on dopamine, since the dopamine neurons that project to the striatum have been shown to play a critical role in mediating motivational and addictive behaviors. These imaging studies successfully measured increased extracellular dopamine released by psychostimulants and physiological reward stimuli in humans with [11C]raclopride (Koepp et al., 1998; Rinne, 2003; Hommer et al., 2011) and [11C]FLB 457 (Narendran et al., 2011). However, some technical difficulties have been encountered for the imaging of changes in the release of other neurotransmitters. Among these are low sensitivity, changes of neurotransmitter release pulse over time during PET imaging and the issue of affinity states, the contribution of carryover mass in the second PET scan, and internalization of receptors (Boecker et al., 2008). Followed by the previous PET measurement of dopamine release, we examined whether neuronal histamine released as a result of mental stress and the circadian rhythm might change the levels of H1 receptors measured *in vivo* by PET and [11C]doxepin. Therefore, we undertook the present study for the purpose of evaluating the test–retest reliability of [11C]doxepin binding in the human brain during different attentive conditions and circadian rhythm in healthy human subjects.

METHODS

SUBJECTS AND STUDY DESIGN

Japanese male volunteers, who were physically and mentally healthy and had no history of allergy or long-term treatment with H1 antagonists, were recruited to participate in this study. They showed no abnormality in brain magnetic resonance imaging (MRI). All subjects gave written informed consent for all study procedures before participation. Concomitant medications, nicotine, caffeine, grapefruit or grapefruit juice, and alcohol were not allowed during the experimental period. The present study was approved by the Committee on Clinical Investigation at Tohoku University Graduate School of Medicine, Japan, and was performed in accordance with the principles of the Declaration of Helsinki. All experiments were performed at the Cyclotron and Radioisotope Center, Tohoku University.

In the first part of this study involving test–retest measurements, six healthy male volunteers (mean age ± SD: 24.6 ± 2.1 years old) were examined twice with [11C]doxepin-PET during a resting condition in the morning (11:00 a.m.) and afternoon (3:00 p.m.) of the same day in order to evaluate the circadian rhythm of H1 receptor binding. In the second part of this study involving investigation of ligand activation, 10 healthy men (22.3 ± 1.0 years old) were examined during resting and attentive waking conditions. They were scanned twice by PET (SET2400W; Shimadzu Co., Kyoto, Japan) on the same day during attentive calculation tasks involving two-digit addition and during resting conditions with their eyes closed after administration of [11C]doxepin. The task protocols are shown in **Figure 1.** The order of resting and calculation conditions was randomized. We performed measurements of subjective feelings five times during PET scans before the scan (pre), at interval 1 (int1), interval 2 (int2), interval 3 (int3), and at the end of scan (end). Subjective feelings including alertness, tiredness, and sleepiness were measured during PET scans using Line Analog Rating Scale (LARS). In LARS measurement, subjects mark a series of 100 mm linear

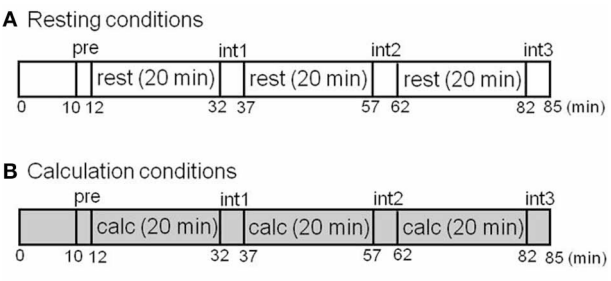

FIGURE 1 | Study protocols of test–retest measurements in the different conditions. (A) Resting condition. **(B)** Calculation condition. The H1 receptors were examined with [11C]doxepin-PET during the resting and calculation conditions. The subjective feelings during PET scans were also measured during the 90 min before the scan (pre), interval 1 (int1), interval 2 (int2), interval 3 (int3), and at the end of the scan (end).

analog scales (+50 to −50 mm), indicating their present feeling with regard to a midpoint, which represents their normal state of mind.

PET IMAGE ACQUISITION AND DATA ANALYSIS

[11C]Doxepin was synthesized by 11C-methylation of desmethyldoxepin with [11C]methyl triflate as described previously. The radiochemical purity of [11C]doxepin was greater than 99%, and its specific radioactivity at the time of injection was 207.5 ± 61.9 GBq/μmol (5608 ± 1673 mCi/μmol). The single injected dose and cold mass of [11C]doxepin were 119.8 ± 10.5 MBq (3.23 ± 0.283 mCi) and 0.577 ± 0.051 nmol, respectively.

For the measurement of H1 receptors, PET scans were carried out with an SET2400W PET scanner (Shimadzu Co., Kyoto, Japan). PET data were acquired 30 s after the administration of [11C]doxepin with the subject's eyes closed for 90 min.

In order to calculate the binding potential (BP) of H1 receptors, brain PET images of each subject during resting and calculation conditions were subjected to inter-frame motion correction and then co-registered to an identical stereotaxic brain coordinate using a corresponding T1-weighted MRI image. MRI images were obtained with a 1.5-T MR scanner (HiSpeed, ver. 9.1; General Electric Inc., WI, USA). Regions of interest (ROI) were first placed on the following brain regions on the T1 images for which precise anatomical information was available: anterior and posterior cingulate gyrus, inferior prefrontal cortex, superior prefrontal cortex, temporal cortex, and cerebellum. ROI was defined for each cortical region by 3–5 concentric circles with a diameter of 5.0 mm for each hemisphere in 4–5 consecutive brain transaxial slices. An averaged value from all ROIs was used as a representative value of each region. In addition, we produced a time-activity curve (TAC) of each region from ROI data. The TACs were obtained by applying the ROIs to the dynamic PET images. A standardized uptake value (SUV) was calculated for the normalization of ROI-TACs as follows:

$$\text{SUV (TAC)} = \text{TAC (MBq/mL)}/[\text{injected tracer dose (MBq)}]/\text{body weight (g)}$$

Subsequently, Logan graphical analysis with the reference tissue input (LGAR) method was applied to calculate BP using PMOD kinetic modeling tool (PKIN) software (PMOD Technologies Ltd., Zurich, Switzerland) and TAC (Suzuki et al., 2005), and we compared the BP between different conditions (resting vs. calculation; morning vs. afternoon). All data were analyzed by a repeated measure of ANOVA followed by multiple comparisons (Tukey–Kramer test, Scheffe's *F* test, and Bonferroni–Dunn test), and $P < 0.05$ was considered statistically significant.

RESULTS

During the performance of the attentive task involving two-digit calculation, the percentage of correct answers was greater than 90%. Subjects felt significantly more tired during the calculation task than in the resting condition (**Figure 2A**). Subjects in the resting condition tended to have significantly higher sleepiness scores than those in the calculation condition (**Figure 2B**).

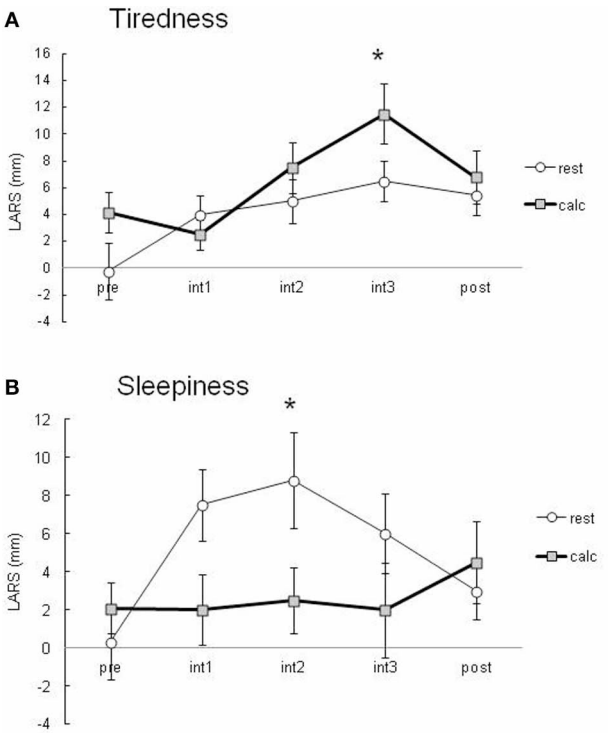

FIGURE 2 | Subjective tiredness (A) and sleepiness (B) evaluated using LARS during PET scans. Data are presented as the means ± SEM of LARS (mm) from 10 healthy subjects in the resting and calculation conditions. All data were analyzed by a repeated measure of ANOVA followed by multiple comparisons and $P < 0.05$ was considered statistically significant.

These data suggest that there was a significant difference in the attention level between the resting and calculation conditions.

Six subjects were tested in the evaluation of the test–retest reliability of [11C]doxepin PET. Given concerns about the possibility that [11C]doxepin binding changes over days and week, the test and re-test trials were performed in the morning (11:00 a.m.) and afternoon (3:00 p.m.) of the same day. As shown in **Figure 3A**, average SUVs over time from test–retest scans show that the tracer gradually entered the brain and the brain activity remained almost stable. The radioactivity in the anterior cingulate gyrus showed a slightly longer elimination phase in the test trial performed in the morning than that in the afternoon, but the difference was insignificant. The radioactivity in the cerebellum with negligible H1 receptor binding was essentially the same between the test and re-test trials. There was an apparent trend for decreased BP in the afternoon, but the BP values in the brain regions including anterior cingulate gyrus were not significantly higher in the morning test trial (**Figure 3B**), demonstrating that [11C]doxepin binding in the brain is essentially the same between in the morning and afternoon.

In order to verify the effects of attentive waking on *in vivo* PET measurements of H1 receptor binding, [11C]doxepin binding was examined twice in the brains of 10 normal volunteers during resting and attentive waking conditions on the same day, as shown in

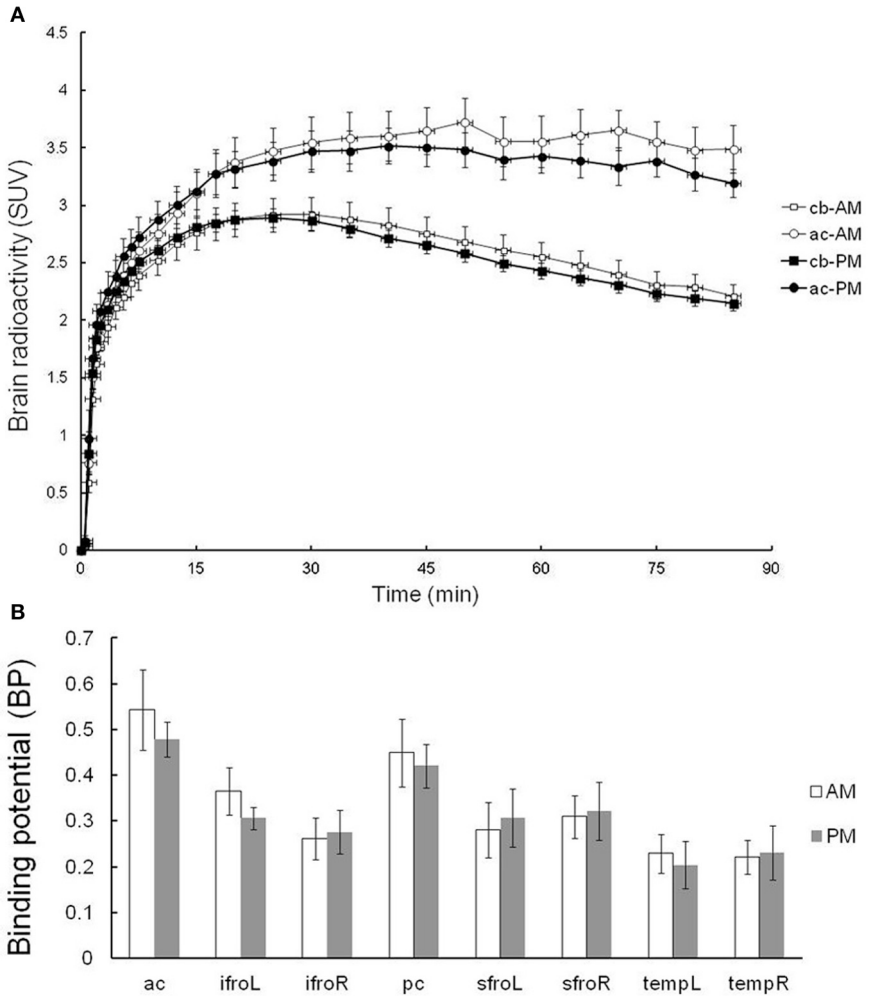

FIGURE 3 | Test–retest trial. (A) TACs in terms of SUVs for the regions of anterior cingulate gyrus (ac) and cerebellum (cb) of test–retest scans. Test–retest scans were performed in the morning and afternoon of the same day, demonstrating a gradual initial increase in SUV followed by a longer elimination phase. Data are expressed as the means ± SEM of six young healthy male volunteers. **(B)** The binding potential (BP) in the test–retest trial. Abbreviations: ac, anterior cingulate gyrus; ifroL, left inferior prefrontal cortex; ifroR, right inferior prefrontal cortex; pc, posterior cingulate gyrus; sfroL, left superior prefrontal cortex; sfroR, right superior prefrontal cortex; tempL, left temporal cortex; tempR, right temporal cortex.

Figure 4. The order of rest and calculation trials of PET studies was randomized to eliminate the effects of circadian rhythm on H1 receptor binding observed in previous experiments. The time courses in the regions of anterior cingulate gyrus (H1 receptor rich region) and cerebellum (H1 receptor null region) were not significantly different between the resting and attentive calculation conditions (**Figure 4A**). There was a trend for decreased BP during the calculation task compared with that in the resting condition, but the difference was not significant. These data suggest that [11C]doxepin binding in the brain is not significantly influenced by the performance of a calculation task as an example of an attentive waking condition.

DISCUSSION

The main objectives of this study were to analyze the specific brain uptake and kinetics of [11C]doxepin in normal volunteers under different conditions, and to assess the test–retest reliability of quantitative PET measurements between morning and afternoon and between resting and attentive waking conditions. The test–retest reliability for estimated BP was found to be sufficiently high to afford reasonable precision in the tracer binding determinations of H1 receptors. Attentive waking and circadian rhythm might have some influences on the BP of H1 receptors, but only within the range of 10% over all regions. The low variability within 10% in the test–retest studies can be compatible to other PET tracers such as [11C]DASB (serotonin transporter), [18F]SPA-RQ (NK-1 receptor), [11C]PIB (amyloid Aβ), and [11C]FLB457 (D2/3 receptor) (Kim et al., 2006; Yasuno et al., 2007; Edison et al., 2009; Narendran et al., 2011).

Evidence from animal studies has implicated the histaminergic neuron system in the pathophysiology of stress-related disorders. Although several antidepressants and atypical antipsychotics are potent H1R antagonists, the significance of their interaction with H1R in a clinical context of efficacy is still unclear. In previous

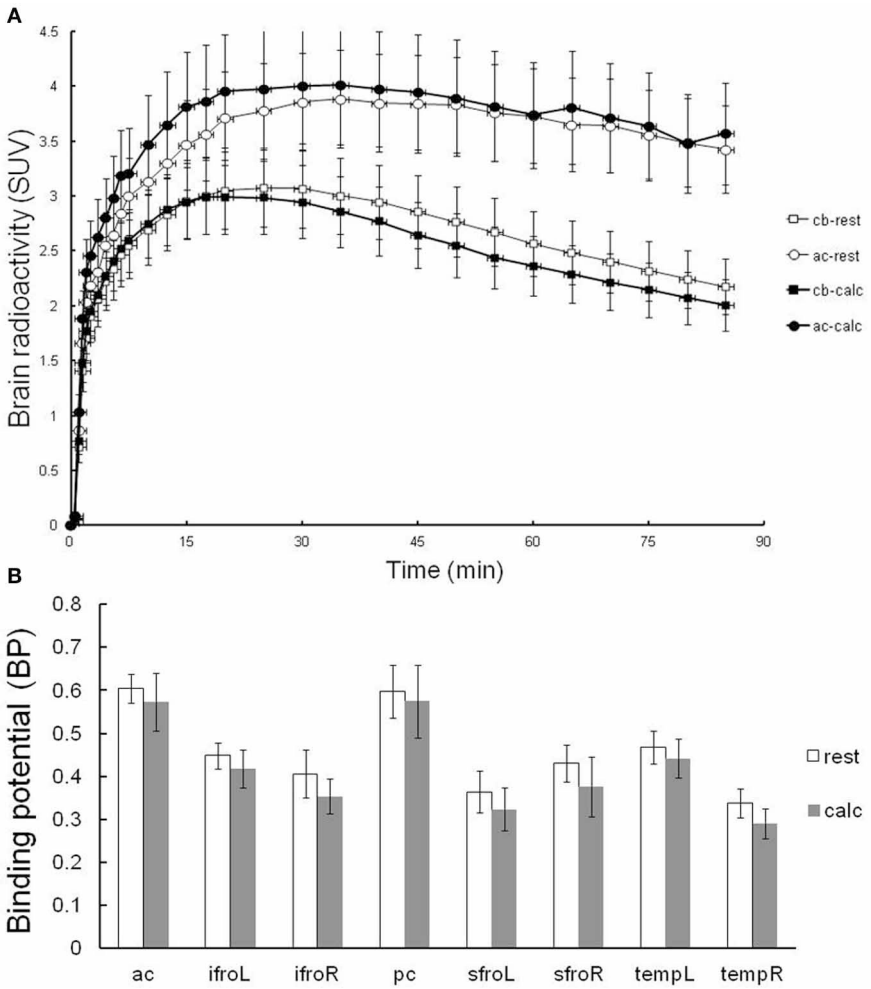

FIGURE 4 | Resting-attentive calculation trial. (A) TACs in terms of SUVs for the regions of anterior cingulate gyrus (ac) and cerebellum (cb) of resting and attentive waking conditions. Data are expressed as the means ± SEM of 10 young healthy male volunteers. **(B)** The binding potential (BP) in the resting and attentive conditions. Abbreviations: ac, anterior cingulate gyrus; ifroL, left inferior prefrontal cortex; ifroR, right inferior prefrontal cortex; pc, posterior cingulate gyrus; sfroL, left superior prefrontal cortex; sfroR, right superior prefrontal cortex; tempL, left temporal cortex; tempR, right temporal cortex.

PET studies, significant reduction in H1 receptor binding was observed in patients with schizophrenia and major depression. It is suggested that prolonged massive histamine release due to repeated stress might lead to the down-regulation and/or internalization of H1R, which may result in decreased binding of [11C]doxepin in stress-related disorders (Endou et al., 2001).

We previously demonstrated that normal female volunteers had significantly higher BP of [11C]doxepin to H1 receptors in the cerebral cortical areas than male volunteers (Yoshizawa et al., 2009). The brain exhibits sexual dimorphism. For example, there are gender differences in the size of the interstitial nucleus of anterior hypothalamus (INAH) (male > female). INAH in homosexual men is only half the size of the nucleus in heterosexual men. The gender difference in human H1 receptors that we observed is reasonable because histamine neurons are exclusively located in the posterior hypothalamus. Sexual dimorphism was also reported for brain histamine in rodents. The density of histamine H1 receptors was higher in female rats than in male rats (Ghi et al., 1999), and hypothalamic histamine release was higher in male rats than in female rats (Ferretti et al., 1998). It is not ruled out that neuronal histamine release and *in vivo* H1 receptor binding are closely correlated. Therefore, we should carefully consider unknown factors influencing *in vivo* [11C]doxepin binding in the brain.

One of the most commonly used types of drug for allergies is the antihistamines. There are many available antihistamines with different sedating properties. Therefore, it is important to develop an objective and reliable method for measuring the strength of such sedative side effects, on which we have conducted numerous PET studies (Tagawa et al., 2001; Yanai et al., 2011, 2012). [11C]Doxepin-PET has been shown to be useful for evaluating their sedating side effects and the mechanisms involved. We succeeded in quantifying the strength of the sedating properties of antihistamines in terms of brain histamine H1 receptor occupancy. We previously reported an age-related decline in H1 receptor binding in normal human brain, especially in the

prefrontal, temporal, cingulate, and parahippocampal regions (Yanai et al., 1992), which are known to be involved in attention and cognition. Therefore, we chose only young male volunteers for these studies of receptor occupancy. This study confirmed the reliability of values in H1 receptor occupancy by antihistamines because different PET studies were summarized to make the figures of occupancy (Yanai et al., 2011, 2012).

This study demonstrates for the first time that subjects' attentive conditions do not affect the reliability of H1 receptor binding measured by [11C]doxepin-PET. This study does not necessarily rule out the feasibility of measuring neuronal histamine release in the living human brain using PET, although PET tracers with better signal-to-noise properties should be developed in the future. For this purpose, H3 receptor binding would be more appropriate because H3 receptors are easily down-regulated by stress-related histamine release. The previous studies reported that histamine release was significantly increased during stressful conditions, and that the H3 receptor density rapidly decreased in response to stress (Ghi et al., 1995; Endou et al., 2001; Westerink et al., 2002). Following the development of other histaminergic PET probes, non-invasive measurement of neuronal histamine release would be feasible in humans by PET ligand-activation study in the future.

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Integrative role of the histaminergic system in feeding and taste perception

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Feeding behavior is regulated by a complex interplay of many endogenous substances, such as peptides and neurotransmitters in the central nervous system. Histamine is a neurotransmitter which expresses an anorectic effect on food intake via histamine H_1 receptors. The histaminergic system exists downstream of leptin, a satiety factor secreted from white adipose tissue. Because direct stimulation of the histaminergic system by histamine H_3-inverse agonists or antagonists can normalize the obese phenotype in which animal models with exogenous leptin resistance, which resembles human obesity, the potential roles of histamine H_3 receptors as a therapeutic target now draw attention. Histaminergic activity is enhanced during feeding, and an oral somatic sensation is thought to affect histaminergic activity while blood glucose levels do not. In addition, gustatory information can modulate histaminergic activity by two mechanisms: by physiological excitation of the chorda tympani nerve, one of the taste nerves and by emotions elicited by taste perception, i.e., taste palatability. Particularly, aversive and hazardous taste stimuli tonically facilitate histaminergic activity, suggesting that the histaminergic system is involved in the response to harmful stimuli. Together with recent findings, it is postulated that the histaminergic system responds to both mechanical and chemical sensory input from the oral cavity during feeding and is exerted as a part of the danger response system.

Keywords: histamine, feeding, taste, palatability, hypothalamus, microdialysis

OVERVIEW OF THE ROLE OF THE HISTAMINERGIC SYSTEM IN THE CONTROL OF FEEDING

Obesity is a prevalent condition which is associated with an increased risk of a consequence of the morbidities of diabetes mellitus, hypertension, and heart disease whose incidence increases with body-mass index (BMI, body-mass in kg/square of the height in meters). Obesity rates is in Europe and North America traditionally high, however, Asian countries such as Japan have recently reported increasing prevalences of obesity, which may reflect changes in dietary patterns and lifestyles. It is considered that life expectancy is maximal when BMI is the ideal level and is reduced 20% or more above the ideal. Thus, controlling food intake and body weight now gathers attention to overcome this worldwide health crisis induced by obesity.

Food intake is necessary for all living organisms to acquire nutrients and energy for the body to grow and repair; it is vital to the survival of every living animal and is subject to intense regulation by brain homeostatic systems (Saper et al., 2002). To ensure feeding behavior takes a high priority in brain function, mammalian brains have evolved several prominent and interrelated neuronal systems that control feeding.

Histamine is regarded as a neurotransmitter and is widely distributed in the mammalian central nervous system (Panula et al., 1984; Watanabe et al., 1984). Histaminergic neurons are confined to the tuberomammillary nucleus (TM), a small region of the posterior hypothalamus. They have wide-spread projection patterns to many different areas with their fibers being found in almost all parts of the brain (Inagaki et al., 1988). The highest density of histaminergic fibers is found in the hypothalamus (Inagaki et al., 1988), which is a brain structure known to play a crucial role in the central regulation of feeding behavior (Oomura et al., 1967).

Because of these morphological features, the involvement of the histaminergic system in the regulation of feeding behavior has been well investigated using histamine-related compounds. It was found that histamine injected intracerebroventricularly produces a long-term suppression of feeding activity in cats (Clineschmidt and Lotti, 1973). Similarly in rats, the administration of histamine into the suprachiasmatic nucleus (Itowi et al., 1988) and an acute injection of histamine in the lateral ventricle (Lecklin et al., 1998) also decreased food intake. The administration of metoprine, which elevates brain histamine content by blocking the conversion of histamine to *tele*-methylhistamine, a methylated metabolite of histamine, can also reduce food intake (Lecklin et al., 1995, 1998; Lecklin and Tuomisto, 1998). Conversely, when neuronal histamine is reduced by α-fluromethylhistidine (FMH), a histamine synthesis inhibitor (Watanabe et al., 1990), significant increases in feeding behavior were produced (Orthen-Gambill

and Salomon, 1992; Ookuma et al., 1993; Tuomisto et al., 1994). Peripheral histamine cannot cross the blood brain barrier, however, L-histidine, a histamine precursor, can and is converted to histamine, resulting in elevated central histamine levels. Studies in rats have shown that peripheral administration of L-histidine by intraperitoneal injection also suppresses food intake (Sheiner et al., 1985; Orthen-Gambill, 1988; Vaziri et al., 1997; Yoshimatsu et al., 2002). The suppressive effect of histidine is attenuated by pretreatment with FMH (Vaziri et al., 1997; Yoshimatsu et al., 2002). Thus, these findings indicate an inverse relationship between histaminergic activity and feeding.

Brain histamine appears to suppress food intake via histamine H_1 receptors. Centrally administered histamine H_1 receptor agonists suppressed food consumption in rats (Lecklin et al., 1998), whereas injection of an H_1-antagonist into the third cerebral ventricle elicited food intake (Sakata et al., 1988). Both H_2-agonists (Lecklin et al., 1998) and antagonists (Sakata et al., 1988) centrally injected had no effect on food intake. Therefore, histamine has been associated with food intake suppression via histamine H_1 receptors, not via histamine H_2 receptors.

Clinically, some antidepressants (Kalucy, 1980; Russ and Ackerman, 1988) and atypical antipsychotics (Deng et al., 2010), which have a high affinity for H_1 receptors, increase food intake and body weight in humans. Similarly, in experimental animal models, tricyclic antidepressants which potently block H_1 receptors, such as doxepin (Orthen-Gambill, 1988) and amitriptyline (Ookuma et al., 1990), can increase food intake, whereas an injection of desipramine, which has a negligible H_1-blockade effect, does not affect food intake (Orthen-Gambill and Salomon, 1990). Recently, the administration of atypical antipsychotics has been shown to be associated with the emergence of metabolic derangements, including body weight increase, dyslipidemia, and type II diabetes (Coccurello and Moles, 2010). Since the orexigenic atypical antipsychotics potently and selectively stimulated hypothalamic AMP-kinase and an action was abolished in H_1 receptor knockout mice (Kim et al., 2007), blockade of H_1 receptors is an important candidate, despite the multiple mechanisms underlying these side effects.

HISTAMINERGIC ACTIVITY DURING FEEDING

How is the actual histaminergic activity during feeding? In order to answer this question, we observed hypothalamic histamine release during feeding by brain microdialysis. Rats were trained to consume standard chow for 1 h a day, and apart from this period, were not given chow but had free access to distilled water. Training was continued for minimum seven consecutive days. In this study using a brain microdialysis technique to measure extracellular histamine levels in the anterior hypothalamus, it was demonstrated that a transient and significant increase in histamine concentration was produced when rats were fed for 1 h, while no significant change in histamine release was observed in the non-fed group, which was similarly trained but was not given chow on the day of microdialysis (**Figure 1**). Another research group also showed similar results when they observed hypothalamic histamine release during feeding in 24 h-fasted rats (Itoh et al., 1991). Valdés et al. (2010) showed histamine release in the posterior hypothalamic area when hungry rats were trying to open a mesh container filled with food. They concluded that histaminergic activation is observed during the appetitive phase of feeding behavior. In contrast, in our experiment histamine release was not altered in the non-fed group, which anticipated meal, but had no access to food on the experimental day. These different experimental conditions seem to cause the different results.

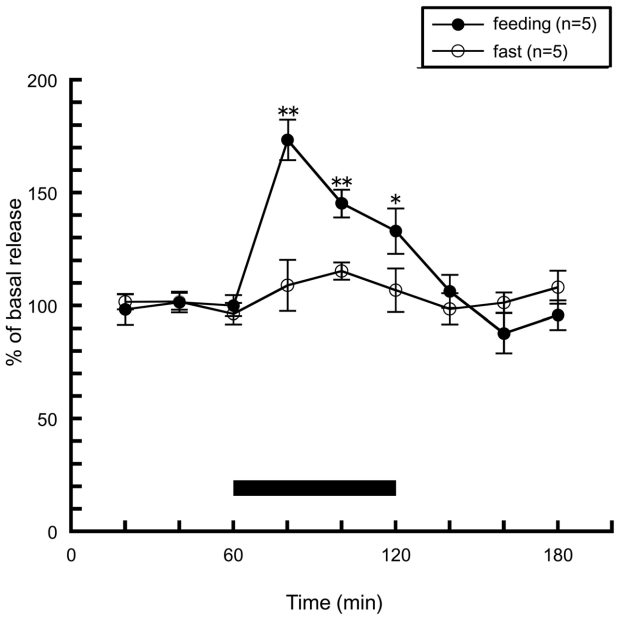

FIGURE 1 | The effects of feeding on the release of histamine from the rat anterior hypothalamus. The black bar indicates the 60 min period when food was available. The mean values of three baseline samples preceding the feeding period were taken as 100%. Values are presented as percentages of the mean basal release ± SEM $^*p < 0.05$, $^{**}p < 0.01$ compared with the basal release of each group.

Together with these observations, it is obvious that histaminergic activity increases during feeding, but what is the factor(s) which affects histaminergic activity during feeding? One possibility is the elevation of blood glucose levels by the post-ingestive effect, thus we studied the effect of the change of blood glucose levels on histamine release. An intraperitoneal injection of D-glucose significantly elevated glucose levels by 140% from basal levels, whereas insulin lowered it to 50% of basal levels about 40 min after the injection (**Figure 2A**). However, histamine release in both groups was unaffected by the change in blood glucose levels (**Figure 2B**), suggesting that the activity of the histaminergic system is not altered by blood glucose levels.

Next, we investigated whether sensory information derived from food influences histaminergic activity. The hardness of food is one of the important physical properties of food, and elicits a somatic sensation received by the masticatory muscle spindles and the periodontal mechanoreceptors in the oral cavity. This information is conveyed to the brain via peripheral sensory receptors, and is thought to play an important role in the regulation of feeding behavior. We examined the effect of the hardness of food

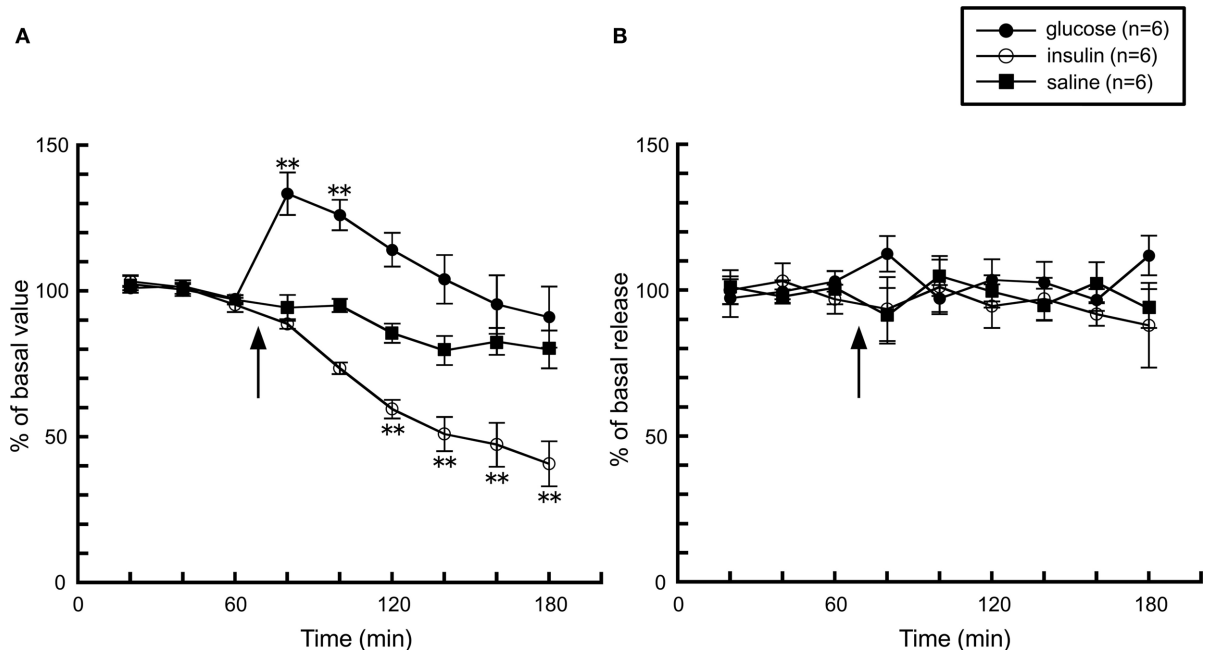

FIGURE 2 | The effects of a 5% D-glucose ($n = 6$) or 200 mU insulin ($n = 6$) injection on (A) blood glucose levels and (B) hypothalamic histamine release in urethane-anesthetized rats. The arrow indicates the time point of injection of each compound. The mean values of three baseline samples preceding the injection were taken at 100%. Values are presented as percentages of the mean basal values ± SEM $^{**}p < 0.01$ compared with the basal values of each group.

on histamine release when rats consumed either of two types of pellets: hard and soft pellets. Hard pellets had similar amounts of tensile stress to commercial pellets but were composed of different ingredients, while the hard and soft pellets withstood different amounts of tensile stress but were made of similar ingredients. The rats were trained similarly in the case of **Figure 1**, but on the last day of training, either hard or soft pellets were presented in order to eliminate neophobia to the food on the day of microdialysis. As shown in **Figure 3**, histamine release was significantly increased in rats fed with hard pellets, which is a similar result to **Figure 1**. By contrast, histamine release was not enhanced in rats fed soft pellets. These observations indicate that the histaminergic activity during feeding is modulated by an oral somatic sensation, but not by blood glucose levels.

LEPTIN AND THE HISTAMINERGIC SYSTEM

Research of the mechanism of feeding has remarkably progressed by the discovery of the *ob* gene product, leptin (Zhang et al., 1994). Leptin is a peptide hormone produced and secreted by white adipose tissue, and consequently, its circulating levels are closely related to body fat mass (Frederich et al., 1995; Maffei et al., 1995). Leptin deficiency in mice homozygous for a mutant *ob* gene (*ob/ob* mice) causes obesity, diabetes, and various neuroendocrine anomalies. Among several splice variants of the leptin receptor, the long form of the leptin receptor, which encodes a protein with a longer cytoplasmic domain (OB-Rb), is highly expressed in the central nervous system (Elmquist et al., 1998) and mediates the action of leptin on feeding and energy

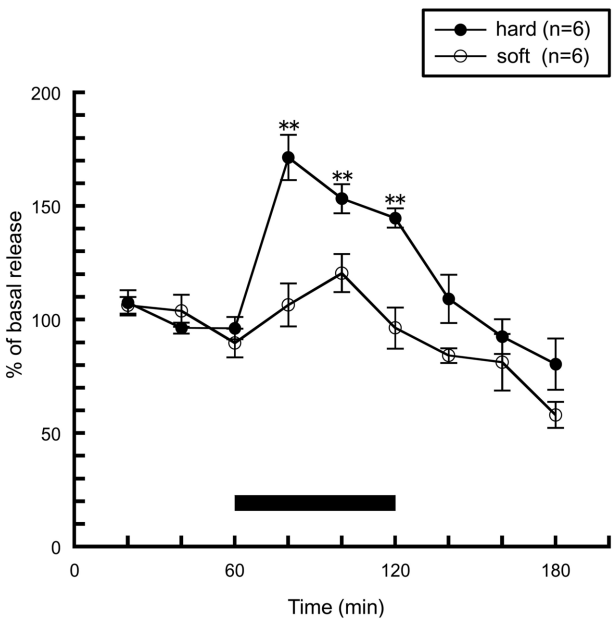

FIGURE 3 | The effects of hard ($n = 6$) or soft ($n = 6$) pellet intake on amygdalar histamine release in freely moving rats [Ishizuka et al. (2010)]. The black bar indicates the 60 min period when food was available. The mean values of three baseline samples preceding the feeding period were taken as 100%. Values are presented as percentages of the mean basal release ± SEM $^{**}p < 0.01$ compared with the basal release of each group, respectively.

expenditure (Friedman and Halaas, 1998). *db/db* mice, which have a mutation in the *db* gene resulting in the abnormal splicing of OB-Rb gene, display a phenotype indistinguishable from *ob/ob* mice (Chen et al., 1996; Chua et al., 1996). A sufficient number of studies proved that leptin acts in the central nervous system to control feeding (Schwartz et al., 1996; Elmquist et al., 1998), and that leptin is the key molecule which links peripheral adiposity levels to the regulation of energy homeostasis in the brain (Campfield et al., 1995).

The interaction of leptin and the histaminergic system has been well investigated by several researchers, including us. Pretreatment of FMH prior to intraperitoneal leptin administration attenuated leptin-induced suppression of food intake in mice (Morimoto et al., 1999; Yoshimatsu et al., 1999; Toftegaard et al., 2003). Moreover, in histamine H_1 receptors knockout mice, the effect of leptin was abolished, whereas leptin remarkably reduced food intake in wild type mice (Morimoto et al., 1999; Mollet et al., 2001; Masaki et al., 2001b). Histamine release was shown to significantly increase when leptin was administered intraperitoneally (Morimoto et al., 2000). Combined with these results, leptin may affect feeding behavior through activation of the central histaminergic system via H_1 receptors, and the histaminergic system exists downstream of leptin signaling in the control of food intake.

