Chapter 2

Development, Characterization, and Validation of Anti-Human H_3 Receptor Isoform-Specific Antibodies

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Abstract

Generation of selective anti-G-protein coupled receptor (GPCR) antibodies is a challenging pursuit and the anti-histamine receptor antibodies are no exception. Furthermore, producing isoform-selective antibodies is even more of a challenge. The first anti-histamine H_3 receptor (H_3) antibodies were developed in 2001 and validated in 2007. The first two antibodies were raised against human H_3 receptor sequences common to most human and rodent isoforms. This chapter describes how a panel of anti-hH_3 receptor isoform-specific antibodies have been generated, characterized, and validated for selectivity [individual rH_3R and hH_3R isoform transient expression in human embryonic kidney (HEK) 293 cells, isoform peptide blockade, and the use of H_3R knock-out (KO) mice], and utility for a range of immunobiochemical techniques (immunoblotting, immunohistochemical, and immunofluorescence techniques).

Key words Antibodies, H_3R, Human, Immunobiochemistry, KO mice, Peptide-directed, Polyclonal, Splice-variants

1 Introduction

1.1 Cloning of the H_3 Receptor

Due to lower than expected homology with histamine receptors H_1 and H_2, the H_3 receptor was not cloned until 1999 [1] more than a decade after it was first described by seminal studies in France [2]. It was discovered as part of an effort to identify orphan G-protein coupled receptors (GPCRs).

Lovenberg and colleagues identified a partial clone (GPCR97) that had significant homology to a range of biogenic amine receptors. The GPCR97 clone was used to probe a human thalamus library, which resulted in the isolation of a full-length clone encoding a putative GPCR. Homology analysis showed the highest similarity to M_2 muscarinic acetylcholine receptors and overall low homology to all other biogenic amine receptors. Subsequent

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analysis revealed a pharmacological profile practically indistinguishable from that for the histamine H_3 receptor. In two analyses of the human gene [3, 4] a 1063 base pair (bp) intron beginning 250 nucleotides downstream of the start codon (corresponding to aa 84) and another 1564 bp intron at position 417 (corresponding to aa 139) suggests a gene with three exons and two introns. By contrast, Cogé and colleagues [5] put forward a gene having a minimum of four exons and three introns. In their analysis, the coding region is interrupted by introns of 1062, 1565, and 240 bp. A further exon may result in an additional eight amino acids at the C-terminus, leading to a protein of 453 aa. The result of these diverse exon/intron junctions is the possibility of alternative splicing to generate multiple splice variants that may potentially lead to several receptor isoforms.

1.2 H_3 Receptor Isoforms

The cloning of the H_3 receptor initiated a new era in histamine research. The existence of multiple H_3 receptor isoforms could contribute to the pharmacological heterogeneity in H_3 receptors, within and across species, which has long been recognized but not understood. In all species tested so far the full-length H_3 receptor encodes a polypeptide of 445 amino acids (predicted polypeptide M_r 47,000). Shorter isoforms with deletions predominantly in the third intracellular loop domain have also been identified (predicted polypeptide M_r 20,000–45,000 range).

1.2.1 H_3 receptor Isoform Anatomical Topology

While the full-length clone is found in most abundance in the CNS in all species studied so far, there is regional variation in the distribution of the mRNAs encoding the different isoforms [5]; this has given rise to speculation that H_3 heterogeneity could underlie different activities and functions of H_3 receptors in specific tissues [6].

The evidence for heterogeneity in anatomical distribution was based initially on the distribution pattern of mRNA coding for the various isoforms. Note that these splicing events yield potentially different protein sequences in rodents and human H_3R orthologs. In order to define the importance of human and rodent H_3 receptor heterogeneity, specific immunological probes are required.

1.3 Anti-Histamine H_3 Receptor Antibodies

Generation of selective anti-GPCR antibodies is a challenging pursuit and the histamine receptors are no exception. Furthermore, producing isoform-selective antibodies is even more testing. The first anti-histamine H_3 receptor (H_3) antibodies were developed in 2001 and validated using knock-out (KO) mice in 2007 [7, 8]. The first two antibodies were raised against human H_3 receptor sequences common to most human and rodent isoforms: anti-hH_3 (349–358) and anti-hH_3 (175–187), respectively. They both detected two specific immunoreactive protein species (M_r 68,000 and 93,000) that were suppressed by prior incubation with their

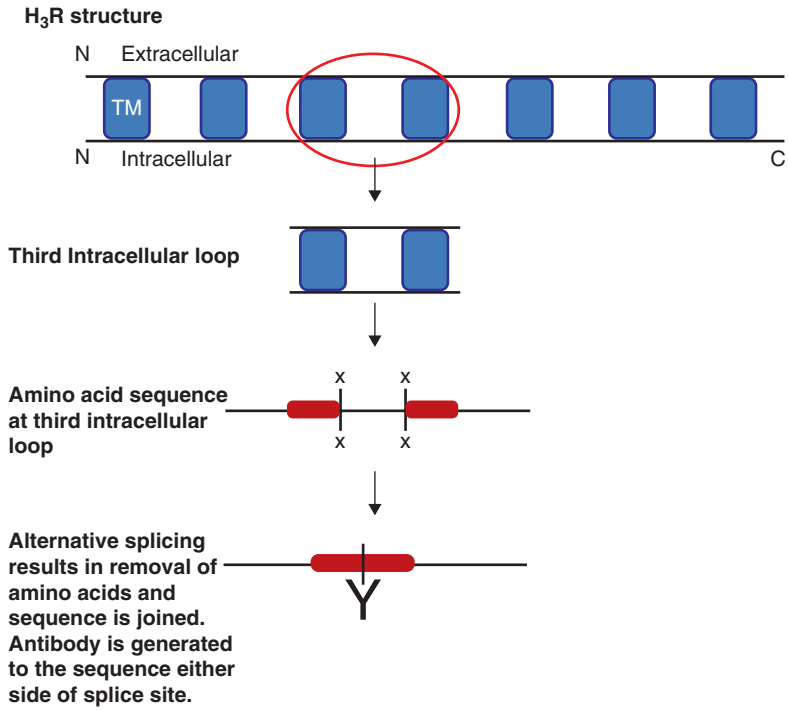

Fig. 1 Schematic showing how peptide sequences were selected in an attempt to produce isoform-specific antibodies against the distinctly spliced third intracellular loop of the human H_3R. Amino acid sequences were chosen that spanned either side of the splicing event so once the sequence was removed and the sequence at either side joined, the antibody generated would be predicted to detect the sequence. The sequence selected (10–15-mer) was long enough so that the antibody was specific but not too long that the antibody would detect other closely related isoforms

respective peptide antigens, in many adult rat and mouse brain regions. These protein species are most likely derived from the dimeric and/or glycosylation variants of the receptor.

In an attempt to generate an antibody specific to the rat $H_{3C\,(397)}$ isoform a peptide sequence was chosen which spanned the deletion within the third intracellular loop rat isoform sequences and should therefore be unique to this isoform (Fig. 1). However, when tested against three of the major human and rat isoforms heterologously expressed in HEK 293 cells namely $hH_{3\,(445)}$, $hH_{3\,(365)}$, and $hH_{3\,(329)}$; $rH3_{A\,(445)}$, $rH3_{B\,(413)}$, and $rH3_{C\,(397)}$, the antibody was found to be selective for both the full-length human and rat isoforms ($hH_{3\,(445)}$ and $rH_{3A\,(445)}$) as well as the expected $rH_{3C\,(397)}$ isoform. Using this same strategy, antibodies specific for three of the other major human H_3R isoforms, $hH_{3\,(415)}$, $hH_{3\,(365)}$, and $hH_{3\,(329)}$ ΔI3 (*see* Fig. 2, human isoform sequences), were also sought.

Peptide sequences chosen for each isoform:

(A) Human sequence showing the H_3R_{365} deletion / isoform 2 **(spliced deletion in red and bold)**
Peptide sequence used to generate: anti-human H_3R_{365} peptide 1 antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa
241 gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv
301 **asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdr**kvaksl
361 avivsifglcwapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk

(B) Human sequence showing the H_3R_{365} deletion / isoform 2 **(spliced deletion in red and bold)**
Peptide sequence used generate: anti-human H_3R_{365} peptide 2 antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa
241 gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv
301 **asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdr**kvaksl
361 avivsifglcwapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk

(C) Human sequence showing the H_3R_{329} deletion / isoform 2 **(spliced deletion in red and bold)**
Peptide sequence used to generate:anti-human H_3R_{329} antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniq**rrtr lrldgareaa**
241 **gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageatlgggggggsv**
301 **asptsssgss srgterprsl krgskpsass aslekrmkmv sq**sftqrfrl srdrkvaksl
361 avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk

(D) Human sequence showing the H_3R_{200} deletion / isoform 5 **(spliced deletion in red and bold).**
Peptide sequence used to generate: anti-human H_3R_{200} antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa**ilsweylsgg**
181 **ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa**
241 **gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageatlgggggggsv**
301 **asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl**
361 **avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr**
421 **raftkllcpq klkiqphssl ehcwk paaprgalrg rahsrgapsr** rrprprwrsa

Fig. 2 The amino acid sequences of the full-length human H_3R and its isoforms and the peptide sequences used to generate specific antibodies to isoforms. (**a**) H_3R_{365} amino acid sequence with the area spliced out highlighted in *red* and selected immunogen sequence in *blue*. (**b**) $H_3R_{(365)}$ amino acid sequence with the area spliced out highlighted in *red* and selected immunogen sequence in *blue*. (**c**) $H_3R_{(329)}$ amino acid sequence with the area spliced out highlighted in *red* and selected immunogen sequence in *blue*. (**d**) $H_3R_{(200)}$ amino acid sequence with the area spliced out highlighted in *red* and sequence highlighted to raise an antibody against selected immunogen sequence in *blue*

Finally, a peptide sequence unique to the heavily truncated $hH_{3\,(200)}$ was selected ([9], Isoform 5). $hH_{3\,(200)}$ has a 170–306 deletion plus a frame shift and a novel stop codon, so that the C terminus is unique to this isoform. Therefore, the last 10 amino acids of $hH_{3\,(200)}$ were selected as the immunogen. Herein, the rationale for peptide selection is described and methods for the preparation of the peptides for immunization, the immunization procedure, antibody purification, and the experiments performed to validate the individual antibody specificities. Results of immunohistochemical analysis using the human/rodent antibody in mouse brain slices from both wild-type (WT) and H_3R KO mice (kind gift of Dr. Tim Lovenberg, Janssens/Johnson & Johnson, USA) are also presented as exemplars.

