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Alterations in the histaminergic system in the substantia nigra and striatum of Parkinson's patients: a postmortem study

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Abstract

Earlier studies showed neuronal histamine production in the hypothalamic tuberomamillary nucleus to be unchanged in Parkinson's disease (PD), whereas the histamine levels and innervation in the substantia nigra (SN) increased. In the present study we used quantitative polymerase chain reaction (qPCR) to assess the changes in the histaminergic system in the SN, caudate nucleus (CN), and putamen (PU) in 7 PD patients and 7 controls. The messenger RNA (mRNA) expression of the histamine receptor-3 (H_3R), which was localized immunocytochemically in the large pigmented neurons, was significantly decreased in the SN in PD, while histamine receptor-4 (H_4R)-mRNA expression showed a significant increase in caudate nucleus and PU. In addition, significantly increased mRNA levels of histamine methyltransferase (HMT), a key enzyme involved in histamine metabolism, were found in the SN and in the PU in PD. Moreover, in the SN, the histamine methyltransferase-mRNA showed a strong negative correlation with PD disease duration. Our observations imply the presence of local changes in the histaminergic system that may contribute to PD pathology, and may thus provide a rationale for possible novel therapeutic strategies.

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1. Introduction

The substantia nigra (SN) receives a strong histaminergic innervation from the tuberomamillary nucleus (TMN), the only site of neuronal histamine production (Lee et al., 2008; Panula et al., 1989; Watanabe et al., 1984). A close functional interaction between the SN and the TMN for motor performance has been demonstrated in rat (Maisonnette et al., 1998). Parkinson's disease (PD) is characterized by a strong degeneration of the large neuromelanin-containing dopaminergic neurons in the SN (Braak and Del Tredici, 2009), and it has been hypothesized that the histaminergic system is involved in the pathogenesis of PD. A denser histaminergic innervation, enlarged axonal varicosities (Anichtchik et al., 2000b), and increased histamine levels (Rinne et al., 2002) were found in the SN of PD patients, while animal studies showed that increased endogenous histamine levels may accelerate degeneration of the dopaminergic neurons in the 6-hydroxydopamine lesioned rat SN (Liu et al., 2007; Vizuete et al., 2000). In addition, in the same animal model for PD, decreased locomotion dysfunctions were observed following injection of a histamine receptor-3 (H_3R) agonist (Liu et al., 2008). It is presumed that H_3R agonist may inhibit γ -aminobutyric acid (GABA) release from SN neurons into the basal ganglia (Liu et al.,

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2008). Moreover, a Thr105Ile polymorphism of histamine methyltransferase (HMT), the main enzyme breaking down histamine, was observed to be associated with PD, suggesting that a changed histamine homeostasis in the central nervous system (CNS) is associated with the risk for PD (Agundez et al., 2008; Ledesma et al., 2008; Palada et al., 2011), although there are also data that are at variance with this (Keeling et al., 2011).

Because of the possible involvement of the histaminergic system in PD, we determined, in an earlier study, the messenger RNA (mRNA) level of histidine decarboxylase (HDC), the rate-limiting enzyme for histamine production, in the TMN by means of quantitative in situ hybridization. However, we did not find any change in histamine production in any of the stages of PD (Shan et al., 2011). In the present study we therefore aimed to investigate whether a possible histaminergic role in PD pathology may take place locally in the SN and striatum, and whether this is reflected in the expression changes of histamine receptors (HRs) and of HMT.

Four types of G protein coupled HRs, i.e., H₁₋₄R, have been found in the human CNS (Haas et al., 2008) but their functional properties were mainly studied in brain structures other than the SN (Haas et al., 2008). Of these four HRs, H₁R excites neurons in most brain regions although its major function is associated with nonneuronal elements, such as blood vessels or glia cells (Haas et al., 2008). The distribution of H₂R in the brain is more consistent with the histaminergic projections than that of H₁R (Ruat et al., 1990; Vizuete et al., 1997). H₃R is an autoreceptor located on TMN somata, dendrites, and axons (Haas et al., 2008) and is to a large degree constitutively expressed. H₃R suppresses cell firing as well as histamine production and release. In addition, H₃R is a presynaptic heteroreceptor that controls the release of many other transmitters (Arrang et al., 2007; Haas et al., 2008). Moreover, H₃R was found as a postsynaptic receptor located on the perikarya of many neuronal populations. However, its physiological role there remains unknown (Arrang et al., 2007). Recent data show that H₃R is also functionally expressed in glia cells (Jurič et al., 2011). Activated H₄R was found to directly stimulate cortical neurons and enhance the input resistance of the cells (Connelly et al., 2009). HMT may, in addition to its neuronal expression, be produced by activated glia cells in the SN in PD (Huszti et al., 1990; Nishibori et al., 2000; Rafałowska et al., 1987).