From these findings, activation of the histaminergic system is considered as an effective strategy to treat obesity. Indeed, Masaki et al. (Masaki et al., 2001a) indicated that direct infusion of histamine into the third ventricle prevents the development of obesity in both *db/db* mice and diet-induced obesity (DIO) mice. DIO mice are hyperleptinemic and considered as an analog of human obesity since exogenous leptin cannot improve adiposity. However, given the clinical use of histamine-related compounds in humans, it is essential that pharmacological manipulation should involve peripheral dosing. Although central histamine H_1 receptors seem to be a valid and feasible target to control food intake, to date, a selective H_1-agonist which can penetrate to the brain and have anti-obesity effect is not available. Therefore, the histamine H_3-autoreceptor, the presynaptic receptor which controls histamine synthesis and release, is thought to be a promising target for the control of feeding besides the control of sleep-wake cycle and cognitive disorders as demonstrated before (Passani et al., 2004).

An H_3-inverse agonist or antagonist injected peripherally can increase central histaminergic activity, and several studies using animals emphasized the utility of an H_3-inverse agonist or antagonist in the regulation of food intake: Intraperitoneal injection of H_3-inverse agonist, clobenpropit, could decrease energy intake for 3 h in DIO mice concomitantly with an increment in hypothalamic histamine release (Ishizuka et al., 2008). In other studies, A-331440, an H_3-antagonist, orally administered at 15 mg/kg for 28 days reduced weight to a level comparable to mice on the low-fat diet (Hancock et al., 2004). Similarly, twice daily oral administration (20 mg/kg) of the H_3-antagonist, NNC 38-1049 (Malmlof et al., 2005) in DIO rats resulted in a sustained reduction in food intake throughout a two week study, and was associated with a significant decrease in body weight compared with controls.

TASTE INFORMATION AND THE HISTAMINERGIC SYSTEM

Taste is a chemical sensation received from the oral cavity other than a somatic sensation, and it is located between external chemical and internal biochemical environments, and thus its primary role is to distinguish what will or will not be swallowed (Scott and Verhagen, 2000). Taste can be viewed as the entrance of a chemosensory tube that extends through the intestines. Chemical stimulation induces receptor potentials in taste cells, followed by impulses in the taste nerve fibers. Although there is a vast array of chemicals that generate receptor potential in taste cells, physiological and psychological evidence have brought about the classification of the four basic tastes: salty, sweet, bitter, and sour. In addition to these four basic tastes, in increasing number of taste researchers are including "umami"—the unique taste elicited by monosodium glutamate.

We first noticed the possible interaction between taste information and the histaminergic system when we observed histamine release induced by peripheral administration of leptin. As described above, an intraperitoneal injection of leptin facilitated hypothalamic histamine release (Morimoto et al., 2000, **Figure 4**), however, this enhancement was abolished by a bilateral transection of the chorda tympani (Morimoto-Ishizuka et al., 2001, **Figure 4**), which is a branch of the facial nerve (seventh cranial nerve) and innervates taste buds in the fungiform papillae on the anterior two-thirds of the tongue and then transports taste information to the central nervous system (Norgren, 1983). In line

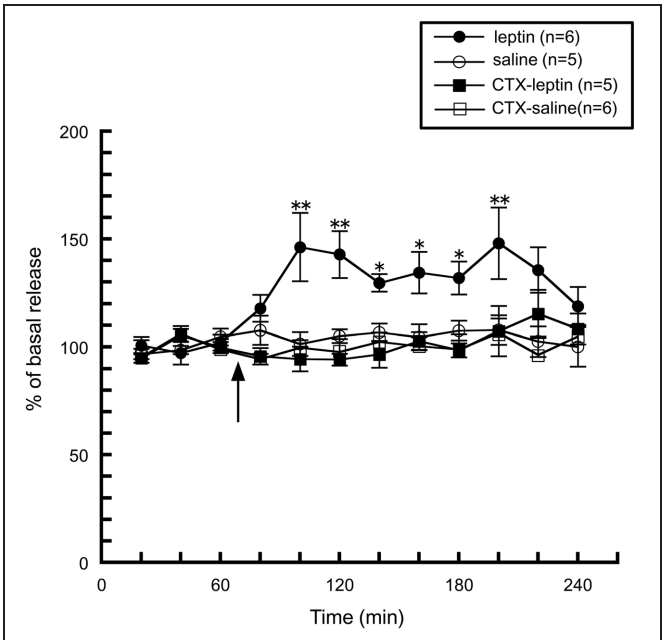

FIGURE 4 | The effect of leptin (1.3 mg/kg, i.p.) on the release of histamine from the anterior hypothalamus in intact ($n = 6$) and chorda tympani-transected (CTX) rats ($n = 5$) (Morimoto-Ishizuka et al., 2001). The arrow indicates the time point of injection. The mean values of three baseline samples preceding the injection were taken at 100%. Values are presented as percentages of the mean basal release ± SEM $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the basal release of each group, respectively.

with these observations, an intracerebroventricular injection of leptin had no effect on histamine release (Morimoto-Ishizuka et al., 2001), or the hypothalamic contents of histamine and *tele*-methylhistamine (Lecklin et al., 2000). Therefore, it is plausible that leptin activates the histaminergic system by peripheral signal inputs via the chorda tympani. These findings led us to study whether direct stimulation of the chorda tympani alters the activity of the histaminergic system.

THE ACTIVATION OF THE HISTAMINERGIC SYSTEM BY TASTE STIMULI VIA THE CHORDA TYMPANI

Because taste stimulation is a more physiological stimulation than electrical stimulation for the chorda tympani, we investigated the effect of gustatory stimuli on the anterior part of the tongue on hypothalamic histamine release using *in vivo* microdialysis in urethane-anesthetized rats (Treesukosol et al., 2003). We used five types of taste solution: a four basic taste mixture [composed of 0.1 M NaCl (salty), 0.5 M sucrose (sweet), 0.02 M quinine HCl (QHCl, bitter), and 0.01 M HCl (sour)] and individual solutions of 0.1 M NaCl, 0.5 M sucrose, 0.02 M QHCl, and 0.01 M HCl. Each taste solution (2 ml) was administered aimed at the anterior tongue directly through the mouth at a rate of 1 ml/min.

The taste stimuli of a four basic taste mixture caused a significant increase in histamine release and this effect was abolished in chorda tympani-transected rats (**Figure 5**), indicating that taste information via the chorda tympani activates the histaminergic system. When each of the components of the four taste mixture was administered separately, a significant increase was observed from administration of 0.1 M NaCl, whereas 0.5 M sucrose and 0.02 M QHCl showed no significant increase in hypothalamic histamine release (**Figure 6A**). Although the HCl solution, 0.01 M HCl, failed to affect histamine release, a higher concentration (0.03 M) increased hypothalamic histamine release (**Figure 6B**). Distilled water was administered as a control and did not cause any effect on hypothalamic histamine release.

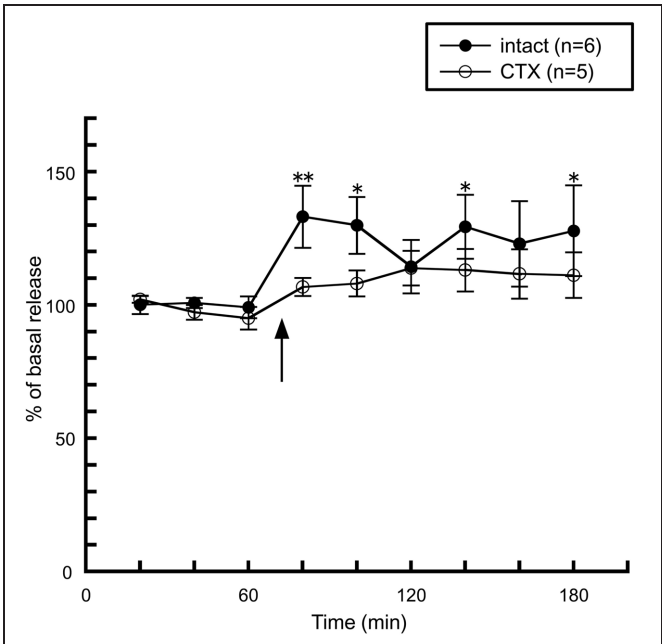

FIGURE 5 | The effect of four taste mixture solutions on the release of histamine in the anterior hypothalamus of intact ($n = 6$) and chorda tympani-transected (CTX) rats ($n = 5$) [Treesukosol et al. (2003)]. The arrow indicates the time point of taste stimuli. The average mean value in the first three samples preceding taste stimuli was taken as 100%. Values are presented as percentages of the mean basal release ± SEM
$^{**}p < 0.01$, $^{*}p < 0.05$ compared with the basal release of each group, respectively.

These results can be explained by electrophysiological properties in which the relative magnitude of the chorda tympani response to 0.01 M HCl, 0.5 M sucrose, and 0.02 M QHCl are 0.61, 0.21, and 0.20, respectively, while the response to 0.1 M NaCl was 1.0 (Beidler et al., 1955, **Table 1**). Thus, it was proposed that hypothalamic histamine release may be proportional to the electrophysiological response of the chorda tympani.

THE EFFECT OF TASTE PALATABILITY ON HISTAMINERGIC ACTIVITY

In the above experiment, we used anesthetized rats in order to stimulate only the region innervated by the chorda tympani, and they did not swallow the taste solution at all. To further investigate the role of the histaminergic system in taste perception, we investigated the effect of taste stimuli on histamine release using freely moving rats (Treesukosol et al., 2005). In this experiment, the taste solution was delivered via an intraoral catheter equipped into the oral cavity beforehand to compare the effects of equal volumes of the solutions (5 ml/20 min).

Consistent with findings from the above study (Treesukosol et al., 2003), application of 0.1 M NaCl and 0.01 M HCl caused significant increases in histamine levels, further supporting the suggestion that this phenomenon is attributed to excitation of the chorda tympani (**Figure 7A**). On the other hand, in freely moving rats, taste stimuli which did not alter histamine release in anesthetized rats dramatically influenced histamine release: when rats were intraorally infused with 0.001 M QHCl solution, a significant increase in hypothalamic histamine release was observed (**Figure 7B**). On the other hand, histamine release was decreased by 0.5 M sucrose and 0.01 M saccharin solutions (**Figure 7B**). Because both the caloric stimulus sucrose and the non-caloric stimulus saccharin produced a decrease in histamine release, the histamine decrease did not seem to be related to caloric content. In rodent experiments, since chemicals which are described by humans as "bitter" or "nauseous" are rejected by rats and those describe as "sweet" or "pleasurable" by humans are avidly accepted (Scott and Verhagen, 2000), it was postulated that histamine increase is produced by the aversive taste stimuli, but not by palatable tastes. Moreover, these findings suggest the possibility that palatable food blunts histamine release resulting in overeating it.

To clarify this hypothesis, we studied the effect of sweet solutions on hypothalamic histamine release in rats which had acquired aversion to sweet solutions by conditioned taste aversion. Conditioned taste aversion is a gustatory long-term memory established after association of the taste conditioned stimulus (CS) with visceral signals of poisoning unconditioned stimulus (US). After the acquisition of conditioned taste aversion,

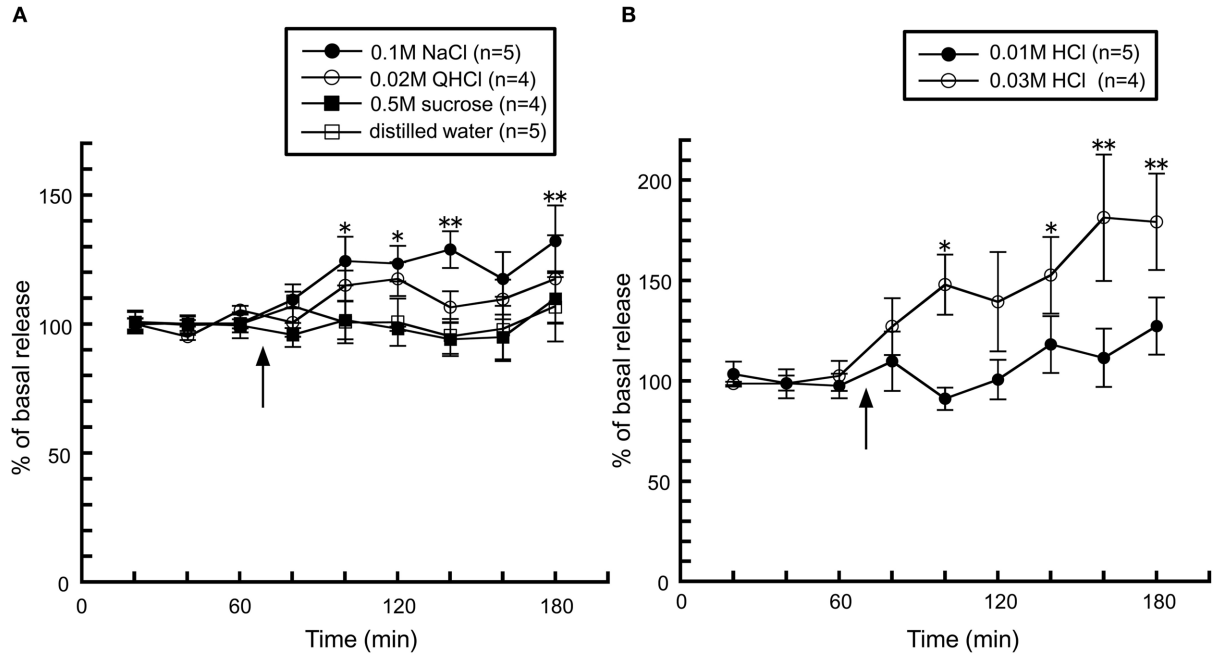

FIGURE 6 | The effects of (A) 0.1 M NaCl ($n = 5$), 0.02 M QHCl ($n = 4$), 0.5 M sucrose ($n = 4$), and distilled water ($n = 5$), and (B) 0.01 M ($n = 5$) and 0.03 M HCl ($n = 4$) on the release of histamine in the anterior hypothalamus of rats [Treesukosol et al. (2003)]. The arrow indicates the time point of taste stimuli. The mean values of the three baseline samples preceding taste stimuli were taken as 100%. Values are presented as percentages of the mean basal release ± SEM $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the basal release of each group, respectively.

Table 1 | The relationship between the chorda tympani response and hypothalamic histamine release induced by taste stimuli.

Taste stimulus	Relative magnitude of response	Histamine release
0.1 M NaCl	1.0	+
0.01 M HCl	0.61	+ (0.03 M)
0.5 M sucrose	0.21	–
0.02 M QHCl	0.20	–

hedonics of the taste CS changes from positive to negative, as indicated by reduced ingestive and increased aversive taste reactivities in response to re-exposures to the CS (Grill and Norgren, 1978).

After rats were conditioned to acquire taste aversion to a sucrose or saccharin solution by one CS (sucrose or saccharin) and US (an intraperitoneal injection of 0.15 M LiCl) pairing, re-exposure to the CS typically elicited active aversive responses such as gapes and chin rubbing (Grill and Norgren, 1978). In both of the conditioned groups, instead of the histamine decrease seen by the palatable solutions (**Figure 7B**), both of the CS significantly increased histamine release and the pattern of histamine release was similar to that seen by the 0.001 M QHCl solution (**Figure 8**). From these observations, it can be concluded that histaminergic activity is modulated not only by the excitation of the chorda tympani, but also by tastant's palatability.

THE PUTATIVE ROLE OF THE HISTAMINERGIC SYSTEM INDUCED BY AVERSIVE TASTE STIMULI

In our studies above, we found some key factors which affect histaminergic activity during feeding. They will be divided into two categories: oral sensation conveyed from the oral cavity to the brain (hardness and taste), and emotion elicited by taste perception. Particularly, the histaminergic system showed robust activation by aversive taste stimuli such as QHCl in naïve rats and the CS in the conditioned rats. What is the physiological role of the histaminergic system under these situations?

Brown et al. hypothesized that the histaminergic system has a role as a danger response system, because the release or turnover of neuronal histamine is enhanced by aversive or dangerous stimuli, such as various kinds of stressors (Brown et al., 2001). More recently, Valdés et al. (2010) expanded their hypothesis and showed that histamine release in the TM is required during motivated behaviors providing the optimal arousal state since the histaminergic system is also known to take part in vigilance (Yamatodani et al., 1996). From this standpoint, it is reasonable to assume that the histaminergic system is promptly activated by aversive or nauseous tastes. Feeding behavior is a complex process which must provide the living organism with a sufficient supply of energy rich edibles as well as certain essential vitamins, minerals, and amino acids from a varied and hazardous external chemical environment. Taste is a very important cue which allows an organism to acquire food selection learning skills, from basic discrimination of edibles from non-edibles to a detailed database of knowledge of a range of foods and their nutritional values or

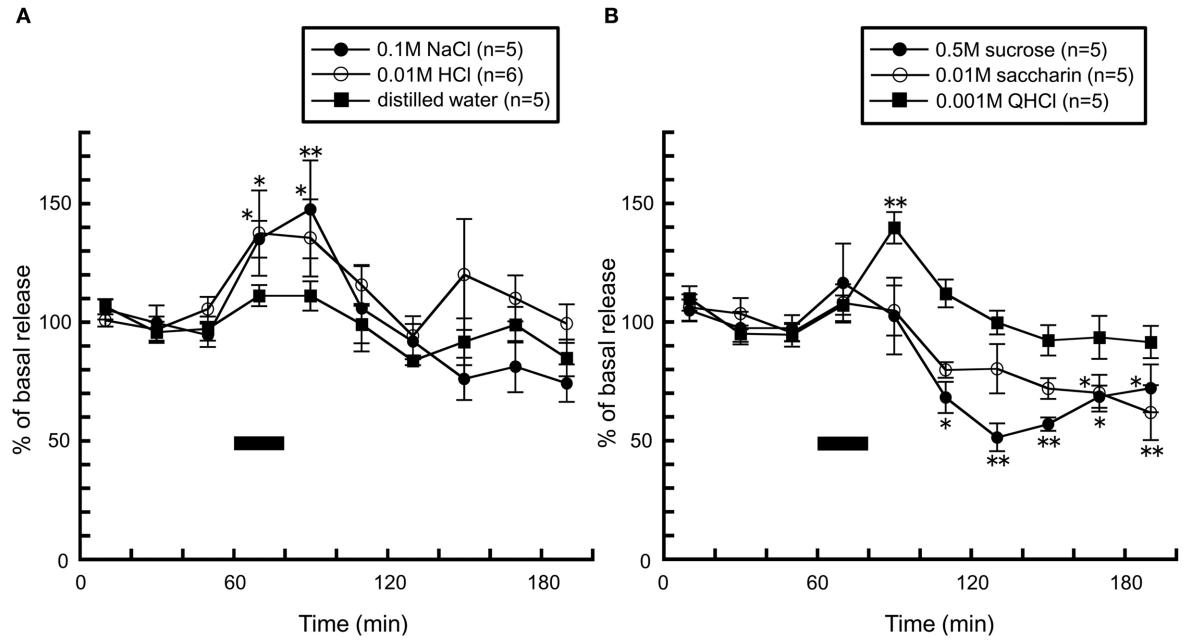

FIGURE 7 | The effect of (A) 0.1 M NaCl ($n = 5$), 0.01 M HCl ($n = 6$), and distilled water ($n = 5$), and (B) 0.001 M QHCl ($n = 5$), 0.5 M sucrose ($n = 5$), and 0.01 M saccharin ($n = 5$) on the release of histamine in the anterior hypothalamus of freely moving rats [Treesukosol et al. (2005)]. The black bar indicates the 20 min period of taste solution infusion. The mean values of the three baseline samples preceding intraoral infusion were taken as 100%. Values are presented as percentages of the mean basal release ± SEM $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the basal release of each group, respectively.

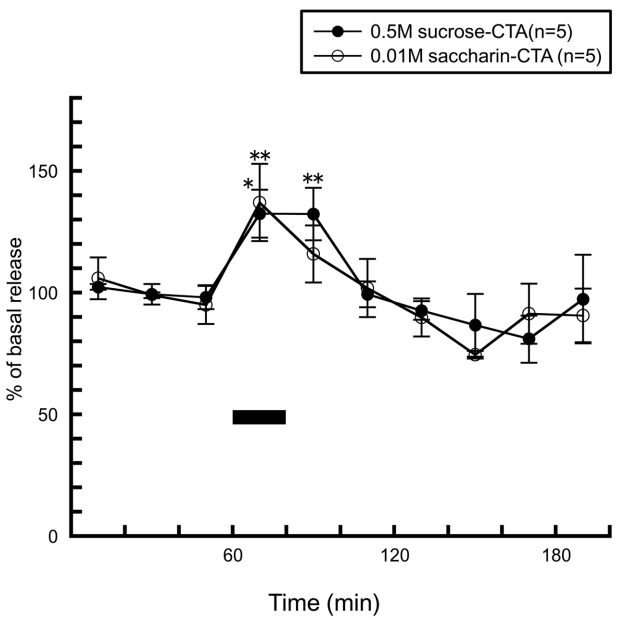

FIGURE 8 | The effects of 0.5 M sucrose ($n = 5$) and 0.01 M saccharin ($n = 5$) to conditioned (CTA) rats on the release of histamine in the anterior hypothalamus [Treesukosol et al. (2005)]. The black bar indicates the 20 min period of taste solution infusion. The mean values of the three baseline samples preceding intraoral infusion were taken as 100%. Values are presented as percentages of the mean basal release ± SEM $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the basal release of each group, respectively.

possible toxic effects (Bures et al., 1998). Generally, bitter or aversive taste stimuli mean a substance is harmful for the animals, and they have to reject such substances in order to protect the internal environment as much as possible. Taken together, the histaminergic system may exert a "biowarning system" effect driven by gustatory information resulting in the rejection of taste solution due to an increased arousal level.

CONCLUSION

The histaminergic system has been shown to be an important neurotransmitter to suppress food intake. Histamine is long considered as a satiety signal, however, recent studies including ours provide new insight into the role of histamine in feeding behavior. The histaminergic system responds to both mechanical and chemical sensory input from the oral cavity and may be exerted as a part of the danger response system. To date, the physiological roles of enhanced histaminergic activity by oral sensation is still unclear, but it is empirically known that the taste or consistency of food affects a variety of aspects of feeding behavior, such as eating speed or jaw movement. Thus, to probe the integrative function of the histaminergic system connecting oral sensory input and these behaviors in detail will be an attractive challenge in the future.

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Histamine and motivation

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Brain histamine may affect a variety of different behavioral and physiological functions; however, its role in promoting wakefulness has overshadowed its other important functions. Here, we review evidence indicating that brain histamine plays a central role in motivation and emphasize its differential involvement in the appetitive and consummatory phases of motivated behaviors. We discuss the inputs that control histaminergic neurons of the tuberomamillary nucleus (TMN) of the hypothalamus, which determine the distinct role of these neurons in appetitive behavior, sleep/wake cycles, and food anticipatory responses. Moreover, we review evidence supporting the dysfunction of histaminergic neurons and the cortical input of histamine in regulating specific forms of decreased motivation (apathy). In addition, we discuss the relationship between the histamine system and drug addiction in the context of motivation.

Keywords: addiction, apathy, appetite, histamine, infralimbic cortex, motivation, tuberomamillary nucleus

INTRODUCTION

Motivations are essential for the preservation of genes, and they provide a rational and structured explanation of behavior and brain organization. Normal and pathological appetites provide guidance; for example, they provide direction in decision-making and influence subsequent behavioral and physiological expression. The scientific study of behavior took an important step forward when the the concept of motivation (sometimes called instinctive behavior, drive or self-regulatory behavior) became widely accepted. It is important to understand why, of all the many possible behaviors in a given context, animals usually perform only a few or just one. In addition, animals in a specific context perform one action and, at other times in the same exact context, perform another action, which may sometimes be opposite to the former. A simple and fixed relationship between the stimulus and response is not a satisfactory model for the behavior of animals with a nervous system. Motivation can be affected by the internal state of the animal, and as other theorists have suggested, arousal or the energizing of behavior is a central component of motivation.

D. Hebb (Hebb, 1955) presented a model of motivation that is particularly useful for discussion; this work is accessible, along with other classic papers in Psychology, at http://psychclassics.yorku.ca/. Briefly, Hebb suggested that motivation provides direction and intensity for behavior; he was probably referring exclusively to higher vertebrates. Motivation is directed toward or away from reinforcers (a functional term for rewards, associated only with positive reinforcers). Positive reinforcers may attract appetitive behavior, while aversions drive behavior away from reinforcers. A historical account of the emergence of the concept of motivation is worth reading (Stellar, 1990).

An additional useful distinction that enriched the concept of motivated behaviors was proposed by Craig in 1917 (Craig, 1918); this distinction is a key element when considering the role of the brain histamine system in behavior. Craig suggested a distinction between the appetitive and consummatory phase of a motivated behavior. Craig stated that "an appetite, so far as externally observable, is a state of agitation which continues so long as a certain stimulus is absent. When the appeted stimulus is at length received it releases a consummatory reaction, after which the appetitive behavior ceases and is succeeded by a state of relative rest, a state of satisfaction." This statement emphasizes that the arousal component characterizes only the appetitive phase of a motivated behavior.

These phases of motivated behavior have counterparts in the physiology of the somatic and visceral output systems that prepare the organism and allow it to better maintain bodily homeostasis and to perpetuate genes. The key role of behavior in long-term homeostatic balance and the preservation of genes has been thoughtfully addressed by Garcia (Garcia et al., 1974) and Blessing (Blessing, 1997), among others.

We will discuss the involvement of brain histamine in appetitive and aversive behaviors; in these behaviors, high arousal and an increased readiness to act and to spend energy predominate in parallel with neuroendocrine and sympathetic activation (Akins and Bealer, 1993). We will also argue that brain histamine may have a negative effect on consummatory behavior.

EFFERENT CONNECTIONS OF HISTAMINERGIC NEURONS

In the mammalian brain, neuronal histamine is exclusively present in the tuberomamillary nucleus (TMN), a loosely packed set of magnocellular neurons located in the posterior and ventral region of the hypothalamus, in close proximity to the posterior recess of the third ventricle (Panula et al., 1989). Histaminergic axons innervate many brain regions, from the prefrontal cortex to the spinal cord, providing an excitatory tone to postsynaptic neurons through H1 and H2 receptors (H1R, H2R) and modulating the release of histamine and other transmitters through H3 receptors (H3R). This widespread distribution of histamine-containing axon terminals and histaminergic receptors helps to clarify the involvement of histamine in many brain functions. However, there have been indications that subsets of TMN neurons, some of which have a specific sensitivity to pharmacological agents, project to defined brain regions (Giannoni et al., 2009) or influence particular brain functions (Miklos and Kovacs, 2003; Valdes et al., 2010).

WHAT INPUTS DRIVE TMN ACTIVITY?

To begin unraveling the roles of histaminergic neurons in a variety of brain functions, it is important to consider the main inputs that might drive, by either increasing or decreasing the activity of TMN neurons. Driver inputs, as opposed to modulatory inputs, are those inputs that are central to the functions of a given cell, whereas modulators may change the expression of those functions (Sherman and Guillery, 1998); for example, a synaptic input may be either a driver or modulator depending on the conditions. We will show the importance of some of these inputs in defining the separate roles of histamine in wake/sleep cycles, feeding-related anticipatory activity, and motivation.

Neuroanatomical studies have addressed the origin of TMN afferents using axonal tract-tracing methods. In general, one can simplify the many inputs ascribed to the ventral TMN by focusing on the most robust (Ericson et al., 1991), considering that some of them may be driver inputs. TMN afferents originate from rostral forebrain limbic structures that include hypothalamic regions involved in food anticipatory activity (Acosta-Galvan et al., 2011), the infralimbic cortex (IL) (Wouterlood et al., 1987), the hypothalamic preoptic region and the lateral septum (Ericson et al., 1991; Sherin et al., 1998). Afferents from other aminergic nuclei and hypothalamic regions, including areas involved in circadian rhythmicity (Deurveilher and Semba, 2005) and the orexin/hypocretin neurons from the lateral/perifornical hypothalamic area, are likely modulatory rather than driver inputs (Ericson et al., 1989; Torrealba et al., 2003) (**Figure 1**).

FOOD ENTRAINABLE CIRCADIAN OSCILLATOR INPUT

The relationship between the motivation for food, arousal, and TMN activity was first studied using a model of restricted feeding. Rats placed on a feeding schedule that is restricted to a few daytime hours wake up in anticipation of mealtime. This anticipatory behavior has an important adaptive value because in nature, food may be available during the same few daily hours (Stephan, 2001), and the anticipatory physiological and behavioral activation prepares the animals to take advantage of this predictable phenomena. It was shown that this anticipatory waking up is related to transient activation of the TMN (**Figure 2A**) but not of other ascending arousal system (AAS) nuclei (Inzunza et al., 2000; Angeles-Castellanos et al., 2004), including the orexin neurons from the lateral hypothalamic/perifornical area (LHA) (Meynard et al., 2005). Increased TMN activation begins approximately one hour before the scheduled mealtime (Meynard et al., 2005), as evidenced by fos mRNA expression; however, the precise relationship between fos expression and electric activity of the TMN neurons remains to be determined. LHA neurons, including non-orexin neurons, become active well after TMN peak activation. We hypothesized that a signal from a food-entrainable circadian oscillator (Meynard et al., 2005) other than the suprachiasmatic nucleus (Mistlberger, 1994) should excite the TMN. Of interest for the present study is the distinction between the histaminergic effects on appetitive versus consummatory behavior. This anticipatory TMN activation quickly disappears (**Figure 2C**) when the animals begin eating (Meynard et al., 2005). Our preliminary data suggest that a bilateral TMN lesion impairs the anticipatory arousal induced by scheduled restricted feeding (Recabarren et al., 2003). Together, these pieces of evidences support the idea discussed that increased histamine levels are important for appetitive behavior and that decreased histamine levels facilitate consummatory behavior. It is likely that the food-entrained circadian input that activates the TMN arises from a component of the intrahypothalamic network that determines food anticipatory activity (Acosta-Galvan et al., 2011) and provides afferents to the TMN. The dorsomedial hypothalamic nucleus, subparaventricular zone, and medial preoptic area are possible candidates for this component (Deurveilher and Semba, 2005).

THE INFRALIMBIC CORTICAL AREA INPUT

Exposure to appetitive food stimuli activates many brain regions in humans (Wang et al., 2004) and rats (Valdes et al., 2010). In both species, there is a marked activation of the frontal cortex, including the anterior insula and orbitofrontal cortices (**Figure 3**), both of which are closely involved in motivation and send projections to the LHA in rats (Gabbott et al., 2005). The activation of these frontal cortices was impaired by a TMN lesion (Valdes et al., 2010).

We view the cortical input to the TMN as essential for the appetitive function of histaminergic neurons, which will subsequently be discussed in more detail. Layer 5 pyramidal cells from the medial prefrontal cortex, which mostly originate from the IL, are the main cortical inputs to the TMN. The close proximity of the axon terminals from the IL to the histaminergic neurons and the glutamatergic phenotype of the pyramidal cells strongly suggest that IL afferents have an excitatory effect on histaminergic neurons. However, it is possible that the IL afferents may have an inhibitory effect if they make synaptic connections with local inhibitory neurons. An electron microscopic study elucidating the type of synaptic contact between the IL and TMN, which is presumably asymmetric and excitatory, similar to the other IL targets (Torrealba and Muller, 1999), is still lacking.

We reasoned that the prefrontal cortex, which is central in decision-making and motivation, is the input that activates the TMN during the appetitive procurement of reinforcers such as food. In fact, Goldman-Rakic demonstrated that the prefrontal

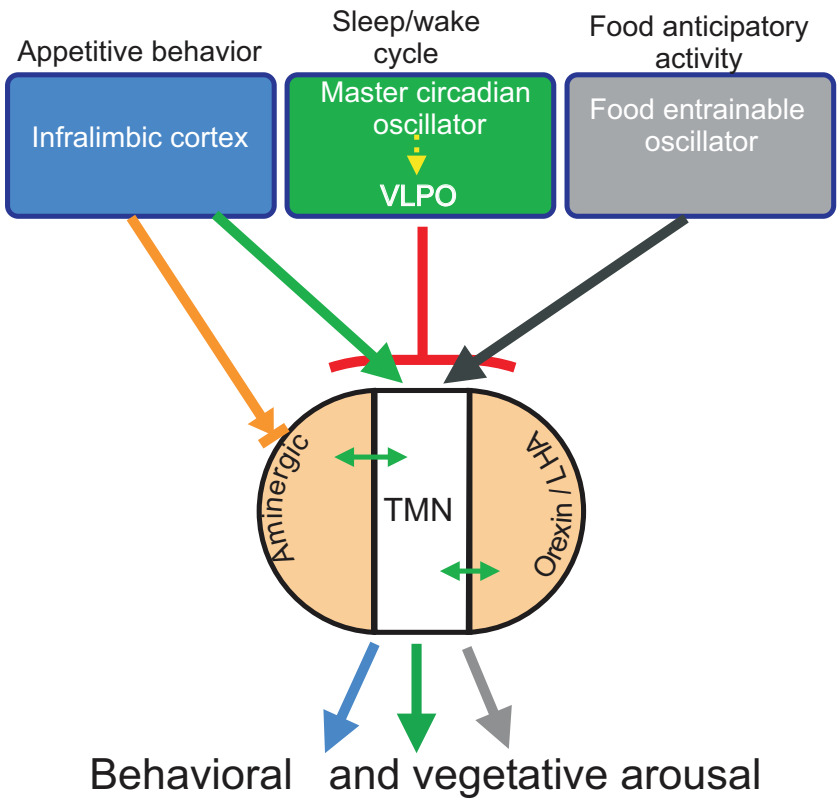

FIGURE 1 | Diagram of the putative drivers (blue, green, and gray) and modulatory (light orange) inputs to the TMN. The presumed role of the driver inputs in the distinct arousal functions of the TMN is indicated on top; in brief, inputs from the prefrontal cortex or the food-entrainable oscillator activate the TMN during appetitive behavior or circadian anticipatory activity, respectively, while input from the VLPO inhibits TMN activity and promotes sleep. The green arrow indicates excitatory input; the black arrow designates presumed excitatory input; the red T-line indicates inhibitory input; the orange arrow designates mixed input to aminergic neurons. Double-headed arrows indicate bidirectional input between the TMN and aminergic and orexin neurons.

cortex is the only source of cortical connections to arousal nuclei and that as such, the prefrontal cortex may control its own level of activity (GoldmanRakic, 1987). To study the functional relationship between IL and TMN neurons in motivated behavior, we devised a method to prolong the appetitive phase by enticing the rats with a wire-mesh box filled with food that was placed within their home cage. The rats may attempt to open the box, but they are unable to obtain the food [see Methods and video in Valdes et al. (Valdes et al., 2010)]. This procedure clearly separates appetitive from consummatory behavior.

Of particular relevance is that a subpopulation of rat IL neurons becomes active during enticement, as demonstrated in single unit recordings in freely moving animals and by c-fos expression (Valdes et al., 2006). A relatively large proportion, 33.3% of IL neurons, increase their firing rate during this enticing stage, while 10% become excited immediately after eating, and 3.3% became active in both conditions. A small percentage (6.7%) of IL neurons decreased their firing rate in response to enticement and after eating. This high proportion of IL neurons that responded to enticement suggests that the IL might use a population code to represent a given behavioral state. Taking into account the many specific functions of the IL during, for example, fear extinction (Quirk and Beer, 2006), stress (Amat et al., 2005), or enticement, and its numerous subcortical targets (Gabbott et al., 2005), one can imagine that subpopulations of IL neurons may participate in several of those behavioral states and that the global "visceral motor" IL (Terreberry and Neafsey, 1987) output may reflect such a combinatorial effect.

IL-TMN AXIS-DEPENDENT MECHANISMS OF BEHAVIORAL RESPONSES TO ENTICEMENT

The increased arousal state made apparent during enticement was measured by polysomnographic recordings (Valdes et al., 2005) or was evaluated by rat motor activity, which in this case and as observed in the video provided by Valdes et al. (Valdes et al., 2010), is goal-directed and not at all non-specific. Animals are motivated to obtain a reward when they detect homeostatic needs (hunger in the present example) and/or to obtain pleasure. One day of fasting ostensibly increased the rats' motivation to open the box more than feeding them *ad libitum*. Additionally, fasted rats familiar with a mixture of plain food pellets and palatable morsels (salami and chocolate cookies) made even more intense attempts to obtain the food compared to rats that were only offered plain pellets (Valdes et al., 2010) (**Figure 2B**). The increase

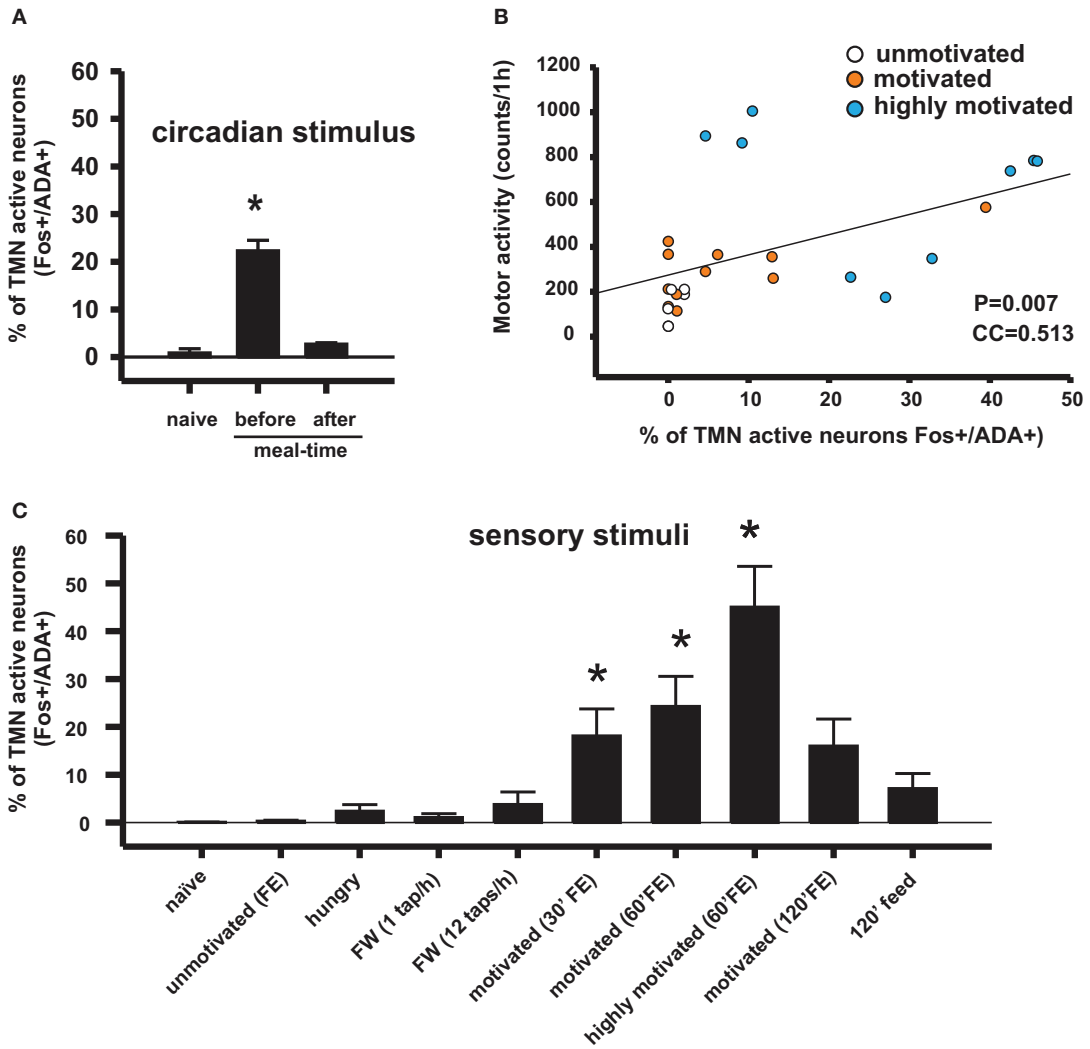

FIGURE 2 | Conditioned sensory signals and circadian signals can activate appetitive behavior and the TMN in rats, depending on the homeostatic state and the hedonic value of the sensory stimuli. (A) TMN activation in anticipation of meal time in food-entrained rats disappeared after having the meal. Naive rats assessed at the same circadian time as rats assessed before meal time (Meynard et al., 2005). **(B)** Correlation coefficient between TMN activation (fos-ir) and goal-directed motor activity during enticing with food. This type of motor activity is used as an arousal index. The Pearson product moment correlation (Valdes et al., 2010). **(C)** Percentage of histaminergic neurons that became active (Fos-ir/ADA-ir) during different conditions that may induce arousal. Kruskal–Wallis One-Way ANOVA followed by multiple comparisons (Dunn's method) versus the naive group; $*p < 0.05$. FE, food enticing; FW, forced wake induced by tapping the rat's cage.

in goal-directed motor activity, and therefore arousal, lasted an average of 30 min in the case of enticement with common pellets and >60 min in the case of enticement with salami and pellets, which was always accompanied by a proportional (in magnitude and duration) increase in the core temperature. These results showed that the enticement procedure used to assess motivation is sensitive to both the homeostatic and hedonic components of appetitive behavior. The use of early gene expression to evaluate the simultaneous activation of arousal nuclei revealed that enhanced motivation (either by hunger or by the anticipation of a more palatable food; **Figure 2B**) corresponded to increased activity of the TMN but not of the other arousal nuclei (**Figures 2C** and **4**).