2 Materials

2.1 Antibody Production

1. Synthetic peptides are purchased commercially (*see* **Note 1**).
2. Keyhole limpet hemocyanin (KLH) or thyroglobulin (Sigma-Aldrich, UK).
3. Imject® Maleimide activated mcKLH kit (Thermo Fisher Scientific, UK).
4. Female Dutch and New Zealand (NZ) white rabbits (suppliers include Harlan or Charles Rivers, UK).
5. Chromatography column with scintered disc (SLS, UK).
6. Phosphate buffered saline (PBS) (Sigma-Aldrich, UK).
7. Spectrophotometer (there are many suppliers, e.g., Jenway Genova).
8. Freund's complete and incomplete adjuvant (Sigma-Aldrich, UK).
9. 1 mL syringes (SLS, UK).
10. 25-gauge needle (SLS, UK).
11. Sterile scalpel blade (SLS, UK).
12. 15 mL and 50 mL falcon tubes (SLS, UK).
13. Microcentrifuge (12,000 × *g*) (e.g., Biofuge Fresco Heraeus, Kendro Laboratory Products).
14. Activated thiol-sepharose beads (Peirce, UK).
15. Rotary Shaker, Stuart™ Just Go SSL5 Shaker (Fisher Scientific laboratories, UK).
16. 0.1 M Tris-HCl pH 8.0, containing 0.3 M NaCl and 1 mM ethylenediamine tetra-acetic acid (EDTA) (Sigma-Aldrich, UK).

17. Tris pH 8.0 (Sigma-Aldrich, UK).
18. 0.1 M citric acid pH 4.5 (Sigma-Aldrich, UK).
19. 0.02% (w/v) sodium azide (Sigma-Aldrich, UK).

2.2 Transfection of Individual Human H_3R Isoforms in HEK 293 Cells

1. 75 cm^3 tissue culture flasks (SLS, UK).
2. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, 1:1 ratio) (Sigma-Aldrich, UK).
3. Mix powdered DMEM/F12 (15 g/L) and 15 mM N-2-hydroxyethylpoiperazine-N′-2-ethane sulfonic acid (HEPES) with 800 mL of sterile water (Sigma-Aldrich, UK). Supplement the media with 40 mL of 10% (v/v) fetal calf serum (FCS) (Cambrex BoioSciences, Belgium), 7.5% (w/v) $NaHCO_3$ (Sigma-Aldrich, UK) giving a final concentration 3 g/L, and 20 mL of penicillin (500 μg/mL)/streptomycin (500 μg/mL) solution (Sigma-Aldrich, UK).
4. 10 M NaOH (Sigma-Aldrich, UK).
5. 0.2 μm Sartorius Satolab-V150 filter unit (SLS, UK).
6. CO_2 incubator (e.g., Shel Lab, Sheldon Manufacturing Inc., USA).
7. Trypsin-EDTA (Sigma-Aldrich, UK).
8. Polyethyleneimine (PEI) (Sigma-Aldrich, UK).
9. pC DNA 3.1 (-) (Promega Corporation, UK) FLAG-tagged H_3R isoforms.
10. 3 mL Petri dish to 75 cm^3 TC flask depending on scale (SLS, UK).
11. Greiner cell scraper (SLS, UK).
12. Dounce glass/glass homogenizer (Sigma-Aldrich, UK).
13. Ice.

2.3 Immunoblotting of Cell Homogenates of HEK 293 Expressing H_3R Isoforms

1. Resolving gel buffer [50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) sodium dodecyl sulfate (SDS) pH 8.8] (Sigma-Aldrich, UK).
2. Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, UK).
3. 30% (v/v) acrylamide (Sigma-Aldrich, UK).
4. 10% (w/v) ammonium persulfate (APS) (Sigma-Aldrich, UK).
5. Hoefer SE 245 dual gel caster (GE Healthcare Biosciences, UK).
6. Saturated water/butanol solution (Sigma-Aldrich, UK).
7. Electrode buffer [50 mM Tris, 384 mM glycine, 1.8 mM EDTA, and 0.1% (w/v) SDS pH 8.8] (Sigma-Aldrich, UK).

8. Chloroform (Sigma-Aldrich, UK).
9. Methanol (Sigma-Aldrich, UK).
10. Sample buffer [30 mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue, and 7.5% (w/v) SDS] (Sigma-Aldrich, UK).
11. Stacking gel buffer [0.5 M Tris-glycine, pH 6.8, containing 8 mM EDTA and 0.4% (w/v) SDS] (Sigma-Aldrich, UK).
12. Prestained standards (protein molecular weight range of 200–6.5 kDa, e.g., Takara Bio Group, USA).
13. Hamilton syringe (SLS, UK).
14. Transfer buffer: 25 mM Tris pH 8.4, 192 mM glycine, 20% (v/v) methanol (Sigma-Aldrich, UK).
15. Hoefer TE 22 tank transfer unit (GE Healthcare, UK).
16. Nitrocellulose membrane (GE Healthcare, UK) cut to size, for 10 cm v 8 cm of gels.
17. PBS or Tris-buffered saline (TBS, 50 mM)/0.9% (w/v) NaCl pH 7.4, containing 5% (w/v) dried milk and 0.02% (v/v) Tween-20 (Sigma-Aldrich (UK).
18. PBS or TBS containing 2.5% (w/v) dried milk (Sainsburys supermarket, UK).
19. Wash buffer: PBS or TBS (50 mM)/0.9% (w/v) NaCl containing 2.5% (w/v) dried milk (Sainsburys supermarket, UK) and 0.02% (v/v) Tween-20 (Sigma-Aldrich, UK).
20. Horseradish peroxidise (HRP) labeled secondary antibody, either anti-rabbit or anti-mouse (GE Healthcare, UK) depending on the host species for primary antibody, at a dilution of 1/2000 in 10 mL of incubation buffer.
21. Chemiluminescence developing solution containing 100 μL of 68 mM p-coumaric acid, 10 mL of 1.25 mM luminal, and 6 μL of 30% (v/v) H_2O_2 (Sigma-Aldrich, UK).
22. Hyperfilm™ (GE Healthcare, UK).
23. Kodak D-19 Developer (Sigma-Aldrich, UK).
24. Kodak Unifix (Sigma-Aldrich, UK).

2.4 Immunohistochemistry (IHC) and Immunofluorescence of H_3R in Wild-Type and H_3R KO Mice

1. 4% (w/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde in 0.1 M phosphate buffer (Sigma-Aldrich, UK).
2. Wild-type and H_3R KO Mice produced by Janssens/J&J, USA on a C5731/6 J mouse background (Lovenberg, Johnson and Johnson, US Patent no. 7151200).
3. 10%, 20%, and 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4 (Sigma-Aldrich, UK).

4. Isopentane (Sigma-Aldrich, UK).
5. Cryostat (e.g., Leica model CM3050S, set to −20C).
6. 10% (v/v) methanol and 3% (v/v) hydrogen peroxide in 50 mM TBS, pH 7.4 (Sigma-Aldrich, UK).
7. PBS, 0.2% (w/v) glycine, and 0.2% (v/v) Tween-20 (Sigma-Aldrich, UK).
8. 2% (v/v) FCS in PBS, 0.02% (v/v) Tween-20 (Sigma-Aldrich, UK).
9. Primary anti-H_3R antibody at a range of concentrations (0–5 μg/mL) (In house).
10. PBS/0.1% (v/v) Triton X 100 (Sigma-Aldrich, UK).
11. Vectastain ABC Elite kit (Promega Ltd., Southampton, UK) including biotin linked, anti-rabbit secondary antibody, and streptavidin-HRP complex (GE Healthcare, UK).
12. 3,3′-diaminobenzidine tetrahydrochloride as the HRP substrate (Sigma-Aldrich, UK).
13. PBS pH 7.4 containing 0.4% Triton X-100, 4% FCS or normal serum from the same species as the secondary antibodies are raised in and 1% bovine serum albumin (BSA) (Sigma-Aldrich, UK).
14. Primary anti-H_3R antibody at a range of concentrations (0–5 μg/mL) (In house).
15. Secondary antibodies: fluorophore linked anti-rabbit antibodies, e.g., Cy3 or Alexa 488, for 1 Citifluor AF4 mountant or equivalent (BDH Laboratories, UK).
16. Peptide is used in molar excess (500 μg/mL) (Cambridge Research Biochemicals, UK) for immunoblotting and IHC/immunofluorescence experiments.

