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Properties of mouse vomeronasal receptor and assessment of its role in pheromone signalling

Soundarapandian Kannan[1]*, Pandi Mareeswaran[1], Muthukalingan Krishnan[2], Shanmugam Achiraman[2], Neil Spencer[3] and Balakrishnan F. Nytingel[4]

[1]Proteomics & Molecular Cell Physiology Laboratory, Department of Zoology, Bharathiar University, Coimbatore-641 046, TN, India
[2]Department of Environmental Biotechnology, Bharathidasan University, Tiruchirapalli-620 024, TN, India
[3]School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[4]Department of Zoology, Govt. Arts College, Melur, Madurai-625106, TN, India

Vomeronasal type 2 receptor (V2Rx) from Swiss mouse (*Mus musculus* (L.)) was analyzed by high-resolution ion-exchange chromatography, reversed-phase high-performance liquid chromatography (RP-HPLC), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), Ion Spray tandem mass spectrometry (MS/MS), 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 1-aminoanthracene (1-AMA) fluorometric assay. Vomeronasal sensory neuronal cell bound proteins were resolved into major protein peaks. Several proteins were identified and subsequently purified as the V2Rx receptor on 10% SDS-PAGE with trace amounts of other protein bands. The molecular weight of the identified V2Rx was 109 kDa. MALDI-TOF and micro-sequencing experiments demonstrated that the identified V2Rx receptor shared considerable sequence similarity with vomeronasal receptor type 2 (NCBI Accession Number AB267725), which is a seven transmembrane peptide with 912 amino acid residues. The molecular characterization revealed that the N-terminus of the V2Rx receptor contained the 11GAEAAE16 domain involved in pheromone signalling. The biometric assay (octanamine-V2Rx binding) showed the identified V2Rx receptor and mouse sex pheromone to 2-octanamine (methyl heptyl) in a 1:1 ratio. Uptake of odourants determined in physiological condition showed enhanced V2Rx receptors as volatile hydrophobic pheromone receptors in the vomeronasal neuron of the Swiss mouse.

Pheromone perception in mammals is mediated by specific olfactory receptor neurons. The mammalian pheromones, existing in the form of small lipophilic or hydrophobic compounds, are bound by olfactory epithelial neurons by which a chain of biochemical events is stimulated to convert the chemical signal into an electric signal in the olfactory neurons.[1] The pheromone binding to the receptor cell is apparently facilitated by soluble binding protein present in the nasal mucosa.[2] The primary sequences of the pheromone binding protein (PBP) of several insects such as moth and other lepidopteran species were the first to be documented. The report related to the sequence analysis of mammalian pheromone binding protein is still scanty. The present investigation was designed to determine the sequential arrangement of amino acids in the PBP. In rodents, pheromone detection starts in a sub-septal region of the vomeronasal organ (VNO). Vomeronasal receptors are considered as potential receptors to volatile and non-volatile pheromone compounds. There are two different forms of G-protein coupled receptors (GPCRs), namely V1R and V2R (~150 members in each), which are effectively expressed in two distinct neuronal cells of the VNO. The V1R is expressed in the apical neurons (luminal side), and V2Rs are secreted from the basal neurons.[3,4]

All basal neurons express supplementary and divergent V2Rs, namely V2R2 and V2Rx.[5] Interestingly, it was pointed out that all V2Rx receptors belong to group III of the GPCR family and exhibit structural and sequence homology with metabotropic glutamate receptors,[6] calcium sensing receptor, gamma-aminobutyric acid type B receptor, taste receptors and fish olfactory receptors. Recently, V2Rx gene cloning and its expression were reported in NCBI (NCBI Acc. No. AB267725), which revealed that V2Rx contains 912 amino acids with seven transmembrane structural identity up to 95%. Besides, the N-terminal domain protrudes outside the cell membrane, whereas the C-terminal domain faces the cytosolic region.[9] The odorant binding proteins (OBPs) are expressed by glands of the nasal cavity and bind to a variety of volatile hydrophobic molecules. They are supposed to mediate non-pheromone-based chemo-signal transudation at molecular levels.[7] Although the VNO is implicated in the detection of pheromones as well as pheromone-based behaviors including inter-male aggression and male sexual activity,[8,9] the role of the main olfactory epithelium (MOE) in these behaviors is not experimentally proved, rather ablation of the VNO has a profound effect on curtailing the reproductive and social behaviours in most rodent species.[2]