Whereas it is known that TMN activation during appetitive behavior depends on an intact IL (Valdes et al., 2006), it is still unclear whether the opposite is true. A TMN lesion (Valdes et al., 2010) decreased the activation of many cortical areas other than the IL (**Figure 3**). However, there is significant histaminergic innervation of the IL. Histaminergic axon terminals are present at a moderate density in the medial prefrontal cortex, including the IL (Panula et al., 1989), and histamine is released within those cortices (Westerink et al., 2002; Giannoni et al., 2009). Highly dense H1R ligand binding is present in the medial prefrontal cortex (Bouthenet et al., 1988), whereas H3R binding is present in the axon terminals of extrinsic origin; radiopharmaceutical ligand binding to H3R, but not H3R RNA, was expressed in the

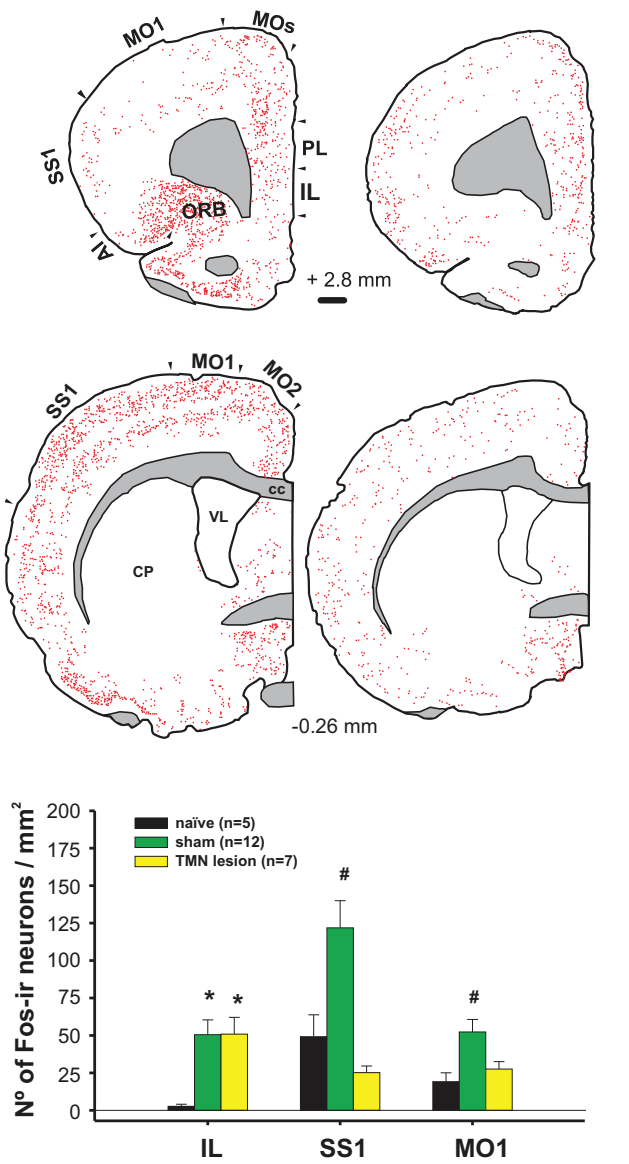

FIGURE 3 | Enticing intact (sham) hungry rats with food strongly activated the frontal cortex (left column). This activation was impaired by a TMN lesion (right column), except for the IL. Lower graph compares the activation of three cortices in the sham and TMN lesioned rats. The naïve group corresponded to the circadian control, i.e., to rats fed *ad libitum*, not enticed and sacrificed at the same time during the day as the other groups (Valdes et al., 2006).

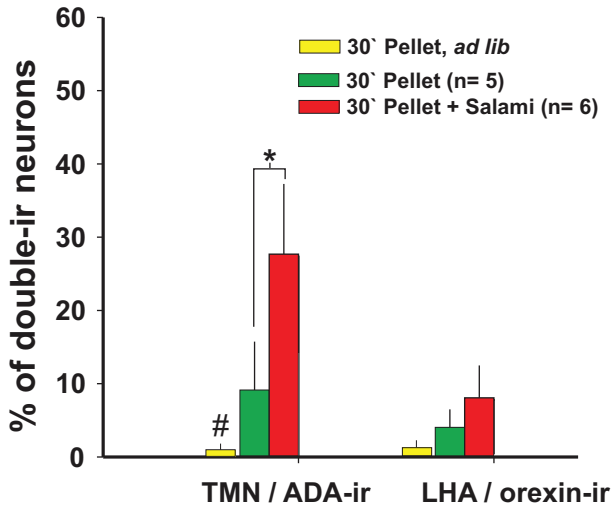

FIGURE 4 | The number of TMN active neurons that responded to enticing was dependent on the homeostatic state and palatability of the stimulus: Orexin neurons from the LHA did not respond to enticing. #Different from the other conditions; *different from the pellet plus salami. Modified from Valdes et al. (2010).

IL (Pillot et al., 2002). It has been shown that H3R activation in the medial prefrontal cortex decreases histamine release in both the TMN and prefrontal cortex of freely moving rats, which is a form of negative feedback (Flik et al., 2011). This finding may help to explain why TMN lesions, which should decrease histaminergic release in the prefrontal cortex, have little effect on IL activity during enticement. When using enticement as a model for appetitive behavior, we found that TMN lesions prevented increases in goal-directed motor activity (Valdes et al., 2010); this decreased appetitive behavior was correlated with the size of the lesion in the dorsal TMN. A bilateral IL lesion also impaired TMN activation during enticement (Valdes et al., 2006). Both the IL and TMN lesions blocked the delayed activation of other AAS nuclei, but not the locus coeruleus (LC) and orexin neurons, suggesting that these neurons contribute to arousal maintenance but not appetitive behavior. It is conceivable that the maintenance of IL activation during enticement after a TMN lesion might be the result of both decreased H3R inhibition and this spared LC activity and the minor activation of orexin neurons (Valdes et al., 2010). In fact, the IL receives a more substantial overlap of noradrenergic and orexinergic input (Baldo et al., 2003) compared with other cortical regions. It is possible that these two inputs maintain IL activity while the other cortical regions are depressed by TMN lesions.

The IL, initially described as a visceral motor cortex (Terreberry and Neafsey, 1987), appears to be essential for the activation of the body and brain that takes place during the anticipation of an event and during appetitive behavior. The IL is a cortical area with more direct connections to subcortical sites involved in neuroendocrine and somatic responses related to appetitive or aversive behaviors (Gabbott et al., 2005). One of those responses is the arousal function of the IL during appetitive behavior, which is importantly mediated by its connection to the TMN. The TMN becomes active and increases arousal (Valdes et al., 2005) before the other AAS nuclei (**Figure 5**), and together with the IL, contributes to the delayed activation of the other AAS nuclei, which in turn maintain and potentially enrich the brain functions that optimize the expression of appetitive behaviors. For example, using the same behavioral task (Robbins and Everitt, 1995), it was shown that cortical cholinergic functions contribute

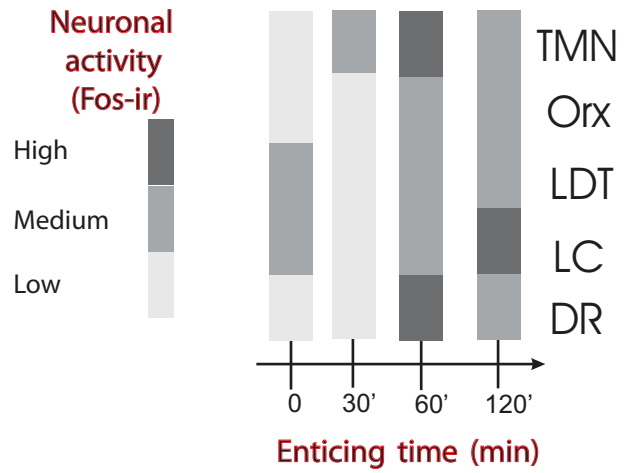

FIGURE 5 | Temporal course of ascending arousal system nuclei activation during appetitive behavior induced by enticing hungry rats with unreachable food. The level of neuronal activity, evaluated by Fos expression, is indicated by the grayscale. The four bar graphs correspond to rats that have fasted for 24 h and were sacrificed immediately before the beginning of the enticing procedure (time 0) or killed after the indicated times (30–120 min). Note that the first nucleus to increase Fos-ir 30 min after the beginning of enticing was the TMN. The other arousal nuclei increased Fos-ir after 60 min and continued with medium to high Fos-ir at 120 min. DR, dorsal raphe; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; Orx, orexin neurons from the lateral/perifornical hypothalamic area; TMN, tuberomamillary nucleus. Modified from Valdes et al. (2005).

to the accuracy of behavioral responses; these functions include the action of dopamine in delaying responses, the action of noradrenaline from the LC in distraction, and the action of serotonin that is related to rats' response impulsivity.

Novelty has reinforcing properties that motivate the exploration of new environments or novel objects. Evidence indicates that this exploration depends on an intact prefrontal cortex (Daffner et al., 2000) and histamine system. It has been shown that mice lacking the enzyme histidine decarboxylase (HDC) show decreased spatial novelty-induced arousal (Parmentier et al., 2002) and reduced exploratory activity in an open field but normal habituation to the same open field (Dere et al., 2004). However, another study found no differences between HDC-KO and wild-type mice in locomotor activity during a 2 h exposure to a new chamber (Nuutinen et al., 2010). Mice lacking H1R (Inoue et al., 1996) show reduced exploratory behavior (ambulation and rearings) in a novel environment. Furthermore, mice lacking H1Rs show reduced emotional responses to a novel environment and do not generate a place preference conditioned by the novel object; however, they do explore (consummatory behavior?) new objects in a familiar setting (Zlomuzica et al., 2008). The nucleus accumbens (NAcc) is one site where histamine increases exploratory behavior through the activation of H1 and H2Rs (Orofino et al., 1999). In contrast, histamine administration into the ventral hippocampus decreased exploration and associated emotionality in an H1- and H2-dependent manner (Ruarte et al., 1997). However, the existence of small regions in the NAcc that may increase or decrease the hedonic response (hotspots) (Smith and Berridge, 2007) makes it difficult to interpret the responses to a NAcc injection, whose precise target within this nucleus is unknown.

IL-TMN AXIS-DEPENDENT MECHANISMS OF VEGETATIVE RESPONSES TO ENTICEMENT

The complexity of the mechanisms underlying the vegetative arousal that characterizes appetitive behavior is illustrated by emphasizing the thermogenic response to enticement. A cell-specific lesion that involves more than 50% of the bilateral IL (Valdes et al., 2006) or a large TMN lesion (Valdes et al., 2010) prevents the thermogenic response to enticement. This effect was correlated with the size of a ventral but not a dorsal TMN lesion (Valdes et al., 2010). However, the normal pattern of thermoregulatory nucleus activation was differentially disrupted by IL versus TMN lesions. A lesion to the IL prevented the activation of all of the thermoregulatory nuclei examined, such as the preoptic area, dorsomedial nucleus of the hypothalamus, and raphe pallidus. In contrast, a TMN lesion only prevented the activation of the raphe pallidus (where sympathetic premotor neurons to brown adipose tissue are located). Other thermoregulatory nuclei were very active despite the absence of a thermogenic response. Importantly, while an IL lesion prevented TMN activation, a TMN lesion did not change IL activation during enticement. Taken together, these results indicate that the thermogenic response during appetitive behavior is controlled by the IL and TMN and that histaminergic input to the raphe pallidus plays a gating or permissive role in the IL thermogenic-related signals to the raphe pallidus. The temperature increase induced by activation of the IL-TMN axis is, in part, mediated by the action of histamine on H1Rs and H3Rs expressed by non-GABAergic and GABAergic neurons of the hypothalamic preoptic region (Lundius et al., 2010). This thermogenic response to histamine injection into the preoptic area is largely mediated by the activation of brown adipose tissue and its sympathetic innervation (Cannon and Nedergaard, 2004). Interestingly and similar to enticement, olfactory stimulation with grapefruit oil excited sympathetic innervation of the brown adipose tissue and adrenal gland and reduced the appetite for food. Moreover, all of these effects were blocked by the systemic administration of an H1 antagonist (Shen et al., 2005).

VENTROLATERAL PREOPTIC AREA INPUT

The best-studied driver input to the TMN is the one central to the wake/sleep function of histaminergic neurons (Saper et al., 2005). This input arises from the ventrolateral preoptic area (VLPO) and adjacent regions in the anterior and ventral hypothalamus (Sherin et al., 1998). These neurons express the inhibitory transmitters GABA and galanin and make symmetrical GABA immunoreactive synaptic connections with the dendrites and cell bodies of TMN neurons and other neurons from the AAS (Sherin et al., 1998). VLPO cells are one of the few cell groups that are more active during sleep than wakefulness (Sherin et al., 1996). In addition, they inhibit AAS neurons, including the TMN, via GABA release, which results in sleep initiation and maintenance (Saper et al., 2005). This VLPO-TMN pathway is essential to understanding the well-described role of histaminergic neurons,

which is to promote wakefulness during the active phase of the day.

The lateral septum is another source of robust, and mostly GABAergic, input to the TMN region. However, there are no published studies on the functional significance of this afferent connection. Nonetheless, histamine in the lateral septum has an anxiogenic effect that is mediated by H1 and H2Rs (Zarrindast et al., 2008).

OREXINERGIC INPUT

Orexin (hypocretin) neurons from the LHA, which are involved in appetitive behavior, in part because of their role in incentive saliency (Harris et al., 2005), form a well studied excitatory (Bayer et al., 2001; Eriksson et al., 2001) input to histaminergic neurons [while histamine has little effect on orexin neurons (Li et al., 2002)]. Orexin neurons may be considered a modulatory input to the TMN. For example, animals unable to synthesize orexin show intense electric activity of histaminergic neurons during cataplexia (John et al., 2004), indicating that inputs other than orexin maintain this high activity of histaminergic neurons. Orexin neurons have a variety of postulated roles, including arousal (Huang et al., 2001), in which histaminergic neurons appear to be the effector. Orexinergic neurons, perhaps under cortical influence (Monda et al., 2004), operate through type B receptors and histamine to increase brown adipose tissue sympathetic nerve activity (Yasuda et al., 2005) and thermogenesis.

HISTAMINE IN AVERSIVE BEHAVIOR

While the focus of this review is centered on the role of histamine in appetitive behaviors, it is useful to note that, in a more general sense, the role of histamine in the activation of the brain (behavioral arousal) and body (increased motor, neuroendocrine, and sympathetic activity) extends to behavioral changes associated with threat situations. Brown and colleagues (Brown et al., 2001) reviewed evidence supporting the idea that the brain histamine system is widely engaged in situations of physiological or existential danger. The anti-nociceptive action of central histamine and its role in water retention and adaptive anorexia highlight the relevance of the histaminergic system in situations of physiological threat, in which licking a wound, drinking, or eating is disadvantageous. For example, axon terminals expressing the hypothalamic anorectic peptide α-MSH, which perhaps originates in the arcuate nucleus, make synaptic contacts with TMN neurons (Fekete and Liposits, 2003) and may contribute to adaptive anorexia. Similarly, the histamine system is involved in situations of potential existential danger, i.e., stressful situations (Westerink et al., 2002; Miklos and Kovacs, 2003), which may elicit anxiety and a behavioral inhibition response (Gray and McNaughton, 2003) that is characterized by increased arousal, changes in behavioral focus, and increased exploratory behavior. For these reasons, Brown and colleagues proposed that brain histamine has a role as a danger response system. We offer a version of Brown's danger hypothesis that extends the range of goal-directed behavior from aversive to appetitive behavior. Histamine is released during goal-directed actions and results in an increase in behavioral and vegetative arousal and a decrease in the drive to consume, which allows the optimal progression of a motivated behavior. This short-lived adaptive anorexia is most likely an important feature of motivated behaviors.

HISTAMINE INVOLVEMENT IN APATHY

Reduced motivation or apathy has been defined as a reduction of self-generated, voluntary, and purposeful behaviors (Levy and Dubois, 2006). There are normal and pathological forms of apathy, depending on the cause, co-morbidity, and duration. Damage to the systems that generate and control voluntary actions located in the prefrontal cortex and basal ganglia will produce apathy. Apathy may be present in some forms of depression, dementia, schizophrenia, Huntington's or Alzheimer's disease, and frontal lobe injury, among others. In addition, apathy itself can also be a syndrome (Marin, 1991).

Levy and Dubois (Levy and Dubois, 2006) distinguish three types of pathological apathy based on the underlying psychological process and neural mechanism. (1) Emotional-affective apathy characterized by difficulties in behavioral modulation on the basis of emotional value, associated with damage to the orbitomedial prefrontal cortex, (2) cognitive apathy characterized by problems in planning and executing goal-directed behavior, associated with dysfunction of the lateral prefrontal cortex, and (3) auto-activation apathy characterized by the loss of spontaneity and a need of an external impulse to initiate actions, associated with injury to the medial dorsal prefrontal cortex, basal ganglia (internal globus pallidus), and/or paramedial thalamus. We think that the auto-activation type of apathy is characterized by a deficit in the energization of motivated behaviors, which we propose depend on an intact IL-TMN axis. Consistent with this idea, this type of apathy is caused by a lesion of the medial Brodmann areas 9/10, 24, 25, and 32; it is thought that the rat infralimbic cortical area is homologous to primate area 25 (Ongur and Price, 2000).

Drug abuse severity is associated with apathy scores (Verdejo-Garcia et al., 2006). Apathy is also associated with poor decision-making and the shortening of self-defined future in patients with frontal lobe damage (Fellows and Farah, 2005). The latter impairments may diminish the likelihood of success for psychological programs that manage drug addiction. Thus, treating apathy with methylphenidate (see below) might be beneficial in the treatment of addiction, as it has proven effective in weight-reducing programs (Desouza et al., 2011).

Interestingly, pathologies that exhibit co-morbidity with apathy have been characterized, from a neurochemical perspective, by dopaminergic (Bressan and Crippa, 2005) and histaminergic (see below) dysfunctions. However, none of these studies focused on the presence of apathy in the subjects analyzed; thus, the link between apathy and histaminergic or dopaminergic dysfunction is tenuous at present.

Regarding the histaminergic system, a reduction in H1R ligand binding in the frontal lobe of depressed patients (Kano et al., 2004) and schizophrenic patients (Iwabuchi et al., 2005) and the frontal and temporal regions of Alzheimer's disease patients (Higuchi et al., 2000) has been observed. However, chronic schizophrenic patients have increased levels of histamine metabolites in their cerebrospinal fluid (Prell et al., 1995). Parkinson's disease patients have increased levels of histamine but do not have increased levels of its metabolite, telemethylhistamine, in

the putamen, substantia nigra compacta, and both divisions of the globus pallidus (Rinne et al., 2002); they also have increased histamine fibers in both divisions of the substantia nigra (Anichtchik et al., 2000). However, no increase in HDC mRNA expression was found in the TMN (Shan et al., 2012b) of Parkinson's disease patients, suggesting that there is no change in histamine production. Reduced H3R mRNA expression and increased histamine methyltransferase mRNA levels in the susbtantia nigra were also found in Parkinson's disease patients (Shan et al., 2012a). In Huntington's disease patients, HDC mRNA is increased in the inferior frontal gyrus and caudate nucleus, while H1 and H3R expression is increased in the inferior frontal gyrus and decreased in the caudate nucleus (van Wamelen et al., 2011). It remains to be determined whether these changes in the brain regions involved in appetitive behavior are a consequence of adaptations to the primary illness or whether they have a causal link to some of the symptoms common to these diseases.

Furthermore, it was found that apathy scores are higher in healthy males than in healthy females (cited in Verdejo-Garcia et al., 2006), while histamine H1R ligand binding is lower in the limbic system of males (Yoshizawa et al., 2009), which further suggests a link between brain histamine and motivation in human. In the same study, the authors found an increase in H1R ligand binding in the amygdala, putamen, and globus pallidus of anorexia nervosa patients (Yoshizawa et al., 2009). It is thought that anorexia nervosa patients are motivated to work around food (some may enjoy cooking, for example) but show a strong aversion to consume it. It was recently suggested that anorexia nervosa patients have a lower sensitivity to natural pleasurable reinforcers (not only to food), a trait that is modulated by cognitive processes focused on thinness (Soussignan et al., 2011).

Pharmacological evidence also indicates a causal relationship between histamine dysfunction and apathy. Methylphenidate strongly increases extracellular levels of dopamine, noradrenaline (Berridge et al., 2006), and histamine in the rat prefrontal cortex (Horner et al., 2007) and improves apathy scores in patients with Alzheimer's disease (Padala et al., 2010), stroke (Spiegel et al., 2009), and dementia (Dolder et al., 2010). It is possible that the increase in the histamine levels by methylphenidate is secondary to the increased extracellular concentration of dopamine, as is the case for systemic methamphetamine administration (Morisset et al., 2002), because D2 brain receptor activation enhances the TMN neuronal firing frequency, histamine release, and wakefulness in freely moving rats (Yanovsky et al., 2011).

We have shown that the IL activity and a consequent increase in histamine release and arousal are necessary for appetitive behavior. We, therefore, hypothesize that damage to the IL-TMN axis could induce a state of apathy that is characterized by a reduction in the disposition to work for rewards or avoid danger. In fact, conditions resulting in high apathy scores are associated with prefrontal damage linked to stroke, depression, or drug addiction (Verdejo-Garcia et al., 2006). The exploration of novel stimuli is a motivated behavior that is dependent on the histamine system (discussed above), which has been shown to be negatively correlated with high apathy scores in patients with Alzheimer's disease or frontal lobe damage (Daffner et al., 2000).

Anorexia may also be a normal response to a variety of physiological or pathological conditions. A well studied example is the reliable and phasic decrease in food intake that follows a cyclic increase in estrogen in rodents and primates, including humans (Geary et al., 2001). While several hypothalamic nuclei, including the TMN, express estrogen receptors, the anorectic effect of estradiol appears to depend on its direct action on TMN neurons, in addition to the effects of corticotropin-releasing hormone on TMN neurons (Gotoh et al., 2005). Estradiol also acts on the paraventricular hypothalamic nucleus (an important anorexigenic region), which releases corticotropin-releasing hormone (Gotoh et al., 2009). Histaminergic neurons may act on ventromedial hypothalamic nucleus (VMH) neurons via H1R to decrease food intake (King, 2006). Female hamsters show changes in brain histamine content following the estrous cycle (Hine et al., 1986); the highest level is reached on proestrous day (day 3) in the hypothalamus and on day 2 in the rest of the brain. During pregnancy, there was an overall decline in histamine content and an increase in food intake. Female rats and humans are more vulnerable to drug abuse in general but particularly on the days with higher estrogen levels (Anker and Carroll, 2011). This vulnerability is facilitated by estrogens.

The site of brain histamine-mediated suppression of food intake is likely the VMH (Ookuma et al., 1989; Malick et al., 2001; King, 2006). The VMH, a hypothalamic site that contains glucose-responsive neurons and descending axonal projections to hindbrain regions that contain premotor sympathetic neurons, is the somatomotor center for motivated behavior-related activities (Simerly, 2004). Blockade of H1R within the VMH, but not in other hypothalamic nuclei such as the paraventricular hypothalamic nucleus or the LHA, increases both meal size and duration and suppresses the activity of glucose-responsive neurons (Fukagawa et al., 1989).

HISTAMINE INVOLVEMENT IN ADDICTION

Addiction is a chronic behavioral disorder characterized by a compulsive and relapsing pattern of drug-seeking and drug-taking behavior that occurs despite the awareness of serious negative consequences. Thus, the behavior and cognitive processes of addicts are centered on drugs, and they have enormous difficulties in attending to other activities (Everitt and Robbins, 2005; Hyman et al., 2006).

Addiction develops after a prolonged period of drug intake in vulnerable individuals (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004) and appears to depend on persistent neuroplastic changes (Kalivas and O'Brien, 2008) that include impairment in the synaptic plasticity of relevant brain circuits such as the NAcc (Kasanetz et al., 2010). Synaptic plasticity (long-term depression) at cortico-striatal synapses is critically controlled by dopamine (Surmeier et al., 2007) and acetylcholine (Wang et al., 2006), and it is thought to underlie the formation of goal-directed behaviors and habits.

It has been proposed that, as with habit formation in general, addiction involves a progression from action-outcome to stimulus-response mechanisms; drug use in the addict is controlled by automatic action patterns interacting with non-automatic cognitive processes (Tiffany, 1990; Everitt and Robbins,

2005; Pierce and Vanderschuren, 2010). This sequence, from behavior controlled by outcome to habitual drug seeking, is paralleled by the gradual recruitment of ventral to dorsal striatal regions (Porrino et al., 2002) that have an anatomical substrate in the spiraling connections between the medial prefrontal cortex, NAcc, and ventral tegmental area (VTA) and the dorsal prefrontal cortex, dorsal striatum, and substantia nigra (Haber et al., 2000; Pierce and Vanderschuren, 2010). The histaminergic system sends projections to the prefrontal cortex and striatum (Panula et al., 1989; Giannoni et al., 2009) and receives inputs from the medial prefrontal cortex, particularly from the IL. This cortical input drives TMN activity and seems to be important in sustaining a high level of arousal during motivated behavior, as discussed above. Because histaminergic axon terminals are present in the prefrontal cortex, dorsal striatum, and NAcc, it is possible that histamine may participate in the initial and late phases of addiction. Consistent with the idea presented in this review, histamine would amplify the incentive salience (Berridge and Robinson, 1998) of reinforcers and reinforcer-associated cues and support the arousal of the appetitive phase of motivated behavior directed toward obtaining drugs.

A link between the activity of the histamine system and the risk of becoming addicted to drugs has been reported. Novelty seeking is a personality trait that is related to an increased risk of addictive behavior in human and animal models (Kampov-Polevoy et al., 2004; Belin et al., 2011). Females have higher levels of brain histamine (Hine et al., 1986; Prell and Green, 1991) and are more vulnerable to addiction than males. High gonadal hormone estrogen levels during the menstrual cycle may facilitate drug abuse in women (Anker and Carroll, 2011). Anorexia nervosa patients show a significantly higher density of histamine H1R than controls, suggesting that the alteration of central histaminergic activity is involved in eating disorders (Yoshizawa et al., 2009). Interestingly, there is a co-morbidity between bulimia nervosa or anorexia nervosa and addiction (Baker et al., 2010). Rats with a strong preference for alcohol have elevated levels of brain histamine and its metabolites, as well as a higher density of histaminergic nerve fibers than rats with a lower preference for alcohol (Lintunen et al., 2001).

Despite these correlation studies, a clear association between histamine activity and the likelihood of engaging in drug seeking behavior is still lacking. The systemic administration of thioperamide to normal mice, which increases the firing of TMN neurons and histamine release in the prefrontal cortex and posterior hypothalamus (Flik et al., 2011) is effective in facilitating the development of conditioned place preference induced by a small (but not a larger) dose of cocaine (Brabant et al., 2005). However, thioperamide interferes with psychostimulant metabolism and maintains higher concentrations (Brabant et al., 2009). In addition, antagonizing H3R alters the release of other neurotransmitters through heterosynaptic mechanisms, making it difficult to ascribe its effects to histamine transmission only. Histamine inhibits the development of morphine-induced conditioned place preference (Gong et al., 2007, 2010); however, HDC-KO mice are not more responsive to the stimulant effect of cocaine and require cocaine doses similar to those required by WT mice to develop conditioned place preference (Brabant et al., 2007).

Drugs that are abused disrupt the neural circuitry involved in motivational processes such as pleasure, incentive saliency, and learning (Robinson and Berridge, 2008). The mesocorticolimbic dopaminergic system arising from the VTA and substantia nigra compacta is important in acquiring a conditioned inclination to stimuli that have been associated with obtaining primary reinforcers and in maintaining habits once a motivation-related task has been learned (Wise, 2004). Addictive drugs share the property of activating this mesocorticolimbic dopaminergic system, and the increase in dopaminergic concentration in the NAcc and frontal cortex appears to be an essential mechanism of drug addiction (Roberts et al., 1980; Koob, 1992). The frontal cortex and NAcc receive input from limbic structures and brainstem autonomic centers related to affective and motivational function (Kelley, 2004). The NAcc is a key region involved in the processing of stimuli reinforcement information and in the selection of the appropriate motor action toward the selected goal (Kelley, 2004; Salamone et al., 2007). Dopaminergic transmission in the NAcc appears to be important for assigning incentive salience to rewards and to conditioned stimulus-reward associations but seems to be irrelevant to hedonic processes (Smith et al., 2011). Brain histamine can also affect the neural operation of structures involved in motivation/reward processes and influence the mesocorticolimbic dopamine system in opposite ways by acting at different levels.

The nuclei of origin of the mesocorticolimbic system (VTA and substantia nigra compacta) receive high to moderate histaminergic fibers (Panula et al., 1989). H1R are present in the VTA and substantia nigra compacta (Bouthenet et al., 1988). In addition, H2R mRNA and protein are present (Vizuete et al., 1997) in the VTA and substantia nigra compacta at moderate levels. H3R mRNA is expressed in substantia nigra compacta but not in VTA neurons (Pillot et al., 2002), suggesting a presynaptic effect of histamine on nigrostriatal but not on VTA axon terminals in the NAcc or the prefrontal cortex. Histamine inhibits the activity of these dopaminergic neurons indirectly via the excitation of GABAergic neurons (Korotkova et al., 2002), although it is not known whether those GABAergic neurons are local or projecting.

The NAcc has a moderate number of histaminergic fibers (Panula et al., 1989) and high densities of H1, H2, and H3R (Bouthenet et al., 1988; Vizuete et al., 1997; Pillot et al., 2002), suggesting that histamine has the potential to exert complex effects on NAcc function but exhibits no effect on VTA dopaminergic terminals, which lack H3R. Intracerebroventricular (icv) administration of histamine stimulates mesolimbic dopamine neurons projecting to the NAcc through an action on H1R, while no such effect was found in the dorsal striatum (Fleckenstein et al., 1993). Local administration of histamine into the NAcc increases or decreases the firing rate of the accumbens neurons (Shoblock and O'Donnell, 2000) and increases local extracellular dopamine via H1 activation of cholinergic interneurons (Prast et al., 1999), which act on presynaptic nicotinic receptors to increase dopamine release (Wonnacott et al., 2000; Galosi et al., 2001). In addition, acetylcholine facilitates the inhibition provided by fast-spiking GABAergic interneurons on medium spiny neurons (MSNs) by activating postsynaptic nicotinic receptors (de Rover et al., 2002).

The dorsal striatum (caudate-putamen) receives a low to moderate density of histaminergic fibers (Panula et al., 1989) and contains high densities of H2R and H3R (Vizuete et al., 1997; Pillot et al., 2002) and a moderate density of H1R (Bouthenet et al., 1988). The substantia nigra sends projections to the dorsal striatum (Beckstead et al., 1979), receives a moderate density of histaminergic fibers (Panula et al., 1989) and has a high density of H3R, particularly in the pars reticulata (Pillot et al., 2002). Histamine H3R are located on nigrostriatal terminals, and their activation inhibits striatal dopamine synthesis (Molina-Hernandez et al., 2000). Histamine excites dissociated cholinergic striatal interneurons through H1 and H2R, which subsequently excite the MSNs, most likely via muscarinic receptors. In contrast, histamine has no direct effect on dissociated MSNs (Munakata and Akaike, 1994). In anesthetized rats, the local iontophoretic injection of histamine into the striatum increased the firing rate in 40% of the neurons (Sittig and Davidowa, 2001). Using a slice preparation from the dorsal striatum of mice, bath-applied histamine depolarized MSNs by acting on H2R and suppressed both cortical (Doreulee et al., 2001; Ellender et al., 2011) and thalamic excitatory inputs (studied by single-pulse stimulation) to MSNs acting on presynaptic H3R (Ellender et al., 2011). Histaminergic depression of excitatory input to the MSNs through H3R can also result from inhibition of glutamatergic release (Brown and Haas, 1999). Interestingly, histamine facilitates the short-term dynamics (studied by paired-pulse stimulation) of thalamostriatal more than the dynamics of corticostriatal synapses, leading to a larger facilitation of thalamic input in this condition (Ellender et al., 2011). Histamine has no effect on fast-spiking GABAergic interneurons but abolishes lateral inhibition between MSNs. Histamine also depolarizes cholinergic interneurons acting at H1Rs in the dorsal striatum (Bell et al., 2000), which increases MSN inhibition, as has been described in the NAcc. These results suggest that increased activity of the histaminergic system in the striatum in general may lead to an amplification of the feed-forward inhibition by interneurons on projection neurons and a facilitated response to thalamostriatal input.

The evidence presented here suggests that the histaminergic system contributes to the regulation of the normal function of the dorsal striatum and highlights the possibility that histamine may exert control over the behavioral and motor output of this region. The effects of histamine on the dorsal striatum suggest that histamine has a role in the later stages of addiction (Everitt and Robbins, 2005), which is when drug intake is driven by habit. While the mechanisms of action are not entirely clear and may influence the intake of specific drugs instead of interfering with addiction in general, it is interesting that pyrilamine significantly reduces nicotine self-administration (Levin et al., 2011) and that H3R antagonists reduce alcohol intake (Lintunen et al., 2001; Galici et al., 2011) and methamphetamine self-administration (Munzar et al., 2004) in animal models of addiction.

Aminergic neurotransmitters act on postsynaptic structures mostly through volume transmission, resulting in longer-lasting and more widespread effects (Torrealba and Carrasco, 2004) compared to fast neurotransmitters. Fast-scan cyclic voltammetry studies report that the half-life in the extracellular space of histamine released by electrical stimulation of the medial forebrain bundle is 4.1 ± 0.9 s (Hashemi et al., 2011). Similar studies have determined that the dopaminergic half-life is 1.12 ± 0.05 s (Park et al., 2010). No high-affinity uptake system for histamine has been reported; the termination of histamine synaptic action appears to require its catabolism to telemethylhistamine by the enzyme histamine N-methyl transferase in the extracellular space (Haas and Panula, 2003). Taken together, these findings suggest that in physiological conditions, histamine signaling could bring about longer-lasting effects compared to dopamine in the same brain region (i.e., NAcc shell), which could contribute to dopaminergic action on incentive salience and learning.

The data reviewed here suggest that the histaminergic system is involved in the motivation for reward and reward-associated stimuli that are relevant to the neural process that mediate addiction through the amplification of the physiological effects of dopamine and by its direct effects on the dorsal striatum and NAcc. However, the functional relationship between the histaminergic and the dopaminergic systems have not been studied in detail, in part because we lack of a clear conceptual framework of the links between the parallel and convergent functions of these two systems in motivated behavior.

CONCLUSIONS

The brain histaminergic system is essential for the appetitive and the aversive phases of motivated behaviors. Such behaviors include the maintenance of homeostatic balance, the exploration of new environments, reproduction and caring of the progeny, and responses to threatening situations. The brain histaminergic system also contributes to successful goal-directed behaviors by decreasing the drive to consume. In addition, dysfunction of the histamine system may underlie some forms of apathy and feeding disorders. Finally, the histaminergic system may have a potentially important function in abnormal appetites for drugs, although this role has not yet been explored in detail.

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Histamine H3 receptor: a novel therapeutic target in alcohol dependence?

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The brain histaminergic system is one of the diffuse modulatory neurotransmitter systems which regulate neuronal activity in many brain areas. Studies on both rats and mice indicate that histamine H3 receptor antagonists decrease alcohol drinking in several models, like operant alcohol administration and drinking in the dark paradigm. Alcohol-induced place preference is also affected by these drugs. Moreover, mice lacking H3R do not drink alcohol like their wild type littermates, and they do not show alcohol-induced place preference. Although the mechanisms of these behaviors are still being investigated, we propose that H3R antagonists are promising candidates for use in human alcoholics, as these drugs are already tested for treatment of other disorders like narcolepsy and sleep disorders.