3 Methods

3.1 Choice of Peptide Sequences

Specific peptide sequences are chosen to immunize rabbits to produce anti-peptide antibodies (Fig. 2). The peptides are chosen based on the peptide specificity being unique to the particular isoform and its likely immunogenicity using the rationale described herein and previously [10]. A cysteine residue is added to one end of each sequence so that the peptide could be *directionally* coupled to a carrier protein, KLH, or thyroglobulin to ensure the appropriate configuration of peptide is maintained.

3.2 Antibody Production

The 10-15-mer peptide alone is too small to stimulate an adequate immune response and is therefore conjugated to a large immunogenic carrier protein before being injected into the rabbit (female

Dutch and NZ white rabbits are commonly used breeds). The peptide is conjugated using Imject® Maleimide activated mcKLH kit to elicit an enhanced humoral immune response. The rabbit serum is collected and purified using peptide affinity chromatography.

3.2.1 The Imject Maleimide Activated mcKLH Method of Coupling Peptides to Carrier Proteins

The method is used to conjugate the peptide to the carrier protein KLH through its carboxyl-terminal cysteine residue. There are other coupling strategies available, including amino- and carboxy-terminal coupling described in other publications [10].

1. Dissolve one vial of maleimide-activated mcKLH by adding 200 μL of distilled water (dd.H_2O), making a 10 mg/mL solution. 200 μL of conjugation buffer is added to 5 mg synthetic peptide (*see* **Note 1**).
2. Immediately add the peptide solution to the reconstituted mcKLH. Incubate the carrier protein and peptide mixture for a further 2 h at room temperature (RT) with gentle agitation.
3. Separate the conjugated peptide from EDTA by desalting. Dissolve one bottle of purification buffer salts in 60 mL of d.H_2O (bottle supplied in Imject® Maleimide activated mcKLH kit). Remove the top and bottom caps from a desalting column and wash three times with 15 mL of purification buffer salts. Add the hapten-carrier mixture to the center of the column disc. Apply to the column and collect each of the fractions sequentially.
4. Read the absorbance of each fraction at 280 nm to detect the fractions containing the conjugate. Pool the peak fractions, and re-read the absorbance. Dilute the peptide protein conjugate with PBS to a final concentration of 1 mg/mL and store in 100 μL aliquots at −20C.

3.2.2 Inoculation Procedure

1. All procedures are performed in accordance with the UK Animals Scientific Procedures Act 1986, or equivalent.
2. Carefully mix 200 μL of sterile PBS with 100 μg of freshly thawed peptide-carrier protein conjugate, and emulsify with an equal volume of Freund's adjuvant (Sigma-Aldrich (UK) using a 1 mL syringe (*see* **Note 2**).
3. Inject the conjugate-adjuvant preparation equally using a 25-gauge needle intramuscularly into both hind legs of a Dutch or NZ White rabbit (*see* **Note 3**). Perform the primary immunization using complete Freund's adjuvant, while for subsequent immunizations (at 1 month intervals) use Freund's incomplete adjuvant.
4. Bleed rabbits (*see* **Note 3**) from the marginal ear vein 7–10 days following the booster injections, and collect 10–15 mL of blood. Allow blood to stand at RT for 2 h, and then for

16 h at 4 °C to complete clot formation and contraction, respectively.

5. Remove cellular material by centrifugation at 12,000 × *g* for 10 min at 4 °C, and store the serum in 1 mL aliquots at −20 °C.

In order to improve the quality and specificity of the antibodies, purification is recommended, particularly for use in immunohistochemical and quantitative immunoblotting experiments.

3.3 Affinity Purification

The method is carried out largely as described previously [10] and is used to couple the peptide to activated thiol-sepharose beads via its C-terminal cysteine residue.

3.3.1 Coupling of Peptides to Sepharose Beads

1. Allow 0.35 g of activated thiol-sepharose to swell in 100 mL dd. H_2O for 15 min at RT. Place the swollen sepharose beads in a 25 mL column containing a scinter filter and wash with 100 mL of 0.1 M Tris-HCl pH8.0, containing 0.3 M NaCl and 1 mM EDTA.
2. Drain the column until 0.7 mL of buffer remains. Dissolve 1 mL of 5 mg/mL peptide in the 0.1 M Tris-HCl pH 8.0, containing 0.3 M NaCl and 1 mM EDTA and add to the swollen sepharose beads and incubate for 2 h at RT with gentle agitation.
3. Terminate the reaction by draining the column and washing the sepharose beads with 25 mL of Tris pH 8.0 followed by 10 mL of 0.1 M citric acid pH 4.5. All remaining un-reacted thiol groups on the sepharose beads are blocked by incubation with 3 mL of 1 mM β-mercaptoethanol in 0.1 M citric acid pH 4.5 for 1 h at RT with gentle agitation.
4. Terminate the blocking reaction by washing the sepharose beads with 25 mL of 0.1 M citric acid pH 4.5. Finally, equilibrate the sepharose column with 25 mL PBS and store in 10 mL PBS containing 0.02% (w/v) sodium azide, at 4 °C until required.

3.3.2 Peptide Affinity Purification of Antibodies

Purification of the anti-peptide polyclonal antibody requires the use of a 1 mL sepharose column linked to the appropriate peptide (5 mg) as described above.

1. Equilibrate the column with 100 mL of PBS, and drain. Then apply 4 mL of the immune serum to the column followed by either a 2 h incubation period at RT or overnight at 4 °C with gentle agitation on a rotary shaker.
2. Drain unbound immune serum from the column (*see* **Note 4**), and then wash the column slowly with 100 mL of PBS. Elute the bound antibody from the column with 8 mL of 50 mM glycine/HCl pH 2.3. Collect the eluate in eight sequential 1 mL fractions, containing 15 µL of 1 M Tris to neutralize the

contents to a final approximate pH 7.4. For each fraction determine the optical density (OD) at λ = 280 nm using a standard bench spectrophotometer and pool the peak fractions (at least 0.1 OD unit). The protein concentration can be calculated using the Beer Lambert law:

C = A/ЄL, where

C is the protein concentration of the antibody.

A is the absorbance at λ = 280 nm.

L is the sample path length = 1 cm.

Є is the molar absorptivity or extinction coefficient of the chromophore at wavelength λ (the OD of a 1 cm thick sample of a 1 M solution). Є is a property of the material and the solvent = 1.35.

3. Pool the fractions containing the highest protein concentrations and dialyze with stirring against 500 mL PBS, overnight at 4 °C.
4. Regenerate the affinity column with 100 mL of PBS and store in 10 mL PBS containing 0.02% (w/v) sodium azide at 4 °C. Affinity columns can be kept for many years without losing their ability to purify antibodies.

3.4 Validation Method 1: hH$_3$R Isoform Selectivity Using Heterologous Expression Methods with Individual Isoforms Transiently Expressed in HEK 293 Cells

In order to confirm the selectivity of the antibodies generated, a series of three types of validation methods can be used.

3.4.1 Cell Culture and Transfection of HEK 293 Cells

Preparation of Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, 1:1 ratio) Media.

All procedures should be performed under sterile conditions.

1. Mix powdered DMEM/F12 (15 g/L) and 15 mM HEPES with 800 mL of sterile water. Supplement the media with 40 mL of 10% (v/v) fetal calf serum (FCS), 7.5% (w/v) $NaHCO_3$ giving a final concentration of 3.0 g/L, and 20 mL of penicillin (500 μg/mL)/streptomycin (500 μg/mL) solution.
2. Adjust the pH of the media to pH 7.6 using 10 M NaOH and the final volume made up to 1 L using sterile dd.H_2O. Filter-sterilize the media using a 0.2 μm Sartorius Satolab-V150 filter unit, and then store at 4 °C.

Sub-culturing of HEK 293 Cells

Perform all procedures under sterile conditions. HEK 293 cells are grown in 250 mL Greiner flasks containing DMEM/F12. The flasks are incubated in an incubator (e.g., Sanyo) at 37 °C, humidified in 5% CO_2. The cells are sub-cultured every 3–4 days, at approx.80% confluence.

1. 30 min before, pre-warm sterile PBS, DMEM/F12 media and trypsin-EDTA to 37 °C.
2. Remove the old medium from the cells and wash the cells in 10 mL of pre-warmed PBS.
3. Remove the PBS and incubate the cells for 2 min in 2 mL of trypsin-EDTA, at 37 °C.
4. Resuspend the cells in 10 mL of fresh pre-warmed DMEM/F12 media by gentle pipetting up and down.
5. Add 2 mL of the cell suspension to a fresh sterile flask with a further 10 mL of fresh media and return back to the CO_2 incubator.

Polyethyleneimine (PEI) Transfection Method.

Mammalian cells can be transfected efficiently (up to 30%) using several different simple techniques, including calcium precipitation, and methods using PEI or lipofectamine (the former two being relatively inexpensive if finances are tight). Herein, the PEI method for the transfection of HEK 293 cells adapted from [11], for a 3 mL petri dish of cells (approximately 80% confluent), is described.

1. Dilute 1 μg cDNA (pC DNA Flag-tagged hH_3R isoform) (*see* **Note 5**) in 100 μL 0.9% (w/v) NaCl in a 1.5 mL eppendorf, then add 2 μL of 1 mg/mL PEI (linear MW ~25,000) and flick gently to mix the contents.
2. Incubate the cDNA/PEI mixture freestanding for 10 min at RT. During the incubation period, remove the old media from the HEK 293 cells and add 2 mL of fresh pre-warmed media.
3. Following the incubation period, add the 1 μg DNA/PEI mixture to the HEK 293 cells, gently mix, and incubate at 37 °C in a standard CO_2 incubator.