There is at present limited and inconsistent information regarding the possible alterations of the HRs in the SN, caudate nucleus (CN) and putamen (PU) in PD. For instance, putative H_3R -binding sites were found to be increased in PD in some studies (Anichtchik et al., 2000a, 2001), but another study, using a different H_3R ligand, concluded that these binding sites were unchanged (Good-child et al., 1999). To obtain insight into the alterations of the 4 metabolic HRs and HMT and their potential relation-

ship to the reported enhanced histaminergic innervation in the SN of PD patients, we used quantitative polymerase chain reaction (qPCR) in postmortem brain material of PD patients to determine the mRNA levels that encode these proteins. Subsequently, we localized H₃R by immunocytochemistry using 2 novel human H₃R isoform-selective antibodies. Previous mRNA studies suggested that the human SN, CN, and PU coexpress the full length H₃R as well as H₃R 365 and H₃R 329 isoforms (Cogé et al., 2001). In previous work we reported on possibilities for heteromeric as well as homomeric H₃R dimers comprising different isoform complements that may have functional significance in rodents (Bakker et al., 2006). The use of the novel H₃R isoform-selective antibodies was, therefore, necessary to address the significance of alternative splicing in the human brain.

2. Methods

2.1. Subjects and sample preparation

The brain samples were obtained from the Netherlands Brain Bank (NBB, Director Dr. I. Huitinga). Permission for a brain autopsy and for the use of the brain material and clinical data for research purposes was obtained by the NBB from the patient or next of kin. Freshly frozen tissue samples of the SN, CN, and PU from 7 clinically diagnosed and neuropathologically confirmed PD patients were studied, together with 7 well-matched controls without neuropsychiatric disorders or neuropathological changes in the brain (for clinicopathological information see Table 1). For the analysis of the PU, control # NBB 01-029 was replaced with control # NBB 00-022, because this area was not available in the samples from the original control. Replacing this subject did not affect adequate matching for possible confounding factors (Table 1). Other aspects of these subjects had been studied by our group before and some detailed molecular information on this material is available (Bossers et al., 2009; Luchetti et al., 2010). Briefly, samples were matched as closely as possible for sex, age, postmortem interval (PMI) and pH of the cerebrospinal fluid (CSF) (Table 1). Due to the stringent tissue selection criteria, the definitive set of samples displayed a small but significant difference for PMI between the PD and control groups (Mann-Whitney U test, p = 0.03) and a trend toward a difference for cerebrospinal fluid-pH (p =0.06). These small differences did, however, not influence our conclusions, as none of the genes showed a significant correlation with PMI or pH, either in our present or in our previous studies (Bossers et al., 2009; Luchetti et al., 2010). Gene expression analysis was performed by qPCR on samples with an RNA integrity number (RIN) value above 6.2 (mean ± SD, 8.36 ± 0.29), which is the minimum quality required for qPCR (Fleige and Pfaffl, 2006). The RIN values of the groups

Table 1 Clinicopathological information of patients with Parkinson's disease and control subjects

	Diagnosis	Sex	Age	PMI	pН	BW	RIN	Cause of death, medications that might affect HA levels
NBB								
00-115	PD/DEM	Μ	70	545	6.33	1258	6.2	Pneumonia, septic shock
04-045	PD/DEM	Μ	71	418	6.55	1358	8.4	Pneumonia
00-139	PD/DEM	Μ	72	435	6.55	1546	6.7	Uremia
02-003	PD	F	75	300	6.52	1218	9.6	Legal euthanasia; entacapone (until last 3 months before death)
02-011	PD	F	79	345	6.37	1203	8.7	Myocardial infarction
00-034	PD	Μ	86	510	6.52	1178	9.2	Unknown
02-064	PD	Μ	87	440	6.37	1166	7.4	Respiratory insufficiency
Median			75	435	6.52	1218	8.4	
98-126	CTRL	Μ	71	360	6.54	1385	8.8	Respiratory insufficiency
00-049	CTRL	Μ	78	415	6.42	1332	9.2	Cardiac failure
97-144	CTRL	Μ	78	240	6.43	1160	9	Pulmonary carcinoma; prednisone (last 24 hours)
00-142	CTRL	F	82	330	6.60	1280	9.2	Myocardial infarction
98-062	CTRL	Μ	85	275	6.95	1332	7.5	Respiratory insufficiency secondary to metastasized adenocarcinoma
99-046	CTRL	Μ	89	310	6.62	1168	9.5	Cardiac arrest
01-029	CTRL	F	90	325	6.58	1066	7.6	Myocardial infarction
00-022 ^a	CTRL	F	83	07:45	6.52	1102	9.2	Acute myocardial infarction
Median			82	325	6.58	1280	9.0	
p value ^b			0.25	0.03	0.06	0.75	0.28	

Key: BW, brain weight (grams); CTRL, control subjects; F, female; HA, histamine; M, male; NBB, Netherlands Brain Bank number; PD, patients with Parkinson's disease; PD/DEM, Parkinson's disease with dementia; pH, pH of cerebrospinal fluid; PMI, post mortem interval (minutes); RIN, RNA integrity number.