Keywords: histamine, H3 receptor, alcohol, self-administration, reward, conditioned place preference, drinking in the dark, addiction

INTRODUCTION

Alcohol dependence is a remarkable health risk for the society. More than 60 diseases and injuries resulting in approximately 2.5 million deaths per year worldwide are linked to heavy alcohol drinking (Edwards et al., 2011). The number of current available medications for the treatment of alcohol dependence is limited, and most treatments show only moderate efficacy. Promising results in the treatment of alcohol dependence have been obtained with some existing drugs such as anti-epileptic drug topiramate, antispasmic baclofen, and nausea relieving ondansetrone (Sellers et al., 1994; Addolorato et al., 2002; Johnson et al., 2003). New therapeutic drug targets have also been launched during the last years including cannabinoid receptors, neuropeptide Y, corticotropin-releasing factor, and ghrelin (Kraus et al., 2005; Thorsell et al., 2006; Lowery and Thiele, 2010; Maccioni et al., 2010). Recent findings from our laboratory using various animal models assessing dependence-like alcohol behaviors as well as binge-like alcohol drinking suggest that histamine H3 receptor could be a potential therapeutic target in the treatment of alcohol dependence (Nuutinen et al., 2010, 2011a,b).

HISTAMINERGIC SYSTEM ALTERATIONS LINKED TO HIGH RISK ALCOHOL CONSUMPTION

Many modulatory neurotransmitter systems converge on the same neurons in the brain. For example, the actions of acetylcholine, histamine, norepinephrine, and serotonin on human neocortical cells are similar: they all increase spiking by reducing spike-frequency adaptation (McCormick and Williamson, 1989). It is thus not surprising that several transmitters or their respective receptor ligands can have similar effects on neural circuits and behavior. Some of the important systems in drug abuse include the mesolimbic dopamine system and corticostriatal and corticoaccumbal glutamate systems (Koob and Volkow, 2010; Kalivas and Volkow, 2011). All these brain regions are also innervated by histamine-containing nerve fibers originating from the posterior hypothalamic tuberomamillary nucleus both in rodents and in humans (Panula et al., 1989; Airaksinen et al., 1991; Jin and Panula, 2005). Following release, histamine is mainly inactivated by methylation in the brain to an inactive metabolite tele-methylhistamine, whereas in the peripheral organs oxidation by diamino-oxidase is predominant (Haas and Panula, 2003). Concentrations of tele-methylhistamine have been shown to correlate well with histamine release in the brain. Very few studies have directly analyzed the role of histamine in alcohol-related behaviors in humans, and animal studies have been carried out only recently. In postmortem analysis, histamine and tele-methylhistamine, the first metabolite of histamine, levels are clearly increased in patients who suffered from extensive liver cirrhosis following alcohol abuse (Lozeva et al., 2003). The histamine levels were elevated in both patients with high alcohol consumption and those with no alcohol use. The mechanism is thus most likely related to the highly increased levels of neutral amino acids in the blood circulation, as reviewed earlier for experimental portocaval anastomosis cases (Fogel et al., 2002). In one study on type 1 (late onset, often females, low degree of association with violence) and type 2 (early onset, often males, high degree of association with violence) alcoholics histamine levels were significantly elevated in the gray matter of type 1 alcoholics and the levels of tele-methylhistamine were elevated in type 2 alcoholics indicating that histamine turnover is altered (Alakarppa et al., 2002).

In alcohol-preferring Alko alcohol (AA) rats developed in Finland in 1960s using a two-bottle choice preference selection method (Eriksson, 1968) several aminergic systems are abnormal. They have higher tyrosine hydroxylase activity and higher levels of dopamine and noradrenaline in striatum and limbic regions (Ahtee and Eriksson, 1975), higher serotonin levels in

many brain areas (Ahtee and Eriksson, 1973), and higher histamine and tele-methylhistamine levels in many brain regions than alcohol non-preferring Alko non-alcohol (ANA) rats (Lintunen et al., 2001). Higher tele-methylhistamine levels indicate also higher histamine release in AA than ANA rats. This high histamine content agrees well with the higher density of histamine-immunoreactive nerve fibers in many regions of the AA rat brain, including nucleus accumbens, septal nuclei, and medial preoptic nucleus. Thus, histamine from the other major storage site of brain histamine, mast cells, is not responsible for the significant difference. Receptor radioligand autoradiography has shown significant differences in histamine H1 receptor binding throughout the brain of AA and ANA rats. The binding densities in AA rat brain were lower, possibly as a result of high histamine release induced downregulation (Lintunen et al., 2001). H3 receptor radioligand binding is lower in AA than ANA rats in the motor cortex, nucleus accumbens, and CA1 area of the hippocampus. Although similar systematic studies have not been carried out in other rat lines selected for high volunteer alcohol intake, an analysis of histamine content in the cortex of the high alcohol preference (HAP) and low alcohol preference (LAP) rats was not different (Kitanaka et al., 2004). However, the histamine induced phosphoinositide hydrolysis was significantly lower in HAP than LAP rats (Kitanaka et al., 2004).

Rat lines selected for high alcohol sensitivity (alcohol non-tolerant, ANT) and low alcohol sensitivity (alcohol tolerant, AT; Rusi et al., 1977) also show distinct differences in histaminergic markers in the brain: the ANT rats have reduced histamine levels in many brain areas, including hypothalamus, septum, cortex, and hippocampus (Lintunen et al., 2002). The alcohol sensitive ANT rats also show higher H3 receptor agonist-induced guanosine 5′-O-(3-[^{35}S]thio)triphosphate binding, indicative of histamine H3 receptor activation, in motor cortex, insula, and caudate-putamen (Lintunen et al., 2002). The behavioral effects of H3 receptor ligands on these rats have not been tested, but administration of a suicide inhibitor of the histamine synthesizing enzyme histidine decarboxylase, α-fluoromethylhistidine (α-FMH), decreases the performance of these rats in the tilting plane test (Lintunen et al., 2002) suggesting higher sensitivity to alcohol in the absence of histamine. Taken together, these results suggest that there are basal differences in the histamine system markers in these rat lines. However, no mutations in genes directly related to histamine synthesis and metabolism or histamine receptors have been identified.

HISTAMINE H3 RECEPTOR-MEDIATED REGULATION OF ALCOHOL DRINKING

Alcohol consumption in experimental animals can be measured with different types of drinking models. One of the most widely used paradigms is the two-bottle choice test where animals are given free-choice access to alcohol and non-alcoholic fluid, typically water (Green and Grahame, 2008). Alcohol consumption in this model, however, can be influenced by other factors than pharmacological action of alcohol, such as taste and calories. Thus control studies with sweet, bitter and high caloric fluids are needed. Using a modified prolonged 8-week version of the two-bottle choice we found that the histamine H3 receptor knockout (H3R KO) mice consumed alcohol at concentrations 10 and 20% significantly less than the wild type animals (Nuutinen et al., 2011a; **Table 1**). Control studies with bitter quinine and sweet saccharin revealed no genotype differences. There was no difference between the H3R KO and control mice in water consumption. However, the food consumption in H3R KO mice was lower than in control animals leaving us with a question whether the H3R KO mice simply drink less alcohol since they need less energy?

A more newly described method for alcohol consumption is so called Drinking in the Dark (DID) method where mice are given access to alcohol for a short period of time during their active time of the day (Rhodes et al., 2005). The DID paradigm is a measure of binge-like alcohol drinking and usually C57BL/6J mice are used in this paradigm due to their high alcohol consumption. The method is based on the finding that rodents consume more alcohol during the first couple of hours of their active period of the day (Goldstein and Kakihana, 1977). In line with the two-bottle choice (dependence-like drinking) study we found that the H3R KO mice drank less alcohol than control mice when given a 4-h access to 20% alcohol in the DID model (Nuutinen et al., 2011a; **Table 1**). Interestingly, H3R KO mice also consumed less 10% sucrose than the wild type mice again pointing to the direction that the lower need of calories could be the underlying reason for the lower voluntary alcohol consumption in H3R KO mice. To study this in more detail we used H3R specific ligands in the DID model and found that an H3R antagonist ciproxifan dose-dependently inhibited alcohol drinking and H3R agonist immepip increased alcohol consumption in wild type mice (Nuutinen et al., 2011a). Importantly, the consumption of 10% sucrose, very high in caloric content, was not modified by ciproxifan or immepip pretreatment suggesting that other reasons than a altered need for calories could underlie the change in alcohol consumption following modification of H3Rs. In agreement with our mouse studies, Galici et al. (2011) found that an H3R antagonist JNJ-39220675 (1 and 10 mg/kg) inhibits alcohol drinking to a similar extent as naltrexone (5 mg/kg) in a two-bottle choice test in selectively bred alcohol-preferring (P) rats. Interestingly, water consumption was increased by JNJ-39220675 treatment. JNJ-39220675 also decreased alcohol preference but not alcohol consumption after a 3-day alcohol deprivation period in P rats. These results suggest that the lack or inhibition of H3R leads to lower alcohol consumption both in mice and rats.

Alcohol drinking can be also studied using self-administration paradigms where an operant response, typically a lever press, is required for the access to drink alcohol. Self-administration models are thought to gauge better the motivation to drink alcohol (Green and Grahame, 2008). Operant alcohol self-administration using oral administration is more commonly used in rats than in mice since mice do not drink alcohol as readily as the rats do with the exception of C57BL/6J mouse strain. Already a decade ago we found that H3R antagonists thioperamide and clobenprobit (**Table 2**) dose-dependently (1, 3, 10 mg/kg for both drugs) decrease operant alcohol self-administration in alcohol-preferring AA rats (Lintunen et al., 2001; **Table 1**). Treatment with H3R agonist R-α-methylhistamine or H1R antagonist mepyramine did not affect alcohol self-administration. Recently researchers at Johnson & Johnson tested the H3R antagonist JNJ-39220675 (10 and 30 mg/kg) in an operant model and found that it significantly reduced lever presses for alcohol (Galici et al., 2011). Thus, data

Table 1 | Summary of the alterations in alcohol consumption and reward followed by manipulations of the brain histaminergic system.

Model	Animal line/strain	Drug treatment	Result	Reference
Two-bottle choice	Alcohol-preferring P rats	JNJ-39220675	Decrease in alcohol drinking	Galici et al. (2011)
Drinking in the dark	C57BL/6J	Ciproxifan	Lower alcohol consumption	Nuutinen et al. (2011a)
Drinking in the dark	C57BL/6J	Immepip	Increased alcohol consumption	Nuutinen et al. (2011a)
Two-bottle choice	H3R KO mice	–	Lower alcohol consumption	Nuutinen et al. (2011a)
Drinking in the dark	H3R KO mice	–	Lower alcohol consumption	Nuutinen et al. (2011a)
Two-bottle choice with 3-day alcohol deprivation	Alcohol-preferring P rats	JNJ-39220675	Decrease in alcohol preference	Galici et al. (2011)
Self-administration	Alcohol-preferring AA rats	Thioperamide	Reduced lever presses for alcohol	Lintunen et al. (2001)
Self-administration	Alcohol-preferring AA rats	Clobenprobit	Reduced lever presses for alcohol	Lintunen et al. (2001)
Self-administration	Alcohol-preferring AA rats	R-α-methylhistamine	Increased lever presses for alcohol	Lintunen et al. (2001)
Self-administration	Alcohol-preferring AA rats	Mepyramine	No effect on alcohol lever presses	Lintunen et al. (2001)
Self-administration	Alcohol-preferring P rats	JNJ-39220675	Reduced lever presses for alcohol	Galici et al. (2011)
Conditioned place preference	H3R KO mice	–	Inhibition of alcohol-induced place preference	Nuutinen et al. (2011a)
Conditioned place preference	HDC KO mice	–	Stronger alcohol-induced place preference	Nuutinen et al. (2010)
Conditioned place preference	DBA/2J mice	Ciproxifan	Inhibition of alcohol-induced place preference	Nuutinen et al. (2011b)
Conditioned place preference	129/Sv mice	Ciproxifan	Stronger alcohol-induced place preference	Nuutinen et al. (2010)
Conditioned place preference	DBA/2J mice	JNJ-10181457	Inhibition of alcohol-induced place preference	Nuutinen et al. (2011b)
Conditioned place preference	DBA/2J mice	Immepip	No effect on alcohol-induced place preference	Nuutinen et al. (2011b)

HDC KO, histidine decarboxylase knockout; H3R KO, histamine H3 receptor knockout.

from oral self-administration studies support the role of H3R antagonism in suppressing motivation to drink alcohol.

INHIBITORY ROLE OF HISTAMINE IN ALCOHOL-INDUCED CONDITIONED PLACE PREFERENCE

Alcohol reward and reinforcement can be also studied using Pavlovian conditioning (Tzschentke, 2007). This approach is advisable to use in parallel with the drinking studies in order to exclude intervening variables such as anxiety (Pohorecky, 1991) or novelty seeking (Cloninger et al., 1988) that might result in altered alcohol drinking. In our laboratory, we are routinely using an unbiased alcohol-induced conditioned place preference (CPP) model in mice originally developed and described by Cunningham et al. (2006). In this model mice are given an alcohol injection paired with an environmental cue (cage floor material) for four to eight times over a period of 2–3 weeks. On alternating days with the alcohol injections mice are given vehicle injections paired with another type of floor material. After the conditioning period mice are tested in a preference test where they can choose between the two cage floor materials. Place preference is indexed by a significant difference in time spent on one of the conditioning cue floors between two groups that have had different cues when conditioned to alcohol. In mice lacking the histamine synthesizing enzyme histidine decarboxylase, the alcohol-evoked CPP was found to be stronger suggesting that the lack of histamine increases the reward and reinforcement by alcohol in mice (Nuutinen et al., 2010; **Table 1**). Next we used different H3R ligands and found that antagonists ciproxifan (3 mg/kg) and JNJ-10181457 (5 mg/kg) totally inhibited the CPP by alcohol in wild type DBA/2J mice (Nuutinen et al., 2011b). Importantly, H3R antagonist ciproxifan did not induce place preference or place aversion alone in 129/Sv mice suggesting that H3R antagonism does not produce place aversion or preference *per se* (Nuutinen et al., 2010). However, studies in other mouse strains and rats and using different H3R antagonists would be important to confirm that the H3R antagonists do not have addictive or aversive properties. The H3R agonist immepip (30 mg/kg; **Table 2**) did not modulate alcohol-induced CPP (Nuutinen et al., 2011b). We think that this was probably due to the difficulty to increase an already strong place preference. When CPP model was applied to H3R KO mice (C57BL/6J background), we found a complete lack of alcohol-evoked CPP (Nuutinen et al., 2011a). In agreement with the alcohol drinking studies these data suggest that inhibition or lack of H3R leads to loss of alcohol reward.

MECHANISMS UNDERPINNING THE BEHAVIORAL FINDINGS

Stronger alcohol-induced CPP in HDC KO mice suggests that histamine could be the key neurotransmitter mediating the inhibitory effects since the lack of histamine leads to stronger CPP. The inhibitory effect of H3R antagonists may, however involve other neurotransmitter systems in addition to histamine due to the heteroreceptor function of H3Rs (Schlicker et al., 1994). Interestingly, brain microdialysis studies have shown that H3R antagonists thioperamide and GSK189254 do not affect histamine release in rat

Table 2 | Examples of drugs used in histamine research and in clinical trials.

	Mechanism of action	Specificity	Affinity at human vs. rat H3 receptor	Use in clinical studies and possible indication(s)	Reference
ANTAGONISTS					
Imidazole-based compounds					
Ciproxifan	Inverse agonist	H3R	$pK_i = 7.0$, $pK_i = 8.9–9.2$	No; experimental drug	Ireland-Denny et al. (2001); Schnell et al. (2010)
Clobenpropit	Inverse agonist	H3R/H4R	$pK_i = 9.1–9.3$, $pK_i = 9.1–9.4$	No; experimental drug	Ireland-Denny et al. (2001); Schnell et al. (2010)
Thioperamide	Inverse agonist	H3R/H4R	$pK_i = 7.1–7.3$, $pK_i = 7.9–8.1$	No; experimental drug	Ireland-Denny et al. (2001); Schnell et al. (2010)
Non-imidazole compounds					
ABT-239	Inverse agonist	H3R	$pK_i = 9.4$, $pK_i = 8.9$	No, experimental drug	Esbenshade et al. (2005); Cowart et al. (2004)
Bavisant (JNJ-31001074)	Neutral antagonist?	H3R?	not disclosed	Yes; ADHD, alcoholism	Kuhne et al. (2011)
GSK189254	Inverse agonist	H3R	$pK_i = 9.9$; $pK_i = 9.17$	Yes, narcolepsy, hyperalgesia	Medhurst et al. (2007)
JNJ-39220675	Neutral antagonist?	H3R	$K_i = 1.4$ nM, $K_i = 23$ nM	Yes; allergic rhinitis	Galici et al. (2011)
Pitolisant (BF2.649)	Inverse agonist	H3R	$IC_{50} = 5.3$ nM, $K_i = 17$ nM	Yes; excessive daytime sleepiness, schizophrenia, epilepsy	Ligneau et al. (2007)
AGONISTS					
Immepip	partial agonist	H3R	$pK_i = 9.6$, $pK_i = 9.0$	No; experimental drug	Ireland-Denny et al. (2001)
R-α-methylhistamine	agonist	H3R	$pK_i = 8.9–9.2$, $pK_i = 8.6–8.7$	No; experimental drug	Ireland-Denny et al. (2001); Schnell et al. (2010)
MODULATORS OF HISTAMINE SYNTHESIS					
l-histidine	precursor of histamine	–	–	No, experimental drug	Verdiere et al. (1975)
α-fluoromethylhistidine	inhibitor of histidine decarboxylase	–	–	No, experimental drug	Kollonitsch et al. (1978); Garbarg et al. (1980)

nucleus accumbens or striatum, the central areas in the regulation of reward and reinforcement (Giannoni et al., 2009, 2010). Further, dopamine release seems not to be affected either in these areas. H3R antagonist ABT-239 did not modify dopamine release in striatum (Fox et al., 2005) and GSK189254 had no effect on dopamine release in nucleus accumbens (Giannoni et al., 2010; **Table 2**). JNJ-39220675 treatment, which was found to inhibit alcohol drinking and self-administration in rats did not change dopamine release *per se* or affect alcohol-induced release of dopamine (Galici et al., 2011; **Tables 1** and **2**). However, many of the above-mentioned drugs as well as many other H3R antagonists do increase histamine and dopamine release e.g., in prefrontal cortex (Brioni et al., 2011), another important area of the meso-cortico-limbic reward circuit which receives dopaminergic input from the ventral tegmental area and sends glutamatergic projections to striatum and nucleus accumbens (Lasseter et al., 2010). In summary, these results demonstrate that H3R antagonists affect neurotransmitter release in a region-specific manner and it seems that the inhibitory effect of H3R antagonism on alcohol consumption and CPP is likely to result from the effects of these drugs on neurotransmitter release on brain areas other than striatum and nucleus accumbens.

Another intriguing mechanism for H3R antagonist-induced inhibition of alcohol reward is the possible modulation of postsynaptic dopamine receptor signaling in striatum and nucleus accumbens. Accumulating evidence suggests that H3Rs form functional receptor heteromers with dopamine D1 and D2 receptors (Ferrada et al., 2008, 2009; Moreno et al., 2011) and that specific ligands that bind to one of the receptor of the heteromer, will affect the affinity and signaling of the other. Thus, it is possible that by binding to postsynaptic H3Rs in striatal areas, the H3R antagonists might interfere with the enhanced dopaminergic signaling induced by alcohol. We have measured the plasma alcohol concentrations after H3R ligand administration and found no difference compared to the corresponding controls thus ruling out the possibility that H3R ligands would alter alcohol metabolism (Nuutinen et al., 2011a).

SIMILARITIES AND CONTRASTING FINDINGS WITH OTHER DRUGS OF ABUSE

Mechanisms leading to addictive behaviors are thought to be common for all drugs of abuse. In line with the inhibitory role of histamine in alcohol-induce place preference, a study using H1 receptor knockout mice found a stronger methamphetamine-induced CPP than in wild type controls (Takino et al., 2009) and H3R antagonists thioperamide and clobenprobit reduced amphetamine self-administration in rats (Munzar et al., 2004). In contrast to our alcohol study the CPP for methamphetamine was not

different in histamine H3R KO mice compared to the control animals (Okuda et al., 2009). However, the authors used a biased CPP design (by using one conditioning cue) and place preference was scored by comparing pre-conditioning and post-conditioning times. In our laboratory we use two distinct conditioning cues that are neutral to the mice meaning that they do not prefer either one of them initially (unbiased design). This is to prevent e.g., the anxiolytic effect of the drug used that could lead to false-positive result (Cunningham et al., 2003). Further, studies have shown that mice change their preference during the conditioning period without any reinforcing drugs, so in our paradigm place preference is scored as time spent in one of the sides of the chamber during the preference test following the conditioning days (Tzschentke, 2007). We compare this time between two groups that received alcohol paired with separate cues. Significant difference between the subgroups indicates place preference.

Studies on histamine's role in morphine dependence are few but support the inhibitory role of histamine in reward and reinforcement. Morphine-induced CPP was attenuated by increasing histamine levels with L-histidine and potentiated by inhibiting histamine synthesis with α-fluoromethyl histidine in mice (Suzuki et al., 1995; **Table 2**). H2 receptor antagonist zolantadine potentiated morphine-induced CPP but also induced place preference alone accompanied by an increase in dopamine turnover suggesting that it has other targets, possibly dopamine transporter, in addition to H2Rs. In accordance with our data with alcohol, the morphine-induced place preference was stronger in HDC KO mice (Gong et al., 2010).

Studies on psychostimulants (cocaine, methamphetamine) are not in line with the suggested inhibitory role of histamine in addictive behaviors. In contrast to other findings in HDC KO mice (Gong et al., 2010; Nuutinen et al., 2010), no difference in cocaine-induced CPP was found between the HDC KO and wild type mice (Brabant et al., 2007). Further, a pretreatment with an H3R antagonist thioperamide enhanced cocaine's effects by inducing place preference in mice with a cocaine dose that did not induce CPP alone (Brabant et al., 2005). However, thioperamide was later found to inhibit cocaine metabolism via inhibition of liver CYP-450 enzymes (Brabant et al., 2009). Amphetamine is also metabolized via liver CYP-450 enzymes. Thus, the observations that H3R antagonists thioperamide and clobenpropit potentiate self-administration and discrimination of methamphetamine might be due to inhibition of methamphetamine metabolism by these imidazole-based H3R antagonists (Munzar et al., 1998, 2004). Further studies with non-imidazole-based H3R compounds and with knockout/transgenic animal models are needed to reveal the role of H3R in psychostimulant-induced reward and reinforcement.

Numerous studies on first generation H1R antagonists support the inhibitory role of histamine in reward (for a review see Brabant et al., 2010). However, these drugs have many targets in addition to H1Rs such as muscarinic receptors and more importantly, the dopamine transporter (Shishido et al., 1991; Oishi et al., 1994). Thus, it is most probably the inhibition of dopamine uptake rather than H1R blockade that explains why H1R blockers can cause *per se* or increase behavioral effects typically induced by addictive substances.

CONCLUSIONS

There is accumulating evidence from alcohol drinking and place preference studies suggesting that alcohol reward could be decreased by treatment with H3R antagonists. More studies are still needed to test the potential of H3R antagonists to prevent alcohol withdrawal symptoms or relapse. Cellular and molecular mechanistic studies are also of great importance in order to more widely increase our understanding of the complex role of H3R in addictive behaviors. Studies on alcohol and morphine suggest that targeting brain H3Rs could be a potential means to treat drug addiction. However, due to conflicting findings with H3R antagonists in psychostimulant addiction-related behaviors, more preclinical studies are needed to really show whether H3R antagonist would be beneficial in other types of drug addictions. One of the key questions to be solved is related to the possible differential expression of H3R in different dopaminergic neurons, and analysis of direct vs. indirect effects of H3R on dopamine release. Whereas it seems that histamine H3 receptor is a key element in alcohol drinking and place preference, the role of histamine in these behaviors is still unclear. The relative importance of histamine and other transmitters regulated by H3 receptor antagonism in several alcohol-related behaviors remains to be studied using specific tools.

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Effects of methimepip and JNJ-5207852 in Wistar rats exposed to an open-field with and without object and in Balb/c mice exposed to a radial-arm maze

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The role of the histamine H_3 receptor (H_3R) in anxiety is controversial, due to limitations in drug selectivity and limited validity of behavioral tests used in previous studies. In the present report, we describe two experiments. In the first one, Wistar rats were treated with an H_3R agonist (methimepip), and exposed to an open-field. In the second one, Balb/c mice were treated with H_3R agonist (methimepip) or antagonist (JNJ-5207852), and exposed to an open space 3D maze which is a modified version of the radial-arm maze. C57BL/6J saline treated mice were included for comparisons. When exposed to an empty open field, Wistar rats spent more time in the outer area and made very low number of brief crossings in the central area. However, when an object occupied the central area, rats crossed frequently into and spent a long time in the central area. Administration of a range of different doses of methimepip (selective H_3R agonist) reduced the entries into the central area with a novel object, indicating enhanced avoidance response. In the 3D maze, both Balb/c and C57BL/6J saline-treated mice crossed frequently onto the bridges that radiate from the central platform but only C57BL/6J mice crossed onto the arms which extend the bridges. This suggests that Balb/c mice are more anxious than C57BL/6J mice. Neither methimepip nor JNJ-5207852 (selective H_3R antagonist/inverse agonist) induced entry into the arms of the maze, indicative of lack of anxiolytic effects.

Keywords: fear, avoidance, anxiety, 3D maze, object novelty, histamine

INTRODUCTION

The biogenic amine histamine is an important neurotransmitter and neuromodulator in the CNS that has been implicated in a variety of biological functions including thermo- and immunoregulation, food intake, hyperexcitability, pain transmission, arousal, reward, memory and emotional responses. The histamine H_3 receptor (H_3R) has been characterized as a presynaptic auto- and hetero -receptor on histaminergic and non-histaminergic neurons, respectively. It modulates histamine synthesis and release through negative feedback (Arrang et al., 1987; Giannoni et al., 2009). The basic organization and functional disposition of the histaminergic system is highly conserved in the vertebrate brain. In the mammalian brain histamine is synthesized and stored in the cell somata and axon varicosities in restricted populations of neurons that originate from the tuberomammillary nucleus (TMN) located in the posterior hypothalamus. Histaminergic neurons projecting from the ventral ascending pathway have strong innervation at the hypothalamus, diagonal band, septum, and olfactory bulb whilst the dorsal pathway has lower density fibers which innervate the thalamus, hippocampus, amygdala, and rostral forebrain structures, many of which play a role in cognitive and emotional behaviors (Haas et al., 2008). They also modulate the release of other neurotransmitters, including acetylcholine, norepinephrine, serotonin, and dopamine, implicated further in emotion (Arrang et al., 1995; Blandina et al., 1996; Threlfell et al., 2004; Haas et al., 2008; Giannoni et al., 2009). Preclinical studies suggest a role of H_3Rs in a variety of cognitive disorders including attention deficit hyperactivity disorder, Alzheimer's disease and schizophrenia (see Esbenshade et al., 2008; Chazot, 2010; Leurs et al., 2011). There is growing evidence for a role of H_3Rs in fear-avoidance (e.g., Baldi et al., 2005). However, a limited number of studies have investigated the role of H_3Rs in anxiety, and these have proved inconsistent and even contradictory (Imaizumi and Onodera, 1993; Frisch et al., 1998; Pérez-García et al., 1999; Bongers et al., 2004; Dere et al., 2004; Rizk et al., 2004; Acevedo et al., 2006; Yokoyama et al., 2009). Both H_3R agonists and antagonists were reported to produce anxiolysis, anxiogenesis or no effects in the current unconditioned tests of anxiety (Imaizumi and Onodera, 1993; Pérez-García et al., 1999; Rizk et al., 2004; Yokoyama et al., 2009). This is likely due to the selectivity of the compounds used in the older studies and/or limitations of the behavioral tests used for anxiety which all provides an option for escape/avoidance and, therefore, cannot distinguish between

fear-induced avoidance and fear-induced anxiety (discussed in detail in Ennaceur, 2012).

It has been reported that rats and mice exposed to an open-field avoid the central area and avoid the presence of an object in this area (Hughes, 2007). It has been also reported that these animals avoid the open arms of the plus-maze (Handley and Mithani, 1984; Pellow et al., 1985) and the lit chamber of the light-dark box (Malin, 1974; Morgan and Kamp, 1980; Crawley, 1981). This avoidance behavior has been interpreted as an indicator of anxiety (discussed in Ennaceur et al., 2009b) though one cannot exclude the possibility that animals express a natural preference for protected and/or unlit spaces (see Malin, 1974; Morgan and Kamp, 1980; Buhot, 1989) and they may have no interest or motivation to venture into unprotected and/or lit spaces. Animals may also express fear from novelty and escape to or avoid from the protected and/or unlit space. In this case too, there is no objective evidence that demonstrate the interest or motivation of animals to approach the source of potential threat.

In the present report, we examined the behavior of Wistar male rats in the presence or absence of an object in the open-field, and we assessed whether this would be affected by methimepip, a selective histamine H_3R agonist (Kitbunnadaj et al., 2005). As stated above, we believe that exposure for the first time to the open-field provides measures of fear-induced avoidance and we expect that methimepip would affect these measures. We also examined the behavior of Balb/c mice which were exposed to a 3D-maze and treated with or methimepip or JNJ-5207852, the latter, a selective histamine H_3R antagonist (Barbier et al., 2004). In this second experiment, we included a group of C57BL/6J mice that were treated with saline for comparisons. The 3D maze is a modified version of the radial-arm maze. It consists of 8 arms attached to bridges that radiate from a central platform. Animals need to cross a bridge to access an arm of the maze. In this test apparatus C57BL/6J mice cross onto arms of the maze on first exposure (low anxiety strain), while Balb/c mice cross only onto the bridges (high anxiety strain), indicative of differential anxiety responses (Ennaceur et al., 2006, 2008; Ennaceur, 2011). If treatments in Balb/c mice induced crossing onto arms of the maze as seen with the control C57BL/6J mice, this would indicate an anxiolytic effect. This test has been validated previously with an anxiolytic agent, diazepam (Ennaceur et al., 2008).

The first experiment used rats because previous experiments in the open-field with and without object were conducted with these animals and demonstrated that the tests provide measures of fear-induced avoidance rather than fear-induced anxiety (Ennaceur et al., 2009a,b). When exposed for the first time to an open-field, rats show natural preference for the walls and corners and avoid the center of the field because there is nothing there to explore. When an object is present in the center they do approach and explore the object.

The second experiment used mice because these were previously assessed for anxiety in the 3D maze and differences in anxiety between strains of mice in this test is well established (Ennaceur et al., 2006, 2008). We have only experience with Wistar rats in this test but not with other strains of rats. It was not worthwhile testing mice in the open-field as in our view it does not provide measures of anxiety.

EXPERIMENT 1—OPEN-FIELD WITH AND WITHOUT OBJECT

MATERIALS AND METHODS

Animals

Forty nine male Wistar rats (six groups) supplied by Charles River Laboratories (Kent, UK) were used in the present study. The animal weight was 190–210 g at the time of the. The colony room was held under a 12 h light/12 h dark cycle (light 0700–1900 h at 180 Lux) and at 23°C ± 1°C. In order to avoid unequal light exposure, the upper shelf was occupied with plastic cages filled with sawdust. Rats were housed four per cage. Individual rats could be identified by their cage number and their color code created with indelible pen marker on their tail. Rats were left to acclimatize for 2 weeks before the start of the experiment. All rats had *ad libitum* access to food and water. During their stay in respective housing conditions, they were removed three times a week from their cages for cleaning the cages and renewing their food and water supply. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK.

Drug treatments

Two groups of male Wistar rats received physiological saline, the other groups received a single injection of one dose of methimepip (1 and 2.5 mg/kg i.p.) 30 min before exposure to the open-field with and without an object (**Table 1**). Methimepip was a kind gift obtained from Professor Rob Leurs (VU, Amsterdam, The Netherlands). Selection of dose concentrations was based on previous *in vivo* studies (e.g., Kitbunnadaj et al., 2005).

Apparatus and testing procedure

The apparatus consists of an open box (width 80 × length 80 × height 50 cm) made of gray PVC. The surface of the open-field was divided into outer, inner, and central areas. Each area was 16 cm wide. The illumination on the floor of the box apparatus was 186Lux. The objects (width 8 × length 8 × height 13 cm) to be explored were identical triplicate and were alternated between animals. They were made of white ceramic. Rats were released from the outer area of the open-field with the head oriented toward a wall. They were left to explore for 10 min.

Tools and recording measures

All sessions were video recorded and the behavior of rats was analyzed with an in-house computer program, EventLog. The recording of the behavior of rats was based on entries into defined areas of the apparatus. An entry was recorded whenever a rat crosses with all four paws into an area. EventLog records in

Table 1 | Groups and number of animals per groups in each test condition.

	Saline	Methimepip 1 mg/kg i.p.	Methimepip 2.5 mg/kg i.p.
Open-field without object	$n = 10$	$n = 7$	$n = 7$
Open-field with object	$n = 9$	$n = 8$	$n = 8$

The drugs were administered i.p. 30 min before the test.

sequential order the start and end of each crossing into an area of the open-field. It provides measures of latency, frequency, and duration of entries.

Measurement and statistical analysis

All data are expressed as mean ± s.e.m. Differences among group means values for each measurement were tested for significance with Two-Way ANOVA followed up with Newman–Keuls *post-hoc* comparisons (Statistica for Windows, version 5.5). Results are considered significant when $p \leq 0.05$.

RESULTS

There were significant differences between groups [$F_{(2, 43)} = 3.30$, $p < 0.05$] and between test conditions [$F_{(1, 43)} = 5.49$, $p < 0.02$] in all test parameters except for latencies of entries into the inner and central areas ($p > 0.10$), and for the number of entries into the outer area ($p > 0.10$). There were, however, significant interactions between groups and test conditions only for the number of entries into the central area [$F_{(2, 43)} = 9.37$, $p < 0.0004$].

The number of entries into the inner and central areas was significantly high in presence of an object than in the absence of an object in all groups ($p < 0.02$; **Figure 1A**). However, the duration of entries into the central area was significantly higher and the duration of entries into the outer area was significantly lower in the presence rather than in the absence of an object in all groups ($p < 0.05$) (**Figure 1B**).

In the absence of an object, there were no significant differences between groups ($p > 0.10$; **Figure 1**). However, in the presence of an object, mice treated with methimepip crossed significantly less into the inner ($p < 0.05$) and central area ($p < 0.0002$) and spent less time in the inner area ($p < 0.05$) compared to control (**Figures 1A,B**). There were no significant differences between the two doses of methimepip ($p > 0.10$; **Figures 1A,B**).

EXPERIMENTS 2 AND 3—3D RADIAL MAZE

MATERIALS AND METHODS

Animals

Sixty four male Balb/c and 16 male C57BL/6J mice were purchased from Charles River Laboratories (Kent, UK). The animal weight was 25–28 g at the time of their arrival. They were housed four per cage. Individual mice could be identified by their cage number and their ear tags. They were left to acclimatize for 1 week before the start of the experiment. The colony room was held and animals were maintained as described in experiment 1.

Drug treatments

In experiment 2, there were two control groups (C57BL/6J, $n = 8$ and Balb/c, $n = 8$) which received physiological saline, and the other groups ($n = 8$ each) received a single injection of one dose each of methimepip (1, 2.5, and 5 mg/kg i.p.). In experiment 3, there were also two control groups (C57BL/6J, $n = 8$ and Balb/c, $n = 8$) which received physiological saline, and three Balb/c groups ($n = 8$ each) which received a single injection of one dose each of JNJ-5207852 (0.5, 1, and 5 mg/kg i.p) 30 min before introduction to the 3D maze. Methimepip and JNJ-5207852 were kind gifts from Professor Rob Leurs and Dr. Nicholas Curruthers (JNJ, USA), respectively. Selection of drug doses was based on previous *in vivo* studies (e.g., Kitbunnadaj et al., 2005; Jia et al., 2006).

Apparatus and testing procedure

The 3D maze is a modification of the classic radial-maze (Ennaceur et al., 2006). It is made from gray PVC (5 mm thick) and consists of eight arms radiating from a central platform. Each arm (51 × 11.2 cm) is made from two segments, extended from an octagonal shaped central hub (30 cm in diameter). The first segment of an arm (15.2 × 11.2 cm) is directly attached to the central platform and constitutes a bridge that allows access to the second segment (35 × 11.2 cm). Each bridge can be independently tilted upward or downward providing three maze configurations. In the present study, all bridges were tilted by 40° providing a configuration in which the arms are raised horizontal above the level of the central platform. Mice need to climb onto the bridges and then cross onto the arms. The floor of the bridges is covered with wire mesh. Sidewalls, about 1 cm high, extended the length of each bridge and arm. The end of each arm is extended with panels of identical size (20.2 × 11.2 cm) which are used to holding cues made of distinctive pattern drawings designed on plastic adhesive material and attached to a PVC board (18 × 11.2 cm). The maze is totally surrounded with a

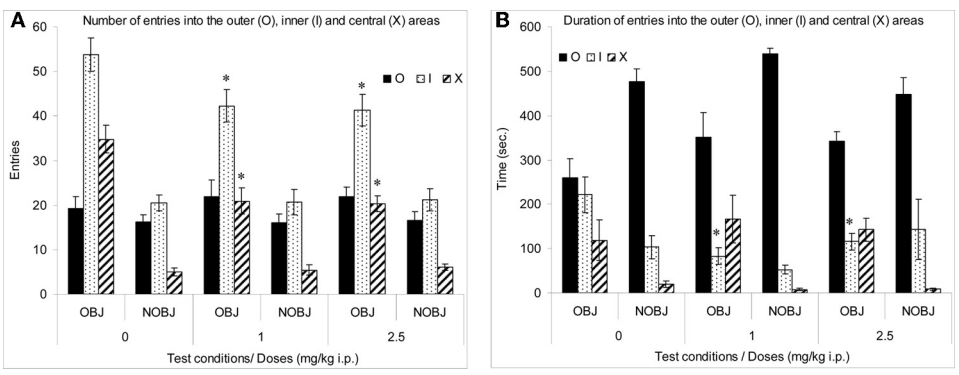

FIGURE 1 | Methimepip experiment with rats. Mean (± s.e.m.) in the open-field with (OBJ) and without object (NOBJ). **(A)** Number of entries: *compared to control in inner area (I), $p < 0.05$ and *compared to control in central area for both doses of methimepip, $p < 0.0002$; **(B)** Duration of entries: *compared to control in the inner area (O) for both doses of methimepip, $p < 0.05$.

heavy beige-light colored curtain. The ambient light at the surface of the central platform is 180 Lux.