Harvesting Cells and Membrane Preparation of HEK 293 Cells

1. 48 h post-transfection, remove/aspirate the culture media and replace with 1 mL of homogenization buffer.
2. Scrape cells from the bottom of the petri dish using a Greiner cell scraper. Homogenize the resuspended cells using a dounce glass/glass homogenizer, kept ice cold.

3. Pellet the homogenate by centrifugation at 18,000 × *g* at 4 °C for 5 min.
4. Discard the supernatant and resuspend the pellet in 1 mL of ice-cold homogenization buffer.
5. Rehomogenize the membranes and divide in 100 μL aliquots and store at −20 °C.

3.4.2 Immunoblots

One of the standard methods to utilize the antibodies is immunoblotting. SDS polyacrylamide gel electrophoresis (SDS-PAGE) is commonly carried out using either 7.5 (v/v) or 10% (v/v) polyacrylamide slab gels [appropriate for analyzing monomeric (M_r 30–45 kDa) and dimeric species (M_r 75–90 kDa)] under reducing conditions. Lower percentage polyacrylamide can be used for higher molecular weight proteins. Immunoblots can then be probed either with anti-FLAG antibody (commercial; Sigma-Aldrich, UK) in the case of the FLAG tagged human H_3R clones, or with the appropriate rabbit anti-H_3R antibodies (untagged human H_3R clones).

Preparation of Resolving Gel

1. Prepare the resolving gel by mixing 6 mL of dd.H_2O with 3 mL of resolving gel buffer [50 mM Tris, 384 mM glycine, 1.8 mM EDTA, and 0.1% (w/v) SDS pH 8.8], 6 μL of TEMED, 3 mL of stock 30% acrylamide, and 60 μL 10% (w/v) APS. Immediately pour the polyacrylamide solution gently into a preassembled Hoefer SE 245 dual gel caster, using two gel plates of 10 × 8 cm and spacers of 1 mm width.
2. Layer 100 μL of saturated water/butanol solution over the top of each gel. Cover the gels with parafilm and allow to polymerize for 40–60 min at RT. The gels can be used immediately or stored by wrapping the gels individually in tissue paper and stored in electrode buffer [50 mM Tris, 384 mM glycine, 1.8 mM EDTA, and 0.1% (w/v) SDS pH 8.8] at 4 °C until required. These can be kept for up to 1 month.

Preparation of Protein Samples for SDS-PAGE.

Protein samples for SDS-PAGE are precipitated using the chloroform/methanol precipitation method.

1. To the protein sample (25–50 μg), add 4× volumes of ice-cold methanol and vortex-mix samples and centrifuge at RT at 18,000 × *g* for 1 min.
2. Add 1× volume of ice-cold chloroform to the samples, vortex-mix and centrifuge at RT at 18,000 × *g* for 1 min.
3. Add 3× volumes of ice-cold water to the samples, vortex-mix and centrifuge at RT at 18,000 × *g* for 1 min.

4. Carefully discard the upper layer and add 1× volume ice-cold methanol to the samples, vortex-mix and centrifuge at RT at 18,000 × *g* for 4 min.
5. Discard the supernatant and air-dry the pellet. Resuspend the dried protein pellet by vortex mixing in 5 μL of sample buffer [30 mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS, 2 μL of 200 mM DL-Dithiothreitol (DTT)] and 8 μL of water to a final volume of 15 μL.
6. Boil the samples at 95 °C in the heat block for 5 min and then centrifuge at 18000 × *g* for 30 s at RT before analysis by SDS-PAGE.

SDS-PAGE

1. Carefully clamp the resolving mini-slab acrylamide gel into a Hoefer mini-vertical gel electrophoresis unit SE260.
2. Prepare the stacking gel by mixing 2.3 mL of water with 1 mL of stacking gel buffer [0.5 M Tris-glycine, pH 6.8, containing 8 mM EDTA and 0.4% (w/v) SDS, 650 μL of stock 30% acrylamide, 5 μL of TEMED, and 80 μL of 10% (w/v) APS].
3. Pour the stacking gel mixture immediately and gently (avoiding air bubbles) into the mini-slab gel on the top of the resolving gel and insert a welled comb into the stacking gel. Polymerization takes up to 10 min.
4. Check and carefully remove the comb and wash the wells with dd.H_2O.
5. Pour approximately 300 mL of electrode buffer (*see* above) into and behind the wells and into the base of the electrophoresis unit.
6. Load using a Hamilton syringe or pipette with gel-loading tip extension 15 μL of protein samples and prestained standards (protein molecular weight range of 200–6.5 kDa, Takara Bio Group, USA) into the ten wells of the stacking gel. Ensure all lanes are loaded.
7. Perform electrophoresis at a constant current 10 mA (per gel) initially and then increase the current by 5 mA or 10 mA (per gel) once the samples reach the resolving gel. Gel run time is approximately 2 h or until the appropriate prestained molecular weight marker (M_r 25 kDa) is at the bottom of the resolving gel.

Immunoblotting stage.

After SDS-PAGE is complete, the proteins from the gels are transferred to a nitrocellulose membrane.

1. In order to do this, construct a transfer cassette in the following order of components each of which has been pre-equilibrated in transfer buffer sponge, two sheets of blotting paper, nitrocellulose membrane, SDS-PAGE gel, two sheets of blotting paper, and finally a piece of sponge.
2. On the addition of each component to the transfer cassette, remove air bubbles carefully using a glass pipette or plastic tube. Transfer proteins at a constant voltage of 50 V for 2 h using a Hoefer TE 22 tank transfer unit containing transfer buffer kept cool with external ice packs.
3. Following the transfer of the proteins, briefly rinse the nitrocellulose and incubate with 15 mL of blocking buffer, which comprises of PBS, containing 5% (w/v) dried milk and 0.02% (v/v) Tween-20, for 1 h at RT with gentle agitation.
4. After blocking of the nonspecific antibody sites, wash the nitrocellulose membrane with 10 mL of PBS. Dilute the appropriate affinity-purified primary antibodies in incubation buffer, which comprises of PBS, containing 2.5% (w/v) dried milk to working concentrations of (0.25–5 μg purified antibody/mL).
5. Incubate the nitrocellulose membranes with 10 mL of the diluted primary antibody solution for 2 h at RT, or overnight at 4 °C with gentle agitation (petri dish or plate).
6. After incubation with the primary antibody, wash the nitrocellulose membranes four occasions in 10 mL of wash buffer, consisting of PBS, containing 2.5% (w/v) dried milk and 0.2% (v/v) Tween-20, for 10 min intervals with gentle agitation on a shaker at RT.
7. Incubate the nitrocellulose membranes for 1 h with gentle agitation with horseradish peroxidise (HRP) labeled secondary antibody, either anti-rabbit or anti-mouse depending on the host species for primary antibody, at a dilution of 1/2000 in 10 mL of incubation buffer.
8. Remove the unbound secondary antibody by washing the membrane as detailed above.
9. Drain the nitrocellulose and briefly rinse with PBS.
10. Develop the immunoreactive bands on the nitrocellulose membrane by processing in a solution containing 100 μL of 68 mM p-coumaric acid, 10 mL of 1.25 mM luminal, and 6 μL of 30% (v/v) H_2O_2, for 1 min at RT (*see* **Note 6**).
11. After incubation, wrap the immunoblot in cling film, and place in a film cassette.
12. Expose the immunoblots to Hyperfilm™ for various times between 1 and 5 min, and develop the film developed in Kodak D-19 Developer until the immunoreactive bands are visible and fix in Kodak Unifix for 5 min at RT.

3.5 Validation Method 2–Omission of the Primary Antibody and a Peptide Block

Controls for immunoblots should include the omission of the primary antibody and a peptide block where the primary antibody is pre-incubated with the relevant peptide immunogen (100-fold excess) to confirm antibody specificity.

3.5.1 Peptide Block to Confirm Antibody Specificity for Individual Immunogen

In order to confirm that immunoreactivity detected either in the immunoblots or in immunohistochemical analysis is specific to the amino acid sequence of the immunizing peptide, a peptide block can be carried out.

1. Pre-incubate the antibody overnight at 4 °C with an equal volume of the relevant peptide. The peptide is used in molar excess (500 μg/mL) to ensure complete blockade of antibody binding. Control irrelevant peptides (same concentration) can be used to confirm the sequence specificity.
2. Adjust the final antibody dilution to take into account the initial 1:2 dilution with the peptide.

Any immunoreactive bands or staining that persists after the antibody block is considered to be due to nonspecific antibody binding. Anti-human H_3R isoform-specific antibodies have been validated against HEK 293 cells transfected with the respective H_3R isoform cDNA.

3.6 Representative Examples of Results for Specificity Demonstration and Control Experiments

Figure 3 shows the peptide sequences selected from the overall sequences in Fig. 2, to produce the H_3R isoform-specific antibodies.