^a The control subject for putamen analysis instead of CTRL NBB 01-029.

^b Mann-Whitney U test.

were well-matched (p = 0.28), so there were no differences in RNA quality that could have influenced the gene expression levels.

2.2. cDNA synthesis and qPCR procedure

Details of SN, CN, and PU tissue dissection and subsequent RNA isolation, cDNA synthesis and qPCR procedure have been extensively described in previous studies on the same samples conducted by our group (Bossers et al., 2009; Luchetti et al., 2010). Details of the primers, the GenBank accession numbers, and the efficiency of each primer pair are given in Table 2. Reference genes were selected by geNorm analysis (Vandesompele et al., 2002) and used for normalization. The relative absolute amount of target genes calculated (Kamphuis et al., 2001) was divided by the normalization factor, which was determined by the geometric mean of the following genes (Vandesompele et al., 2002): ACTB, MRPL24, and DHX16 for SN, GAPDH, PRPSAP1, and UFM1 for CN, and ACTB, GAPDH, DHX16, GOT2, and FAM96B for PU. The specificity of amplification was checked by melting curve analysis and electrophoresis of the products on an 8% polyacrylamide gel. Negative controls included a nontemplate water control and a nonreverse transcriptase control, from which SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) was omitted during cDNA synthesis. None of these processes resulted in any kind of product formation.

2.3. Specificity of H_3R antibodies

Two novel polyclonal rabbit anti-human (h) H_3R isoform-specific antibodies were generated as described previously, using Cys-coupled thyroglobulin conjugates (Cannon et al., 2007). Expression of all the a polypeptide protein tag (FLAG)-tagged isoforms (kind gift of Servier, France; Cogé

Table 2

Sequence of the primers, gene bank accession numbers, the amplified efficiency, the size of amplified product for target genes and reference

Gene	Accession code	Forward primer	Reverse primer	Amplification efficiency	Amplicon length (base pairs)
HMT	NM_006895	AATGGAGACCTGCTTTGGG	ATCAGGTGGTGCTGTGGC	2.2	66
HDC	NM_002112	GGCAGCAAGGAAGAACAA	GAGTCGGGCATTTAGGC	1.9	100
$H_{I}R$	NM_001098213	CTGGGAGGTTCTGAAAAGG	GCTGAAGACAACTGGGGATT	2.1	103
H_2R	NM_022304	GGAACAGCAGGAACGAGAC	AGTAGCGGGAGGTAGAAGGT	1.9	115
$H_{3}R$	NM_007232	GAAGATGGTGTCCCAGAGC	CCAGCAGAGCCCAAAGAT	1.9	103
$H_{4}R$	NM_021624	CAAAATGGGTTCCTTCTC	TGACTTGGCTAATCTCCTG	1.9	100
MRPL24	NM_024540	GGAGACCCGGAAATACAAGAAGGT	TGGCATCTGAAAAAGAAGTGCC	1.9	111
β-Actin	NM_001101	TCCCTGGAGAAGAGCTACGA	AGGAAGGAAGGCTGGAAGAG	2.0	98
DHX16	NM_003587	TTGACTCGGAGTGGCTACCG	AGCGTGGCTGTTGCTCAAAGA	2.0	87



Fig. 1. (A) Isoform specificity of the anti-human (h) histamine receptor-3 (H_3R) 365 peptide 1 (268-281) antibody. Human H_3R 365 peptide 1 (268-281) EAMPLHRKVAKSLA, sequence is specific to the i3 region in the human H_3R 365 isoform. This antibody was shown to be selective for the human H_3R 365 and H_3R 445 isoforms (anti-h H_3R 365/445 antibody). (B) Isoform specificity of the anti-h H_3R 329 (222-231) antibody. Human H_3R 329 (222-231) YLNIQSFTQR, sequence is specific to the i3 region in the human H_3R 329 isoform. This antibody was shown to be selective for the human H_3R 329 isoform and not to cross-react with the H_3R 365 and H_3R 445 isoforms. (C) Western blot, performed on homogenates from human tuberomamillary nucleus (TMN) and substantia nigra (SN) tissue samples, revealed 2 main bands (solid arrowheads pointing at approximately 93 kDa and 68 kDa) stained more strongly with the anti- H_3R 365 (the first 2 lanes on the left), compared with the bands stained with the anti- H_3R 329 antibody (the adjacent 2 weaker lanes), which confirmed the specificities of these 2 h H_3R antibodies used. "-" lane indicated that omission of anti- H_3R antibodies failed to show bands on a lysate from human TMN tissue.