Pheromone binding proteins (PBPs) belong to a super-family, the lipocalins. In rodents, the lipocalins are reported

* *Correspondence to*: S. Kannan, Department of Zoology, Bharathiar University, Coimbatore-641 046, TN, India.
E-mail: sk_protein@buc.edu.in

to be the third class of label molecules largely used as complex chemical signals.[8] Three-dimensional structures of five lipocalins have so far been resolved by X-ray crystallographic[9] and ^{13}C and ^{15}N NMR spectroscopic analyses.[10] Evidence indicates that the lipocalins are a large, disparate and diverse group of small proteins with molecular weights ranging between 20 and 200 kDa. Most of the lipocalins are extracellular and display selective binding pocket affinity for small hydrophobic molecules.[11] All lipocalins share a common structure which is a central eight standard, β-barrel with simple back and forth repeated topology lining a hydrophobic cavity, with an N-terminal helical turn and long C-terminal helix.

The secretory lipocalin may be used as a chemical label for animal communication. Several reports are in favour of this hypothesis. Secretory lipocalins are well characterized by species, sex, strain and possibly individual link polymorphism.[12] For instance, β-lactoglobulin is excreted in large amounts in the milk of cow, buffalo, sheep and pig, but not in guinea pig, camel or primates. It is very surprising to note that in many cases one end of the β-barrel lipocalin is open and is the entrance to the internal ligand-binding pocket. The loops bordering this entrance have been proposed to be the recognized sites for the receptor, but the opposite end of the β-barrel has also been suggested for this function.[13] The physiological function of these soluble proteins is thus generally defined as pheromone carrier.[14] Pelosi [7] reported that odourant binding proteins (OBPs) are expressed by glands of the nasal cavity that bind to a variety of volatile hydrophobic molecules and are supposed to mediate transudation at the molecular level. However, the behavioural biology of chemical communication in mouse has been known for a longer time and hence an investigation has been initiated to decipher the primary PBP sequence and its structural characterization.

EXPERIMENTAL

Swiss mice (*Mus musculus*) were reared under controlled conditions (light on from 06.00 to 18.00 h, temperature 28 ± 1°C) and fed *ad libitum* with mouse feed purchased from Sai Durga Feeds (Bangalore) with water. The bedding materials were changed twice a week.

Receptor extraction and purification

Four-week-old Swiss mice (*Mus musculus*) were sacrificed under deep anaesthesia. The vomeronasal epithelial cells were removed and washed in NaCl/Pi, 180 mM NaCl (pH 7.3). The tissue samples were stored in liquid nitrogen until further use. The nasal epithelial wash solution was centrifuged at 10 000 *g* for 20 min at 4°C and used as the source of V2Rx receptor and other soluble proteins. Soluble proteins were subjected to anion-exchange chromatography using a Vydac QAE column (300 VHP, 0.75 i.d. × 5 cm; Interchim, France) pre-equilibrated with 20 mM Tris buffer (pH 8.2). Unbound protein was removed by washing and elution was achieved with the same buffer using a linear gradient from 0.1 to 0.5 M NaCl in 40 min. The flow rate was 0.5 mL/min. The eluent from the column was monitored at 215 nm in a flow cell of 9 mm path length where suitable fractions were collected manually directly after passage through the flow cell. All aspects of data acquisition and processing were regulated via the computer-aided packages and programmes supplied with the instrument. The V2Rx-receptor-containing fractions were processed for further analysis.

Purity checking by RP-HPLC

The V2Rx-containing fraction was pooled and submitted to reversed-phase high-performance liquid chromatography (RP-HPLC) on an Aquapore (C8) RP 300 column (0.21 i.d. × 3 cm Browalee, Perkin Elmer, France) equilibrated with 0.1% trifluoroacetic acid (TFA) in distilled H_2O and eluted with a linear gradient from 0 to 27% methyl cyanide in 6 min, then up to 100% of solvent mixture (60% CH_3CN, 40% H_2O), and finally 0.09% TFA in 60 min. The flow rate was 0.2 mL/min and the absorbance was recorded at 215 nm as described by Braind *et al*.[15] with slight modifications.