3.6.1 Isoform Specificity of the Anti-$rH_{3A/C}/hH_3R_{(445/453)}$ (268–277) Antibody

The sequence chosen is specific to the third intracellular loop region of the $rH3_{C\,(397)}$ isoform. The antibody is shown to be selective for the $rH3_{A\,(445)}$ and $rH3_{C\,(397)}$, but not rat $rH3_{B\,(413)}$ (not shown). Herein, it is shown that the antibody in terms of human isoform also detects both the full-length $hH_{3\,(445)}$ and $hH_{3\,(453)}$ isoforms (Fig. 4). The $hH_{3\,(453)}$ isoform is the result of an additional

Ab 1 Rat H_{3C} isoform rH_{3C}	**(268 - 277)**	**EAMPLHRGSK-Cys**
Ab 2 Human $H_3R_{(365)}$ peptide 1	**(268 - 281)**	**EAMPLHRKVAKSLA-Cys**
Ab 3 Human $H_3R_{(365)}$ peptide 2	**(268 - 278)**	**Cys-EAMPLHRKVAK**
Ab 4 Human $H_3R_{(329)}$ Δ/3	**(222 - 231)**	**Cys-YLNIQSFTQR**
Ab 5 Human $H_3R_{(200)}$	**(191 - 200)**	**Cys-RRPRPRWRSA**

Fig. 3 Anti-human H_3R isoform antibody panel

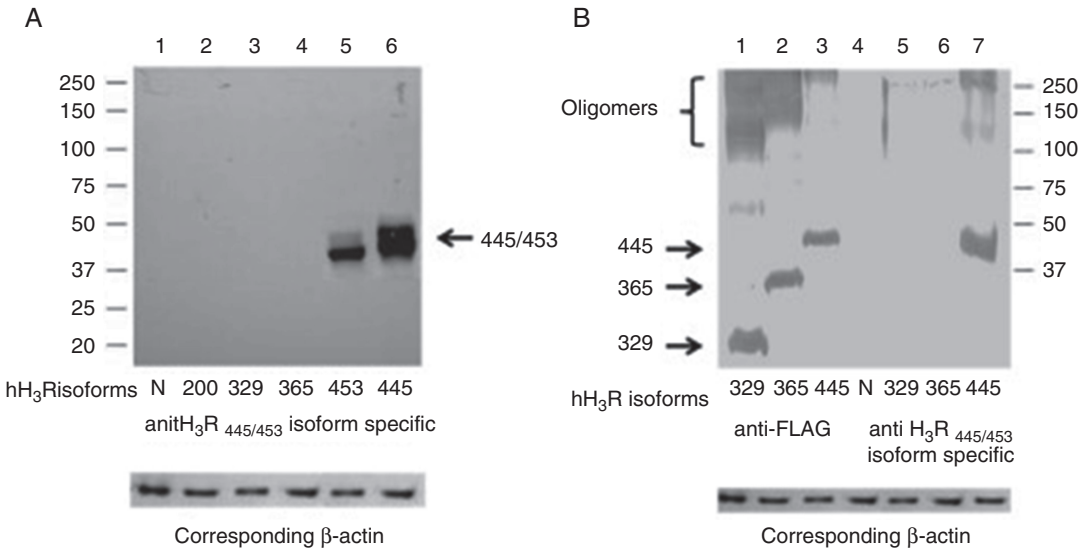

Fig. 4 (**a**) Immunoblot of five different human H_3R isoforms probed with anti-$rH_{3AC}/hH_3R_{(445/453)}$-specific antibody. HEK 293 cells were transfected with individual hH_3R isoforms and processed for immunoblotting 24 h-post-transfection. Approximately 25 μg of protein/well was loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-$rH_{3AC}/hH_3R_{(445/453)}$ antibody (1 μg/mL concentration). The antibody detects only the full-length $hH_3R_{(445)}$ and $hH_3R_{(453)}$ isoforms with no cross reactivity with the shorter isoforms. The lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). *Lane 1*, HEK 293 cells untransfected but still contain PEI; *Lane 2*, HEK 293 cells expressing $hH_3R_{(200)}$; *Lane 3*, HEK 293 cells expressing $hH_3R_{(329)}$; *Lane 4*, HEK 293 cells expressing $hH_3R_{(365)}$; *Lane 5*, HEK 293 cells expressing $hH_3R_{(453)}$; *Lane 6*, HEK 293 cells expressing $hH_3R_{(445)}$. (**b**) Immunoblot of three different FLAG tagged human H_3R isoforms probed with anti-FLAG (*lanes 1–3*) and the anti-$rH_{3AC}/hH_3R_{(445/453)}$ specific antibody (*lanes 4–7*). Homogenates of HEK 293 cells transfected with three different human H_3R isoforms (329, 365, and 445), all epitope tagged with FLAG, were prepared. Approximately 25 μg of protein/well was loaded onto a 7.5% PAGE gel. Identical sample were run on both left and right-hand sides of the gel. Once transferred onto a nitrocellulose membrane, the left-side panel was probed with a monoclonal mouse anti-FLAG antibody (1:5000 dilution) while the right-side panel probed with affinity purified anti-$rH_{3AC}/hH_3R_{(445/453)}$ antibody (1 μg/mL concentration). The FLAG antibody reacts with all three isoforms with monomeric species migrating at approximately M_r 33, 36, 44 kDa. The anti-$rH_{3AC}/hH_3R_{(445/453)}$ antibody detects only the full-length 445 isoform with no cross reactivity with the two shorter isoforms. Lower panel shows corresponding β-actin signal, probed with monoclonal mouse anti β-actin antibody (1:5000). *Lanes 1* and *5*, HEK 293 cells expressing $hH_3R_{(329)}$; *Lanes 2* and *6*, HEK 293 cells expressing $hH_3R_{(365)}$; *Lanes 3* and *7*, HEK 293 cells expressing $hH_3R_{(445)}$; *Lanes 4*, HEK 293 cells untransfected but still contain PEI. All blots shown are representative blots from at least eight similar experiments

exon utilized resulting in an additional eight amino acids added at the C-terminus [9]. The significance of this very rare isoform is yet to be determined.

3.6.2 Immunoblot Showing Labeling of the Human $H_3R_{(445/453)}$ Isoforms Using the Anti-$rH_3R_{AC}/hH_3R_{(445/453)}$ Antibody

The sequence specific to the i3 region in the $rH3_{C(397)}$ isoform is selected. This antibody is shown to be selective for the rat H_{3A} and $rH3_{C(397)}$ isoforms and, surprisingly only the full-length human $H_{3(445/453)}$ isoform (Fig. 4a, b). Antibodies purified from this sequence in different rabbits and different bleeds consistently showed the same human $H_{3(445)}$ selectivity.

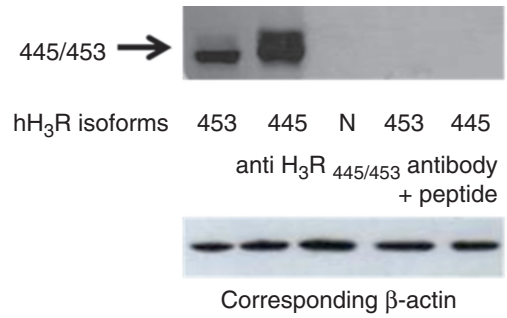

Fig. 5 Selectivity of the anti-rH_{3AC}/ $hH_3R_{(445/453)}$ antibody using the corresponding peptide sequence: Immunoblot confirming the selectivity of the anti-rH_{3AC}/ $hH_3R_{(445/453)}$ antibody. HEK 293 cells were transfected with individual hH_3R isoforms and processed for immunoblotting 24 h post-transfection. Approximately 25 μg of protein/well was loaded onto a 7.5% PAGE gel. Identical samples were run on both left and right-hand sides of the gel. Once transferred onto a nitrocellulose membrane, the left side was probed with the affinity purified anti-rH_{3AC}/ $hH_3R_{(445/453)}$ antibody (1 μg/mL concentration) while the right side was probed with affinity purified anti-rH_{3AC}/$hH_3R_{(445/453)}$ antibody pre-incubated with the antigen peptide (1 μg/mL concentration)

Once the reactivity of antibody against the human H_3R isoform transfected cells was determined, the selectivity of the antibody was determined using peptide blockade (Fig. 5). The major immunoreactive bands labeled in HEK 293 expressing the $hH_3R_{(453)}$ or $hH_3R_{(445)}$ (lanes 1 and 2, respectively) were greatly suppressed by pre-incubation with the antigen peptide (lanes 4 and 5), demonstrating the sequence selectivity of the antibody. Lane 3, HEK 293 cells mock transfected. Lower panel shows corresponding β-actin signal, where the immunoblot is re-probed with monoclonal mouse anti β-actin antibody (1:5000). The anti-rH_3R_{AC}/$hH_3R_{(445/453)}$ antibody has been shown to detect only the full-length human and rat isoforms as well as a truncated $rH3_{A(445)}$ isoform. Therefore, this antibody is useful for looking at the full-length H_3R in both rodent and human tissue.

Occasionally, the peptide-directed antibody production approach yields unexpected results. One such example is the following where an attempt was made to generate an anti-$hH_3R_{(365)}$ selective antibody. However, when screened it was found to only selectively identify the $hH_3R_{(445)}$ isoform.

3.6.3 Isoform Specificity of the Anti-$hH_3R_{(365)}$ Peptide 2 (268–278) Antibody

The sequence chosen is specific to the third intracellular region in the human $H_3R_{(365)}$ isoform. This antibody was shown to be selective for the human $H_3R_{(445)}$ isoform only (Fig. 6).

In contrast, the following antibody was found to selectively label the isoform to which it was directed, namely the $hH_3R_{(329)}$ isoform (Fig. 7).