et al., 2001) was confirmed by parallel immunoblotting using an anti-FLAG antibody (Sigma, Dorset, UK) and by [³H] Glaxo Smith Kline (GSK) 189294 binding assays (not shown). Based on immunoblotting of FLAG-tagged hH₃R isoforms individually expressed in HEK293 cells, the antihH₃R 329 (sequence CysYLNIQSFTQR) antibody was shown to selectively detect the hH₃R 329 isoform, but not the other major hH₃R 365 or 445 isoforms (Fig. 1B). In contrast, the anti-hH₃R 365 (peptide sequence EAMPL-HRKVAKSLACys) antibody detected both the hH₃R 365 and 445 isoforms, but not the 329 or 200 isoform (Fig. 1A). The 3 major H₃R isoforms in CNS were covered by these 2 antibodies (Drutel et al., 2001; Morisset et al., 2001). In the present study, specificity of the antibodies was further confirmed by (1) Western blotting and (2) staining following solid phase preadsorption of the anti-h H₃R 365/445 and 329 antibodies, with homologous peptides of human H₃R receptor.

2.3.1. Western blotting

Total protein was isolated from snap-frozen human TMN and SN through homogenization using an Ultra Turrax (Staufen, Germany) in suspension buffer, followed by 1-minute centrifugation at 14,000 rpm at 4 °C. Soluble fractions were subsequently used for Western blotting. Protein was fractionated on Precast 4%-12% gradient gels (cat. no. NP0323BOX, Invitrogen) and transferred onto nitrocellulose membranes (cat. no. 10401196, Whatman PRO-TRAN, Dassel, Germany) using the wet blotting method. After a 10-minute blocking step in supermix (0.05 M Tris-HCl pH 7.6, 0.15 M NaCl, 0.25% gelatin, 0.5% Triton X-100), part of the blot was incubated overnight at 4 °C with either of the H₃R antibodies (1:200 in supermix), while the remaining part was incubated without addition of the primary anti-H₃R antibodies, as a negative control. The next day, blots were rinsed in Tris-buffered saline (TBS)-0.05% Tween and incubated with anti-rabbit Cy5 coupled antibodies at 1:5000 in supermix for 1 hour at room temperature. Finally, after a few rinses, blots were scanned on an Odyssey infrared Imager (LI-COR, Inc., Lincoln, NE, USA) to visualize bands.

2.3.2. Solid phase preadsorption

Solid phase preadsorption was performed as previously described (van der Sluis et al., 1988). The synthetic peptides corresponding to the 2 H₃R antibodies were each dissolved $(2 \ \mu g/\mu L)$ in medium (10% glycerol, 10% dimethylformamide, 2.5% nonidet [Sigma]) and spotted on 3 strips of gelatin (0.2%)-coated nitrocellulose (0.1 μ m pore size, Whatman PROTRAN), each containing 10 μ g of peptide. Fixation of the peptide to the nitrocellulose was performed overnight with 4% formaldehyde in a press block, followed by rinsing in water (10 minutes), TBS (10 minutes), and Super mix (0.25% gelatin and 0.5 ml Triton X-100 in 100 ml TBS, pH 7.6) (10 minutes). Each adsorption cycle consisted of an overnight incubation at 4 °C of a peptidespotted strip with either the anti-H₃R 365 or 329 antibodies at a 1:100 dilution. Consecutive cycles (3 in total for each antibody) followed one another by replacing the antibodybound strip with a new strip. To check for possible antibody deterioration during the relatively long process of preadsorption, we took along unspotted nitrocellulose strips in the adsorption procedure. After the final adsorption cycle, binding of the antibody to the peptide was visualized by staining the spotted strips using the avidin-biotin-complex (ABC) method as described above. Finally, TMN, SN, and locus coeruleus (LC) sections were immunostained using the adsorbed anti-H₃R 365 or anti-H₃R 329 antibody solutions according to the procedure described above.

2.4. H_3R immunocytochemistry

Because H₃R-mRNA levels were found to be decreased significantly in the SN of the PD group, H₃R immunocytochemistry was performed on formalin-fixed paraffin-embedded 6- μ m sections of SN from the same subjects in order to define the detailed location of H₃R. One SN section per subject was used. Staining of H₃R in the TMN—where its location has been confirmed (Giannoni et al., 2009; Haas et al., 2008)—was used as positive control. After deparaffinization in xylene and rehydration through a graded ethanol series, SN and TMN sections were rinsed in distilled water and in TBS (0.05 M Tris, 0.15 M NaCL, pH 7.6; 3 × 5 minutes). Sections were microwave-treated in Tris-HCl (pH 9.0, 2×5 minutes) at 700 W for antigen retrieval. Two anti-H₃R antibodies were used in a 1:200 concentration, i.e., anti-H₃R 365 and anti-H₃R 329 (for details see below). To reduce background staining for the anti-H₃R 365 staining, blocking with milk was performed by incubating the antibody in supermix-5% milk (0.25 g of gelatin, 0.5 mL of Triton X-100, and 5 g milk in 100 mL TBS), while this was not necessary for anti-H₃R 329. Detection was done with the avidin-biotin-complex (Vector, Burlingame, CA, USA) and the signal was visualized by incubating sections in TBS containing 3.3'-diaminobenzidine (DAB; Sigma) at 0.5 mg/ mL, 0.23% (wt/vol nickelammoniumsulfate [Merck]) and 0.01% vol/vol H₂O₂ (Merck, Whitehouse Station, NJ, USA) for approximately 5 minutes (room temperature). It should be noted, however, that we could not perform immunocytochemistry for the localization of H1, H2, H4, or HMT in the SN, due to the lack of validated antibodies (H₁, H₂, HMT) or due to the fact that the signal was below the detection limit (H_4) (data not shown).