Proteolysis of the V2Rx receptor

Purified protein was proteolysed by trypsin (Bio Labs). Approximately 100–200 μL of V2Rx receptor (in PBS pH 7.2) was treated with 10 mM 2-mercaptoethanol for 60 min at room temperature. Then the protein sample was incubated with 50 mM final concentration of iodoacetamide for 45 min at room temperature. The reaction mixture was then subjected to TCA (tricarboxylic acid) precipitation using an equal volume of 20% TCA. After centrifugation at 12 000 *g* for 10 min at 4°C, the supernatant was discarded and the precipitate was washed with diethyl ether. Excess ether was removed by keeping the sample in an oven at 40°C for 5 min and the precipitate was resuspended in its starting volume of 100 mM Tris/2 mM $CaCl_2$ (pH 8.5). Protease solution (2 μL of 0.1 mg/mL) was added to the resuspended precipitate which was then incubated overnight at 37°C. At the end of the proteolytic reaction approximately 10 μL of stopping solution (formic acid) was added. The end product of protein digestion was stored at –20°C until further use.

MALDI-TOF and FTICR-MS analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the V2Rx receptor was carried out on a HP G2025A instrument (Hewlett Packard). The digested V2Rx sample was mixed in a 1:1 ratio with a matrix solution of saturated α-cyano-4-hydroxycinnaminic acid (Sigma) in 50% (v/v) acetonitrile/0.2% v/v TFA. Approximately 3–5 mL of this sample was loaded on the sample holder of the MALDI and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers and dried at room temperature. Spectra were subsequently recorded between 0 and 2200 Th with the laser energy optimized to give the best signal-to-noise ratio for each sample. The laser firing range was between 3 and 7 Hz and spectra were collected within a few seconds. The final mass spectrum was a combination of 3–6 such campaigned data sets, illustrating 100–150 individual laser shorts. All aspects of data acquisition, processing and machine management were controlled through the MassLynx Software (version 4.0).

Electrospray ionization (ESI)-MS

ESI-MS and tandem mass spectrometry (ESI-MS/MS) were performed on a Micromass Q-TOF micro instrument, attached to a nano-spray source. The electron spray was generated using a silver-coated glass capillary with a 10 μm orifice, held at a potential of +2000 V relative to the sample concentration. During mass analysis, desalted sample was loaded into the mass spectrometer by syringe pump infusion (Harvard Instruments Ltd., Edenbridge, UK) at a rate of 0.5 μL/min. The instrument was operated in time-of-flight (TOF) mode only with the quadrupole analyzer operating in radio-frequency (RF)-only mode to permit transmission of all product ions fragmented from the V2Rx receptor. Raw data were collected between 200 and 900 at the scanning speed of 2.4/0.1 s. These raw data were appropriately processed using MassLynx (version 4.0).

Electrophoretic analysis of the V2Rx receptor

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) was performed using a Genei vertical gel electrophoretic apparatus (Bangalore Genei Pvt. Ltd., India) according to the method of Schagger and Von Jagow[16] with minor modifications.[17] Molecular mass calibration kits LMW and PMW (Pharmacia) were used and the proteins stained with Commassie Brilliant Blue G^{250} and a similar electrophoresis protocol was adopted to check the purity of the proteins.