A mouse was removed from its cage, put in a small bucket in which it was weighted, and then tilted gently on the center platform of the maze. It was left to explore the test apparatus for 12 min. The surface of the maze was cleaned to minimize the effects of lingering olfactory cues. Any feces and urine were removed with paper towels, then cleaned with antibacterial solution followed by 90% ethanol and left to dry before the introduction of the next mouse.

Tools and recording measures

See similar section in experiment 1.

Measurement and statistical analysis

Differences among group means values for each measurement were tested for significance with One-Way ANOVA. Anything else as described in experiment 1.

RESULTS

In both experiments, only C57BL/6J mice (comparator low anxiety strain) crossed onto the arms of the maze (not shown). Their number of crossings onto the bridges (**Figures 2B,E**) and duration of entries onto the bridges (**Figures 2C,F**) was significantly higher than in the other groups. Balb/c mice treated with methimepip (2.5 mg/kg i.p.) took significantly longer time to cross onto the bridges compared to Balb/c mice treated with saline ($p < 0.02$) and to Balb/c mice treated with methimepip at 1 and 5 mg/kg i.p. ($p < 0.02$; **Figure 2A**). There were no significant differences between Balb/c saline treated mice and either methimepip- or JNJ-5207852-treated mice in any other measures (**Figures 2C–D** and **F**).

DISCUSSION

We demonstrated in previous studies that C57BL/6J mice cross onto the distal arms of the 3D maze (Ennaceur et al., 2006; Ennaceur, 2011) and onto the steep hanging slopes of a novel elevated platform (e.g., Ennaceur et al., 2010; Michalikova et al., 2010). This demonstrates that these mice are able to take risks when exposed to unfamiliar open spaces (low anxiety strain). However, when a shelter is provided in the central platform of the maze or in the central area of the elevated platform, they stop crossing onto the arms and slopes, and spend most of the time inside the shelter. We have also confirmed this with rats in an enclosed and open space test with and without an object (Ennaceur et al., 2009a,b). The avoidance or preference responses observed in the presence of a shelter compare to those observed in the open-field, the plus-maze and the light/dark box. They do not provide unequivocal measures of fear-induced anxiety responses.

In the first experiment, using rats, the number of crossings into the central area of the open-field was significantly higher in the presence of an object than in the absence of an object

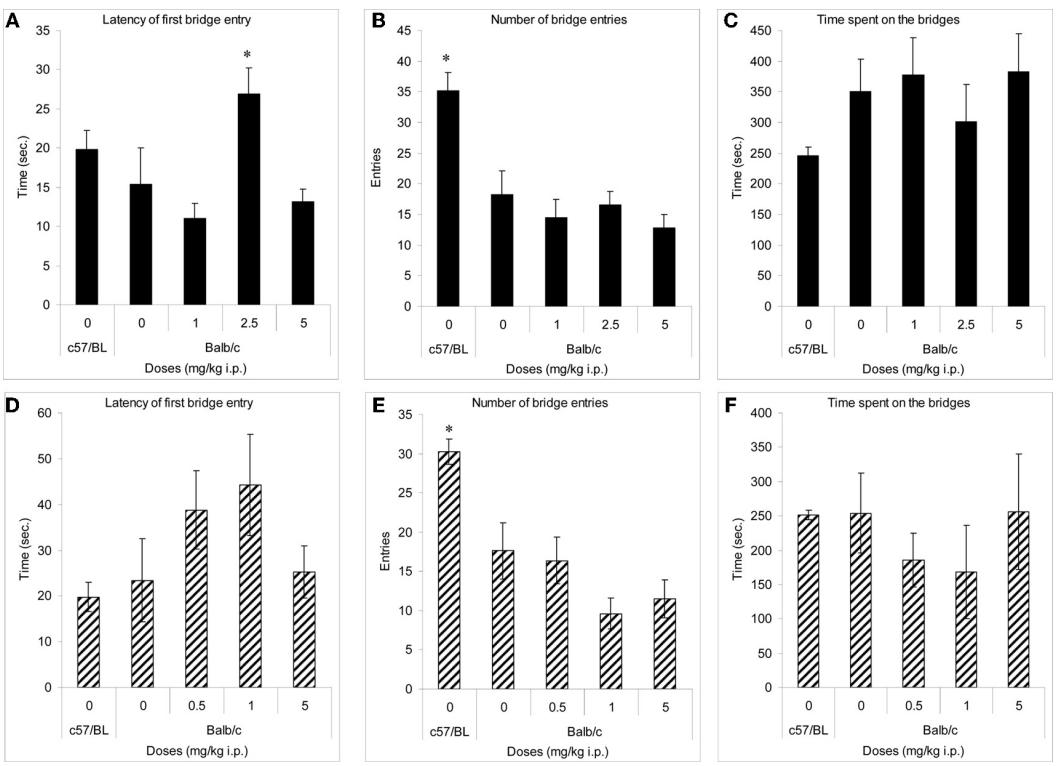

FIGURE 2 | Methimepip experiment with mice (A–C), JNJ-5207852 experiment with mice (D–F). Mean (± s.e.m.) in the 3D-maze with mice. **(A)** Latency: *compared to Balb/c saline and methimepip treated mice, $p < 0.05$; **(B)** number of entries, and **(E)** duration of entries: *compared to Balb/c saline and all methimepip treated mice, $p < 0.0002$; (**C**, **D,** and **F**) no significant differences between groups, $p > 0.10$.

in agreement with our earlier report (Ennaceur et al., 2009a,b). This avoidance of the empty central area cannot be attributed to a state of anxiety in animals. It is accounted for by animals' preference for walls and corners that form the open-field and also by the fact that there is nothing to encourage animals to stop and explore the central area (see Ennaceur et al., 2009a,b). In the open-field with an object, methimepip reduced the number of entries into and time spent in the inner and central areas. This not due to the effect of the drug on motor or exploratory activity as this is still higher than in saline and drug treated rats exposed to the open-field without an object. The presence of an object seems to further increase this avoidance response. This could be due to methimepip facilitating or exacerbating fear response when exposed to novelty. Indeed, it has been suggested that histaminergic neurotransmission in the brain is increased in stressful situations (Dere et al., 2010). In agreement, administration of histamine H_3R agonists has been shown to increase the level of fear avoidance responses in fear conditioning paradigms (e.g., Baldi et al., 2005), in the plus-maze (Pérez-García et al., 1999). However, in a number of studies histamine H_3R antagonists were also shown to increase the level of fear-induced avoidance responses in the plus-maze (Pérez-García et al., 1999; Bongers et al., 2004) and the light/dark box (Imaizumi and Onodera, 1993).

In order to evaluate the role of the H_3R in anxiety, we adopted our recently developed and intensively characterized open space 3D maze test (Ennaceur et al., 2006, 2008; Ennaceur, 2011) with mice. In this test, animals are exposed to an open-space environment where the option to escape/avoid and explore are of equal valence. We reported that strains of mice display different levels of anxiety in this test, with Balb/c displaying consistently higher anxiety than C57BL/6J strains (Ennaceur et al., 2006, 2008; Ennaceur, 2011). Indeed, Balb/c mice alternate between the central platform and the proximal part of the arms (the bridges) and only C57BL/6J mice cross onto the distal part of the arms. In the present study, C57 mice crossed onto the arms of the maze which is in agreement with our previous findings (Ennaceur et al., 2006, 2008; Ennaceur, 2011) while all Balb/c treated with saline, methimepip or JNJ-5207852 did not cross onto the arms. These results do not suggest any effects of a selective H_3 agonist and antagonist on measures of anxiety. This was confirmed (unpublished) using another selective H_3 antagonist/inverse agonist, GSK334429B in a novel elevated platform open space test (Ennaceur, 2012). The differential effects of H_3R modulation on avoidance and anxiety behaviors may be explained by the growing anatomical and functional evidence for H_3 auto- and heteroreceptor heterogeneity (Giannoni et al., 2009, 2010; Passani and Blandina, 2011).

In most studies, the effects of different doses of a drug treatment are compared to saline as control. In experiments 2 and 3, we included C57BL/6J as a second control group. This is simply because in order to assess the anxiolytic effect of a drug one would have to choose the strain of animals with high anxiety and if the drug produces a reduction in anxiety one would need to demonstrate that this reduces anxiety below or at least to the level of low-anxiety strains of animals.

As stated in our introduction, avoidance of the central area or an object in the central area may be indicative of fear response but also a preference response of walls and corners. If the definition of anxiety is based on the conflict between the drive to explore and the drive to avoid, one must demonstrate evidence in animals of the drive to explore in this test. In the 3D maze, all parts of the maze are open and unprotected. When released on the central platform, animals explore the bridges but do not venture further onto the arms; they alternate between the bridges and the central platform. The drive to explore is clearly evident by the crossings onto the bridges.

In previous studies, we demonstrated the difference between test conditions that promotes fear-induced escape/avoidance and fear-induced anxiety (Ennaceur et al., 2006; Michalikova et al., 2010; Ennaceur, 2012). When exposed for the first time to a 3D-maze, C57BL/6J mice venture onto the arms of the maze while Balb/c mice explore only the central platform and the bridges. However, if the central platform is enclosed they behave like Balb/c mice; they do not cross onto the arms of the maze. Comparable behavior was observed in the elevated platform with slopes. C57BL/6J mice stop crossing onto the slopes when a hiding place is provided in the middle of the platform (Michalikova et al., 2010; Ennaceur, 2012). In the presence of a protected space, animals may not feel the need to take risks away from the protected space; this is the case in the current unconditioned tests of anxiety for rats and mice.

In summary, the results of our present experiments with selective agonist and antagonist drugs provide new evidence that the H_3R may have a role in fear-induced avoidance responses, but not in anxiety.

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Histamine H3 receptor antagonists/inverse agonists on cognitive and motor processes: relevance to Alzheimer's disease, ADHD, schizophrenia, and drug abuse

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Histamine H3 receptor (H3R) antagonists/inverse agonists possess potential to treat diverse disease states of the central nervous system (CNS). Cognitive dysfunction and motor impairments are the hallmark of multifarious neurodegenerative and/or psychiatric disorders. This review presents the various neurobiological/neurochemical evidences available so far following H3R antagonists in the pathophysiology of Alzheimer's disease (AD), attention-deficit hyperactivity disorder (ADHD), schizophrenia, and drug abuse each of which is accompanied by deficits of some aspects of cognitive and/or motor functions. Whether the H3R inverse agonism modulates the neurochemical basis underlying the disease condition or affects only the cognitive/motor component of the disease process is discussed with the aim to provide a rationale for their use in diverse disease states that are interlinked and are accompanied by some common motor, cognitive and attentional deficits.

Keywords: histamine H3 receptor, Alzheimer's disease, ADHD, schizophrenia, drug abuse

Histamine H3 receptor (H3R) antagonists/inverse agonists have revealed potential to treat diverse disease states of the central nervous system (CNS) including Alzheimer's disease (AD), attention-deficit hyperactivity syndrome (ADHD), schizophrenia, obesity, pain, epilepsy, narcolepsy, substance abuse, etc. (Leurs et al., 2011; Passani and Blandina, 2011). The histamine H3R was first identified in the rat brain (Arrang et al., 1983), its presence in the human brain was confirmed a few years later (Arrang et al., 1988); and it was successfully cloned and functionally expressed by Lovenberg et al. (1999) following which there was spurt of activities by several academic groups and pharmaceutical companies to develop a flurry of compounds that could therapeutically modulate the functions of this receptor. Though there is no direct pathophysiological mechanism linking any disease condition of the CNS with histamine, the distinct localization of H3Rs in the CNS coupled with the fact that it modulates the release of other neurotransmitters in the brain via its action on heteroreceptors on non-histaminergic neurons led to evaluation of its ligands in various brain diseases (Nuutinen and Panula, 2010).

H3R, a G-protein coupled receptor (GPCR, coupled to Gi/o proteins), is able to signal on its own i.e., without activation by an agonist, and thus displays constitutive activity. H3R activation inhibits synthesis of histamine through adenylate cyclase/protein kinase A and calcium/calmodulin-dependent protein kinase type II (CaMKII) pathways. In addition, it can activate phospholipase A2 (PLA2) mediated release of arachidonic acid (AA) and phosphoinositol-3-kinase activity resulting in activation of the Akt/glycogen synthase kinase (GSK)-3β axis (Leurs et al., 2005). Evidence linking how these signaling pathways relate to the efficacy of H3R antagonists in various disease states is scarce. Though the role of histamine H3Rs as therapeutic target in brain diseases have been the subject of many recent reviews, here we have tried to bring together various neurobiological/neurochemical evidences available so far following H3R ligands in the pathophysiology of AD, ADHD, schizophrenia, and drug abuse each of which is accompanied by deficits of some aspects of cognitive and/or motor functions. Whether the H3R inverse agonism modulates the neurochemical basis underlying the disease condition or affects only the cognitive/motor component of the disease process is discussed.

ALZHEIMER'S DISEASE

AD is a chronic and progressive neurodegenerative brain disease and one of the most prevalent forms of dementia affecting nearly 20 million people worldwide (Chen et al., 2012). Role of histamine in AD is well documented. But, owing to variable findings concerning the histamine levels in brain compartments of AD patients, a direct correlation between histaminergic neurotransmission and AD pathology cannot be made and hence the rationale of using H3R antagonists in the treatment of AD is rather complex (Fernández-Novoa and Cacabelos, 2001; Brioni et al., 2011). Few studies by a group of Spanish researchers claim that histamine levels in AD patients is increased in areas such as temporal and frontal cortex, basal ganglia, and hippocampus (Cacabelos et al., 1989) also, together with its metabolites, in the cerebrospinal fluid (CSF) and serum of AD patients (Cacabelos et al., 1992; Fernandez-Novoa et al., 1997), although this rise in histamine level has been vaguely attributed to its non-neuronal pool i.e., mast cells in the CNS [reviewed by Fernández-Novoa and Cacabelos (2001)].

On the contrary to the above findings, several independent studies show decline in histamine levels in AD brains. The tuberomamillary nucleus (TMN) seems to be affected in AD as occurrence of high density of neurofibrillary tangles (NFT) surrounding tuberomammillary histaminergic neurons was observed in AD patients (Saper and German, 1987), while, the distribution and number of histaminergic cell bodies remained similar to that of normal brains (Airaksinen et al., 1991). Braak et al. (1993) suggested that during the course of AD, diminished histamine production is paralleled by build up of NFT in the TMN in the early stages. In agreement with this, a significant loss of large-sized histamine containing neurons in the rostral TMN was observed where numerous NFT were found, indicative of a central histaminergic dysfunction (Nakamura et al., 1993). Similarly, high performance liquid chromatography results in some studies have shown reduction in histamine content in the hypothalamus, hippocampus, and temporal cortex of AD brains (Mazurkiewicz-Kwilecki and Nsonwah, 1989; Panula et al., 1998). But clearly, the number studies that evidenced a decline in histamine level is more than the studies that observed an excess of brain histamine in AD. These discrepancies may have arisen from the putative confounding factors like post-mortem delays and differences in subjects assessed e.g., control subjects from a different population than AD patients (Panula et al., 1998).

Central histaminergic fibres originating from the TMN in the posterior hypothalamus (Brown et al., 2001) widely projects into different brain areas including the cerebral cortex, thalamus, basal ganglia, amygdala, and hippocampus, where histamine is crucially associated with a large number of basic physiological functions including sensory and motor functions, cognition, attention, learning, and memory (Haas et al., 2008). Blockade of human H3 autoreceptor by thioperamide evokes the increase of the neuronal histamine release (Flik et al., 2011), and the neurotransmitter modulates cognition processes via both human H1 and H2 receptors or via cholinergic and GABAergic interneurons either directly via excitation of neocortical pyramidal neurons and thalamic relay neurons or indirectly via excitation of ascending cholinergic neurons (Bacciottini et al., 2001; Haas and Panula, 2003; Haas et al., 2008). The H3R heterogeneity was revealed in a microdialysis study, where local TMN administration of H3R antagonist yielded variable histamine release in different brain areas: whereas the concentration increased in TMN, nucleus basalis magnocellularis (NBM), and prefrontal cortex (PFC), it remained unchanged in striatum and nucleus accumbens (NAc). There was differential regulation of neurotransmitter release in a region-specific manner in the brain (Giannoni et al., 2010). The degeneration of histamine neurons in AD does not parallel with the extent of H3R expression. In fact, only a very minor portion of brain H3Rs are located in histaminergic neurons and are largely expressed in deep cortical and thalamocortical projection neurons and in striatal neurons among many other neuron populations. Thus, despite a strong AD related neurodegeneration of the TMN in its severe late stages, H3R densities are either preserved in the brains of amyloid overexpressing TASTPM transgenic mice as well as of AD patients as revealed by receptor binding data (Medhurst et al., 2007, 2009) or increased expression of H3R mRNA (in brains of female AD patients) is evidenced by PCR studies (Shan et al., 2012), signifying that they constitute an adequate target to improve the cognitive disorders encountered in AD.

Recently H3R ligands are being extensively investigated for their potential as a therapeutic agent for cognitive deficits (Sander et al., 2008; Tiligada et al., 2009; Brioni et al., 2011; Leurs et al., 2011). In preclinical studies, H3R antagonists enhance histamine neuron activity and a simultaneous improvement in cognition and learning was observed (Passani et al., 2004). Clearly, the promising preclinical findings have given strong impetus for numerous pharmaceutical companies to embark on proof-of-concept clinical studies with a number of H3 antagonists for diverse neurological conditions including AD (Drahl, 2009; Abbott-press release, 2011; GSK-pipeline, 2011). Recently phase II trials of H3R antagonists ABT-288 (NCT-ID NCT01018875), GSK239512 (NCT-ID NCT01009255), and MK0249 (NCT-ID NCT00420420) for mild to moderate AD have been completed. The outcomes of these trials have not yet been publicly divulged. Another compound PF-03654746 has completed phase I clinical trial (NCT-ID NCT01028911) to evaluate its safety, tolerability and blood levels in mild to moderate AD patients and is subjected to phase II trial (http://www.pfizer.com/files/research/pipeline/2010_0927/pipeline _2010_ 0927.pdf).

Degeneration of the basal forebrain cholinergic neurons occurs early in the course of AD and has been correlated well with the observed cognitive decline in such patients (Coyle et al., 1983). Reduced acetylcholine (ACh) levels and function in the brain is considered to be the classical attribute to cognitive deficits in AD. Researchers have found that besides controlling histamine release, H3R antagonists may alleviate AD associated cognitive deficits by augmenting release of other neurotransmitters including ACh (Passani et al., 2004; Haas et al., 2008). H3R antagonists enhanced ACh levels in the PFC and in the dorsal hippocampus accompanied by an improvement of cognitive functions in behavioral studies in rodents (Fox et al., 2005). However, the progressive cholinergic cell loss in basal forebrain linked with AD (Whitehouse et al., 1982) probably confines the therapeutic efficacy of drugs to be reliant on endogenous ACh synthesis and doesn't provide disease-modifying efficacy in addition to symptomatic improvement.

The two cardinal features of AD pathology speculated to be the potential targets of disease-modifying drugs are β-amyloid (Aβ), a product of aberrant amyloid precursor protein (APP) leading to production of extracellular Aβ plaques, and NFT arising from hyperphosphorylation of tau, a microtubule-associated protein (Giacobini and Becker, 2007). Activation of cellular pathways that inhibit tau kinase signaling and subsequent tau hyperphosphorylation is considered to be the most feasible strategy to prevent tau aggregation and associated pathological effects. Suppression of tau protein can also block Aβ-induced apoptosis thereby reducing cognitive deficits (Martin et al., 2011). A recent study using rat PC12 tumour cell line demonstrated neuroprotective effects of clobenpropit, an H3R antagonist, on injury produced by Aβ42 toxicity (Fu et al., 2010). Though there are several mechanisms that are thought to be involved in Aβ toxicity, a glutamate mediated mechanism was suggested in

this study. An encouraging preclinical evidence with the H3R antagonist ABT-239 suggests that H3R antagonism might not only deliver symptomatic treatment in AD, but also possess disease-modifying benefits (Bitner et al., 2011). Acute administration of the H3R antagonist ABT-239 in CD1 mice increased cortical CREB (cAMP response element binding protein) and Ser9-GSK-3β phosphorylation (GSK-3β phosphorylated at serine 9) at cognitive enhancing doses. Furthermore, donepezil (an acetylcholinesterase inhibitor) at clinical doses induced CREB phosphorylation consistent with a pro-cognitive action, but unlike ABT-239, did not alter Ser9-GSK3β level (Bitner et al., 2011). Together, these findings indicated that increased Ser9-GSK3β phosphorylation induced by ABT-239 is independent of increased ACh release. In an earlier study by the same group, both CREB and Ser9-GSK3β phosphorylation were shown to be down-regulated in the Tg2576 (APP/Aβ) transgenic mouse model of AD (Bitner et al., 2009). However, a 2-week infusion of ABT-239 in Tg2576 mice restored reduced cortical CREB and hippocampal pSer9-GSK3β phosphorylation. In parallel studies conducted in female TAPP mice, an AD transgenic line expressing both APP and tau transgenes, ABT-239 infusion reversed tau hyperphosphorylation in the spinal cord and hippocampus (Bitner et al., 2011). Mechanistically, ABT-239 produced pSer9-GSK3β changes in α7 nicotinic ACh receptor (nAChR) knockout mice, an effect also observed in normal mice that exhibit α7 nAChR agonist-induced phosphorylation (Bitner et al., 2009), suggesting that H3R antagonist-mediated signaling (increased pSer9-GSK3β) does not appear to require ACh-stimulated α7 nAChR activation (Bitner et al., 2011). These *in vivo* signaling studies boost the exciting prospect that H3R antagonists activate multiple signaling pathways that may translate into improved disease-modifying efficacy in patients with AD, along with symptomatic alleviation (Bitner et al., 2011; Brioni et al., 2011). Thus, it can be hypothesized that H3R antagonist-evoked neurotransmitter release (e.g., ACh) leads to activation of postsynaptic receptor pathways such as phosphorylation-activation of CREB, a transcription factor relevant to cognitive function, and phosphorylation of inhibitory residue Ser9 of GSK3β, a primary tau kinase in AD responsible for tau hyperphosphorylation (Hooper et al., 2008; Bitner et al., 2011). This, together with the disease-modifying capacity of H3R antagonist might also impact the underlying disease pathology (e.g., tau phosphorylation) beyond mere symptomatic alleviation (reviewed by Brioni et al., 2011). In line with the above view, Abbott has suggested a combinatorial treatment of cognitive disorders consisting of a nAChR ligand (either α4β2 or α7 subtype) and a H3R antagonist e.g., ABT-239 (Abbott laboratories, WO2009082698; 2009) which can also include psychostimulants (e.g., methylphenidate) or monoamine re-uptake inhibitors (e.g., atomoxetine) to achieve greater clinical efficacy (Lazewska and Kiec-Kononowicz, 2010).

ATTENTION-DEFICIT HYPERACTIVITY DISORDER

ADHD is a disorder most prevalent in children characterized by persistent carelessness, hyperactivity, and impulsivity. The current pharmacological treatments of ADHD include stimulants (methylphenidate, amphetamines, etc.), non-stimulant (atomoxetine), α2 agonists (clonidine and guanfacine) etc. However, these treatments (mainly stimulants) are associated with significant adverse effects and abuse liability. The potential usefulness of H3R antagonists in this pathology is buttressed by their pro-attentional and pro-cognitive activity in a number of rodent models [such as object recognition task, social recognition task, spontaneous hypertensive rats (SHR), and five-choice stimulus reaction time test (5-CSRTT)] which is devoid of any psychomotor activation and abuse liability (Gemkow et al., 2009; Kuhne et al., 2011; Passani and Blandina, 2011). ADHD involves interplay of multiple neurotransmitter systems mainly of dopaminergic and noradrenergic systems but also of cholinergic and serotonergic systems (Curatolo et al., 2009; Cortese, 2012). While stimulants block the reuptake of dopamine (DA) and norepinephrine (NE) into presynaptic neuron (amphetamine in addition also promotes release), atomoxetine, a non-stimulant drug, blocks NE transporter thereby increasing concentrations of NE throughout the brain but DA only in PFC (Cortese, 2012). In agreement, H3R antagonists have been shown to elevate the release of neurotransmitters involved in cognition e.g., ACh and DA in the PFC (Fox et al., 2005; Ligneau et al., 2007), ACh, DA, and NE in the anterior cingulate cortex (Medhurst et al., 2007; Southam et al., 2009), and AChh in the hippocampus (Fox et al., 2005).

In preclinical models, pharmacological alterations that antagonize the cholinergic system or enhance the various neurotransmitter systems like DA, orexin, cannabinoids systems including histamine cause hyperactivity [an increase in locomotor activity (LA)] that accompanies various neurological disorders including ADHD The LA can be decreased by genetic alterations that reduce the level of histamine (e.g., in HDC KO mice) or by lesions of the TMN (Viggiano, 2008). Recently, H3R antagonist (carnicine, a stable analog of the naturally occurring dipeptide carnosine) attenuated hyperlocomotion in an ADHD-specific model with neonatal habenula lesion without having an effect on attention-deficit (Goto and Lee, 2011). In other studies, antagonists of H3R have demonstrated pro-attentional effects in various ADHD-specific animal models including five-trial inhibitory avoidance in SHR pups (thioperamide, ABT-239, GT-2331, and ciproxifan) (Fox et al., 2002; Komater et al., 2003) and impairment in a 5-CSRTT (ciproxifan) (Day et al., 2007). In addition, CEP-26401 (irdabisant), antagonized H3R agonist R-α-methylhistamine-induced drinking response in the rat dipsogenia model, improved performance in the rat social recognition model of short-term memory, and showed wake-promoting properties (Raddatz et al., 2012). Recently, a single-blind trial with pitolisant (BF2.649) in 28 adult ADHD patients yielded a progressive improvement in clinical scores. However, the placebo also showed some effect in this trial, so the clinical efficacy is unclear which merits confirmation in a double-blind trial in adults and children (Schwartz, 2011). In addition, MK-0249 (NCT-ID NCT00475735) has completed phase II clinical trials for ADHD but the results are still awaited in public domain (Brioni et al., 2011; Kuhne et al., 2011). PF-03654746 recently completed a phase II trial (NCT00531752) in adult ADHD patients, though no efficacy was observed vs. placebo (Brioni et al., 2011). Likewise, in a randomized clinical study, ADHD-RS-IV score (primary efficacy end point of ADHD) of bavisant, a highly selective, wakefulness-promoting H3R antagonist, though displayed an improved trend, was not statistically

significant compared with placebo in ADHD patients (Weisler et al., 2012). These data accumulating from rodent models and clinical settings underpin the idea that modulation of H3R represents a novel therapeutic target for the treatment of ADHD especially due to its wider therapeutic role in CNS and the fact that ADHD is frequently co-morbid with sleep disorders, learning difficulties, substance abuse, anxiety, depression and other neuropsychiatric disorders (Cortese, 2012).

In addition to impaired motor functions and vigilance, ADHD is also associated with impairment of cognitive functions (Bidwell et al., 2011) even though the direct clinical relevance of interventions enhancing cognitive functions in the treatment of ADHD appears limited. Nevertheless, several classes of anti-ADHD drugs have uniformly shown evidence of acute cognitive enhancement and improvement in the core symptoms of ADHD. As described in AD section, a wide array of neurobiological mechanisms have been attributed for the observed pro-cognitive and pro-attentional effects of H3R antagonists in rodent models including H3R antagonists mediated enhancement of neurotransmitter release (Passani et al., 2004; Giannoni et al., 2010) or modulation of electrophysiological activity (Hajos et al., 2008; Andersson et al., 2010) in the hippocampus, induction of immediate early gene expression in brain regions, such as the NAc (Southam et al., 2009) or motor cortex (Bonaventure et al., 2007), that directly regulates locomotor behavior; disruption of dopaminergic activity related to hyperactivity induced by DA agonists, such as apomorphine, cocaine, and methamphetamine (Fox et al., 2005; Ligneau et al., 2007) although paradoxes (Ferrada et al., 2008; Brabant et al., 2009) have also been reported. Recently ciproxifan, H3R antagonist, alleviated hyperactivity and memory impairment in an amyloid-precursor protein transgenic (APPTg2576) mouse model of AD (Bardgett et al., 2011). APP (Tg2576) mice displayed significantly increased LA and longer escape latencies in the swim maze than wild-type mice. In probe trials, ciproxifan-treated APP (Tg2576) mice spent more time near and made more crossings of the previous platform location than did saline-treated APP (Tg2576) mice. Acute ciproxifan treatment also reversed impaired object recognition task in APP (Tg2576) mice (Bardgett et al., 2011). It was hypothesized that the loss of synapses in the hippocampus of APPTg2576 mice possibly caused dysregulation in the NAc and H3R antagonist via increased neurotransmitter release in the hippocampus or by induction of early gene expression in NAc or motor cortex regulated LA.

SCHIZOPHRENIA

Schizophrenia, a chronic debilitating neurological syndrome with 0.5–1% prevalence worldwide, is characterized by positive (e.g., hallucination and delusion), negative (e.g., paucity of emotion and motivation), and impaired cognitive symptoms. Though basic knowledge at the level of molecular etiology and psychopathology is inadequate to delineate molecular targets for drug development, dysregulation in DA and other neurotransmitter systems are involved in the development of the disease (Gross and Huber, 2008). Current pharmacotherapy for schizophrenia consists of first generation (FGA) and second generation (SGA) antipsychotics, which mainly act by DA antagonism in CNS mediated mainly by DA D2- receptors but also by D3R and/or D4R, serotonin receptor (5-HTR) subtypes (5-HT2AR/5-HT2CR) and/or via modulation of the glutamatergic system (Sander et al., 2008) which may evoke extrapyramidal (mainly FGAs) and/or serious metabolic disorders (Deng et al., 2010). Due to several adverse effects of antipsychotic therapy, rising therapeutic resistance and inadequate treatment of negative symptoms, H3R antagonist could be a pervasive therapeutic strategy owing to its pro-cognitive property (Ito, 2009).

Histaminergic innervations into the brain areas closely associated with the development of schizophrenia raises the possibility of H3R antagonists influencing its pathophysiology. The clinical relevance of H3R in schizophrenia is bolstered by their high density presence, mostly postsynaptically, in the GABAergic striatal efferent neurons. There they are co-localized with DA D1 and D2 receptors and form heterodimers with the D1 receptor in GABAergic dynorphinergic and with the D2 receptor on GABAergic enkephalinergic neurons, respectively (Pillot et al., 2002a; Ferrada et al., 2008). This intracellular cross-talk results in modified signaling directed by the heterodimer which may underlie the pathogenesis of schizophrenia: e.g., H3-D1 receptor heteromer induces a Gi protein mediated activation of MAPK pathway (Ferrada et al., 2009) whereas H3-D1/D2 heteromers exhibited an antagonistic interaction as H3R agonism negatively modulated D1 or D2 receptor function (Ferrada et al., 2008). H3R antagonists are anticipated to mediate their action by exploiting these functional cross-talks. Ciproxifan, an H3R antagonist, potentiated locomotor hypoactivity and catalepsy induced by haloperidol, a widely used antipsychotic, via enhanced activation of striatopallidal neurons of the indirect movement pathway, upregulation of proneurotensin, proenkephalin, and c-*fos* (Pillot et al., 2002b). Though ciproxifan potentiated haloperidol induced catalepsy, it had no effect on catalepsy when administered alone implicating that no extrapyramidal side effect would result from their therapeutic use when used alone. The same team, a year later, revealed that ciproxifan suppressed the methamphetamine-induced upregulation of striatal neuropeptides like prodynorphin and substance P, the D_1-receptor-mediated responses; attenuated methamphetamine induced locomotor hyperactivity possibly by inhibition of striatonigral neurons in NAc involved in regulation of locomotor function (Pillot et al., 2003). Thus H3R cross-talk with dopaminergic receptor system has opened a new avenue for designing anti-schizophrenics. Further, BF2.649 activated DA turnover in the PFC but not in striatum, thus possessing a favorable profile similar to SGAs with low liability to induce extrapyramidal symptoms (Ligneau et al., 2007).

As a proof of concept, numerous data has accumulated from schizophrenia cases studied in both rodent models and in human subjects: increased H3R radioligand binding was found in dorsolateral PFC of schizophrenic subjects and bipolar subjects with psychotic symptoms (Jin et al., 2009) and antipsychotic-like profile of H3R antagonists observed in animal models. The latter included improvement of prepulse inhibition (PPI) deficits in rodent schizophrenia models by H3R antagonists/antagonists such as ABT-239 in DBA/2 mice (Fox et al., 2005), pitolisant in Swiss mice (Ligneau et al., 2007), GSK-189254 in Wistar rats (Medhurst et al., 2007), and more recently irdabisant in rats (Raddatz et al., 2012). In DBA/2NCrl mice, CEP-26401 increased

PPI of the acoustic startle response alone and also showed synergistic effect when co-administered with risperidone at subefficacious doses (Raddatz et al., 2012). The PPI deficits model the impairment of sensorimotor gating in acoustic startle response in schizophrenic patients. Stimulation of DA activity is critical for attenuation of such deficits induced by amphetamines and other DA agonists and NMDA antagonists. In addition to PPI, antagonism of dopaminergic stimulants (amphetamine, apomorrphine)-induced hyperactivity is important mechanism in pathophysiology of diseases associated with hyperactivity of dopaminergic pathway including schizophrenia and drug abuse. Animal studies demonstrated an antagonism of amphetamine and MK-801 (NMDA R antagonist)-induced motor activation by BF2.649 in mice (Clapham and Kilpatrick, 1994; Ligneau et al., 2007) and attenuation of amphetamine, methamphetamine and apomorphine-induced locomotor stimulation and/or stereotypy by thioperamide and ciproxifan (Clapham and Kilpatrick, 1994; Morisset et al., 2002) in rodent models. While acute amphetamine induced locomotor stimulation is known to represent the euphoric and abusive effects rather than psychosis which is mainly modeled by chronic amphetamine induced locomotor sensitization models (Trujillo et al., 2004), H3R antagonist ciproxifan also reduced LA in methamphetamine induced chronic sensitization model (Motawaj and Arrang, 2011).

Contrary to these, reports ranging from partial or no suppression of apomorphine induced stereotypies (Burban et al., 2010) and no improvement in sensorimotor gating deficits in rats (Southam et al., 2009; Burban et al., 2010) by H3R anatgonists to even exacerbation of hyperactivity (Ferrada et al., 2008; Burban et al., 2010) has also been documented. Cross-talk between H3R and D1/D2 receptor (Ferrada et al., 2008; Burban et al., 2010); interaction between the severity of endpoints (startle magnitude and changes in PPI) used in experimental paradigm that modeled schizophrenia, different levels of PPI among different species and strains (Burban et al., 2010), H3R antagonist used, route administered, specific memory task studied may partially account for the discrepancies (Bardgett et al., 2009) are some of the confounding factors which needs to be taken into account.

Cognitive impairment, a hallmark trait of schizophrenia, is the central determinant of functional outcomes associated with this disease. Although current antipsychotic drugs, mainly targeting the positive psychotic symptoms of the illness, treat psychosis in schizophrenia rather well, their impact on cognitive dysfunction is minimal or largely untreated. In recent years there has been growing interest in developing novel treatments for cognitive deficits in schizophrenia (Ibrahim and Tamminga, 2012). H3R antagonists increased hippocampal gamma (Andersson et al., 2010) and theta oscillation power (Hajos et al., 2008). Deficits of cognition-relevant neuronal oscillations are observed in individuals with schizophrenia which would result in erroneous higher-order cognitive processes, including sensory perception, coherent feature binding, attention, memory, and object representation, that manifest as the positive and negative symptoms of the disease (Shin et al., 2011). Augmentation of DA release in the PFC is key to minimize the negative symptoms and cognitive impairment elicited by antipsychotics. H3R antagonists, enjoy the advantage of enhancing DA release from PFC (Fox et al., 2005; Ligneau et al., 2007; Medhurst et al., 2007), thus may have a sparing effect on prefrontal function over strong DA antagonizing antipsychotics.

Although, inconsistent findings on animal models mimicking schizophrenia does not bring any full proof evidence for antipsychotic properties of H3R antagonists, their waking and pro-cognitive properties uphold their therapeutic interest and can be exploited to adequately address the cognitive impairment central to schizophrenic patients which is resistant to current neuroleptics in hand, hence better clinical outcome. A recent study on ciproxyfan in mice showed a reversal of methamphetamine-induced LA, reversal of downregulation of brain-derived neurotrophic factor (BDNF) and NMDA receptor subunit 1 genes in various regions of mice brain, reinforcing the interest of H3R antagonists in the treatment of cognitive deficits in psychotic patients (Motawaj and Arrang, 2011). Further, the results of clinical studies undertaken by numerous pharmaceutical companies are now eagerly awaited to establish the proof of concept. Pitolisant has completed Phase II of clinical trials showing efficacy in patients suffering from antipsychotic-induced weight gain and hence might be useful for co-treatment (WO2006084833A1). A study designed to assess the pro-cognitive potential of tiprolisant in people with schizophrenia and schizoaffective disorder is currently underway (NCT-ID NCT00690274). A Phase I trial of PF-03654746 as an add-on treatment of cognitive deficits in schizophrenic patients has been completed [87] without publicly disclosed results. H3R antagonists ABT-288, GSK-239512, and MK0249 have recently completed Phase II trials for cognitive impairments in schizophrenia (NCT-ID NCT01077700, NCT01009060, and NCT00506077) although the outcomes of these studies remain undisclosed so far. The findings of ongoing clinical trials in this area will be invaluable in guiding the future course of action.