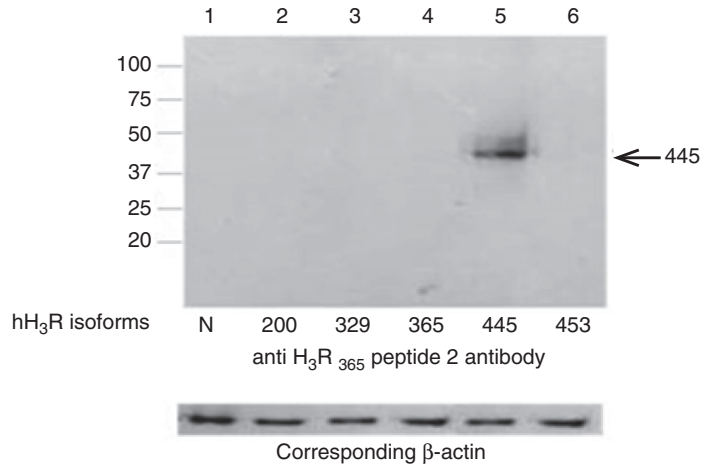

Fig. 6 Immunoblot of five different human H_3R isoforms probed with anti-$hH_3R_{(365)}$ peptide 2 specific antibody. HEK 293 cells were transfected with individual hH_3R isoforms and processed for immunoblotting 24 h post-transfection. Approximately 25 μg of protein/well was loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-$hH_3R_{(329)}$ antibody (3 μg/mL concentration). The anti-$hH_3R_{(329)}$ antibody detects only the $H_3R_{(329)}$ isoform. No cross reactivity with the other shorter or longer isoforms was detected. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). *Lane 1*, HEK 293 cells mock transfected; *Lane 2*, HEK 293 cells expressing $hH_3R_{(200)}$; *Lane 3*, HEK 293 cells expressing $hH_3R_{(329)}$; *Lane 4*, HEK 293 cells expressing $hH_3R_{(365)}$; *Lane 5*, HEK 293 cells expressing $hH_3R_{(445)}$; *Lane 6*, HEK 293 cells expressing $hH_3R_{(453)}$

3.6.4 Isoform Specificity of the $H_3R_{(200)}$/Isoform 5 (191–200) Antibody

The sequence was generated to the C terminus of the human $H_3R_{(200)}$ because of its unique C terminal sequence. The antibody was shown to detect immunoreactivity in both human putamen (not shown) and recombinant HEK 293 cells expressing the $H_3R_{(200)}$ cDNA (Fig. 8).

3.7 Validation Method 3

Immunohistochemical analysis is carried out as essentially described previously [7, 8].

3.7.1 Immunohistochemical Analysis

Diaminobenzidine IHC

1. Perfusion-fix adult mouse brains with 4% (w/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4.
2. Remove brains, post-fix in 4% (w/v) paraformaldehyde (0.05% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 overnight, and then cryoprotect the tissue by incubation in 10%, 20%, and 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4 at 4 °C for 48 h in an even step-wise-manner.
3. Freeze the tissue at −80 °C in isopentane for 1.5 min, and cut coronal sections (25 μm thick) on a cryostat set to −20 °C.

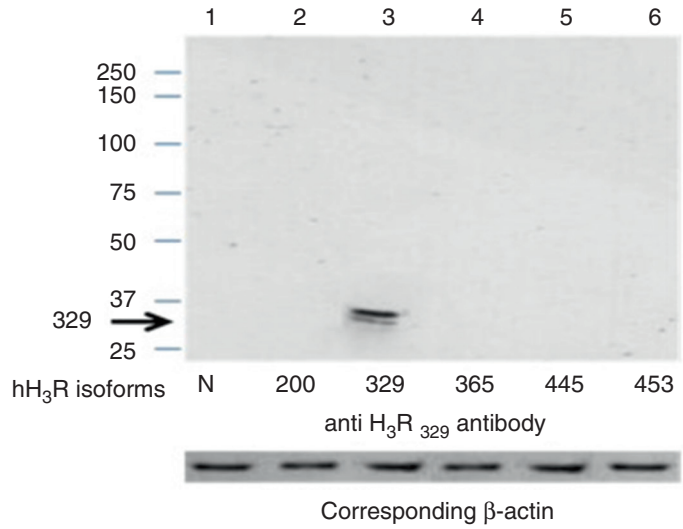

Fig. 7 Immunoblot of five different human H_3R isoforms probed with anti-$hH_3R_{(329)}$ specific antibody. HEK 293 cells were transfected with individual hH_3R isoforms and processed for immunoblotting 24 h post-transfection. Approximately 25 μg of protein/well was loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-$hH_3R_{(329)}$ antibody (3 μg/mL concentration). The anti-$hH_3R_{(329)}$ antibody detects only the $H_3R_{(329)}$ isoform. No cross reactivity with the other shorter or longer isoforms was detected. *Lower panel* shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). *Lane 1*, HEK 293 cells mock transfected; *Lane 2*, HEK 293 cells expressing $hH_3R_{(200)}$; *Lane 3*, HEK 293 cells expressing $hH_3R_{(329)}$; *Lane 4*, HEK 293 cells expressing $hH_3R_{(365)}$; *Lane 5*, HEK 293 cells expressing $hH_3R_{(445)}$; *Lane 6*, HEK 293 cells expressing $hH_3R_{(453)}$

Pick up sections using a fine paintbrush and store in PBS (with 0.05% sodium azide) for not more than 2 weeks at 4 °C. Care should be taken to remove the sodium azide prior to completion of IHC methods as this can inactivate the peroxidase enzyme.

4. Treat free-floating sections initially with 10% (v/v) methanol and 3% (v/v) hydrogen peroxide in 50 mM TBS, pH 7.4, for 10 min to quench endogenous peroxidise activity.
5. Incubate sections in PBS, 0.2% (w/v) glycine and 0.2% (v/v) Tween-20, for 15 min to mop up residual un-reacted aldehyde groups from the fixative.
6. Block nonspecific antibody binding sites on the tissue by incubating with 2% (v/v) FCS in PBS, 0.02% (v/v) Tween-20 for 1 h.
7. Incubate sections overnight at 4 °C in the primary anti-H_3R antibody at a range of concentrations (0–5 μg/mL) in 1% (v/v) FCS/PBS.

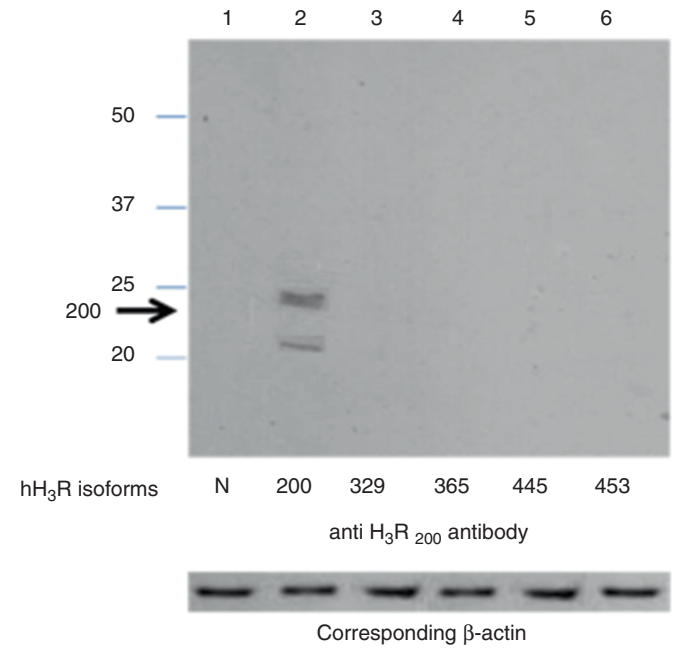

Fig. 8 Immunoblot of five different human H_3R isoforms probed with anti-$hH_3R_{(200)}$ specific antibody. HEK 293 cells were transfected with individual hH_3R isoforms and processed for immunoblotting 24 h post-transfection. Approximately 25 μg of protein/well was loaded onto a 10% PAGE gel. Once transferred the membrane was probed with affinity purified anti-$hH_3R_{(200)}$ antibody (3 μg/mL concentration). The anti-$hH_3R_{(200)}$ antibody detects only the $hH_3R_{(200)}$ isoform. No cross reactivity with the longer isoforms was detected. *Lower panel* shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). *Lane 1*, HEK 293 cells mock transfected; *Lane 2*, HEK 293 cells expressing $hH_3R_{(200)}$; *Lane 3*, HEK 293 cells expressing $hH_3R_{(329)}$; *Lane 4*, HEK 293 cells expressing $hH_3R_{(365)}$; *Lane 5*, HEK 293 cells expressing $hH_3R_{(445)}$; *Lane 6*, HEK 293 cells expressing $hH_3R_{(453)}$

8. After washing the sections on three occasions in PBS/0.1% (v/v) Triton X 100, detect antibody binding using the Vectastain ABC Elite kit.
9. Incubate the sections with a biotin linked, anti-rabbit secondary antibody for 2 h followed by incubation for 1 h with streptavidin-horse radish peroxidase (HRP) complex.
10. Visualize the immune reaction using 3,3′-diaminobenzidine tetrahydrochloride as the HRP substrate.

Immunofluorescence Protocol

1. Perfuse-fix adult mouse brains (wild-type and H_3R KO) with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.
2. Remove brains, post-fix in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 overnight, and then cryoprotect the

tissue by incubation in 10%, 20% and 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4 at 4 °C for 48 h in an even step-wise-manner.