2.5. Cell density in the SN

Data on cell densities of neuromelanin-positive neuronal profiles in the SN of the subjects were available from earlier work (Bossers et al., 2009). In brief, neuron counting was performed in hematoxylin-eosin stained SN sections, 5 sections per patient. To prevent double counting, the large neuromelanin-positive neuronal profiles were counted only when the neuron contained a nucleolus. The estimated cell densities per cubic millimeter were calculated by dividing the number of identified structures by the measured area, and by correcting for section thickness. The final cell density in the SN per subject was defined as the average density of 5 sections. The density of neuromelanin-containing neurons in the SN declined by 54% in PD as compared with the controls (p = 0.018; see Bossers et al., 2009).

2.6. Statistical analysis

Because the distribution of data was not entirely regular, the differences between the groups were statistically evaluated by the nonparametric Mann-Whitney U test. Correlations were tested by Spearman's correlation coefficient. Tests were 2-tailed and values of p < 0.05 were considered to be significant. Percentage changes of mRNA levels or cell densities were calculated using the median values.

3. Results

3.1. Alterations in histaminergic gene expression in SN

HMT-mRNA showed a significant 51% increase (p = 0.02; Fig. 4A), and H1-(p = 0.11), H2R-(p = 0.23) and H4R-mRNA (p = 0.14) were unaltered in the SN of PD patients compared with the control group (Fig. 4B, C, and E), while H3R-mRNA showed a significant 40% decrease (p = 0.01; Fig. 4D) in the SN of PD patients.

001	1		5		
	HMT	H_1R	H_2R	H ₃ R	H_4R
Putamen (median)					
Control $(n = 7)$	0.0176	0.5073	2.722	5.1132	0.0007
Parkinson $(n = 7)$	0.0266	0.5801	2.015	3.9641	0.0008
p value, Mann-Whitney U test	0.025*	0.482	0.142	0.064	0.013*
Caudate (median)					
Control $(n = 7)$	0.0175	0.0002	0.0947	0.1175	0.0007
Parkinson $(n = 7)$	0.3679	0.0004	0.0530	0.0657	0.0012
p value, Mann-Whitney U test	0.142	0.153	0.406	0.565	0.007**

Table 3 Alteration of histaminergic gene expression levels in patients with Parkinson's disease and control subjects

Key: H₁R, histamine receptor 1; H₂R, histamine receptor 2; H₃R, histamine receptor 3; H₄R, histamine receptor 4; HMT, histamine methyltransferase. * p < 0.05.

** p < 0.01.

HMT-mRNA levels showed a significant negative correlation with disease duration (years between diagnosis and death, from 3 to 14 years, n = 7, $\rho = -0.893$; p = 0.007; Fig. 6A), while its expression was independent of age (n = 7, $\rho = -0.464$; p = 0.294).

A very low—negligible—expression of HDC-mRNA signal cycle of threshold (Ct) value was observed not only in the SN, but also in the PU and CN of PD patients (Ct 34 ± 1.6 , 34 ± 1.28 , 34 ± 0.7 , respectively) as well as in the controls (Ct 34 ± 2.7 , 35 ± 1.4 , 34 ± 0.8).

3.2. Alterations in histaminergic gene expression in the CN and PU

In the CN, the H₄R-mRNA expression showed a significant 6.3-fold increase (p = 0.007) in PD subjects, while the other receptors (H₁R p = 0.153, H₂R p = 0.406, H₃R p = 0.565) and HMT (p = 0.142) were unaltered in PD (Table 3). In the PU, on the other hand, there was a significant 51% increase of HMT-mRNA expression (p = 0.025) and a significant 4.2-fold increase of H₄R-mRNA expression (p = 0.013) in PD (Table 3), whereas mRNA expression levels of H₁R (p = 0.482), H₂R (p = 0.142), and H₃R (p = 0.064) remained unaltered. Moreover, a significant positive correlation was observed between SN and PU HMT-mRNA levels in the control group (n = 6, $\rho = 0.829$, p = 0.042; Fig. 6B), but not in the PD group (n = 7, $\rho = 0.714$, p = 0.071).