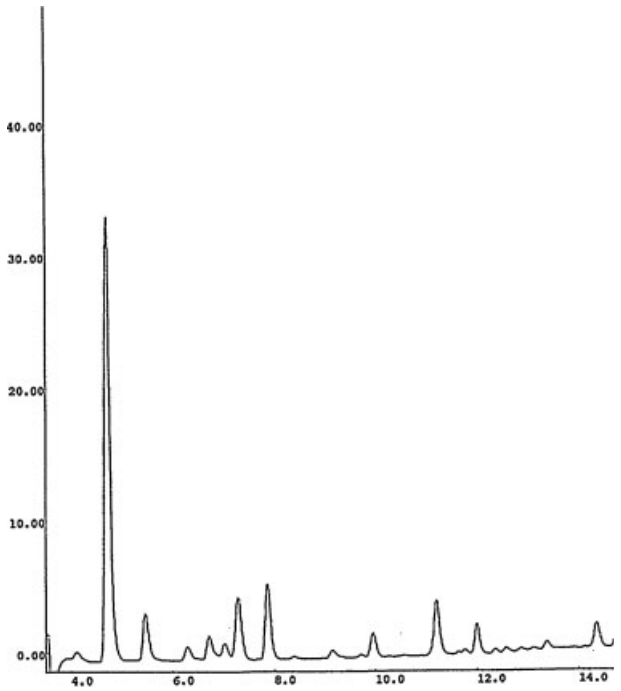

Figure 1. Chromatographic profile of the V2Rx receptor. Ion-exchange chromatography was performed on a Vydac QAE column mounted on a Bio LC platform (Dionex, Camberly, UK). The column was equilibrated with 20 mM Tris buffer, pH 8.5, at 0.5 mL/min before loading the sample of the V2Rx protein. After loading, the sample was washed for 5 min after which the sample was eluted with a linear NaCl gradient of 0.1 to 0.5 M in 40 min. Proteins eluted from the column were detected by monitoring the absorbance at 280 nm of the column eluate in a flow cell of 1 cm path length.

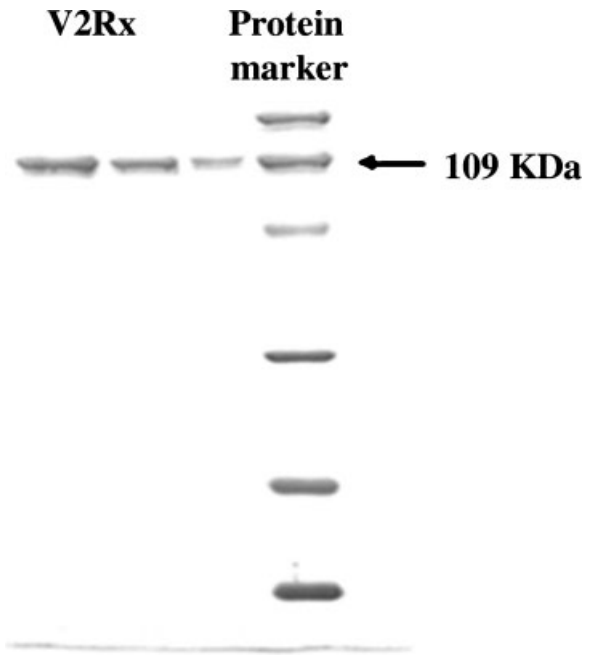

Figure 2. The V2Rx receptor clones such as full length (912 aa residues) expressed in HEK cells were purified and checked in 10% SDS-PAGE.

Micro-sequencing of the V2Rx receptor using mass spectrometry

Further, Ion Spray MS analysis was performed using an API 100 Sciex apparatus (PE Biosystems) linked to a 173 Micro-blotter (PE Biosystems). N-Terminal sequencing was performed by following the modified protocol of Briand *et al.*[18] The oligomerization of the V2Rx protein was studied by ion-exchange chromatography on a 24 m, bed volume Sepharose G-150 column (Pharmacia). A 100-μL sample of purified V2Rx was loaded at 1.0 mg/mL onto the Sepharose column and the elution profiles were obtained from on-line UV detection at 280 nm. The concentration of V2Rx was determined using a UV spectrophotometer. Protein samples

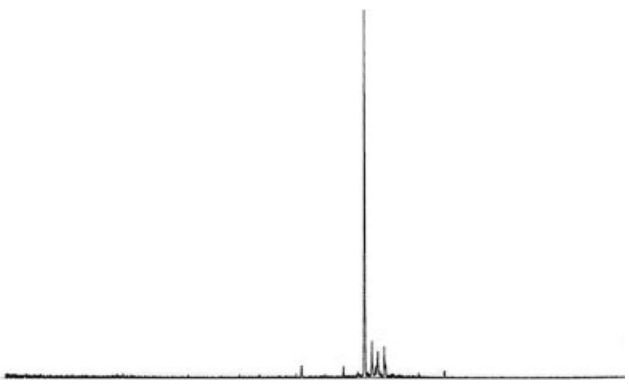

Figure 3. Purification of the pheromone binding protein fragment (N-terminal domain) of the V2Rx receptor with the assistance of LC/MS.