3. Freeze the tissue at −80 °C in isopentane for 1.5 min and cut coronal (or other appropriate orientation) sections (14 μm thick) on a cryostat set to −20 °C. Free-floating sections may be collected as above, alternatively if thinner sections are preferred (8–16 μm), they can be collected onto poly-l-lysine coated slides, air dried, and stored at −80 °C in air-tight boxes until use.
4. On the day of the assay remove the slides from the freezer and air dry again for 1 h.
5. Block the sections for 45 min at RT in PBS pH 7.4 containing 0.4% Triton X-100, 4% FCS or normal serum from the same species as the secondary antibodies are raised in, and 1% BSA.
6. Following the blocking stage, rinse the sections briefly in PBS and then incubate for 48 h at 4 °C with primary anti-H_3R antibody at a range of concentrations (0–5 μg/mL). The antibody diluent for both the primary and secondary antibodies is PBS pH 7.4 containing 0.1% Triton X-100, 1% FCS (or normal serum as before), and 1% BSA.
7. Rinse sections and wash in PBS (3 × 10 min), and then apply secondary antibodies: fluorophore linked anti-rabbit antibodies, e.g., Cy3 or Alexa 488, for 1 h.
8. Rinse sections and wash in PBS as before and mount in Citifluor AF4 or equivalent.

3.7.2 Demonstration of Selectivity Using H_3R KO Mice

Immunoblotting Studies in Mice.

As an illustration of this approach, the specificity of the anti-$rH_{3A/C}$/anti-$hH_{3\,(445)}$ antibody for murine H_3Rs was confirmed by using it to probe immunoblots of H_3R wild-type (WT) and H_3R KO mouse brain. Two bands at Mr 50,000 (±2000) and Mr 44,000 (±2000) are consistently detected in WT mouse brain but not in the KO material. Occasionally, a Mr 40,000 species is also selectively detected (Fig. 9).

IHC Studies in the Mouse.

IHC studies using anti-$rH_{3A/C}$/anti-$hH_{3\,(445)}$ antibody to compare immunoreactivity in H_3R KO mice with WT mice clearly demonstrate the specificity of this antibody. The anti-$rH_{3A/C}$/$hH_{3\,(445)}$ isoform-specific antibody is used to carry out a detailed mapping of the anatomical distribution of the H_3R in mouse brain. IHC is carried out in WT mouse brain slices alongside identical slices obtained from H_3R KO animals. Additional controls include the prior incubation of the anti-$hH_{3\,(445)}$ antibody

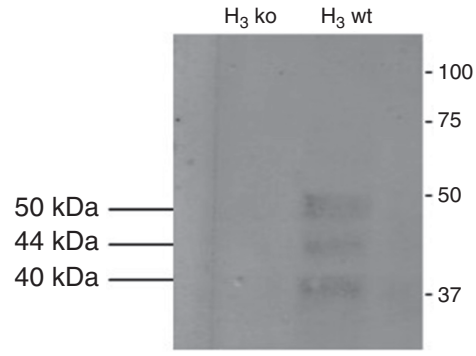

Fig. 9 Immunoblot using anti-rH$_{3A/C}$/anti-hH$_{3\,(445)}$ to compare H$_3$ receptor immunoreactivity in H$_3$R −/− knockout (KO) mice with H$_3$R +/+ wild-type (WT) mice. KO and WT mouse forebrain membranes (10 μg protein) were probed in parallel with anti-rH$_{3A/C}$/hH$_{3\,(445)}$ R used at 1 μg/mL. Two bands at Mr. 50,000 ± 2000 and 44,000 ± 2000 were consistently detected in WT but not in KO material (n = 3 separate mice). After each immunoblot the nitrocellulose sheet was stained with Ponceau S to confirm that the total protein loading was the same for both the KO and WT lanes

with its respective peptide such that specific immunoreactivity would be blocked. To achieve this, the primary antibody is incubated with the peptide at a final concentration of 500 μg/mL overnight at 4 °C. Before use the antibody/peptide mixture is diluted such that the final antibody concentration is the same as for the unblocked antibody. As a second control, IHC is carried out with the secondary antibody alone.

A descriptive summary of the immunoreactivity in selected brain regions is shown in Tables 1 and 2 for both wild-type (WT) and H$_3$R KO mice. Tables 1 and 2 show results using the pan anti-H$_3$R (346–358) antibody and the isoform-specific anti-rH$_{3A/C}$/anti-hH$_{3\,(445)}$ antibody, respectively. The data are from three different mice and the findings are in agreement with those from an independent laboratory using our antibodies to screen at least four further WT and KO animals (Dr Keri Cannon, Albany, USA). Intensity of staining is graded 0–4, with 0 being equivalent to background and 4 representing intense labeling. The strongest specific staining (that is not present in knockout animals) is seen in cortical laminae II, somatosensory cortex (Fig. 10), piriform cortex, and the amygdaloid complex (Fig. 10). Moderate reactivity is also noted in the stratum terminalis, str: caudate putamen, fimbria of the hippocampus, and the cingulum. Weak nonspecific reactivity is observed in some of the brain structures of knockout animals using the anti-rH$_{3A/C}$/anti-hH$_{3\,(445)}$ antibody (Fig. 10). This nonspecific staining was not seen with an immunofluorescence detection system using rhodamine linked anti-rabbit secondary antibodies.

Table 1
Summary of some key brain structures using pan-H_3 receptor antibody

Brain structure	H_3R wild-type mice	H_3R KO mice
Striatum	+3–4	0
Substantia nigra	+3	0
S1/S2 cortex	+3–4	+1
Cingulate cortex	+3	0
Entorhinal cortex	+3	0
Thalamus	+3	0
Amygdaloid complex	+3–4	0
Hippocampus CA1	0–1	0–1
Medial septal cortex	0	0

Wild-type (WT) and H_3R KO Mice produced by Janssens/J&J, USA on a C57Bl6 mouse background. 0 = no detectable labeling, +1 = weak labeling, +2 = moderate labeling, +3 strong labeling, +4 very strong labeling

Table 2
Summary of some key brain structures using anti- $rH_{3A/3C}/hH_{3\,(445)}$ antibody

Brain structure	H_3R wild-type mice	H_3R KO mice
Substantia nigra	+4	+1
Striatum	+3	+1
Cingulum	+3	+2
Somatosensory cortex	+3	0
Fimbria of the hippocampus	+3	+2
Hippocampus: CA1/CA2/CA3 subfields	0	0
Cortex layer II	+4	0
Piriform cortex	+4	0
Stratum terminalis	+4	+1
Amygdaloid complex	+4	0

Wild-type (WT) and H_3R KO Mice produced by Janssens/J&J, USA on a C57Bl6 mouse background. 0 = no detectable labeling, +1 = weak labeling, +2 = moderate labeling, +3 strong labeling, +4 very strong labeling

The background strain of the H_3R KO mice is C5731/6J (US Patent no. 7151200). In contrast to results using the pan anti-H_3 (346–358) in B6C3Fe mice [7], there is negligible immunostaining in the deeper cortical layers and little detectable signal above

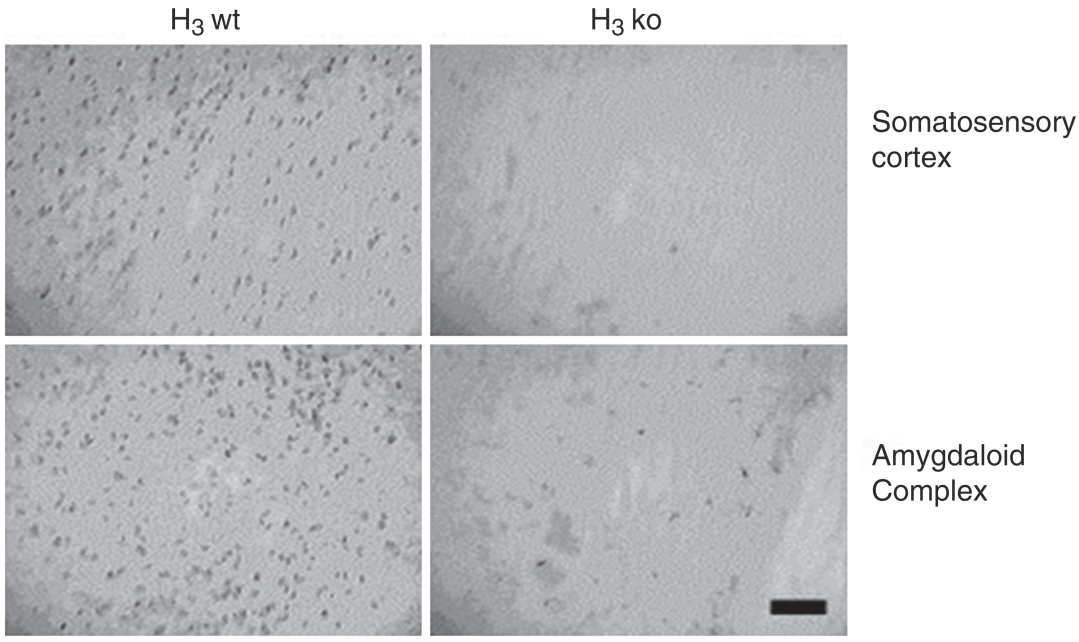

Fig. 10 Immunohistochemical detection of H_3R immunoreactivity in a couple of representative brain structures. Immunohistochemical analysis was performed using anti-r$H_{3A/C}$/anti-h$H_{3\ (445)}$ antibody to compare immunoreactivity in H_3R KO mice with wild-type (WT) mice to clearly demonstrate the specificity of this antibody. 10 μm brain cryoslices were probed with anti-H_3R antibody at 1 μg/mL for 16 h at 4 °C and developed using the DAB/peroxidase method. Scale bar is 100 μm

background in the CA1, CA2, and CA3 regions of the hippocampus. In B6C3Fe mouse brain slices pan anti-H_3 (346–358) immunoreactivity is detected in cortical layers II and V, and in the CA3 region of the hippocampus [7].