ADDICTION

Addiction is a persistent, compulsive dependence on a behavior or substance. One common mechanism for addiction caused by various drugs of abuse is thought to be the activation of brain's reward circuitry which mainly involves mesolimbic dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their targets in limbic forebrain especially NAc (Nestler, 2005). However, other neurotransmitter systems including histamine are known to modulate the psychotropic effects of rewarding drugs [role of histamine reviewed recently by Brabant et al. (2010)]. The fact that histamine also modulates mesolimbic DA transmission suggests that histaminergic drugs may be tried therapeutically in drug addiction (Munzar et al., 2004; Brabant et al., 2010). Though NAc, the main region involved in addiction, receive only weak histaminergic innervations, very high densities of H3Rs are present here. Striatum contains one of the highest densities of H3Rs in the brain (Pollard et al., 1993; Pillot et al., 2002a). Mainly, striatal H3Rs are located postsynaptically in the GABAergic efferent neurons where they are co-localized with D1 and D2 receptors (Ferrada et al., 2008). They are also located presynaptically on dopaminergic terminals in the striatum where they have an inhibitory role on DA release. The central administration of histamine is known to produce a biphasic effect on LA, initially a brief reduction (hypoactivity) followed by a prolonged increase

in LA (hyperactivity) (Bristow and Bennett, 1988). The initial hypoactivity is presumed to be due to stimulation of presynaptic H3Rs (heteroreceptors) reducing DA activity and release (Chiavegatto et al., 1998). Thus, histamine is known to have an inhibitory effect on reward by inhibition of mesolimbic dopaminergic system. Histamine can also activate mesolimbic dopaminergic system possibly via presynaptic H3Rs located on DA terminals or postsynaptically on GABAergic neurons in striatum or through H1 Rs located on striatal cholinergic interneurons (reviewed by Brabant et al., 2010). Activation of H3Rs that were found to form heteromers with D2 Rs on GABAergic neurons attenuated stimulant (quinpirole)-induced hyperactivity (Ferrada et al., 2008).

Addictive drugs modulate the histamine levels in the brain. For instance, alcohol affects histamine levels in the brain by modulating histamine synthesis, release and turnover (Zimatkin and Anichtchik, 1999), acute injection of cocaine increases histamine levels and histamine-N-methyl transferase activity in the striatum and NAc (Ito et al., 1997) and opioids (e.g., morphine) increases turnover of neuronal histamine via opioid receptors (Nishibori et al., 1985). On the other hand, histaminergic system can modulate behavioral effects of various drugs of abuse including cocaine, amphetamines, opioids, and alcohol. All addictive drugs stimulate LA (psychomotor stimulant effects). Several studies demonstrate that H1-antagonists produce behavioral and/or neurochemical effect similar to addictive drugs possibly by blocking DA uptake thereby facilitating the activity of mesolimbic dopaminergic system (Brabant et al., 2010; Levin et al., 2011). However, a pharmacokinetic interaction between H1-antagonists (inhibition of CYTP450 CYP2D enzymes) and methamphetamine leading to elevated levels of the latter (Okuda et al., 2004) could not be ruled out. Locomotion activation by amphetamine/methamphetamine and other dopaminergic agonists was attenuated by H3R blockade by thioperamide (Clapham and Kilpatrick, 1994), ciproxifan (Motawaj and Arrang, 2011), ABT-239 (Fox et al., 2005), or BF2.649 (Ligneau et al., 2007) in rodents in acute and chronic behavioral sensitization models. The latter is repeated administration of drug leading to an augmentation of behavioral effects of psychostimulants on re-administration (Celik et al., 2006). However, evidence contradicting the same is also available. For instance, there are cases where H3R antagonists (GSK-207040, Southam et al., 2009 and JNJ-5207852, JNJ-10181457, Komater et al., 2003) did not reverse amphetamine-induced hyperactivity or H3R antagonist thioperamide increased cocaine-induced hyperlocomotion in mice (Brabant et al., 2010). Indeed, the potentiating effects of imidazole-based H3R antagonists like thioperamide, clobenpropit on stimulants could also be due to their inhibitory effects on cytochrome P450 activity as the non-imidazole based antagonist doesn't enhance cocaine-induced stimulant effects (Brabant et al., 2009). Repeated administration of methamphetamine increases HDC activity in striatum and cortex in rats (Ito et al., 1996) and release of histamine in mice (Dai et al., 2004). Consistently, antagonism of H3Rs increases methamphetamine-induced stereotypic hyperactivity in young male mice (Acevedo and Raber, 2011), an effect contradicting the so-called antipsychotic profile of H3R antagonists. There are also reports where thioperamide potentiated the locomotor activation induced by D1 or D2R agonist (Ferrada et al., 2008) suggesting an antagonistic interaction between postsynaptic H3 and D1 or D2 receptors.

Self-administration and conditioned place preference (CPP) paradigms are widely employed to study reinforcing or rewarding effects of drugs of abuse. Histamine has a negative effect on reinforcement because H1-antagonists can act as reinforcers either alone or with other reinforcers like opiates, cocaine, or amphetamine (Brown et al., 2001). Going by this hypothesis, H3R antagonists should prevent reinforcement. Indeed, H3R antagonists (ciproxifan and JNJ-10181457) were found to inhibit the ethanol-evoked CPP whereas H3R agonist immepip did not alter ethanol-induced place preference in male DBA/2J mice. This is further supported by a study where H3 R knock-out (H3RKO) mice did not develop alcohol-induced CPP (Nuutinen et al., 2011). Inhibition of ethanol reward by H3R antagonism implies that H3R might be a noble target to suppress compulsory ethanol seeking (Nuutinen et al., 2011). H3R antagonists also reduced the ethanol drinking in alcohol-preferring (AA) rats (Lintunen et al., 2001; Galici et al., 2011) and blocked rewarding and reinforcing effects of ethanol in DBA/2 mice (Nuutinen et al., 2011). However, they increased acute stimulatory effects of ethanol. The same authors also reported that ciproxifan increased ethanol CPP in mice (Nuutinen et al., 2010) but reduced ethanol-induced locomotor activation in C57BL/6 mice. Consistent to this, thioperamide and clobenpropit increased methamphetamine self-administration and reward possibly through H3 heteroreceptors located on dopaminergic terminals increasing DA release in VTA (Munzar et al., 2004). H3R antagonists also increased cocaine CPP (Brabant et al., 2005) but suppressed LA induced by amphetamine.

Repeated exposure to cocaine, amphetamine or opiates induces phosphorylation and activation of CREB in several reward-related brain regions including NAc whereas alcohol and nicotine reduces CREB phosphorylation in this region. In NAc, psychostimulants induce CREB via activation of DA D1 receptors (Goodman, 2008). Acute administration of ABT239 increased cortical CREB while its continuous infusion normalized reduced cortical CREB phosphorylation in AD transgenic mice (Bitner et al., 2011). In another study, sensitization by methamphetamine-induced reduction of BDNF mRNA in the hippocampus and NMDA R subunit 1 (NR1) mRNA in cerebral cortex, hippocampus and striatum, was reversed by ciproxifan (Motawaj and Arrang, 2011). The effects of H3R antagonists have not been mechanistically evaluated for their effects on CREB phosphorylation or BDNF in brain regions relevant for drug abuse.

CONCLUSION

Cognitive dysfunction and motor impairments are the hallmark of multifarious neurodegenerative and/or psychiatric disorders including AD, ADHD, schizophrenia, and drug abuse. The neurochemical basis interlinking these complex behavioral traits in these diseases conditions is complex and it is unclear whether they derive from alterations in a common neuronal circuit. H3R antagonists/inverse agonists, through H3 heteroreceptors, enhance the release of various important central neurotransmitters in brain

in addition to histamine and hence can modulate various processes of the CNS including cognition. In addition, postsynaptic H3Rs are present in striatum at GABAergic cell bodies of the medium spiny neurons both on the striatonigral neurons of the direct movement pathway and on the striatopallidal neurons of the indirect movement pathway modulating motor activity (Nuutinen et al., 2011). Locomotor hyperactivity accompanies not only ADHD but also AD and schizophrenia (Viggiano, 2008). Likewise, attentional impairments are observed not only in ADHD but also in Schizophrenia, AD, and drug abuse. It is noticeable that the latter accompanies cognitive and attentional deficits too (Potenza et al., 2011). Such deficits observed in chronic alcohol, cocaine, and methamphetamine abusers is considered to be a particular challenge for treatment-seeking users who require intact cognitive functioning to stop drug abuse. Thus, various cognition enhancing strategies and anti-ADHD drugs have been tried in drug abuse, e.g., atomoxetine, a selective norepinephrine transporter (NET) inhibitor. Similarly, cognitive enhancers are useful in improving attention in ADHD (Bidwell et al., 2011; Levin et al., 2011). Histamine H3R antagonists, though not directly linked to the pathophysiology of disease states, improve cognitive functions and increase alertness, wakefulness, learning, memory without significantly impairing motor functions and thus provides a rationale for their evaluation and research on various neurological conditions where impairment of such functions is paramount especially AD, the most common cause of progressive decline of cognitive functions, and others including ADHD, schizophrenia, epilepsy, narcolepsy, and drug abuse. The broad spectrum of activities of H3R antagonists continues to expand as more and more novel therapeutic roles have been investigated including Parkinson's disease, multiple sclerosis, cerebral ischaemia, depression, etc., and hence identification of potential clinical targets. Nevertheless, many issues such as paucity of clinical data and specific studies evaluating the molecular mechanisms involved in the role of H3R antagonists as well as the conflicting nature of the data gathered so far in pharmacological/biochemical evaluations, remains a challenge that remains to be answered with more rigorous evaluations to develop H3R specific therapeutic agents for human use.

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APPENDIX

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Histamine and neuroinflammation: insights from murine experimental autoimmune encephalomyelitis

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Multiple sclerosis (MS) is a chronic inflammatory, neurodegenerative disease of the CNS whose pathogenesis remains largely unknown, and available therapies are rarely successful in reversing neurological deficits or stopping disease progression. Ongoing studies on MS and the widely used murine model of experimental autoimmune encephalomyelitis (EAE) are focused on the many components of this complex and heterogeneous neurodegenerative disease in the hope of providing a mechanism-based characterization of MS that will afford successful strategies to limit and repair the neuronal damage. Recently, histamine has been postulated to have a key regulatory role in EAE and MS pathogenesis. Histamine is a mediator of inflammation and immune responses, exerting its many actions through four G protein-coupled receptors ($H_{1,2,3,4}R$) that signal through distinct intracellular pathways and have different therapeutic potentials as they vary in expression, isoform distribution, signaling properties, and function. Immune cells involved in MS/EAE, including dendritic cells (DCs) and T lymphocytes, express H_1R, H_2R and H_4R, and histamine may have varying and counteracting effects on a particular cell type, depending on the receptor subtypes being activated. Here, we review evidence of the complex and controversial role of histamine in the pathogenesis of MS and EAE and evaluate the therapeutic potential of histaminergic ligands in the treatment of autoimmune diseases.

Keywords: multiple sclerosis, EAE, autoimmune diseases, H_1 receptor, H_2 receptor, H_4 receptor

MS: A COMMON INFLAMMATORY-DEGENERATIVE CNS DISEASE

Multiple sclerosis (MS) is the most common, non-traumatic cause of neurological disability among young adults in Western Europe and North America. The current hypothesis asserts that MS is triggered by environmental factors in individuals with complex genetic-risk profiles. As with other autoimmune diseases, MS shows moderate polygenic inheritability and may be caused or exacerbated by environmental exposure such as viral infections or vitamin D deficiency (Correale et al., 2009). It is characterized by clinical and genetic heterogeneity and with individuals with MHC class II complex genes being most susceptible (Ballerini et al., 2004; Gourraud et al., 2011). Out of the MHC locus a plethora of genes have been described as minor contributors to genetic risk, among which are those encoding for IL-2 receptors and IFN-γ (Blankenhorn et al., 2011).

MS is characterized by inflammation of the central nervous system (CNS) in which focal lymphocytic infiltrations lead to damage of myelin and axons associated with neurological dysfunction. Initially, the inflammatory response is transient and remyelination occurs, but this is not durable and over time widespread microglial activation ensues along with extensive and chronic neurodegeneration.

The observation that histamine may be implicated in MS dates back to the early 1980s when Tuomisto et al. (1983) showed that patients with remitting or progressive disease have histamine levels about 60% higher than controls. Another clinical study, though, did not show elevated concentrations of histamine and its metabolite methylhistamine in MS patients when compared, in this case, with individuals affected by other neurological diseases (Rozniecki et al., 1995). More recently, gene-microarray analysis has shown that histamine H_1 receptor (H_1R) expression is upregulated in MS lesions (Lock et al., 2002), and epidemiological studies suggest a protective effect of brain penetrating H_1R antagonists (Alonso et al., 2006). Furthermore, in a small pilot study, a cohort of MS patients treated with an H_1R antagonist showed signs of neurological amelioration (Logothetis et al., 2005). The H_1R has long been associated with inflammatory responses and for decades, the antiallergic and antiinflammatory activities of H_1R antagonists have been used in therapy. Possible explanations of the therapeutic potential of H_1R antagonists come from preclinical results in experimental allergic encephalomyelitis (EAE) in mice.

Abbreviations: APCs, antigen presenting cells; BBB, blood brain barrier; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; HDC, histidine decarboxylase; MOG_{35-55}, Myelin Oligodendrocyte Glycoprotein; MS, multiple sclerosis; $PLP_{139-151}$, myelin proteolipid protein.

EAE IS A GOOD MODEL FOR STUDYING MS

Pathological features of MS are studied in three main animal models: toxic induction of disease, viral induction, and several types of EAE. Cuprizone and lysolecithine, for example, are used to investigate demyelination and remyelination in the CNS (reviewed in Woodruff and Franklin, 1999; Kipp et al., 2009), and to investigate how viral infections can induce CNS autoimmunity; Theiler's murine encephalomyelitis virus (TMEV) inoculated intracerebrally is a currently used experimental protocol (Olson et al., 2001). EAE may be actively induced by immunization of susceptible mouse strains with myelin proteins or myelin protein immunodominant peptides in the presence of complete Freund's adjuvant (CFA), or may be passively induced by transfer of myelin specific T cells. EAE, in most animal models, is mainly driven by MHC class II restricted, autoimmune $CD4^+$ T cells and the clinical course depends on the immunization protocol, as not all combinations of genetic background, antigen, and adjuvant induce EAE. Classically, EAE immunized animals develop an ascending flaccid paralysis from tail to head with variable disease score; manifestation of clinical symptoms may be relapsing remitting, monophasic, or chronic. In active disease pathogenesis of EAE, two main steps are usually described: priming of myelin-reactive T cells and CNS invasion through the blood brain barrier (BBB). Once cells invade the CNS, local, and infiltrated antigen presenting cells (APCs) will present myelin peptides for full activation. Many different types of cells contribute to the development of the disease: APCs, mainly dendritic cells (DCs), B cells, microglia, macrophages, and astrocytes, although astrocytes have an unclear function in EAE development (Volterra and Meldolesi, 2005).

For many years the principal paradigm of EAE pathogenesis has been centered on IFN-γ producing T cells (Th1). These cells differentiate upon exposure to IL-12 and IFN-γ, are characterized by transcription factors T-bet, STAT1, STAT4 and are found as infiltrates in CNS lesions. With time this paradigm has been partially revised and a recently described T cell subpopulation has been shown to have a central role in disease pathology: Th17 cells that differentiate in the presence of IL-6 and TGF-β and need RORγt as a transcription factor (Gutcher and Becher, 2007). Th17 cells have been extensively studied in several autoimmune diseases and there is wide agreement on the instability of the phenotype that may switch between Th1 and Th17. This plasticity derives from epigenetic factors with Th17-derived Th1 cells promoting autoimmune diseases (Annunziato et al., 2007; Mukasa et al., 2010). Altogether, the studies on $CD4^+$ T cells involved in EAE do not ascribe a particular role to one specific T cell population, and T helper cells may undergo alterations. Furthermore, recent observations suggest that EAE can elicit both Th1 and Th2 immune responses in the same subjects, i.e., elements of the Th2 cell-mediated allergic response are associated with autoimmune demyelination (Pedotti et al., 2003).

In conclusion, EAE has provided mechanistic insights into the complex pathogenesis of MS and has proven to be a good model for the preclinical testing of new diagnostic or treatment modalities. In addition, it is considered a well-suited model because of its histopathological and immunological similarities to MS (Schreiner et al., 2009). Consequently, the large majority of studies looking for a possible implication of histamine in autoimmune diseases have been performed using the murine EAE experimental model.

HISTAMINE IN AUTOIMMUNE DISEASES

Histamine has long been known to be a major promoter of allergic inflammatory conditions and gastric acid secretion. Synthesized by histidine decarboxylase (HDC) from histidine, histamine was later described as a neurotransmitter in the CNS that regulates several physiological processes and homeostatic functions including cognition, arousal, circadian, and feeding rhythms (Haas et al., 2008). These effects are mediated through four distinct G protein-coupled receptors (H_1R, H_2R, H_3R, and H_4R) with very low sequence homology. The H_1R couples to G_q proteins, leading to phospholipase C activation and calcium mobilization (Bakker et al., 2002). The H_2R activates $G\alpha_s$ and increases camp formation, whereas the H_3R mediates its function through $G\alpha_{i/o}$, inhibits cAMP synthesis (Bakker et al., 2002), activates MAP kinases and the AKT/GSK3β axis (Bongers et al., 2007; Mariottini et al., 2009).

Inflammatory responses consequent to histamine release have long been thought to be mediated by the H_1R, and antihistamines commonly used to treat allergies are H_1R antagonists. The discovery of a fourth histamine receptor (H_4R) and its expression on virtually all inflammatory and immune cells, though, has prompted a reassessment of the role of histamine in inflammatory and immune disorders and widened the spectrum of potential therapeutic interventions (Thurmond et al., 2008). The H_4R is coupled to $G\alpha_{i/o}$ proteins (Liu et al., 2001) and to the β-arrestin pathway (Rosethorne and Charlton, 2011), and signals via intracellular increases of calcium. Its functions include mediation of calcium mobilization, shape change, actin polymerization (Barnard et al., 2008) chemotaxis of mast cells, and eosinophils (Buckland et al., 2003), and up-regulation of adhesion molecules (Buckland et al., 2003).

All histamine receptors are expressed on the complement of cells involved in autoimmune diseases, with the exception of the H_3R that is normally not expressed by hematopoietic cells, but is mostly confined to the CNS (Passani et al., 2011b). Histamine participates in the development and progression of EAE as it controls accessibility to the site of inflammation by modulating vasopermeability and adhesion molecule expression, chemotaxis, and the cytokine profile of DCs and T lymphocytes, the main players in autoimmune diseases.

ROLE OF HISTAMINE RECEPTORS IN EAE: CONTROVERSIAL RESULTS

The regulatory functions of histamine relevant to the onset and progression of neuroinflammatory diseases and in particular EAE, are being studied in genetically modified mice lacking histaminergic receptors, and with relatively selective agonists and antagonists. Hence, the contribution of each histamine receptor in autoimmune diseases has been identified. Histamine plays a complex role with varying and counteracting effects, depending on the receptor subtypes being activated and the targeted tissue (see **Table 1**). *In vitro* experiments have shown that activation of H_1R and H_2R on DCs modulates cytokine and chemokine

Table 1 | Histamine receptors and EAE.

Histamine receptor	EAE	Investigated cell types	Pharmacological target	Disease outcome	Reference
H_1	SJL mice $PLP_{139–151}$	Increased H_1R expression on Th1 cells	H_1R antagonism	Less severe disease	Pedotti et al., 2003
		Humoral immunoresponses			El Behi et al., 2007
	$MOG_{35–55}$ H_1R-KO mice	$CD4^+$T cells		Reduced IFN-γ, increased IL-4 Less severe disease	Ma et al., 2002 Noubade et al., 2007
	$MOG_{35–55}$ H_1R-KO mice	Endothelial cells	H_1R overexpression	Restored BBB integrity Less severe disease	Lu et al., 2010
	$PLP_{139–151}$ SJL mice	$CD3^+$T cells	H_1R activation	Reduced IFN-γ Decreased endothelial adhesiveness	Lapilla et al., 2011
H_2	$MOG_{35–55}$ H_2R-KO mice	APC Th1		Reduced cytokines Inhibition of cell polarization Less severe disease	Teuscher et al., 2004
	$MOG_{35–55}$ C57/Bl6	Proinflammatory cells	H_2R activation	Less severe disease	Emerson et al., 2002
	$PLP_{139–151}$ SJL mice	$CD3^+$T cells	H_2R activation	Reduced IFN-γ Decreases endothelial adhesivness	Lapilla et al., 2011
H_3	$MOG_{35–55}$ H_3R-KO mice	Th1 Endothelial cells		Increased expression of chemokines/chemokine receptors BBB deregulation More severe disease	Teuscher et al., 2007
H_4	$MOG_{35–55}$ H_4R-KO mice	Treg		Lower frequency	del Rio et al., 2012
		Th17		Higher frequency More severe disease	
	$MOG_{35–55}$ C57/Bl6 mice	Th1 Mdc	H_4R antagonism	Increased IFN-γ, reduced IL-10 More severe disease	Passani et al., 2011a
	$MOG_{35–55}$ HDC KO mice	$CD3^+$T cells		Increased IFN-γ, TNF, More severe disease	Musio et al., 2006

APC, antigen presenting cells; BBB, blood brain barrier; mDC, myeloid dendritic cells; $MOG_{35–55}$, Myelin Oligodendrocyte Glycoprotein; $PLP_{139–151}$, Myelin Proteolipid Protein.

production and their ability to drive $CD4^+$ T-cell differentiation to the Th2 phenotype. On the other hand, H_4R activation modulates chemotaxis (reviewed in Schneider et al., 2010). Depending on the receptor engaged on polarized T cells, histamine can promote Th1 responses through H_1R and down-regulate both Th1 and Th2 responses through H_2R (Jutel et al., 2001). Similarly to DCs, H_1R and H_4R activation on $CD4^+$ T cells induces chemotaxis *in vitro*, whereas H_1R and H_2R modulate cytokine production (Schneider et al., 2010).

Mast cells are generally thought to be the major sources of histamine and can themselves be modulated by histamine as they express H_1R, H_2R, and H_4R. There is ample correlative and direct evidence that supports mast cell involvement in amplifying the severity of both MS and EAE. Mast cell-deficient W/W(v) mice exhibit significantly less severe disease than wild type littermates in both progressive (Sayed et al., 2011) and relapsing-remitting (Secor et al., 2000) models of EAE.

Susceptibility to EAE requires expression of *Hrh1*, the gene encoding the H_1R (Ma et al., 2002). The H_1R is expressed on Th1 cells in EAE mice brain lesions (Pedotti et al., 2003), where its presence is necessary for full encephalitogenic expression (Noubade et al., 2007). Furthermore, expression of the H_1R is up-regulated on encephalitogenic $PLP_{139–151}$ specific Th1 compared to Th2 cell lines (Pedotti et al., 2003). Not surprisingly, specific pharmacological targeting of H_1R results in amelioration of EAE (Pedotti et al., 2003; El Behi et al., 2007) and H_1R-deficient (H_1R-KO) mice exhibit a significant delay in the onset of EAE and a reduction in the severity of the clinical signs compared with WT mice (Ma et al., 2002; **Table 1**). In fact, $CD4^+$ T-cells from H_1R-KO mice produce significantly less IFN-γ and more IL-4 (that induces differentiation of naive $CD4^+$ T cells to Th2 cells) in *in vitro* assays compared to wild-type controls, indicating that H_1R signaling in $CD4^+$ T cells plays a central role in regulating pathogenic T-cell responses (Ma et al., 2002).

The H_2R also seems to partially regulate encephalitogenic Th1-cell responses and EAE susceptibility, as H_2R-KO mice develop a less severe disease than wild-type littermates during the acute, early phase (Teuscher et al., 2004). The failure of H_2R-KO mice to generate encephalitogenic Th1 effector cell responses is attributed to H_2R-mediated regulation of cytokine production by DCs that affects T-cell-polarizing activity. In conclusion, H_1R and H_2R seem to have a pro-inflammatory role and disease-promoting effect, but the story is not quite as simple as that, as H_1R or H_2R activation may also play an important role in limiting autoimmune responses.

Adhesion of T cells to the inflamed microcirculation precedes their penetration in the brain parenchyma, following breakdown of BBB integrity. H_1Rs are expressed on endothelial cells where they increase vascular permeability (Owen et al., 1980); however, functional expression of the H_1R on endothelial cells does not restore EAE susceptibility in H_1R-KO mice, rather, overexpression of the H_1R further suppresses the residual symptoms shown by H_1R-KO mice, suggesting that endothelial H_1R signaling is needed to maintain cerebrovascular integrity (Lu et al., 2010). Pedotti and colleagues (Lapilla et al., 2011) have demonstrated that histamine activating H_1R and H_2R, inhibits *in vitro* the proliferation of mouse $CD3^+$ T cells reactive against $PLP_{139–151}$, and their adhesiveness to the inflamed endothelium. Also, treatment with an H_2R agonist reduces the clinical signs of murine EAE (Emerson et al., 2002; **Table 1**). As pointed out by the authors (Pedotti et al., 2003; Lapilla et al., 2011) methodological differences may account for the discrepancies observed in *in vivo* and *ex vivo* experiments, such as the immunization protocols adopted. Also, *ex vivo* experiments may not necessarily recapitulate the integrated action of signals relevant to EAE and components of an integrated system maybe lost. On the other hand, genetically modified mice may carry alterations of systems other than the targeted ones, and activation of vicarious mechanisms may hinder the effects related to the deleted gene(s). In fact, the complete lack of HDC and histamine synthesis in HDC-KO mice exacerbates EAE and increases the production of proinflammatory cytokines (Musio et al., 2006). This phenotype does not seem to summarize the lack of signaling at histamine receptors observed in H_1R- and H_2R-deficient mice, rather a reduced effect of histamine at H_3R and H_4R (see below).

As mentioned earlier, H_3R are normally not expressed by hematopoietic cells, but are mostly confined to the CNS where they limit histamine synthesis and release (Arrang et al., 1983), as well as regulate other neurotransmitters' release (reviewed in Passani and Blandina, 2011). It was recently shown that deletion of the H_3R leads to more severe EAE, an effect associated with altered BBB permeability and an unexpected increased expression of chemokines/chemokine receptors that promote entry into the CNS on peripheral T cells that do not themselves express H_3R (Teuscher et al., 2007). The authors suggest that neuronal H_3R may serve as a central control of cerebrovascular tone, and negatively regulate susceptibility to neuroinflammatory diseases. Their proposed mechanism states that in H_3R-KO mice the absence of a presynaptic inhibition would lead to increased release of neurotransmitters and postsynaptic activity that would exert neurogenic control of BBB permeability and T cell chemokine profile.

THE COMPLEX ROLE OF H_4R DURING INFLAMMATORY RESPONSES IN EAE

The distribution of the H_4R on immune cells and its primary role in inflammatory functions has made it a very attractive target for the treatment of asthma and autoimmune diseases (Bhatt et al., 2010). Recent evidence has also shown the topological and functional localization of the H_4R in the CNS of both humans and rodents (Connelly et al., 2009; Strakhova et al., 2009). Immunohistochemical detection revealed that H_4Rs are expressed on the soma of sensory neurons with intense staining of small and medium diameter neurons, as well as lamina I-II of the lumbar spinal cord, where the immunoreactivity pattern suggests localization with terminals of primary afferent neurons (Connelly et al., 2009). These findings widen the range of therapeutic potentials of compounds targeting the H_4R, as antagonists might relieve itching by decreasing not only inflammation, but also the urge to scratch. The H_4R is detected on hematopoietic progenitor cells (Petit-Bertron et al., 2009), and its activation before exposure to growth factors leads to a drastic decrease in the percentage of cycling cells (Schneider et al., 2011). The H_4R expression is dynamic as it is up-regulated during the differentiation from human monocytes to DCs (Gutzmer et al., 2005). In addition, receptor levels may change with the progression of pathophysiological responses, e.g., inflammatory stimuli can up-regulate the expression of H_4R in monocytes (Dijkstra et al., 2007). The anti-inflammatory effect of H_4R antagonists in asthma and pruritus is consolidated (reviewed in Thurmond et al., 2008; Zampeli and Tiligada, 2009). The use of selective antagonists demonstrated a pro-inflammatory role for this receptor in several paradigms and suggested a facilitating action on autoimmune diseases (Jadidi-Niaragh and Mirshafiey, 2010). Furthermore H_4R are expressed on Th17 cells where their activation increases IL-17 production (Mommert et al., 2012). Quite surprisingly though, mice with a disrupted H_4R gene develop more severe EAE together with increased neuroinflammatory signs and increased BBB permeability compared to wild type mice (del Rio et al., 2012). In this paradigm, H_4R-KO mice have a lower frequency of infiltrating Treg cells that possess disease suppressive activity, more precisely during CNS invasion at day 10 post immunization (10 dpi), and a higher proportion of inflammatory Th17. Preliminary data in our laboratory are corroborating these findings (**Table 1**), as H_4R antagonists such as JNJ7777120 administered daily for eight days to EAE mice at the onset of clinical signs exacerbate the clinical and neuropathological signs of the disease (Passani et al., 2011a). In our model, a decreased release of regulatory cytokines such as IL-10 is accompanied with augmented production of IFN-γ in $MOG_{35–55}$-specific T cells during the acute phase of the disease (18–20 dpi), suggesting a more complex role of H_4R not only on regulatory mechanisms of the immune response but also on T effector cells.

In conclusion, the use of different inflammatory and immune models is producing conflicting results about the role of the H_4R in allergic and immune responses. For example, recent data show that the activation, and not antagonism, of H_4R leads to reduced pro-inflammatory capacity of a subpopulation of DCs found in inflamed tissues in atopic dermatitis (Gschwandtner et al., 2011). Also, in a murine model of allergic asthma, the administration

of H_4R agonists mitigated airway hyperreactivity and inflammation with a suggested direct effect on T regulatory cell recruitment (Morgan et al., 2007). It is clear that in the context of the conflicting activities of the H_4R that depend on its activation on different hematopoietic cells, additional research is needed to clarify whether H_4R agonists can yield promising drugs in the treatment of autoimmune diseases.

CONCLUSIONS

Histamine receptors play multiple roles in immune reactions and autoimmune disorders. Strategies aimed at interfering with the histamine axis may have relevance in the therapy of autoimmune diseases of the CNS as histamine may determine, through different receptor activation pathways, a shift in T helper cell subpopulation, may influence migration of lymphocytes and myeloid cells during CNS invasion, interfere with antigen presentation at the immune synapse level and finally, determine variations in normal neuronal functions. It will be of paramount importance to define the temporal sequence of histamine receptor activation during disease initiation in peripheral tissues and during CNS invasion. Hopefully, this will help the scientific community to put the sometimes confusing and contradictory observations reviewed here into better focus and provide a perspective for evaluating potential therapeutic interventions using histaminergic compounds.

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Proton- and ammonium-sensing by histaminergic neurons controlling wakefulness

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The histaminergic neurons in the tuberomamillary nucleus (TMN) of the posterior hypothalamus are involved in the control of arousal. These neurons are sensitive to hypercapnia as has been shown in experiments examining c-Fos expression, a marker for increased neuronal activity. We investigated the mechanisms through which TMN neurons respond to changes in extracellular levels of acid/CO_2. Recordings in rat brain slices revealed that acidification within the physiological range (pH from 7.4 to 7.0), as well as ammonium chloride (5 mM), excite histaminergic neurons. This excitation is significantly reduced by antagonists of type I metabotropic glutamate receptors and abolished by benzamil, an antagonist of acid-sensing ion channels (ASICs) and Na^+/Ca^{2+} exchanger, or by ouabain which blocks Na^+/K^+ ATPase. We detected variable combinations of 4 known types of ASICs in single TMN neurons, and observed activation of ASICs in single dissociated TMN neurons only at pH lower than 7.0. Thus, glutamate, which is known to be released by glial cells and orexinergic neurons, amplifies the acid/CO_2-induced activation of TMN neurons. This amplification demands the coordinated function of metabotropic glutamate receptors, Na^+/Ca^{2+} exchanger and Na^+/K^+ ATPase. We also developed a novel HDC-Cre transgenic reporter mouse line in which histaminergic TMN neurons can be visualized. In contrast to the rat, the mouse histaminergic neurons lacked the pH 7.0-induced excitation and displayed only a minimal response to the mGluR I agonist DHPG (0.5 μM). On the other hand, ammonium-induced excitation was similar in mouse and rat. These results are relevant for the understanding of the neuronal mechanisms controlling acid/CO_2-induced arousal in hepatic encephalopathy and obstructive sleep apnoea. Moreover, the new HDC-Cre mouse model will be a useful tool for studying the physiological and pathophysiological roles of the histaminergic system.

Keywords: mGluR, histamine, HDC-Cre, waking, ASIC

INTRODUCTION

The mammalian brain depends on a constant supply of glucose and oxygen. Lack of either one of them rapidly leads to failure of neuronal function. Extracellular levels of CO_2 change in response to alterations of blood oxygen levels and are fundamental physicochemical signals controlling breathing and wakefulness. However, the mechanisms connecting changes in extracellular CO_2 to arousal are unclear.

Arousal-inducing systems are modulated, either directly or indirectly, in response to hypercapnia or acidification. For instance, serotonin neurons from the dorsal raphe respond to CO_2 and genetic deletion of serotonin neurons abolishes the ventilatory response to hypercapnia (Corcoran et al., 2009; Buchanan and Richerson, 2010). Similarly, previous studies have shown that the orexin (hypocretin) neurons in the perifornical area of the posterior hypothalamus are highly sensitive to minor changes in the extracellular pH (Williams et al., 2007), displaying a 100% increase in firing rate in response to a 0.1 unit acidic shift. Furthermore, in wild type but not in orexin-deficient mice augmented ventilation is observed during the 2 h following a brief hypercapnia episode (Terada et al., 2008), implicating the orexin system in the ventilatory response.

The tuberomamillary nucleus (TMN) in the posterior hypothalamus contains the wake promoting histamine system, which provides a complementary and synergistic control of wakefulness together with the orexin neurons (Anaclet et al., 2009). The ventrolateral TMN group of neurons shows increased c-Fos expression indicating increased neuronal activity (Johnson et al., 2005) in response to hypercapnia and consistent with the increased arousal response. It is unclear however, whether CO_2 or protons can directly affect TMN neurons or this is the consequence of a system response. In particular, it is possible that histaminergic neurons are excited indirectly via other arousal systems since they receive excitatory afferents from orexinergic neurons (Eriksson et al., 2001) in addition to inputs from other arousal centers of the brain (Haas and Panula, 2003; Haas et al., 2008).

Acid-induced excitation is a hallmark of chemosensing neurons (Williams et al., 2007) and contrasts with the majority of brain neurons which respond to acidification with a hyperpolarization. The cellular mechanisms underlying acid-induced

excitation are diverse. In addition to direct activation of acid-sensitive ion channels (ASICs), acidification or hypercapnia may cause ATP-release from astrocytes. In brainstem, ATP-activation of respiratory neurons through P2Y1 receptors is a core event in the fundamental reflex controlling breathing (Gourine et al., 2010). In addition, some acid-sensing mechanisms can be activated by ammonium (Pidoplichko and Dani, 2006) and hyperammonemia leads to intracellular acidification (Kelly and Rose, 2010). In hepatic encephalopathy with ammonia as a main pathogenic factor, brain histamine concentration is increased (Lozeva et al., 2003).

This study investigates the role of the histaminergic system in acid/CO_2-sensing and characterizes the mechanisms involved. We focus in detail on the ventrolateral part of the TMN since it is located close to the brain surface and may contact the cerebrospinal fluid. The expression of possible effector systems in the ventrolateral and the medial subdivisions of TMN are compared. The action of pH 7.0 is also compared with that of ammonium chloride at 5 mM. Despite similarity of time course and magnitude, we show that different mechanisms are involved in the effects of protons and ammonia. We identify glutamate acting through mGluR I as a mediator of acid-induced excitation in rats but not in mice.

MATERIALS AND METHODS

PATCH-CLAMP RECORDINGS FROM RAT OR MOUSE TMN NEURONS IN SLICES

Housing of rats, mice, and all animal procedures were in accordance with the Animal Protection Law of the Federal Republic of Germany and European Union: EEC (86/609/EEC) directives. They obtained approval of the UTSW Medical Center Institutional Animal Care and Use Committee (Dallas, TX, USA). All efforts were made to reduce the number of animals used. Recordings from histaminergic neurons in male Wistar rat (22–26 day old) or mouse hypothalamic slices were performed as previously described (Chepkova et al., 2012; Yanovsky et al., 2011). Cell-attached recording was used to register action potential currents (Perkins, 2006). Visual identification of histaminergic neurons was confirmed at the end of experiments by application of the histamine 3 (H3) receptor agonist R-α-methylhistamine (0.2 μM) (Yanovsky et al., 2012) (in mM): KCl 130, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 0.25, glucose 5, Hepes 5, Mg-ATP 5, EGTA 10, Na-GTP 0.3, pH 7.3 adjusted with 1 M KOH. Temperature was held at 33 ± 0.5° if not mentioned otherwise. For gramicidin-perforated patch-clamp recording, (Akaike, 1996) pipettes (4.5–6.0 MΩ) were tip-filled with (in mM): K-gluconate 138, KCl 11, Hepes 10, $MgCl_2$ 2, pH 7.3, and back-filled with the same solution containing 50 μg/ml of gramicidin (Sigma). Gramicidin was dissolved in dimethyl sulfoxide (5 mg/0.1 ml), and then was diluted to the final concentration with recording solution. Gramicidin-perforated patch experiments were started when series resistance was below 100 MΩ. After identification of neurons properties, the holding potential was set to −50 mV. Voltage signals and synaptic currents were filtered at 3 kHz with a four-pole Bessel filter, sampled at 10 kHz using pCLAMP9 software (Axon Instruments, Union City, CA, USA), and stored on a PC for off-line analysis. Cells were discarded if their capacitance transients changed during recordings by more than 10%. Solutions of different pH were applied for 7 min through the bath perfusion system. They were obtained by changing $NaHCO_3$ concentrations in the basal recording solution according to the protocols of Jarolimek et al. (1990). Briefly, to achieve pH 6.8 saturated with carbogen and heated to 33.5°C the control solution was supplemented with 5 mM $NaHCO_3$; for pH 7.0 ± 0.05 with 9 mM $NaHCO_3$, for pH 7.2 ± 0.05 with 14 mM $NaHCO_3$. Given variations of pH are averages of measurements done on different experimental days. The control recording solution (ACSF: artificial cerebrospinal fluid, pH 7.4) was composed of (in mM): NaCl 125, KCl 3.7, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO4 1.3, $NaHCO_3$ 25, D-glucose 10.