(a)

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        10         20         30         40         50         60
MASRQISLAL GAEAAEHAVL GAQNKTEEVQ CRLMAKFNLS GYVDAKNHSL VIAGLFPIHS

        70         80         90        100        110        120
RIIPVDEAIL EPVSPMCEGF NFRGFRWMKT MIHTIKEINE RKDILPNHTL GYQIFDSCYT

       130        140        150        160        170        180
ISKAMESSLV FLTGQEEFKP NFRNSTGSTL AALVGSGGSS LSVAASRILG LYYMPQVGYT

       190        200        210        220        230        240
SSCSILSDKF QFPSYLRVLP SDNLQSEAIV NLIKHFGWVW VGAIAADDDY GKYGVKTFKE

       250        260        270        280        290        300
KMESANLCVA FSETIPKVYS NEKMQKAVKA VKTSTAKVIV LYTSDIDLSL FVLEMIHHNI

       310        320        330        340        350        360
TDRTWIATEA WITSALIAKP EYFPYFGGTI GFATPRSVIP GLKEFLYDVH PNKDPNDVLT

       370        380        390        400        410        420
IEFWQTAFNC TWPNSSVPYN VDHRVNMTGK EDRLYDMSDQ LCTGEEKLED LKNTYLDTSQ

       430        440        450        460        470        480
LRITKQCKQA VYAIAHGLDH LSRCQEGQGP FGSNQQCAYI PTFDFWQLMY YMKEIKFKSH

       490        500        510        520        530        540
EDKWWILDDN GDLKNGHYDV LNWHLDDEGE ISFVTVGRFN FRSTNFELVI PTNSTIFWNT

       550        560        570        580        590        600
ESSRRPDSFC TQVCPPGTRK GIRQGQPICC FDCIPCADGY VSEKSGQREC DPCGEDDWSN

       610        620        630        640        650        660
AGKSKCVPKL VEFLAYGEAL GFTLVILSIF GALVVLAVTV VYVIHRHTPL VKANDRELSF

       670        680        690        700        710        720
LIQMSLVITV LSSLLFIGKP CNWSCMARQI TLALGFCLCL SSILGKTISL FFAYRISVSK

       730        740        750        760        770        780
TRLISMHPIF RKLIVLVCVV GEIGVCAAYL VLEPPRMFKN IEIQNVKIIF ECNEGSVEFL

       790        800        810        820        830        840
CSIFGFDVLR ALLCFLTTFV ARQLPDNYYE GKCITFGMLV FFIVWISFVP AYLSTKGKFK

       850        860        870        880        890        900
VAVEIFAILA SSYGLLGCLF LPKCFIILLR PKRNTDETVG GRVPTVDRSI QLTSASVSSE

       910
LNSTTVSTVL DE
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(b)

Conserved lipocalin motif (CXXXC)

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SFCTQVCPPGTRKGI      Identified by MS micro-sequencing
PICCFDCIPCADGYV      Identified by MS micro-sequencing
KPCNWSCMARQITLA      Identified by MS micro-sequencing
QCCSGFCIDLLEKFA      gi 3292888 slr1257
KCCYGYCIDLLERLA      gi 3025446 ANF_receptor EG:80H7.7
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Figure 4. (a) Full length sequence of the V2Rx receptor protein extracted from HEK-293 cells transfected with the v2rx/pCDN3 plasmid. (b) V2Rx protein sequence subjected to BLAST search and multiple sequence alignment programme which revealed that it contains the conserved domain of lipocalin motif CXXXC. The occurrence of CXXXC is structurally and functionally significant in lipocalin family members.