3.3. Specificity of H_3R antibodies

Western blots, performed with the anti-H₃R 365 and 329 antibodies on protein extracts from fresh frozen human TMN or SN, revealed the expected dimeric H₃R protein bands (93 and 68 kDa, respectively) (Fig. 1), the same as has been reported in rodents (Cannon et al., 2007; Chazot et al., 2001). In addition, a solid phase adsorption test showed that the anti-H₃R antibodies recognize the corresponding synthetic peptides on nitrocellulose sheets. After adsorption, staining on TMN, SN, and LC sections disappeared entirely for both the anti-H₃R 365 (Fig. 2) and 329 antibodies (Fig. 3). However, the native anti-H₃R antibodies, not subjected to peptide adsorption, did show positive staining in these areas (Figs. 2 and 3). Moreover, to rule out the possibility of antibody deterioration during the relatively long process of adsorption as an explanation for the elimination of staining on sections afterward, we included an additional control, whose antibodies were adsorbed to blank nitrocellulose sheets so they did not contain any peptide spots. These sham-adsorbed antibody solutions did display positive staining, albeit in a slightly weaker form than originally, in adjacent sections of all areas (Figs. 2 and 3). Concluding: our data, together with the available literature (Chazot et al., 2001), support the specificity of the anti-H₃R 365 and anti-H₃R 329 antibodies in the human brain.

3.4. H₃R immunocytochemistry

The present study does not only report on the generation, validation, and use of the first panel of anti-hH₃R isoformselective antibodies in the human brain but also provides the first evidence that H₃R isoform proteins exist in the human brain. A strong, positive cytoplasmic H₃R immunoreactivity (ir) and H₃R-ir fiber staining, dominantly present in dendrites and sparsely in axons, showed up in the TMN with both H₃R antibodies (Fig. 5). H₃R-ir was observed almost exclusively in the pigmented SN neurons, as a strong cytoplasmic staining, while sporadically a weak H₃R-ir was seen in the fibers in the SN by anti-H₃R 365 (Fig. 5). A weaker staining, localized in a similar way, was found by the anti-H₃R 329 antibody (Fig. 5), in addition only a few sporadic axons were stained with the H₃R antibodies in the SN in both PD and controls (Fig. 5).

4. Discussion

In the present study we found a significant decrease of H_3R -mRNA and a significant increase in HMT-mRNA expression in the SN of PD patients. The latter showed a significant negative correlation with disease duration. Immunocytochemistry in the SN of the same subjects revealed a nearly exclusive localization of H_3R -ir in the large neuromelanincontaining neurons. The lower density of these neurons in PD thus offers an explanation for the decreased H_3R expression levels in the SN in PD. In addition, HMT-mRNA levels were significantly higher in the PU. Moreover, this was the first time



Fig. 2. Anti-histamine receptor-3 (H₃R) 365 immunoreactivity in human tuberomamillary nucleus (TMN), substantia nigra (SN), and locus coeruleus (LC) neurons using the native, unadsorped (A, B, C), sham-adsorped (D, E, F), and homologous peptide-adsorped (G, H, I) antibody. Images from each brain area are depicted in the columns of panels (A), (D), and (G) (TMN), (B), (E), and (H) (SN), and (C), (F), and (I) (LC). Note that in all 3 areas, the black cytoplasmic H₃R 365-staining is completely eliminated after preadsorption with the homologous peptide. Staining remains after sham-adsorption, although slightly weaker compared with using the native antibody. The brown staining in the SN and LC is endogenous neuromelanin. Scale bar represents 25 μ m.

that H_4R -mRNA expression was reported to be present in the SN, CN, and PU, and its expression appeared to be significantly higher in PD, both in the PU and CN. In general, our data implicate clear local alterations of the histaminergic system in the nigrostriatal circuitry in PD. An extremely low HDC-mRNA expression level was detected in SN, CN, and PU, possibly from the microvascular endothelial cells (Yamakami et al., 2000). This observation agrees with the concept that the exclusive neuronal histamine source lies in the TMN (Watanabe et al., 1984).

Despite the fact that we did not find significant changes in neuronal histamine production in the TMN in PD (Shan et al., 2011), an increased density of histaminergic fibers was reported in the SN and enhanced histamine levels were found in both the SN and PU (but not CN), in PD patients (Anichtchik et al., 2000b; Rinne et al., 2002).