ELECTROPHYSIOLOGY IN ACUTELY ISOLATED NEURONS FROM SLICES

The TMN was dissected from posterior hypothalamic slices (450 μm thick) and incubation with papain in crude form (0.3–0.5 mg/ml) for 20–30 min at 37°C. After rinsing the tissue was placed in a small volume of recording solution with the following composition (in mM): NaCl 150, KCl 3.7, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 10, glucose 10 (pH 7.4). Cells were separated by gentle pipetting and placed in the recording chamber. TMN neurons were recorded and identified as previously described (Sergeeva et al., 2010; Yanovsky et al., 2012). Briefly, patch electrodes were sterilized by autoclaving and filled with the electrode solution for whole-cell recordings (in mM): KCl 130, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 0.25, glucose 5, Hepes 5, Mg-ATP 5, EGTA 10, Na-GTP 0.3, pH 7.3 adjusted with 1 M KOH. The cells were voltage-clamped by an EPC-9 amplifier. The holding potential was −50 mV. An acutely isolated cell was lifted into the major chute of the application system, where it was continuously perfused with the sterile control bath solution. Substances were applied through a glass capillary (application tube), 0.08 mm in diameter. All solutions flowed continuously, gravity-driven, at the same speed and lateral movements of the capillaries exposed a cell either to control- or test-solutions. Solutions with different pH were prepared by the addition of different amounts of HCl to the recording solution (see above). In some experiments solutions at pH < 6.5 contained 10 mM MES [2-(N-morpholino) ethanesulfonic acid, Sigma] instead of HEPES. As the obtained pH_{50} did not differ between the two buffers data were pooled. To evaluate the proton sensitivity of the cells the mean of the peak current amplitude was fitted with a dose-response curve using the equation:

$$y = [A1 - A2/1 + (x/x_0)^p] + A2, \quad (1)$$

where A1 and A2 are the bottom and top asymptote, x_0 is the half-activation between A1 and A2 and p is Hill's slope.

ASIC—EXPRESSION ANALYSIS (SINGLE-CELL RT-PCR)

Amplification of cDNAs encoding for histidine decarboxylase was performed as previously described (Sergeeva et al., 2002, 2005, 2010). The expected size of the amplification product was 457 bp. For the first amplification round of the ASIC family we used following degenerate primers Dglo: 5′-ccRatRaacaRBcccatctg-3′ and Dgup: 5′-agatYcacagYcagKMKgaRcc-3′. In the second amplification round the primers varied for each ASIC-channel: ASIC 1 up: 5′-cctagtggagaaagaccaggaata-3′ was used

in combination with Dglo (size of PCR product: 276 bp.), ASIC 2 lo: 5′-ccttttctgccagtagaccga-3′ was taken with Dgup (321 bp), ASIC 3 up: 5′-gggtgactgcaataccgcat-3′ with ASIC 3 lo: 5′-tcattcgacagccacacttc-3′ (164 bp) and ASIC 4 up: 5′-gaacagcggctaacttatctgc-3′ with ASIC 4 lo: 5′-catgggccctcagagccc-3′ (263 bp). For the analysis of ASIC1 splice variants (ASIC1a and ASIC1b) amplification was done with the following primers: ASIC1up: 5′-tccctgggcctgctgctg-3′ and ASIC1lo: 5′-gtggccgcccatcttg-3′ (size of PCR product for the ASIC 1b: 324 bp and for the ASIC 1a: 360 bp). Digestion of ASIC1a with the restriction endonuclease Eco RI (Invitrogen) confirmed presence of both splice variants in cDNA derived from the trigeminal ganglion: undigested amplimer of 324 bp size (ASIC1b) was seen together with ASIC1a restricted cDNA fragments of 210 bp und 150 bp size. Thin-walled PCR tubes contained a mixture of first strand cDNA template (1–1.5 μl), 10× PCR buffer, 10 pM each of sense and antisense primer, 200 μM of each dNTP and 2.5 units Taq polymerase. The final reaction volume was adjusted to 10 μl with nuclease-free water (Promega, Mannheim, Germany). ($MgCl_2$) was 2.5 mM. Amplifications were performed on a thermal cycler (Perkin Elmer, GeneAmp 9600, Weiterstadt, Germany). In each round, 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 s, annealing at 50–53°C for 48 s, and extension at 72°C for 1 min. For the second amplification round, 1.1 μl of the product of the first PCR was used as a template. The Taq enzyme, PCR buffer, Mg^{2+} solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Products were visualized by staining with ethidium bromide and analyzed by electrophoresis in 2% agarose gels. Randomly selected PCR products were purified in water (PCR purification kit, Qiagen, Erkrath, Germany) and sequenced on an automatic sequencing machine (ABI, model 377, Weiterstadt, Germany). Comparison with corresponding GENBANK sequences revealed no mismatches (ASIC 1: NM_024154.2, ASIC 2: NM_012892.2, ASIC 3: NM_173135.1, ASIC 4: NM_022234.2).

SEMIQUANTITATIVE REAL-TIME RT-PCR

The TMN was dissected from hypothalamic slices and total cellular mRNA was isolated using an mRNA isolation kit (Pharmacia Biotech) according to the manufacturer's protocol. Total mRNA was eluted from the matrix with 30 μl of RNase-free water. For the reverse-transcription 8 μl of eluted mRNA was added to 7 μl of reagents mixture prepared according to the protocol of the "first strand cDNA synthesis kit" (Pharmacia Biotech). After incubation for 1 h at 37°C the reverse transcription reaction was stopped by freezing at –20°C. The reverse-transcription reactions were not normalized to contain the equivalent amounts of total mRNA. The PCR was performed in a PE Biosystems GeneAmp 5700 sequence detection system using the SYBR green master mix kit. Each reaction contained 2.5 μl of the 10xSYBR green buffer, 200 nM dATP, dGTP, and dCTP and 400 nM dUTP, 2 mM $MgCl_2$, 0.25 units of uracil N-glycosylase, 0.625 units of Amplitaq Gold DNA polymerase, 10 pM forward and reverse primers, 5 μl of 1:4 diluted cDNA, and water to 25 μl. All reactions were normalized to β-actin expression. Primers for the β-actin, peripherin and mGluR I cDNA amplification were published previously (Chepkova et al., 2009; Yanovsky et al., 2011). Primers for the HDC—cDNA amplification (Sergeeva et al., 2002) and for the ASIC-cDNAs were the same as those used in the second round of single-cell RT-PCR. Primers for the histamine *N*-methyl-transferase were: HNMT up: 5′-ttcttggcacaaggagaca-3′and HNMT lo: 5′-gctaagagaccatggaaaaat-3′. The reactions were performed in tubes with MicroAmp optical caps. The reactions were incubated at 50°C for 2 min to activate uracil N′-glycosylase and then for 10 min at 95°C to inactivate the uracil N′-glycosylase and activate the Amplitaq Gold polymerase followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. The PCR reactions were subjected to a heat dissociation protocol (PE Biosystems 5700 software). Following the final cycle of the PCR, the reactions were heat-denaturated over a 35°C temperature gradient at 0.03°C/s from 60°C to 95°C. Each PCR product showed a single peak in the denaturation curves. Standard curves for real-time PCR protocols with all primer-pairs obtained with sequential dilutions of one cDNA sample (till 1:64) were found optimal (linear regression coefficients were > 0.95). Semiquantitative analysis of target gene expression relative to the β-actin endogenous control was performed according to the "$2^{-\Delta\Delta Ct}$"(ΔFold) method as described previously (Chepkova et al., 2009).

GENERATION OF HDC-Cre BAC TRANSGENIC MICE AND HISTOCHEMICAL VALIDATION OF THE TRANSGENE EXPRESSION WITH THE HELP OF LacZ- OR Tmt-REPORTER MICE

We generated several lines of transgenic mice that express cre recombinase (Cre) within histidine decarboxylase-expressing cells. These animals were made by first using various ET-cloning "recombineering" technologies (Lee et al., 2001; Muyrers et al., 2001); to construct an HDC-Cre transgene-containing bacterial artificial chromosome (BAC). The original HDC BAC (RP24-141N14), which was purchased from BACPAC Resources Center at Children's Hospital Oakland Research Institute, spanned the entire coding region of HDC and furthermore contained approximately 92.42 kb sequence upstream of the HDC start codon and approximately 13.91 kb sequence downstream of the HDC stop codon. This BAC was transformed into EL250 cells by electroporation. EL250 cells were provided by N. Copeland; they contain heat-inducible recE and recT recombinases for homologous recombination and arabinose-inducible Flp-recombinase for site-specific recombination at frt sites (Lee et al., 2001). Next, a DNA fragment containing the coding sequence of Cre recombinase followed by an SV40 polyadenylation (polyA) signal and a kanamycin resistance gene flanked by frt sites (FKF) was inserted into the HDC BAC, at the translational start site of HDC, by ET-cloning. The construction of the Cre-polyA-FKF cassette was described previously (Dhillon et al., 2006). This insertion resulted in the removal of 2194 bp of the HDC gene (this included the latter portion of exon 1 and the first portion of exon 2—which encodes the first 47 amino acids of histidine decarboxylase—and the intervening intron 1). Finally, the kanamycin resistance gene was removed by arabinose induction of Flp-recombinase, and the Cre recombinase coding region was sequenced to ensure that no mutations had been introduced. The Cre-modified HDC BAC was submitted to the UTSW Medical

Center Transgenic Core Facility for microinjection into pronuclei of fertilized one-cell stage embryos of C57Bl6/J mice. We were successful in generating multiple potential HDC-Cre founder mice, of which one was shown to contain the expected Cre expression and activity within the CNS. Oligonucleotide primers used to confirm the genotype of mice harboring the HDC-Cre transgenes were as follows: M249: 5′-CAGGGAGTGCACAGCACAGACAA-3′ and M247: 5′-TGCGAACCTCATCACTCGTTGCAT-3′. The mice used in this study were on a pure C57BL6/J genetic background. Animals were housed under 12 h of light/12 h of dark per day in a temperature-controlled environment. They were fed standard chow diet (details) and had free access to water.

For histological validation of Transgene expression, HDC-Cre mice were bred to Rosa26-lox-STOP-lox-lacZ reporter mice [B6.129S4-Gt(ROSA)26–Sor^{tm1Sor}/J; stock#003474; The Jackson Laboratory, Bar Harbor, Maine], in which a transcriptional stop cassette is removed only in the presence of Cre activity, allowing for visualization of cells with Cre activity by assaying for β-galactosidase or for lacZ-IR, as below. Mice derived from the HDC-Cre X Rosa26-lox-STOP-lox-lacZ crosses were deeply anesthetized with an intraperitoneal injection of chloral hydrate (500 mg/kg) and subsequently perfused transcardially with diethylpyrocarbonate (DEPC)-treated 0.9% saline followed by 10% neutral buffered formalin. Brains were removed, stored in the same fixative for 4–6 h at 4°C, and then immersed in 20% sucrose in DEPC-treated phosphate buffered saline (PBS), pH 7.0 at 4°C overnight. Brains were sectioned coronally, using a sliding microtome, into five equal series at a 25 μm thickness. Brain sections were permeabilized and blocked in 3% normal goat serum/0.3% Triton X-100 for 1 h, and then incubated for 48 h at 4°C in 1:5000 guinea pig anti-HDC antiserum (Catalog number: B-GP 265-1, Lot: HS4061; ALPCO Diagnostics, Salem, NH). After washing in PBS, sections were incubated in Alexa Fluor 594 goat anti-guinea pig IgG (Invitrogen, Carlsbad, CA; 1:300) for 1 h at room temperature, followed by more PBS washes. For lacZ staining, sections were subsequently incubated for 24 h at 4°C in 1:10000 chicken anti-lacZ antiserum (Catalog number: ab9361, Lot: 713063, Abcam, Cambridge, MA). After washing in PBS, sections were incubated in Alexa Fluor 488 goat anti-chicken IgG (Invitrogen, Carlsbad, CA; 1:300) for 1 h at room temperature, followed by more PBS washes. Finally, the sections were mounted in Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and viewed using microscopy (Axioskop 2, Carl Zeiss). Images were taken using both the red filter and green filter at each level, and were placed side-by-side. All the cells at each level with either HDC-immunoreactivity alone, lacZ-immunoreactivity alone, or both were counted. An imaging editing software program, Adobe PhotoShop 7.0 (San Jose, CA) was used to adjust contrast, brightness, and color of the photomicrographs, and to merge the images taken using the red and green filters.

To be able to visualize TMN neurons in slices we crossed the HDC-Cre mouse to the Tomato reporter mouse B6J/N. Cg-Gt(ROSA) 26Sor$^{tm14(CAG-tdTomato)}$ (Jackson Laboratory, #007908) yielding the HDC/Tmt mice. Offspring (both genders) carrying both transgenes were used for the slice recordings. Histamine immunostainings were done according to the previously published protocols (Yanovsky et al., 2011). Wilde type littermates of HDC-Cre breeders were used as a control in electrophysiological and molecular biological experiments (comparative gene expression analysis).

DATA ANALYSIS

Experiments were conducted and analyzed with commercially available software (TIDA for Windows, HEKA, Lambrecht, Germany) and with Clampex 9.0. All graphs were assembled in GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) and Photoshop.

Data are presented in text as the mean ± SEM. Statistical analysis was done with the non-parametrical Mann-Whitney *U*-test (real-time PCR) or unpaired Student *t*-test (comparison of magnitude of excitation between groups in cell-attached firing rate recordings). Comparison with the baseline activity in each individual experiment was done using paired Student *t*-test. Differences were considered statistically significant if $p < 0.05$.

RESULTS

ACSF ACIDIFICATION EXCITES RAT E2 TMN NEURONS

As it was shown previously that only the ventrolateral TMN of the rat shows increased c-Fos expression in response to systemic hypercapnia (Johnson et al., 2005), we performed now patch-clamp recordings from ventrolateral TMN, which corresponds to the E2 group (**Figure 1A**). Acidification of the bath solution for 7 min increased the firing rate of most rat TMN neurons recorded in cell-attached mode (23 out of 28 neurons, 82%). Unresponsive neurons were not further investigated. On average pH 7.0-induced excitation amounted to 166 ± 15% of control firing rate ($n = 23$ at 33°C). The mean firing rate in these experiments under control conditions was 1.14 ± 0.05 Hz ($n = 28$). The excitatory response did not show a significant temperature-dependence: at room temperature it reached 157 ± 40% of control ($n = 6$). The period of high initial excitation in response to the acidification was followed by a reduction of activity with subsequent suppression of activity below control level in the beginning of the washout period (**Figure 1B**). The response to pH 7.0 in perforated patch whole-cell voltage-clamp recordings (Vh = −50 mV) perfectly matched the time course of responses seen in the cell-attached configuration (**Figure 1C**). The initial inward current of 22 ± 7 pA (starting after 1.9 ± 0.3 min, maximum after 4.6 ± 0.6 min) was followed by an outward current of 8.5 ± 1.4 pA (0.9 ± 0.1 min after beginning the washout) (**Figure 1C3).** Note a slight and delayed decrease in membrane resistance measured in parallel experiments (180 ± 10 MΩ versus 190 ± 4 MΩ in control, $n = 4$, $p > 0.05$), which did not correspond with respect to time to the fast rising phase of the inward current (**Figure 1C4**). Moreover, no robust membrane depolarisation was observed during the first phase of the response ($n = 5$, **Figure 1C2**), whereas the second phase was characterized by reduced firing frequency, outward current and membrane hyperpolarization. The magnitude of facilitation was dependent on the degree of acidification. The firing rate measured in cell-attached configuration was changed by 17 ± 2% for each 0.1 pH unit (**Figure 2A**).

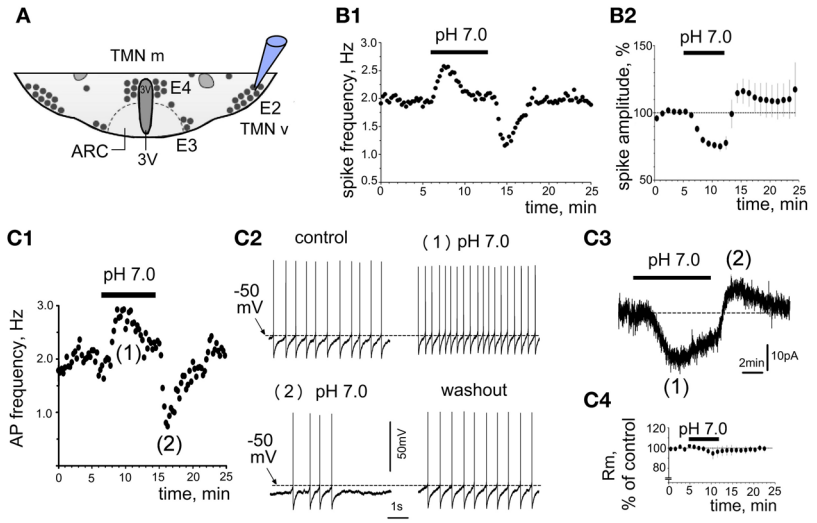

FIGURE 1 | Proton-induced increase in TMN neuron firing. (A) Schematic presentation of the coronal hypothalamic slice indicates the TMN groups (E2 corresponds to ventrolateral TMN, whereas medial TMN contains the E3, E4 groups). 3V, third ventricle; ARC, arcuate nucleus. Electrode points to the recording site E2. **(B1,2)** Firing of TMN neuron recorded in cell-attached mode in control at pH 7.4 and at pH 7.0. **(B1)** Time course of firing-frequency in representative neuron. Every point on the histogram represents the frequency averaged for 15 s of recording. **(B2)** Change in the amplitude of action potential currents during acidification (each point: average recorded during 1 min in 6 neurons). **(C1)** Current clamp recording from one TMN neuron illustrates the action potential (AP) frequency in response to low pH. Each point is the average of 15 s recording. **(C2)** fragments of voltage trace recordings in the same neuron. Note no deflection of the control membrane potential ∼–50 mV during the first phase of the response and a slight hyperpolarization during the second phase. **(C3)** Biphasic current (voltage-clamp mode) recorded from the same neuron as in (C1,2). Note that phase (1) and phase (2) correspond timely to the excitation and inhibition phase in (C1). **(C4)** membrane resistance (average of 4 neurons) measured from current deflections in response to brief voltage steps (–10 mV for 10 ms from holding potential at 10 s intervals) in experiments like the one shown in **(C3)**.

METABOTROPIC GLUTAMATE RECEPTORS BUT NOT P2Y1 RECEPTORS MEDIATE ACID-INDUCED EXCITATION

Previous studies have shown that glial cells respond to acid/CO_2 by the release of ATP, which excites respiratory neurons in brain stem through the P2Y1 receptor (Gourine et al., 2010). We have shown that ATP/ADP excites TMN neurons through P2X (Vorobjev et al., 2003) and P2Y (Sergeeva et al., 2006) receptors. We also found that activation of P2Y1 receptors in hypothalamic slices containing TMN causes release of glutamate which amplifies the excitatory response through type I metabotropic glutamate receptors (mGluR I). Acid-induced excitation of TMN neurons in the present study was not affected by the P2Y1 receptor antagonist MRS 2179 (90 μM, $n = 5$; **Figure 2B**). The metabotropic glutamate receptor type I (mGluR I) antagonists MPEP (10 μM, mGluR5 antagonist) combined with LY 367385 (20 μM, mGluR1 antagonist) (**Figure 2C**) significantly reduced acid-induced excitation ($p < 0.05$). In the presence of mGluR I antagonists the excitation amounted to 123 ± 10% ($n = 6$) of baseline activity versus 211 ± 31% ($n = 10$) in the parallel control experiments. The AMPA and NMDA receptor antagonists CNQX (10 μM) and D-AP5 (50 μM) neither affected proton-induced excitation (199 ± 30%, $n = 3$) nor increased the block of excitation by metabotropic glutamate receptor antagonists when all glutamate receptor antagonists were applied together (138 ± 28% of control, $n = 4$, **Figure 2C**). Thus proton-induced excitation is largely (∼70%) mediated by metabotropic glutamate receptors.

EXPRESSION OF ACID-SENSORS IN VENTROLATERAL VERSUS MEDIAL TMN

Electrophysiological experiments revealed that mGluR mediate most of the acid-induced excitation of TMNv. Therefore, we investigated differential expression of these receptors with respect to functional diversity between TMNv and TMNm (**Figure 2D**). Semiquantitative RT-PCR was performed from mRNA isolated from two parts of TMN as shown in **Figure 2D**. The lateral part contained largely the E2 dense group of TMN neurons, whereas in the medial part only a minority of cells were histaminergic (groups E3 and E4, see **Figure 1A**). Therefore, histaminergic markers, such as histidine decarboxylase (HDC) or peripherin (prph) were expressed significantly higher in the ventrolateral part compared to the medial TMN (**Figure 2D**). Expression of mGluR1 did not differ between ventrolateral and medial TMN, whereas mGluR5 expression in the ventrolateral TMN represented 79% of that in the medial TMN ($p < 0.05$).

We then investigated the expression of classical acid-sensors, such as acid-sensing ion channels (ASICs) which others found expressed in the majority if not in all central neurons (Lingueglia, 2007; Grunder and Chen, 2010). We found the expression of ASIC3 significantly higher in ventrolateral TMN compared to the medial subdivision (**Figure 2D**). Interestingy, this member of the ASIC family responds to acidic pH with a fast inactivating followed by a sustained sodium current and is sensitive to physiological changes in proton concentration (Waldmann et al., 1997).

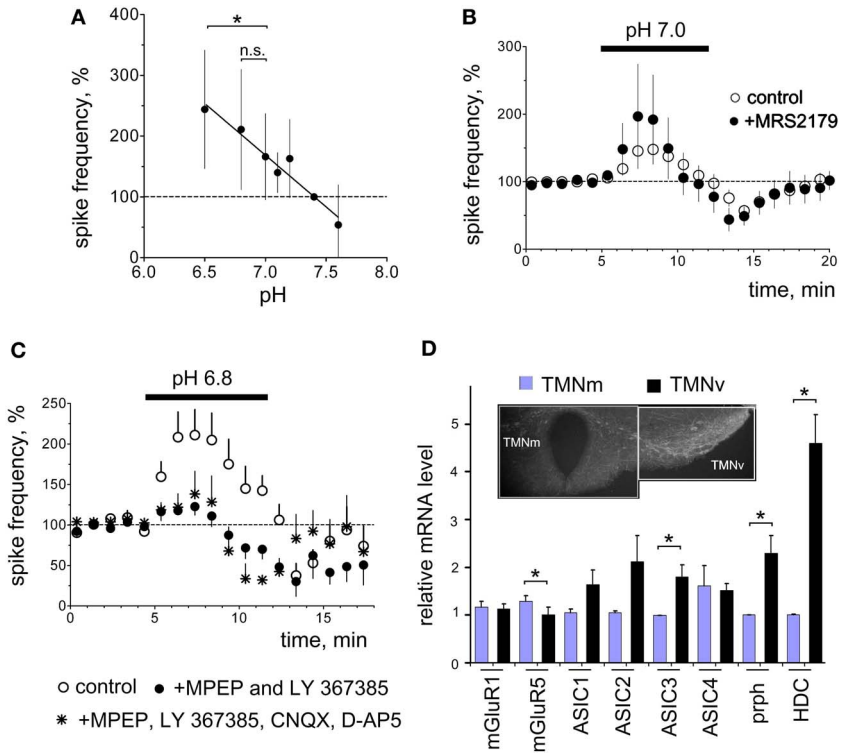

FIGURE 2 | Functional identification and expression analysis of acid-sensors in TMN. (A) Acid-induced excitation depends on the degree of acidification. Each data point is an average of 4–23 neurons. The difference in excitation between pH 6.5 and 7.0 but not between pH 6.8 and 7.0 is significant ($*p < 0.05$). **(B)** The P2Y1 receptor antagonist MRS2179 ($n = 5$) does not prevent pH 7.0-excitation. Control diagram represents the average of 23 neurons. **(C)** Facilitation of firing in control and in the presence of the metabotropic GluR (mGluR) antagonists MPEP (mGluR5, 10 μM) and LY367385 (mGluR1, 20 μM) or mGluR antagonists combined with inotropic glutamate receptor antagonists CNQX (10 μM, AMPA) and D-AP5 (50 μM, NMDA). **(D)** mRNA levels normalized to the minimal expression level for mGluR1, mGluR5, ASIC (1–4), peripherin (prph) and histidine decarboxylase (HDC) detected with real-time RT-PCR (eight rats aged from 23 to 27 days). Hypothalamic slice shows regions dissected for the analysis. TMN neurons stained with the rabbit-anti-histamine antibody are visualized with the secondary donkey-anti-rabbit antibody conjugated to Alexa fluor 488. $*p < 0.05$ Mann–Whitney.

Expression of ASIC3 in the hypothalamus was shown recently (Meng et al., 2009). Members of ASIC 1 and ASIC2 subfamilies are activated by much lower pH values ($pH_{50} \sim 4.1$–6.2) except for ASIC 1a (Lingueglia, 2007), which also responds to ammonium (Pidoplichko and Dani, 2006).

TMN NEURONS ISOLATED FROM SLICES DO NOT RESPOND TO pH 7.0

Solutions with pH ranging from 7.0 to 4.0 were applied for 2 s at 30 s intervals in 17 acutely isolated TMN neurons. In none of the investigated neurons did pH 7.0 evoke measurable currents, whereas pH 6.5 evoked currents of 37 ± 8 pA in 82% of neurons (**Figure 3B**). Half-maximal current amplitude was evoked by pH 5.8 ± 0.1. In 12 neurons a solution with pH 6.0 was applied alone or in the presence of the ASIC blocker benzamil hydrochloride (bzm). Bzm 10 μM suppressed the peak current amplitude to $59 \pm 8\%$ of control ($n = 4$) without effects on plateau current. Bzm 20 μM suppressed peak current to $30 \pm 6\%$ and plateau to $57 \pm 11\%$ of control ($n = 12$)(see **Figure 3**). The reversal potential of the acid-induced current was determined in 3 TMN neurons. Currents in response to acidic pH were inward at negative membrane potentials and became outward at potentials more positive than $+64 \pm 4$ mV (the predicted reversal potential for the sodium ion under our recording conditions is +63.4 mV). Previous studies have shown that ASICs are mainly permeable for sodium ions (Bassilana et al., 1997; Waldmann et al., 1997). Single-cell RT-PCR analysis was performed from 32 TMN neurons. We found variable expression of all four known ASICs in histaminergic cells: thus ASIC1 transcripts were detected in 71%, ASIC2 in 56%, ASIC3 in 63% and ASIC 4 in 47% of TMN neurons ($n = 32$, **Figures 3C,D**). Restriction analysis of PCR fragments containing the splice region of ASIC1 showed that only the ASIC1a subtype is expressed in TMN. The whole TMN region was analyzed from four rats. This result was confirmed by sequencing. Thus, similar to dopaminergic neurons of the midbrain (Pidoplichko and Dani, 2006) TMN neurons express ammonium-sensitive ASIC1a.

Na^+/Ca^{2+} EXCHANGE AND Na^+/K^+ ATPase ARE ESSENTIAL FOR ACID-EVOKED TMN EXCITATION

To test the participation of ASICs in the acid-induced excitation of TMN, we applied pH 7.0 in the presence of bzm (20 μM, $n = 3$) and found that the firing increase was virtually abolished (**Figure 4A**). This can be explained by the

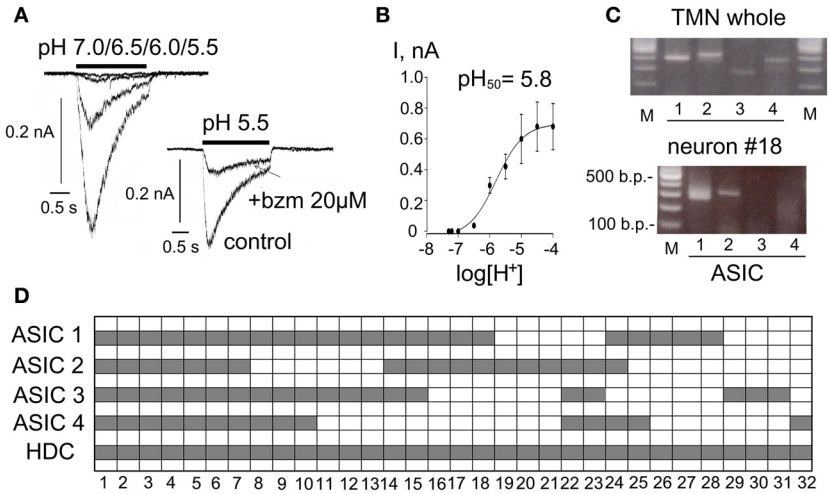

FIGURE 3 | Expression and function of ASIC (acid sensing ion channels) in TMN neurons. (A) Acid-induced currents in two representative neurons. Neurons are moved to the test solution with indicated pH for 2 s from the control ACSF (pH 7.4). Benzamil hydrochloride (bzm) abolishes the fast inactivating phase of the response and reduces the non-desensitizing component. **(B)** Averaged current-pH histogram represents data obtained from 17 neurons. Data points are fitted with the logistic equation. **(C)** Photographs of gels illustrate RT-PCR analysis of ASIC (1–4) expression in whole TMN and in one representative neuron. **(D)** Floating bar histograms show co-expression of 4 ASICs in 32 TMN neurons positive for histidine decarboxylase (HDC).

simultaneous antagonism toward both: ASICs and Na^+/Ca^{2+} exchange, which works in a reverse (depolarizing) mode downstream to mGluR activation. In addition, bzm blocks Na^+/H^+ exchange, preventing intracellular acidification during exposure to acid.

Despite ubiquitous expression of ASICs in TMN neurons, the following findings argued against a massive ion flow through such membrane channels in response to pH 7.0 and favor the idea that the sodium gradient is reduced during the excitation phase of the response, possibly through inhibition of Na^+/K^+ ATPase by protons, as described previously for cardiomyocytes (Fuller et al., 2003): (1) lack of a pronounced membrane depolarisation during increased neuronal firing frequency; (2) decreased amplitude of action potential currents during the excitation phase; (3) no significant changes in R_{in} seen in perforated patch configuration. Fuller et al. (2003) demonstrated that Na^+/K^+ATPase subtypes expressed in brain and heart are inhibited by a proton-activated factor during the initial phase of sodium influx in response to ischemia, which leads to membrane depolarisation. Increased intracellular sodium concentration turns on the Na^+/Ca^{2+} exchange in the reverse mode leading to the extrusion of sodium, intracellular acidification and rise of calcium (Fuller et al., 2003). Therefore, the second, inhibitory phase of the response to acidification recruits calcium-activated potassium channels acting synergistically with the reverse electrogenic Na^+/Ca^{2+} exchange (3 sodium ions are exchanged for 1 calcium ion) to produce a hyperpolarization. Upon return to the control ACSF (pH 7.4) the Na^+/K^+ ATPase regains its functionality and contributes to the hyperpolarization. This scenario, schematically depicted in **Figure 4**, could explain the biophysical characteristics of the first (excitatory) and second (inhibitory) phase of the TMN response to the mild (physiological) acidification. Indeed, Na^+/Ca^{2+} exchange and Na^+/K^+ ATPase were found to be essential for the first excitatory phase of the acid-induced excitation.

The Na^+/K^+ ATPase blocker ouabain (0.5 μM) increased the firing frequency of TMN neurons to 214 ± 20% of control ($p < 0.0001$; $n = 8$). In the presence of ouabain acidification of the extracellular solution produced neither excitation (**Figure 4B**) nor the rebound inhibition upon return to the control medium. When pH 7.0-induced currents were recorded in the presence of ouabain (perforated patch-clamp), outward currents were seen instead of inward currents in control experiments (**Figure 4B**). This can be explained by the activation of the reverse mode of Na^+/Ca^{2+} exchange which leads, together with calcium-activated potassium currents to hyperpolarization.

AMMONIUM-INDUCED EXCITATION DIFFERS FROM ACID-INDUCED EXCITATION OF TMN

As ASIC1a responds to ammonium (Pidoplichko and Dani, 2006) we compared the pharmacology of ammonium-induced excitation with the acid-induced excitation in the following experiments. In cell-attached recordings NH_4Cl evoked a transient increase in firing rate to 177 ± 14% of control ($n = 16$). In some neurons an inhibition similar to that seen in experiments with pH 7.0 was present at the beginning of the washout period (**Figure 5**). In contrast to the acidification-induced increase in firing rate, the excitation by ammonium was not prevented by mGluR I-antagonists ($n = 4$), by benzamil hydrochloride ($n = 4$) or by ouabain ($n = 3$, **Figure 5**).

Ammonium can be transported instead of K^+ by the Na^+/K^+ ATPase (Kelly and Rose, 2010) or interact with KCC2 (potassium/chloride co-transporter) (Liu et al., 2003). Thus mechanisms of action of ammonium and of protons are different in TMN neurons.

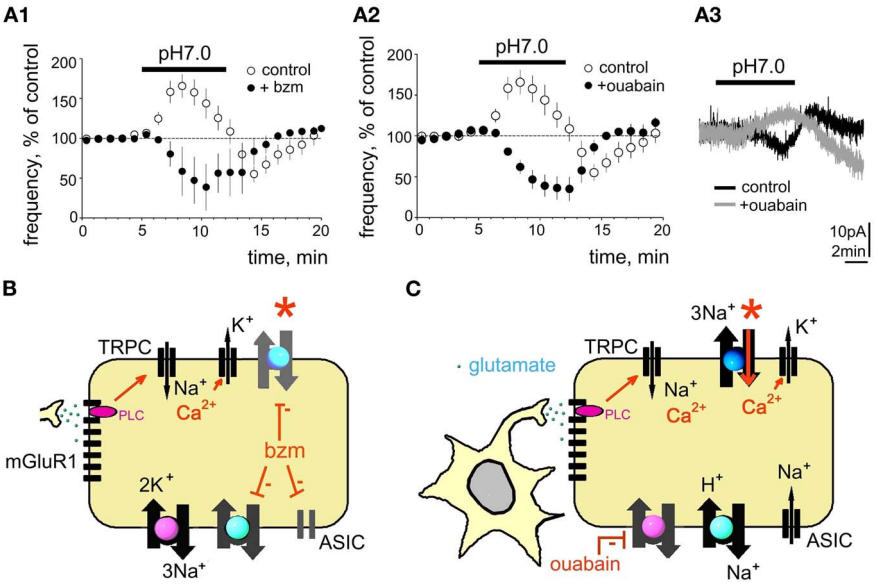

FIGURE 4 | Benzamil or ouabain abolish acid-induced excitation. **(A1)** Benzamil (bzm, 20 μM) blocks acid-induced excitation. **(A2)** Time course of firing frequency in control and in ouabain (0.5 μM). **(A3)** gray trace shows acid-induced outward current recorded in ouabain (perforated patch-clamp mode) superimposed to the black (control) response recorded from the same neuron. **(B,C)** Models explaining action of bzm or ouabain. Bzm inhibits ASIC, Na^+/H^+ and Na^+/Ca^{2+} exchangers. Ouabain **(C)** blocks Na^+/K^+ ATPase, which leads to the compensatory sodium removal by Na^+/H^+ and Na^+/Ca^{2+} exchangers, which is accompanied by a rise of intracellular Ca^{2+} and calcium-activated potassium conductance. Red stars point to block of the direct mode of Na^+/Ca^{2+} exchange in both experimental sessions.

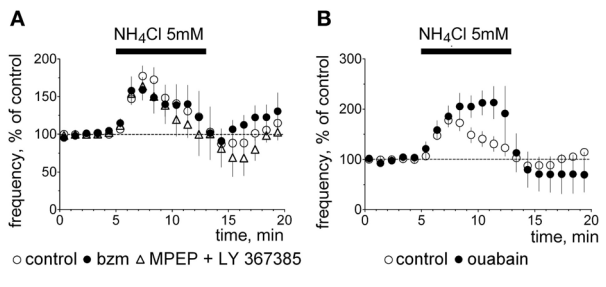

FIGURE 5 | Ammonium-induced excitation. **(A)** Averaged time course diagrams show normalized firing frequency (cell-attached voltage-clamp mode) in control experiments and in the presence of indicated blockers, which abolish pH 7.0-induced excitation. **(B)** Ouabain does not block ammonium-induced excitation.

Table 1 | Co-expression of Cre recombinase activity and HDC within the ventrolateral tuberomamillary nucleus of HDC-Cre transgenic line[1].

Approximate distance of section to Bregma[2]	% lacZ-IR[3] neurons co-expressing HDC-IR	% HDC-IR neurons co-expressing lacZ-IR[3]
−2.30 mm	100	76.3 ± 2.9
−2.70 mm	100	71.1 ± 2.5
−2.92 mm	100	75.7 ± 2.9

[1] *The percentage of HDC-IR/LacZ-IR co-expressing neurons was determined at three different levels through the ventrolateral tuberomamillary nucleus. The data are reported as the mean percentage ± SEM for three different brains.*
[2] *These approximate distances were determined by comparison to figures within the mouse brain atlas of (Paxinos and Franklin, 2004).*
[3] *lacZ-IR is a marker for the presence of Cre recombinase activity.*

ARE METABOTROPIC GLUTAMATE RECEPTORS ESSENTIAL FOR THE ACID-INDUCED EXCITATION? GENERATION AND CHARACTERIZATION OF HDC-Cre MICE

Our experiments in rats showed that metabotropic glutamate receptors of type I, NCX and Na^+/K^+ ATPase are the major players in proton-induced excitation of TMN, but not in response to ammonia. In order to identify the role of the histaminergic system in the response to hypercapnia or/and hyperammonemia it would be important to delete the major sensors only in histidine-decarboxylase (HDC) expressing neurons and see the behavioral response according to the previously published protocols (Johnson et al., 2005). For this purpose an HDC-Cre mouse was generated. A schematic diagram showing derivation of the HDC-Cre mouse is shown in **Figure 6**. In initial experiments a LacZ-reporter mouse was used for the visualization of HDC-Cre transgene in TMN. Results of this analysis are summarized in **Table 1** and **Figure 6**.

For the electrophysiological recordings another reporter mouse was used which allowed us to visualize living TMN neurons in the slices. The co-localization pattern of transgene and histamine in the HDC/Tmt-mouse was similar qualitatively and quantitatively to that seen in the LacZ-reporter mouse. Thus, in the ventrolateral TMN (our recording site) all Tmt+ neurons contained histamine (**Figure 7A**). Among 1101 histaminergic neurons counted in similar fields as shown in **Figure 7A** (21 fields in slices obtained from five mice were investigated) 298 neurons were negative for the Tmt-protein (25.7 ± 2.8% of total in each mouse).