Table 1. Results of the conserved motif analysis and sequence comparison

Mass (in Da)	Acc. Number	Conserved domain	Functional properties
1231.3640	gi 27374525	VDFTVPYYDL	glutamate receptor family
1198.2915	gi 3080421	VDFTQPYIES	putative protein
1183.3201	gi 4185738	VDFTLPYTDI	putative glutamate receptor
1171.2659	gi 3482941	VDFTLPYTES	putative ligand-gated ion channel protein
1306.4365	gi 18087723	VDFTEPWLYH	NMDA-type ionotropic glutamate receptor NMR1
1125.2402	gi 2191183	VDFSLPYTPS	similar to the ligand-gated ionic channels family
1196.4232	gi 103181	VDFTVPFMQL	glutamate receptor GluR-II precursor
1201.3101	gi 3292888	VDFSEPFMET	slr1257
1147.2465	gi 3025446	VDFTSPFFST	ANF_receptor EG: 80H7.7

($\sim$1 mg.mL^{-1} in 50 mM sodium phosphate buffer, pH 7.2) were placed in the 0.01 cm path length of the cell. Baseline was recorded with phosphate buffer. Structure proportions were computed with the algorithm of Deleage and Geourjon.[19]

Pheromone binding assay using the 1-AMA probe

Pheromone and protein binding assay (PPBA) was performed with 5 μL of V2Rx in 50 mM potassium phosphate buffer (pH 7.5) using 1-AMA dissolved in 10% methanol of 1 mM stock solution. Spectra were recorded at 25°C using a SFM 25 Kontron fluorometer with a 5 nm bandwidth for both excitation and emission. No cut-off filter was used in the excitation at 255 nm. Calculations were performed from a plot of fluorescence intensity vs. ligand concentration obtained with a non-linear regression method[20] using Delta-graph software (version 4.5). The competitive binding assay buffer 50 mM potassium phosphate buffer (pH 7.5) contained 2 μM of V2Rx and other proteins. The competitor ligands in methanol and dimethyl sulfoxide were used in pure form and 2-octamine (methyl heptyl) was dissolved in 10% methanol.

Purified V2Rx was dissolved in 50 μL of 500 mM potassium phosphate buffer (pH 7.5) to a final concentration of 1.65 mM. Twelve 500 μL glass vials, containing control buffer, V2Rx solutions, was incubated overnight at 25°C in a 2-L sealed glass chamber containing a pre-undiluted odourant (10 μL in the chamber) which was allowed to evaporate freely. The proteins and the control buffer were then extracted at room temperature with 50 μL hexane and analyzed by GC