4 Notes

1. Synthetic peptides can be purchased commercially from various companies (e.g., Cambridge Research Biochemical, Billingham, UK), at >98% purity that is sufficient. Ideally at least 15 mg is required for all procedures.
2. Complete emulsification is very important for a successful protocol. Routinely, this is checked by leaving the sample to stand for at least 30 min at RT and ensuring that the adjuvant and conjugate do not separate into two layers. If this occurs further mixing is required and retested prior to inoculation.
3. In order to ensure that both inoculation and bleeding procedures are stress-free, rabbits are withstrained gently by an experienced handler in a dedicated clean lab coat with holes cut out for ears to protrude, and back legs supported to ensure the animals do not injure themselves. Rabbits are routinely

housed in small groups in large cages or free-range, the latter being more stress-free.

4. Retain the unbound fraction and store in case the affinity column is overloaded and does not retain all the available antibody. This is rare but does happen occasionally.
5. This CMV promoter cDNA construct, ideal for expression in mammalian cells, was a kind gift from [5], epitope tagged with a FLAG sequence (DYKDDDDK) which offers an alternative method of detection using commercial anti-FLAG antibodies (Sigma-Aldrich (UK), UK).
6. This is a homemade recipe developed in the laboratory. It has a drawback in that it lacks a stabilizer and has a use limit of approx. 10 min. There are a number of commercial products that can be used which contain stabilizers and can be utilized for longer.

5 Conclusions

This chapter describes the development and characterization of a selection of novel anti-human H_3R isoform selective immunoprobes. These probes can be used to investigate expression of three of the human H_3R isoforms 445, 365, and 329 in native human tissue. Different isoforms have been shown for the first time to be expressed at the protein level in the human CNS (e.g., [12; 13]). Future studies can now explore whether there are changes in human H_3R isoforms expression in ageing or disease.

5.1 The Rat $H_{3C}/H_{3\,(397)}$ Isoform Antibody is a Specific Human $H_{3\,(445)}$ Antibody

A peptide sequence unique to the rat $H_{3C\,(397)}$ isoform was chosen and used to immunize rabbits for the generation of sequence-specific antibodies. The antibody was affinity purified and screened against rat and human H_3R isoforms expressed in HEK 293 cells. Immunoblotting of cell homogenates revealed that in addition to detecting the $rH_{3C\,(397)}$ isoform it also labeled the full-length 445 isoforms of *both* the human and the rat H_3R. The peptide sequence EAMPLHRGSK is found within the third intracellular loop of the rat $rH_{3C\,(397)}$ isoform, aa 268–277. The sequence is present in both the human and rat full-length forms but in the $rH_{3C\,(397)}$ isoform it is split: EAMPLHR appearing on the 5′ side of the deletion site and GSK on the 3′ side. Presumably, the tertiary structure of the receptor is such that the two halves are brought together in a structural form that the antibody can recognize. One might have expected the antibody to react with $hH_{3\,(365)}$ (deletion 275–354) and $rH_{3B\,(413)}$ (deletion 275–306) since only the last two amino acids are missing from the EAMPLHRGSK sequence in these two isoforms. In addition in the full-length mouse H_3R the sequence is EAMPLHR**YGV**, with the last three amino acids being different

from the immunizing peptide; nevertheless, specific anti-H$_3$R immunostaining was detected with both the pan anti-H$_3$ and the anti-rH$_{3A/3C}$/anti-hH$_3$R$_{(445)}$ in mouse brain slices (validated by H$_3$ KO mice). However, the tertiary structure of the hH$_{3\,(365)}$ and rH$_{3B\,(423)}$ may have been unfavorable, with the peptide sequence presumably not accessible to the antibody within these isoforms. The hH$_{3\,(329\ \Delta IC)}$ (deletion 227–342) does not possess any of this particular peptide sequence and, as expected, no reactivity was seen with this expressed clone.

5.2 Oligomerization of Human H_3R

Immunoblots of receptors expressed in HEK 293 cells revealed the presence of higher molecular weight species in addition to bands running at the expected molecular weight of the respective receptor. The receptor is not being expressed in its natural environment and expression levels are generally considerably higher than in native tissue. However, higher molecular weight species were also observed in immunoblots of native tissue, especially in the human material. Oligomerization of rat H$_3$R has been described by us and others [14, 15] and there is a growing consensus that many GPCRs, possibly all of them, function as dimers or higher molecular weight oligomers. With rat isoform, rH$_{3\,(397)}$ expressed in HEK 293 cells, higher molecular weight species were less apparent than with the full-length rH$_{3\,(445)}$ isoform.

5.3 Human H_3R Isoform-Specific Antibodies

5.3.1 Immunoblotting Methods Probing Heterologously Expressed Human H_3R Isoforms

Attempts were made to generate antibodies against three more human H$_3$R isoforms using an identical strategy. This strategy yielded in some cases unexpected results, for example Ab2 designed to be a selective hH$_{3\,(365)}$ antibody actually yielded a H$_{3\,(445/365)}$ specific antibody, Ab3 designed to produce a hH$_{3\,(365)}$ selective antibody surprisingly yielded a H$_{3\,(445)}$ selective antibody. In contrast, Ab4 designed to produce an anti-H$_{3\,(329)}$ antibody, proved successful in yielding such an antibody; finally, Ab5 directed to a unique sequence with the highly truncated H$_{3\,(200)}$ receptor again proved successful despite the limited available selective immunogen sequence. Therefore, to date, a panel of anti-H$_{3\,(200)}$, H$_{3\,(329)}$ and H$_{3\,(445)}$ antibodies have been successfully generated; the mixed specificity H$_{3\,(365)}$ /H$_{3\,(445)}$ antibody may have some utility using immunoblotting methods.

5.3.2 Immunohistochemical Techniques with KO Mice

Immunohistochemical (IHC) studies using wild-type and H$_3$R KO mice showed a marked ablation in immunoreactivity in the knockout animals compared to the wild-type, demonstrating the specificity of the antibodies. The immunoreactivity in wild-type animals was similar for both the original pan anti-hH$_3$ (346–358) antibody (not shown) and the new isoform selective antibody. The findings also confirmed those of a previous study using the pan anti-hH$_3$ (346–358) antibody [7] to map the anatomical distribution of the H$_3$R in mouse brain slices; with the exception of

cortex layer V that showed moderate/strong staining in the first study but only light staining in the present investigation, and the hippocampus (regions CA1–CA3 and dentate gyrus) where there was some moderate reactivity, not found here (Table 1). It is possible that these differences might be accounted for by the fact that different mouse strains were used (B6C3Fe, [7]; C5731/6 J in the more recent study); however, further work is necessary to validate these observations and confirm their reproducibility. IHC using the anti-rH_{3C}/h$H_{3\,(445)}$ antibody demonstrated clear specific immunoreactivity in striatum, substantia nigra, cortical laminae II, somatosensory cortex, piriform cortex, and the amygdaloid complex. Moderate reactivity was also noted in the str terminalis, fimbria of the hippocampus, and the cingulum (Table 2). Background labeling was evident in some brain areas in knockout animals, notably the fimbria of the hippocampus and cingulum, and to a lesser degree in the striatum; nevertheless, it was at a significantly lower level than in equivalent areas from wild-type mice. This background labeling was not apparent using an immunofluorescent detection system, e.g., basal ganglia (not shown). A similar picture was observed in the pain circuits study with the pan-H_3R antibody; clear labeling was observed in wt mice for a subpopulation of A delta fibers, and was completely absent in H_3R KO mice; however, a small subset of A beta fibers were labeled nonspecifically as evidenced by maintenance of this immunoreactivity in H_3R KO mice [8]. Furthermore, in immunoblots comparing mouse brain homogenates from WT and KO the animals there was an indication of some immunoreactivity in the KO material [8]. Care should be taken with screening antibodies in KO mice. In the H_3R KO mice, the H_3R gene is disrupted by homologous recombination (US Patent no. 7151200). A 0.7 kb region covering part of the first intron and the 5′ end of the second exon is replaced. Although the gene is rendered nonfunctional by this disruption with no detectable [^{3}H] RAMH binding in KO animals [16] (US Patent no. 7151200), there could possibly be some low level expression of nonfunctional H_3R protein remaining. This was a significant issue in some of the early H_3R KO mice (personal communication). This is also unlikely to be due to the closely related H_4R; as the EAMPLHRGSK sequence is not found within the H_4R aa sequence and the anti-rH_{3C}/h$H_{3\,(445)}$ antibody does not detect recombinant H_4R expressed in HEK 293 cells. The high expression in the basal ganglia (Fig. 10), particularly in the striatum and substantia nigra, is consistent with recent double labeling confocal studies using these H_3 receptor antibodies. These have shown that the H_3 receptor is expressed on subpopulations of GABA-ergic neurons in the sustantia nigra and frontal cortex, and furthermore on subpopulations of histaminergic neurons in the tubermammilary nucleus, which suggests distinct control of output neurons to other brain regions by the H_3 receptor [17].

5.4 Overall Conclusion

In conclusion, generating human H_3R isoform-specific antibodies requires a high level of knowledge and expertise, plus a little bit of fortune. Thorough validation of the antibodies, as described herein, with multiple methods and in combination with in situ hybridizaton and ligand autoradiographical studies is essential before they can be used as reliable probes to investigate the physiological relevance of alternative splicing in the human H_3 receptor.

Dedication

P.L.C. would like to dedicate this chapter to the memory of Sheila Chazot for her love and support.

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