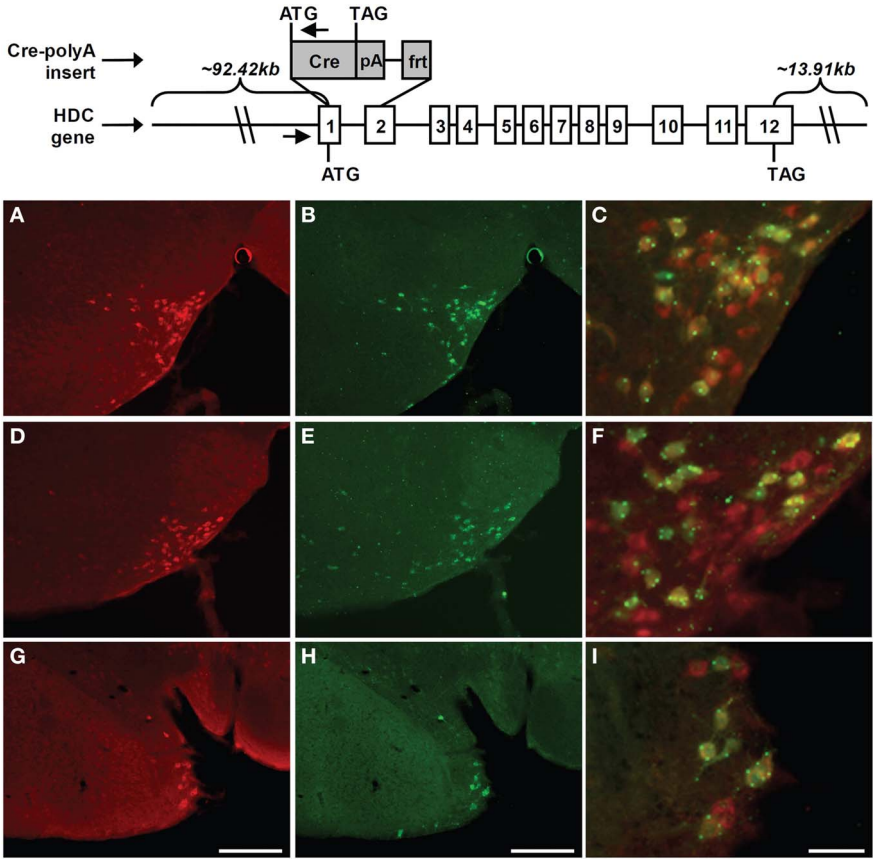

FIGURE 6 | Schematic diagram of the derivation of HDC-Cre transgenic mice and immunohistochemical analysis of transgene expression in TMN. Mice expressing Cre recombinase under the control of the HDC promoter were generated by inserting a Cre recombinase expression cassette into a BAC genomic clone containing HDC. In particular, a 2194-bp region of HDC was replaced by the coding sequence of Cre recombinase followed by an SV40 polyadenylation signal (pA) and an frt site. HDC's exons (denoted by numbered rectangles), and the start codons (ATG) and stop codons (TAG) for HDC and Cre are all indicated. The amounts of mouse chromosomal sequence present in the BAC either upstream of the HDC start codon or downstream of the HDC stop codon also are indicated. Small arrows denote the locations of oligonucleotide primers used for genotyping. Expression of Cre activity within the tuberomamillary nucleus of the HDC-Cre transgenic mouse line was examined in coronal brain sections of Rosa26-lox-STOP-lox-lacZ reporter mice harboring the HDC-Cre transgene. **(A,D,G)** Rostrocaudally arranged brain sections [**(A)** Bregma −2.30 mm, **(D)** Bregma −2.70 mm, **(G)** Bregma −2.92 mm)] processed for HDC-immunoreactivity (red). **(B,E,H)** The same sections processed for lacZ-immunoreactivity (green). **(C,F,I)** Merged HDC (red) and lacZ signals (green), within cells containing co-localized HDC- and lacZ-immunoreactivity, appearing as yellow (yellow dots and green dots reflect highly localized collections of lacZ). Scale bars = 200 μM in **G** and **H** (applies to **A, B, D, E, G, H**); 50 μM in I (applies to **C, F, I**).

The ventrolateral TMN (E2 group) in the mouse is not as compact as in the rat. Moreover, spontaneously firing cells in this region are not always histaminergic. Among 10 recorded cells in wild type (WT) mice ($n = 3$) only five reduced their firing activity in response to the histamine receptor 3 agonist R-α-methylhistamine (RAMH, 2 μM) and were considered histaminergic neurons (**Figure 7**). All red (Tmt+) TMN neurons from HDC/Tmt mice responded to RAMH ($n = 6$) with an inhibition of firing not different from that seen in TMN neurons of WT mice (**Figure 7B**). Orexin A (100 nM) excited mouse TMN (Tmt+) neurons to 255 ± 63% of control frequency ($n = 4$, not shown) similar to our previous results in the rat (Eriksson et al., 2001).

Relative mRNA levels encoding for HDC, peripherin, histamine *N*-methyl-transferase (HNMT) and vesicular monoamine transporter (VMAT2) in the TMN region were found similar between WT and transgenic HDC/Tmt mice (**Figure 7C**). In contrast to the rat neither WT nor Tmt+ mouse TMN neurons were excited by pH 7.0 (**Figure 7D**). Another difference was the significantly smaller excitation by the mGluR I agonist DHPG in mice (to 125 ± 12% of control, $n = 5$) compared to rats (to 220 ± 26% of control ($n = 5$), $p < 0.05$).

There was no difference between ammonium-induced excitation between rat and mouse TMN neurons: 177 ± 14 ($n = 16$) and 240 ± 42% ($n = 6$) of control, respectively (**Figure 8**). Thus the HDC-Cre mouse offers a good model for exploring the role of the histaminergic system in hepatic encephalopathy, characterized by the hyperammonemia and increased histamine level in the brain (Lozeva et al., 2003).

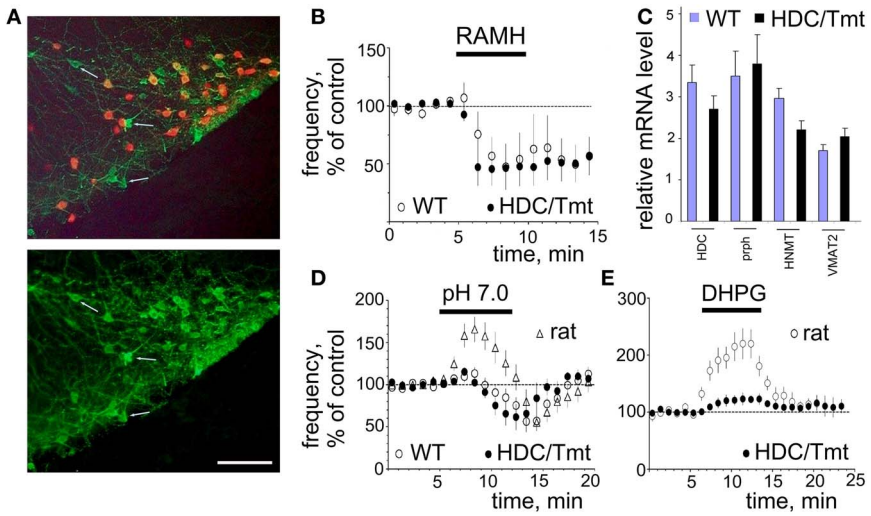

FIGURE 7 | Mouse TMN neurons resemble those of the rat but hardly respond to pH 7.0 and DHPG. (A) Histamine (AF488) co-localization with Tmt protein (expressed in HDC-Cre positive neurons). Note, that virtually all red cells are histamine-positive, but some histamine+ cells do not express HDC-Cre (few cells are indicated by arrows). Scale bar 100 μm. **(B)** The H3 receptor agonist R-(α)-methyl histamine (RAMH, 2 μM) similarly decreases firing rate of mouse WT ($n = 5$) and Tmt+ ($n = 6$) TMN neurons. **(C)** Relative levels of mRNAs encoding for the markers of histaminergic activity in TMN region of 4 WT and 6 HDC/Tmt mice. HDC, histidine decarboxylase; prph, peripherin; HNMT, histamine *N*-methyl-transferase; VMAT2, vesicular monoamine transporter 2. **(D)** Acid-induced excitation is hardly seen in mouse WT ($n = 6$) or Tmt+ ($n = 6$) TMN neurons. Data points are superimposed to the time course diagram of acid-induced excitation from rats ($n = 23$). **(E)** The metabotropic glutamate receptor agonist DHPG (0.5 μM) excites rat TMN neurons to a significantly larger extent ($n = 5$) than mouse TMN neurons ($n = 5$).

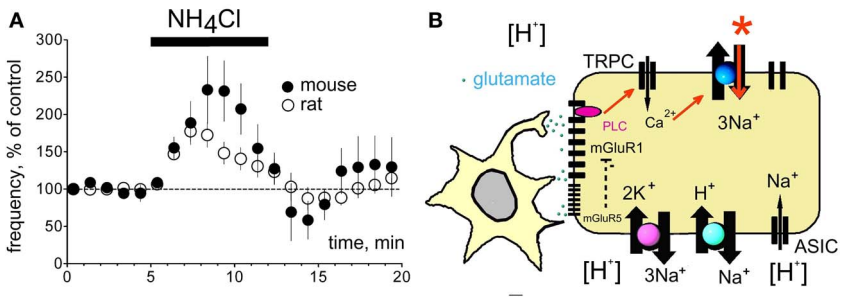

FIGURE 8 | Ammonium and proton-sensing in histaminergic TMN neurons. (A) Excitation by ammonium chloride (5 mM) is not significantly different between mouse and rat. None of the effectors depicted in **(B)** participates in ammonium-sensing. **(B)** Proton sensing cascade in TMN neuron, see discussion.

DISCUSSION AND CONCLUSIONS

The rat histaminergic neurons in the TMN respond to acidification in the physiological range (pH 7.0) with an excitation resembling that of chemosensors in the brainstem. A similar effect has also been described for the neighboring orexin/hypocretin-containing neurons (Williams et al., 2007) which express glutamatergic markers (Torrealba et al., 2003). Optogenetic stimulation of their axons results in glutamatergic currents in some TMN neurons (Schöne et al., 2011), which may add to the acid-response of TMN neurons. In principle, ATP release from glia in response to acid/CO_2 could have excited TMN neurons, however antagonists at purinergic receptors failed to affect the excitation observed here.

We describe an unusually high sensitivity to glutamate of type I metabotropic receptors in TMN neurons. We applied the metabotropic glutamate receptor agonist DHPG at 0.5 μM, much below the concentrations used in previous studies (50–200 μM, Bonsi et al., 2005; Hartmann et al., 2008; Chepkova et al., 2009). Mice lack the pH 7.0-induced excitation and show much smaller responses to DHPG compared to the rat.

Our previous single-cell RT-PCR analysis of mGluR I receptor expression in the rat TMN showed that almost all histaminergic neurons express mGluR1 but only 78% of them are mGluR5-positive (Sergeeva et al., 2006). The rat ventrolateral but not the medial TMN is activated by hypercapnia (Johnson et al., 2005). We show now a lower level of mGluR5 transcripts in this TMN part than in the non-responsive medial TMN. In striatal cholinergic neurons mGluR5 receptors mediate an increased desensitization rate of mGluR1 receptors (Bonsi et al., 2005), thus antagonizing the mGluR1-response. Although different effector systems are involved in mGluR I signaling in striatum and in TMN, such antagonistic interactions between mGluR5 and

mGluR1 may provide an explanation for the lacking pH 7.0-mediated excitation in the medial TMN with its higher expression level of mGluR5. Thus our current model explaining the proton-induced excitation of TMN (**Figure 8B**) depicts mGluR1 as a primary chain. The mGluR1 activates (through PLC) non-selective cation channels, most likely of the TRPC-type, which increase intracellular Ca^{2+}. This is removed by the electrogenic Na^+/Ca^{2+} exchanger (NCX) leading to depolarisation and increased firing. We have previously described NCX as the major effector for several excitatory neurotransmitters in TMN (Eriksson et al., 2004; Parmentier et al., 2009; Yanovsky et al., 2011). Block of ASIC, Na^+/H^+ and Na^+/Ca^{2+} exchangers by benzamil (20 μM) also abolished the pH 7.0-induced excitation (**Figure 4A**), indicating that the direct mode of Na^+/Ca^{2+} exchange is essential for the maintenance of low intracellular calcium level and for the neuronal excitation in response to pH 7.0. TMN neurons express variable types and splice variants of Na^+/Ca^{2+} exchangers (NCX) (Sergeeva et al., 2003, 2004), their functional diversity remains to be elucidated. We have shown previously that serotonin, orexins/hypocretins, the dopamine receptor 2 agonist quinpirole and thyrotropin releasing hormone (TRH) excite TMN neurons by activation of NCX (Eriksson et al., 2004; Parmentier et al., 2009; Yanovsky et al., 2011). These transmitters are coupled to phospholipase C, like glutamate through type I metabotropic receptors (Wang et al., 2007), which are involved in acid-induced activation of TMN (see above). In our previous studies (Parmentier et al., 2009; Yanovsky et al., 2011) we found that non-selective cation channels (most likely of TRPC type) make the major contribution and are likely activated by the phospholipase C. Intracellular calcium rise allows sodium influx through the electrogenic (depolarizing) NCX. We did not observe pronounced depolarization in response to pH 7.0 in the present study most likely due to the dissipation of sodium gradient and subsequent reversal of Na^+/Ca^{2+} exchange.

Benzamil but not metabotropic glutamate receptor antagonists blocks proton-induced excitation entirely. Furthermore, acid-induced excitation does not disappear in TMN neurons in slices at room temperature, where NCX function is dramatically reduced (Eriksson et al., 2001; Parmentier et al., 2009). Therefore, we assume contributions from ASICs in pH 7.0-excitation, which is seen only in slices where a larger fraction of dendrites and axons is preserved than in the acutely isolated cells. Such a strategically important location of ASICs may have no impact on somatic membrane conductances but can influence local sodium concentrations and action potential generation. Dissipation of the sodium gradient after blockade of Na^+/K^+ ATPase may also abolish or reduce ASIC-currents. ASIC1a and ASIC3 are most likely candidates as they are highly sensitive to small, physiological changes in pH (Waldmann et al., 1997; Lingueglia, 2007). Na^+/K^+ ATPase, Na^+/H^+ exchanger and the direct mode of Na^+/Ca^{2+} exchange play supportive roles for the pH 7.0-induced excitation maintaining sodium and calcium homeostasis. Impairment of their function abolishes the excitatory response, most likely by turning on the reverse mode of Na^+/Ca^{2+} exchange, which leads to accumulation of intracellular calcium and recruits calcium-activated potassium channels. The inhibition of Na^+/K^+ ATPase by protons (Fuller et al., 2003) can contribute to the observed excitation of TMN. A block of this pump by ouabain (0.5 μM) causes an acidification and an increase in firing rate comparable to that achieved by pH 7.0. The effects of ouabain and pH 7.0 are not additive: acidification in the presence of ouabain does not produce an excitatory response, indicating that preservation of the sodium gradient under basal conditions is necessary for the excitatory response.

The mechanisms of proton- and ammonium-evoked excitation of the histaminergic neurons are different: blockers of the excitation by protons (ouabain, benzamil hydrochloride, antagonists of mGluRI) did not influence ammonium (5 mM)-induced excitation. Moreover, ammonium-induced excitation did not differ between rat and mouse, whereas proton-induced excitation was only observed in rat, with the large DHPG (0.5 μM)-responses. As ammonium, similar to extracellular protons, causes intracellular acidification (Kelly and Rose, 2010) we believe that the increase in intracellular protons plays a role for both, acid- and ammonium-sensing. We demonstrate now that blockade of proton entry into the cell (Na^+/H^+ exchanger) eliminates acid-induced excitation, but does not affect ammonium-induced excitation. Kelly and Rose (2010) have shown that ammonium enters hippocampal neurons through the Na^+/K^+ ATPase, leading to intracellular acidification. We failed to block ammonium-induced excitation with the Na^+/K^+ ATPase antagonist ouabain.

The novel HDC-Cre mouse presented here will provide unique possibilities to investigate physiological functions of the histaminergic system in normal and pathological brain. In particular, the role of the histaminergic system can be studied now under conditions of hyperammonemia, where it is poorly understood. In hepatic encephalopathy brain concentrations of histamine are elevated (Lozeva et al., 2003). The resulting changes in the histaminergic system are unknown; it could be protective or deleterious. Our recent experiments in histamine-deficient mice ($HDC^{-/-}$) indicated that histamine may play a protective role against damaging effects of ammonia on hippocampal long-term potentiation (Chepkova et al., 2012).Thus, ammonium but not proton-induced excitation can be studied in transgenic mice which allow manipulating receptors and signaling cascades selectively in histaminergic neurons. This mouse will shed more light on the role of the histaminergic system in health and disease, for instance on obstructive sleep apnea and hepatic encephalopathy.

In conclusion, acidification excites the histaminergic neurons which are prominent keepers of the waking state. Physiological and pathophysiological changes of pH are thus bound to influence many functions of the hypothalamus including sleep-waking, energy homeostasis and the release of hormones.

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Histamine neurons in the tuberomamillary nucleus: a whole center or distinct subpopulations?

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Histamine axons originate from a single source, the tuberomamillary nucleus (TMN) of the posterior hypothalamus, to innervate almost all central nervous system (CNS) regions. This feature, a compact cell group with widely distributed fibers, resembles that of other amine systems, such as noradrenaline or serotonin, and is consistent with a function for histamine over a host of physiological processes, including the regulation of the sleep-wake cycle, appetite, endocrine homeostasis, body temperature, pain perception, learning, memory, and emotion. An important question is whether these diverse physiological roles are served by different histamine neuronal subpopulation. While the histamine system is generally regarded as one single functional unit that provides histamine throughout the brain, evidence is beginning to accumulate in favor of heterogeneity of histamine neurons. The aim of this review is to summarize experimental evidence demonstrating that histamine neurons are heterogeneous, organized into functionally distinct circuits, impinging on different brain regions, and displaying selective control mechanisms. This could imply independent functions of subsets of histamine neurons according to their respective origin and terminal projections.

Keywords: *c-fos*, $GABA_A$-R, GABA, histamine, H_3-R antagonist, stress

HISTAMINERGIC CELL BODIES ARE RESTRICTED TO THE TUBEROMAMILLARY NUCLEUS

In neurons histamine is synthesized through decarboxylation of L-histidine by a specific histidine decarboxylase (E.C.4.1.1.22; HDC) (Green et al., 1987). In a comparable manner to the architecture of noradrenergic, dopaminergic, and serotonergic systems (Mason and Fibiger, 1979; Jacobs and Azmitia, 1992), histamine neurons are restricted to discrete cell clusters in the tuberomamillary nucleus (TMN) of the posterior hypothalamus (Watanabe et al., 1983; Panula et al., 1984), and send mostly unmyelinated axons to the entire central nervous system (CNS; Inagaki et al., 1988; Airaksinen et al., 1989). Catecholaminergic and serotonergic nuclei are clustered in distinct compartments with respect to projection fields, as sets of axons innervating separate brain regions originate from separate subgroups of noradrenergic (A1–A7), dopaminergic (A8–A17) and serotonergic (B1–B9) neurons (Mason and Fibiger, 1979; Jacobs and Azmitia, 1992). This pattern implies independent functions of sets of neurons according to their origin and terminal projections. This does not seem to be the case for the histaminergic system, as retrograde tracers injected into different CNS regions labeled histaminergic somata scattered throughout the TMN without a strict topographical pattern (Köhler et al., 1985; Inagaki et al., 1990). Moreover, histaminergic efferents present diffuse varicosities containing synaptic vesicles, but form synaptic contacts only infrequently (Takagi et al., 1986; Tohyama et al., 1991; Michelsen and Panula, 2002). This feature and the lack of a high-affinity uptake mechanism specific for histamine in the CNS suggest that histamine may diffuse from the site of release and has widespread effects, thus acting as a local hormone affecting not only neuronal, but also glial activity and blood vessel tone (Wada et al., 1991b). These features are compatible with reports of mismatches between distribution of histaminergic fibers and histaminergic receptors (Pillot et al., 2002; Haas et al., 2008), and have led to the concept that the histaminergic regulation of a wide range of brain functions, ranging from basic homeostasis such as sleep-wake rhythm, thermoregulation, food intake to emotions, learning, and memory (Haas et al., 2008; Passani et al., 2011), is achieved by the histaminergic system as a whole (Wada et al., 1991b).

HISTAMINE IS INVOLVED IN MANY BRAIN FUNCTIONS

Although several characteristics of histaminergic neurons emphasize their broad modulatory function, namely their diffuse projections as well as their capacity to release histamine at non-synaptic sites, other features, such as the existence of synaptic contacts and the demonstration of a tight temporal control, suggest that histamine can exert more discrete actions at specific brain loci (Inagaki et al., 1987; Haas et al., 2008). Indeed, the histaminergic system can implement many functions, including the arousal state, brain energy metabolism, locomotor activity, neuroendocrine, autonomic, and vestibular functions, feeding, drinking, sexual behavior, and analgesia, presumably by engaging independent subpopulations of histaminergic neurons according to the brain regions required for a particular behavioral outcome. Consistent with this hypothesis, heterogeneity within the histaminergic neuronal population in response to stress (Miklos and Kovacs, 2003) and pharmacological stimulations was recently reported (Blandina et al., 2010; Passani and Blandina, 2011).

HETEROGENEITY OF HISTAMINE NEURONS IN RESPONSE TO STRESS

In the rat brain histaminergic, neuronal somata are grouped within the TMN in five clusters, E1–E5, each of which sends overlapping projections throughout the neuroaxis with a low level of topographical organization, and bridged by scattered neurons (Ericson et al., 1987; Inagaki et al., 1988, 1990). A similar pattern of distribution has been reported in the brains of other mammals and non-mammalian vertebrates (Wada et al., 1991a). Histamine release is a sensitive indicator of stress (Verdiere et al., 1977; Westerink et al., 2002). Indeed, restraint and/or metabolic stress are among the most potent activators of histamine neurons. Different types of stress (Miklos and Kovacs, 2003), as well as hypercapnic loading (Haxhiu et al., 2001) activated only selected subgroups of histaminergic neurons. These findings rely on the measurement of *c-fos* immunocytochemistry, a marker of cell activation, in histamine neurons identified with *in situ* hybridization of HDC mRNA. Under stress-free, basal conditions *c-fos* expression was detected in a negligible number of histamine neurons (less than 1%) distributed uniformly in the TMN (Miklos and Kovacs, 2003). Following restraint, up to 36% of histamine neurons in E4 and E5 regions became *c-fos* positive, but significantly fewer, less than 10%, were activated in E1, E2, or E3 regions (Miklos and Kovacs, 2003). Moreover, different types of stress, foot shock or insulin-induced hypoglycemia, activated E4 and E5 histamine neurons, but failed to induce any significant *c-fos* activation in the histamine neurons of the three remaining clusters. Therefore, stress-sensitive histamine neurons were detected in the rostral (E4–E5) subgroups rather than in the caudal ones (E1–E3), clearly indicating that histaminergic neurons of distinct TMN clusters are recruited in a stressor- and subgroup-specific manner (Miklos and Kovacs, 2003).

In line with the concept of heterogeneity of these neurons, only a subset of histaminergic neurons (E2) responded to hypercapnic stress (CO_2 exposure) with an increase of *c-fos* expression (Haxhiu et al., 2001; Johnson et al., 2005). Consistently, recordings in rat brain slices revealed that acidification within the physiological range excited E2 neurons localized in the ventrolateral TMN. The excitation is mediated by both metabotropic glutamate receptors and acid sensing ion channels (ASICs; Yanovsky et al., 2012) that are expressed at significantly higher density in the ventrolateral TMN than in the medial part (Yanovsky et al., 2012). The functional significance of chemosensory traits in histaminergic neurons is not well known. However, it is expected that activation of histamine neurons by an increase in CO_2 and/or H^+ may affect central respiratory drive through activation of neurons in the nucleus tractus solitarius, a region displaying a dense network of histaminergic fibers (Airaksinen and Panula, 1988; Airaksinen et al., 1989).

HETEROGENEITY OF HISTAMINE NEURONS IN RESPONSE TO GABA AND GLYCINE

Heterogeneity within the histaminergic neuron population was shown also using a different approach. It is well established that the activity of histamine neurons is closely associated with the behavioral state: in behaving cats, rats, and mice, the firing is more variable during waking and absent upon drowsiness and during sleep. This is the most wake-selective firing pattern identified in the brain to date (Lin, 2000; Lin et al., 2011). Sleep-active, GABAergic neurons in the ventrolateral preoptic nucleus (VLPO) provide a major input to the TMN, and may play a key role in silencing the ascending histaminergic arousal system during sleep (Sherin et al., 1998). Interestingly, GABA release in the posterior hypothalamus increased during SWS, and microinjection of the $GABA_A$-receptor ($GABA_A$-R) agonist muscimol, into the same area increased SWS time (Nitz and Siegel, 1996). Hence, GABA release in the posterior hypothalamus inhibits directly histaminergic cells firing rate (Yang and Hatton, 1997), thereby facilitating SWS, whereas $GABA_A$-R inhibition increases significantly the release of histamine from the TMN, as measured with microdialysis (Cenni et al., 2006). Electrophysiological studies using whole-cell recording identified two subpopulations among histamine neurons isolated from the TMN according to their sensitivity to GABA (Sergeeva et al., 2002), with GABA concentrations for threshold and maximal responses ranging between 0.5 and 5 μM in one subpopulation, and between 50 and 500 μM in the other one (Sergeeva et al., 2002). GABA responses were completely blocked by gabazine, a selective antagonist of $GABA_A$-R (Sergeeva et al., 2002). This receptor is a protein complex assembled from a family of 19 homologous subunit gene products that form mostly hetero-oligomeric pentamers. The major isoforms contain alpha, beta, and gamma subunits and show differential sensitivity to GABA, to modulators like steroids, to physiological regulation, and to disease processes (Olsen and Sieghart, 2009). Sergeeva and coworkers (Sergeeva et al., 2002), using whole-cell recording and single cell RT-PCR from isolated rat histamine neurones, characterized $GABA_A$-R evoked currents and correlated them with the expression patterns of 12 $GABA_A$-R subunits. They identified three different groups of histamine neurons on the basis of their gamma subunits expression. The occurrence of each gamma subunit was correlated with GABA EC_{50}. The group expressing both gamma1 and gamma2 subunits displayed a high sensitivity to GABA, whereas the group expressing only the gamma2 subunit displayed a low sensitivity. Histaminergic neurons are also heterogeneous with respect to their sensitivity to glycine that correlates with their size. Indeed, the maximal glycine response (1 mM) in histaminergic cells with larger somata (25 μ) was about half of the maximal GABA response whereas in the cells with a smaller soma size (19.5 μ) the glycine response was absent or very small (Sergeeva et al., 2001).

HISTAMINE NEURONS ESTABLISH FUNCTIONALLY DISTINCT PATHWAYS ACCORDING TO THEIR TERMINAL PROJECTIONS

In our laboratory we addressed the question of whether histaminergic neurons are organized into distinct functional circuits impinging on different brain regions. We used the double-probe microdialysis technique in freely moving animals, which provides a powerful means for defining the dynamics regulating histamine release in discrete brain regions. Rats were implanted with one probe in the TMN, to deliver drugs and measure histamine release locally, and another probe to measure histamine release from histaminergic projection areas such as the prefrontal cortex, the nucleus basalis magnocellularis (NBM), the nucleus accumbens (NAcc) or the dorsal striatum. By applying compounds targeting

receptors expressed on histamine neurons and measuring histamine output in different brain areas, we demonstrated that the same drug influenced differently the release of histamine from distinct brain regions. Bicuculline, a $GABA_A$-R antagonist, acts directly onto histaminergic neurons to augment cell firing (Haas et al., 2008). We found that intra-hypothalamic perfusion of bicuculline increased histamine release from the TMN, the NAcc and the prefrontal cortex, but not from the striatum (Giannoni et al., 2009) (**Table 1**). Different subunit composition and stoichiometry of $GABA_A$-Rs among histaminergic neurons (Sergeeva et al., 2002, 2005) may account for these results. It is worth noting that functional heterogeneity of responses to bicuculline among histaminergic neurons relates to TMN neurons heterogeneity with respect to projection fields. Also, responses to histamine 3 receptor (H_3-R) antagonists differentiate histaminergic neurons according to their projection areas. When applied to the rat TMN, H_3-R antagonists, such as thioperamide or the more recently synthesized, non-imidazole compounds as GSK-189254, invariably augmented histamine release from the TMN, from the prefrontal cortex and from the NBM, but not from the NAcc, nor the striatum (Giannoni et al., 2009, 2010) (**Table 1**). The H_3-R is present on presynaptic terminals and varicosities of histaminergic axons, where it moderates histamine synthesis and release (Arrang et al., 1983, 1985). H_3-Rs are also located on histaminergic neuronal somata, and provide a tonic inhibition (Haas and Panula, 2003) by modulating inward Ca^{++} currents (Reiner, 1987; Takeshita et al., 1998; Stevens et al., 2001). Therefore, blockade of somatic and presynaptic H_3-autoreceptors increase cell firing (Haas and Panula, 2003) and augment histamine levels in the synaptic cleft. In the TMN, histamine is released from short projections that form extensive axonal arborizations in the posterior hypothalamus. Thioperamide or GSK-189254 were applied locally through the microdialysis probe to the TMN (Giannoni et al., 2009, 2010), hence histamine output increased as a consequence of both somatic and presynaptic H_3-autoreceptors blockade, although the participation of only one component cannot be excluded. Thioperamide or GSK-189254, applied locally into the TMN, significantly increased histamine release also from the prefrontal cortex, and the NBM, but histamine levels remained stable in the dorsal striatum and NAcc. Increases in the prefrontal cortex and NBM were likely due to discharge potentiation of histamine neurons sending efferents to these regions, in a way similar to the effects of TMN perfusion with prostaglandin E_2 (Huang et al., 2003), or Orexin-A (Huang et al., 2001). On the other hand, the lack of increase in histamine release during TMN perfusion with H_3-R antagonists observed in the dorsal striatum and NAcc, despite the fact that these brain areas receive histaminergic innervation (Panula et al., 1989), indicates that histaminergic neurons projecting to these regions are insensitive to H_3-R blockade. We further demonstrated that histaminergic neurons are not a homogenous neuronal population using cannabinoid receptor 1 (CB1) agonists. Administration of methanandamide (mAEA) or ACEA in the TMN facilitated histamine release from the TMN itself, from the NBM and striatum as well (Cenni et al., 2006). However, perfusion of the posterior hypothalamus with mAEA did not change significantly histamine release from the perirhinal cortex (Passani et al., 2007; **Table 1**) despite the profuse histaminergic innervation of this region (Panula et al., 1989) and the presence of histaminergic receptors (Pillot et al., 2002).

Spatial segregation due to probe localization does not explain the lack of response, as retrograde tracing with dye injections into the striatum or prefrontal cortex showed that most histaminergic somata are within the medial part of the ventral TMN (Köhler et al., 1985). This proximity suggests that histaminergic somata projecting to the striatum and prefrontal cortex had the same exposure to H_3-R antagonists, but were not affected in the same way. In conclusion, H_3-R antagonists may discriminate groups of histaminergic neurons impinging on different brain regions, thus suggesting that these neurons are organized into functionally distinct circuits that influence different brain regions, and display selective control mechanisms. In keeping with these results, activation of *c-fos* after GSK189254 administration occurred in cortical areas and the TMN, but not in striatum (Medhurst et al., 2007). To explore the local effects of H_3-R blockade in the histaminergic projection areas, single-probe microdialysis experiments were performed. Local perfusion with H_3-R antagonists in the NBM or prefrontal cortex augmented significantly histamine release within these regions, an effect that can be explained by blockade of local H_3-autoreceptors. The same drugs administered locally to the striatum or NAcc did not modify histamine release, thus indicating that the whole somatodendritic domain of histaminergic neurons projecting to these regions is insensitive to H_3-R antagonists. Accordingly, lesion experiments indicate that the vast majority of H_3-Rs in the NAcc and striatum are not associated with histaminergic fibers (Pollard et al., 1993), which is consistent with high densities of H_3-R

Table 1 | Influence of drug administration into the TMN on histamine release from different brain areas of freely moving rats.

Drugs administered into the TMN	Histamine release					Reference
	TMN	Cortex	NBM	Striatum	NACC	
Thioperamide	*Increase*	*Increase*	*Increase*	*No change*	*No change*	Giannoni et al., 2009
GSK189254	*Increase*	*Increase*	*Increase*	*No change*	*No change*	Giannoni et al., 2010
Bicuculline	*Increase*	*Increase*	*No change*	*No change*	*Increase*	Giannoni et al., 2009
ACEA	*Increase*	NA	*Increase*	*Increase*	NA	Cenni et al., 2006
R(+)Methanandamide	*Increase*	*No change*	*Increase*	*Increase*	NA	Cenni et al., 2006; Passani et al., 2007

NA, not available

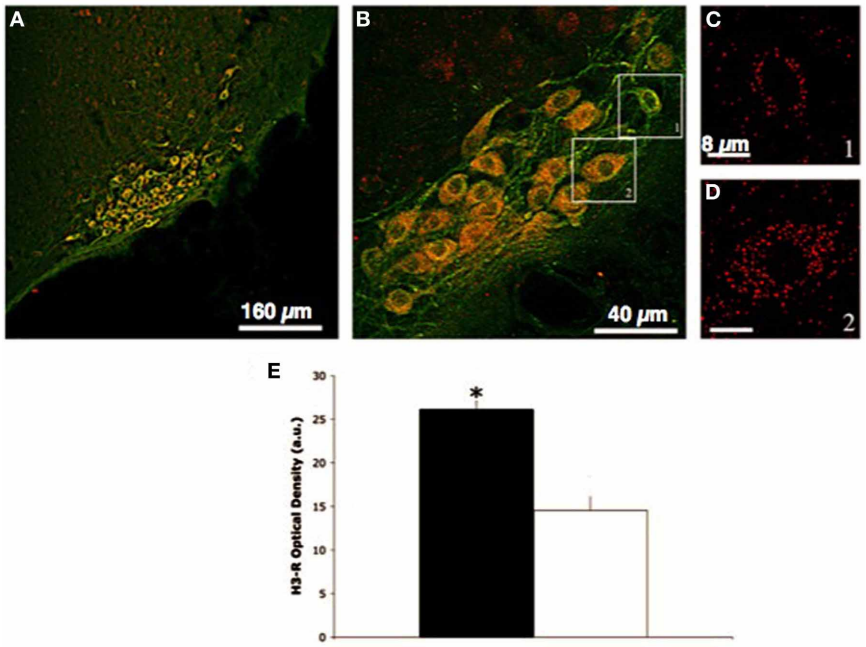

FIGURE 1 | Distribution of H_3-R on TMN histaminergic neurons. (A,B) HDC-positive cells in the E2–E3 subdivision of the TMN showing H_3-R fluorescence; **(C,D)** high resolution z-projections of H_3-R expression in the HDC-positive neurons selected in **(B)**; **(E)** Average intensity of colocalised signals. Analysis of immunocolocalization density was carried out on regions of interest (ROI) of identical area (576 pixels), identified on randomly chosen HDC-positive neurons that where entirely within the thickness of the specimen and were not overlapping. An arbitrary level was set at an optical density value of 20 arbitrary units. Shown are means ± SEM, $^*P < 0.05$ (unpaired *t*-test). Observations were repeated in three rats with similar results. Calibration bar; **(A)** 160 mm; **(B)** 40 mm; **(C,D)** 8 mm. Modified from Giannoni et al. (2009).

mRNAs levels in the same regions (Chazot et al., 2001; Cannon et al., 2007), and suggests a postsynaptic localization of these receptors. Indeed, H_3-R are not restricted to histaminergic neurons (Pollard et al., 1993), and they act also as heteroreceptors modulating the release of neurotransmitters such as acetylcholine (ACh), dopamine, glutamate, noradrenaline, or serotonin from brain regions crucial for the maintenance of alertness or the storage of information (Passani et al., 2004; Haas et al., 2008). Although it is generally assumed that all histaminergic neurons express H_3-R, several isoforms displaying strong differences have been described (Bongers et al., 2007). Hence, *in vivo* insensitivity to H_3-R antagonists may depend on high expression of particular isoforms. In this regard, another observation is intriguing. The distribution of H_3-R on TMN neurons was examined by performing double immunofluorescence labeling with a combination of anti-H_3-R and anti-HDC antibodies (Giannoni et al., 2009). Polyclonal H_3-R antibodies are directed against residues 349–358 of human and rat H_3-R and were previously validated (Chazot et al., 2001; Cannon et al., 2007). In the posterior lateral hypothalamus, both HDC-positive and -negative cells show H_3-R immunostaining (**Figure 1**) The density of H_3-R immunolabeling was very high in the cytoplasm and on cell membranes of some HDC-positive cell and very low in others, as revealed by confocal microscopy (for immunocytochemical procedures see Cenni et al., 2006). A quantitative analysis was carried out counting the number of pixels with colocalized signal on randomly chosen neurons in hypothalamic brain sections from three rat brains. Setting at 20 the optical density expressed in arbitrary units, two histaminergic neuronal populations that differed significantly for H_3-R expression levels were found (**Figure 1**). Since the magnitude of neuronal responses to extracellular signals may depend, at least in part on membrane receptor density, one might envisage that HDC-positive cells displaying very low levels of H_3-R immunoreactivity are those innervating the NAcc or striatum.

CONCLUSIONS

Much has been learned over the past 25 years about the role of histamine as a neurotransmitter. Histamine is found in TMN neurons that are active during wakefulness and exert multiple functions. Indeed, histamine has a major role in maintenance of arousal and contributes to modulation of circadian rhythms, energy, and endocrine homeostasis, motor behavior, and cognition. The extent to which these diverse physiological roles are served by different functional histamine systems is unclear, however, there is much experimental evidence suggesting that the histaminergic system is organized into distinct pathways modulated by selective mechanisms. This could imply independent functions of subsets of histaminergic neurons according to their respective origin and terminal projections. Consequences could be relevant for the development of specific compounds that affect only subsets of HA cells, thus increasing the target specificity.

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