The present study reports on increased HMT-mRNA levels in the SN and PU of PD patients. A positive correlation between the PU and the SN in control subjects was observed in HMT-mRNA levels. This relationship was absent in PD. HMT-mRNA expression was not only found in neurons, but also in glia cells (Huszti et al., 1990; Nishibori et al., 2000; Rafałowska et al., 1987) (Table 4). Because both microglia and astrocytes become activated in the SN in PD (McGeer and McGeer, 2008), the activated glia subpopulations may contribute to the increased HMT-mRNA expression detected in the present study. Whether the upregulation of HMT-mRNA reflects increased levels of local histamine (Duch et al., 1978; Fogel et al., 2002) is yet to be determined, but should it turn out to be indeed the case, then it may be that the increased expression of HMT acts as protection against neurodegeneration by metabolizing histamine in PD (Anichtchik et al., 2000b; Rinne et al., 2002). Finding such a protective effect may have important implications, because animal experiments have shown that increased histamine levels in the SN may cause a degeneration of dopaminergic neurons (Liu et al., 2007; Vizuete et al., 2000). In addition, the inverse correlation found be-



Fig. 3. Anti-histamine receptor-3 (H_3R) 329 immunoreactivity in human tuberomamillary nucleus (TMN), substantia nigra (SN), and locus coeruleus (LC) neurons using native, unadsorped (A, B, and C), sham-adsorped (D, E, and F), and homologous peptide-adsorped (G, H, and I) antibody. Images from each brain area are depicted in the columns of panels (A), (D), and (G) (TMN), (B), (E), and (H) (SN), and (C), (F), and (I) (LC). Note that in all 3 areas, the black cytoplasmic H3R 329-staining is completely eliminated after preadsorption with the homologous peptide. Note that the anti-H₃R 329 staining is weaker than the anti-H₃R 365 staining (see Fig. 2). The brown staining in the SN and LC is endogenous neuromelanin. Scale bar represents 25 μ m.

tween HMT-mRNA expression and disease duration in the SN of PD patients suggests that the more serious (thus the shorter lasting) the disease, the more HMT-mRNA is expressed, which also indicates a relationship between the histaminergic system and the pathogenesis of PD.

Our qPCR results showed that in controls H₃R-mRNA expression level was about 50 times higher in the PU relative to the SN and CN, which is in agreement with previous reports showing H₃R-mRNA and protein levels to be higher in the striatum (Anichtchik et al., 2001; Chazot et al., 2001; Pillot et al., 2002). Our finding of a decreased H₃R-mRNA and H₃R-ir in the SN of PD patients concurs with a previous study demonstrating a tendency toward lower H₃R receptor activity in the SN in PD, as determined by the guanosine triphosphate (GTP)- γ -[³⁵S] assay (Anichtchik et al., 2001). In contrast to our results, a significant increase of H₃R binding sites (i.e., receptor density) was found in the SN in PD, using [³H] N^{α}-methylhistamine as the ligand (Anichtchik et al., 2000a, 2001; Ryu et al., 1994). However, this ligand seems to bind only to a subtype of H₃R, as evidenced

by its different binding pattern from other antagonist/inverse agonist ligands such as $[^{125}I]$ -iodophenpropit (Mezzomo et al., 2007). This may explain the seeming discrepancy between their H₃R-binding observations and our own, which address the overall H₃R changes.

The main type of the H_3R -mRNA assayed by our qPCR is most probably the heteroreceptor H_3R as expressed in pigmented neurons in SN. In principle a potential additional type of H_3R , the autoreceptor localized on the fibers of the histaminergic projecting neurons from the TMN or striatonigral neurons (Anichtchik et al., 2001; Haas and Panula, 2003) cannot be absolutely excluded. However, based upon our observation of a strong H_3R -ir in the neuromelanin neurons and weak and sporadic H_3R -ir in the fibers in the SN, the proportion of the latter is likely to be very small or even negligible. H_3R regulates the release of GABA (Garcia et al., 1997) and serotonin (Threlfell et al., 2004) in the direct and indirect striatonigral pathways and may therefore disturb neurotransmission in PD. A positive correlation was found between glutamic acid decarboxylase (GAD)-1



Fig. 4. Transcript levels of histamine methyltransferase (HMT) and histamine receptor-1-4 ($H_{1-4}R$), as determined by quantitative polymerase chain reaction (qPCR) in the substantia nigra (SN). Box plots show the median, 25th–75th percentiles and the range of the amount of individual gene expression. The asterisk(s) indicate a significant increase (A) or decrease (D) of HMT or H_3R mRNA, respectively, in the SN in Parkinson's disease (PD) patients versus controls. * p = 0.018; ** p = 0.009. Transcript levels of H_1R , H_2R , H_4R remained unchanged in PD (B, C, and E).



Fig. 5. Representative photomicrographs of histamine receptor-3 (H_3R) staining in the tuberomamillary nucleus (TMN) from a control (A, and D) and substantia nigra (SN) (B, C, E, and F). (A, B, and C) illustrate black staining with the anti- H_3R 365 antibody, while (D, E, and F) show black staining with the anti- H_3R 329 antibody. (C) and (F) depict images from PD patients, (B) and (E) from controls. Please note the clear staining in the TMN shown by both antibodies. The anti- H_3R 365 antibody generally showed stronger labeling of cells in both TMN and SN brain areas. Differences in the staining intensity of SN neurons are not evident between Parkinson's disease (PD) and control subjects with either antibody. The brown staining in the SN is endogenous neuromelanin. Scale bars (A–F) = 5 μ m.



Fig. 6. A highly significant negative correlation was present between histamine methyltransferase (HMT)-mRNA levels and disease duration in Parkinson's disease (PD) patients (A). In the group of control subjects, HMT-mRNA levels in the substantia nigra (SN) and putamen (PU) are positively correlated (B).

(Luchetti et al., 2010) and H₃R-mRNA in the SN, but only in controls (n = 6, $\rho = 0.943$, p = 0.005), which confirms the possibility of such a relationship. This relationship was apparently disturbed in PD. Whether the association between H₃R- and GAD-1-mRNA is due to colocalization requires further study.

 H_1R , H_2R , and H_4R did not show a significant change in the SN of PD patients. There are at least 2 possibilities for the "unaltered" receptor expression: (1) the mRNAs for the receptors are lost in the PD SN together with the melanin pigmented neurons but their expression is increased in the nonpigmented neurons and/or nonneuronal compartments as compensation; or (2) they are localized in the nonpigmented neurons or nonneuronal compartments, which remain largely intact during the course of PD (see Table 4). Because specific and sensitive antibodies for these receptors are not available, their localization within the SN remains at present unresolved. H_4R expression was considerably higher in the CN and PU in PD compared with matched controls. However, only limited information exists on the function of H_4R in the brain (Connelly et al., 2009). The possible role of this receptor in the CN and PU in PD is an intriguing topic for further investigation.

One limitation of the present study is the relatively low number of patients. In addition, the PD patients had been taking medication, specifically levodopa, and 1 patient had been taking entacapone (catechol-*O*-methyltransferase inhibitor) (see Table 1). A recent report on a rat study showed that acute levodopa treatment may increase the release of histamine (Yanovsky et al., 2011). Nevertheless, in our recent study, the levels of HDCmRNA of 2 patients who had stopped taking PD medication 1-3 months before death fell well within the range of the other PD subjects who took PD medication until their demise (Shan et al., 2011). Moreover, none of the

Table 4 Summary of regional and cellular localization of the histaminergic genes in brain

Gene	Summary of regional and cellular localization in brain
HMT	Localized in neuron membrane and endothelial cells (Barnes et al., 2002; Nishibori et al., 2000). Glia compartment plays a main role in histamine inactivation (Huszti et al., 1990).
HDC	Mainly expressed in posterior hypothalamus, tuberomamillary nuclear (Ericson et al., 1987; Pænula et al., 1984; Panula et al., 1990), and sporadic expression in endothelial cells (Yamakami et al., 2000), or master cells (Goldschmidt et al., 1985).
$H_I R$	Cerebral cortex, claustrum, hippocampal formation, and thalamus, and the two segments of the globus pallidus presented high Levels H ₁ R binding (Martinez-Mir et al., 1990).
	Expressed in neurons, glia, blood vessels, and nonneuronal elements (Haas et al., 2008).
H_2R	H ₂ R binding showed higher density in basal ganglia, amygdale, hippocampus, and cerebral cortex in both primates and rodents (Martinez-Mir et al., 1990; Ruat et al., 1990; Vizuete et al., 1997).
	Expressed both in neurons and glia such as astrocytes (Haas et al., 2008; Jurič et al., 2011). H ₂ R localized pre- and post- synaptically in the brain (Chen et al., 2005; Poli et al., 1994; Timm et al., 1998).
H_3R	The high levels presented in deep layers of the cerebral cortex, dentate gyrus, subiculum of hippocampal formation, and striatum (Anichtchik et al., 2001; Chazot et al., 2001; Pillot et al., 2002; Pollard et al., 1993).
	H_3R localized pre- and postsynaptically in the brain (Arrang et al., 2007).
	Expressed in neurons and astrocytes (Haas et al., 2008; Jurič et al., 2011).
H_4R	The high expression of H_4R was found in cerebral cortex, entorhinal cortex, and thalamus and mainly expressed in neurons (Connelly et al., 2009).

Key: H1R, histamine receptor 1; H2R, histamine receptor 2; H3R, histamine receptor 3; H4R, histamine receptor 4.

PD patients showed a significant change in HDC mRNA compared with control subjects (Shan et al., 2011), indicating that levodopa probably does not significantly influence histamine production in humans. It should be noted, moreover, that it is quite impossible to obtain brain material from PD patients not taking medication.

In conclusion, the observed increased expression of HMT-mRNA in both the SN and PU and the decreased expression of H_3R -mRNA and protein in the SN support the hypothesis of local alterations of the histaminergic system that may contribute to nigrostriatal pathology in PD. This provides a rationale for therapeutic strategies based upon the use of H_3R agonists and/or HMT modulators in PD.

Disclosure statement

The authors disclose no conflicts of interest.

Appropriate approval was obtained and procedures followed concerning the human material used in the current report.

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