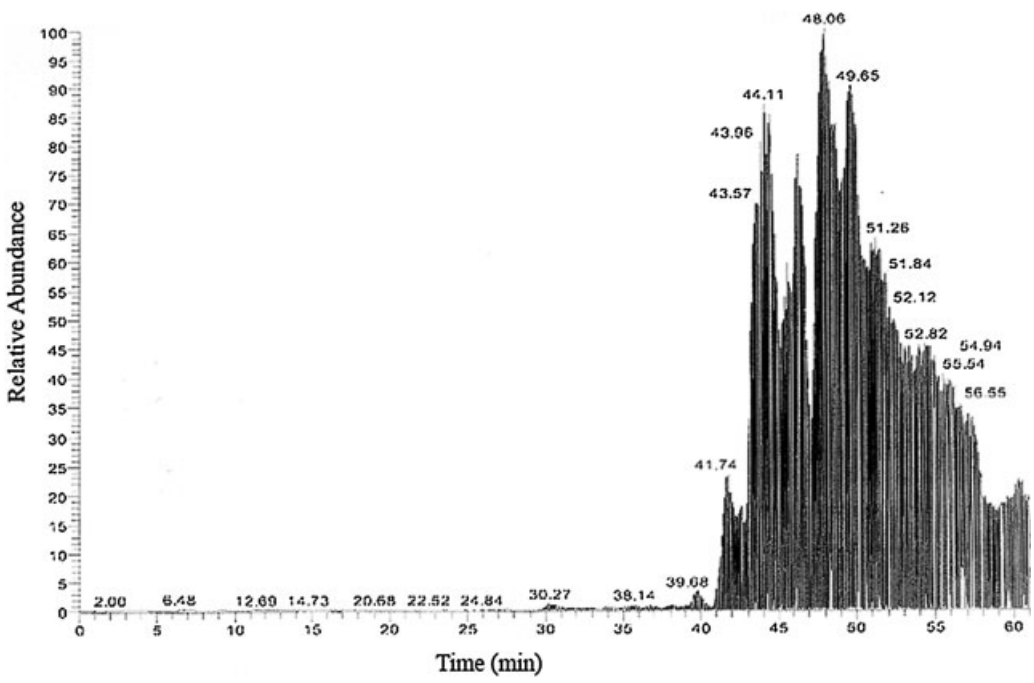

Figure 5. FTICR-MS reveals the relative abundance of the amino acid composition of the V2Rx receptor. Ion spray mass spectrum for proteolysed and fractionated N-terminal region of the V2Rx receptor expressing in the VNO neuron. The V2Rx receptor was digested, separated and diluted 1:50 with a solution of 50% (v/v) acetonitrile/0.2% formic acid. This was introduced into a FTICR mass spectrometer at 0.5 μL/min from the injection port. The mass spectrometer was operated in TOF-only mode and raw data were generated between *m/z* 200 and 900 with scan and inter-scan times of 2.4/0.1 s. Finally, raw data were deconvoluted and transformed to a true mass scale using MasEnt1 software.

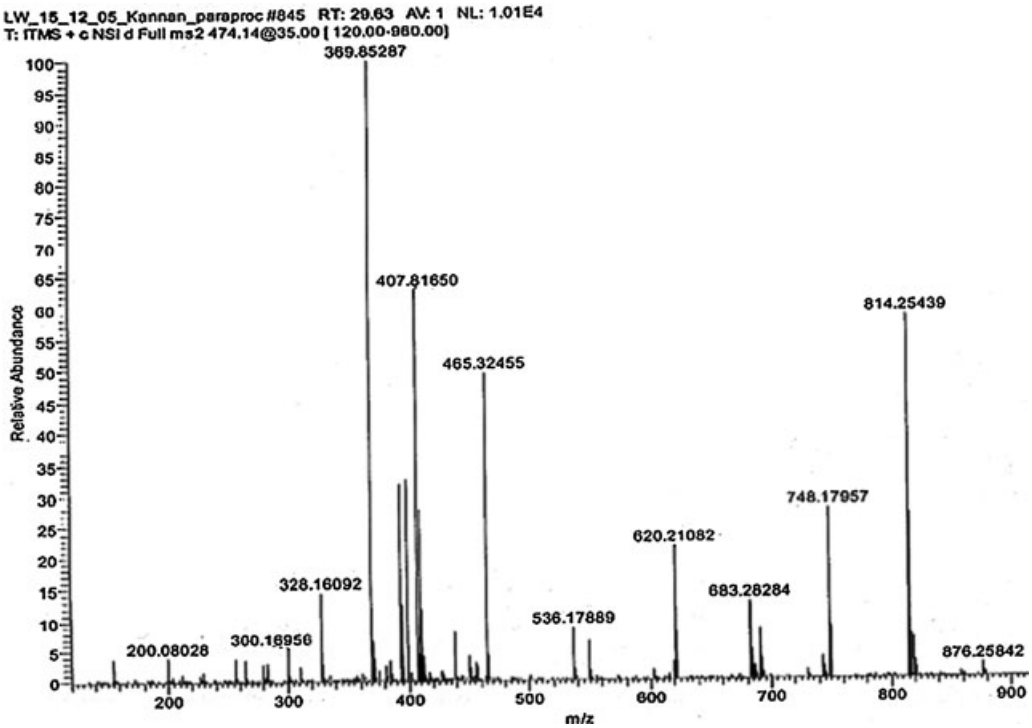

Figure 6. Ion spray mass spectrum of the V2Rx receptor. Ion spray mass spectrum for proteolysed and fractionated N-terminal region of the V2Rx receptor expressing in vomeronasal neurons. The V2Rx receptor was digested, separated and diluted 1:50 with a solution of 50% (v/v) acetonitrile/0.2% formic acid.

using a QP-5000 Series 8180 (Shimatzu, Japan) equipped with an anion-column injector and flame ionization detector (FID) (150°C). The analytical column used was a DB-1 column (30 m × 0.32 mm, i.d., 0.25 μm; J & W Scientific, Interchim, France) with a deactivated precolumn. The oven temperature gradient was applied from 60°C to 150°C at 10°C min^{-1} and then raised to 200°C. The carrier gas was helium at 5 mL/min. Odourants diluted in hexane were used for calibration.

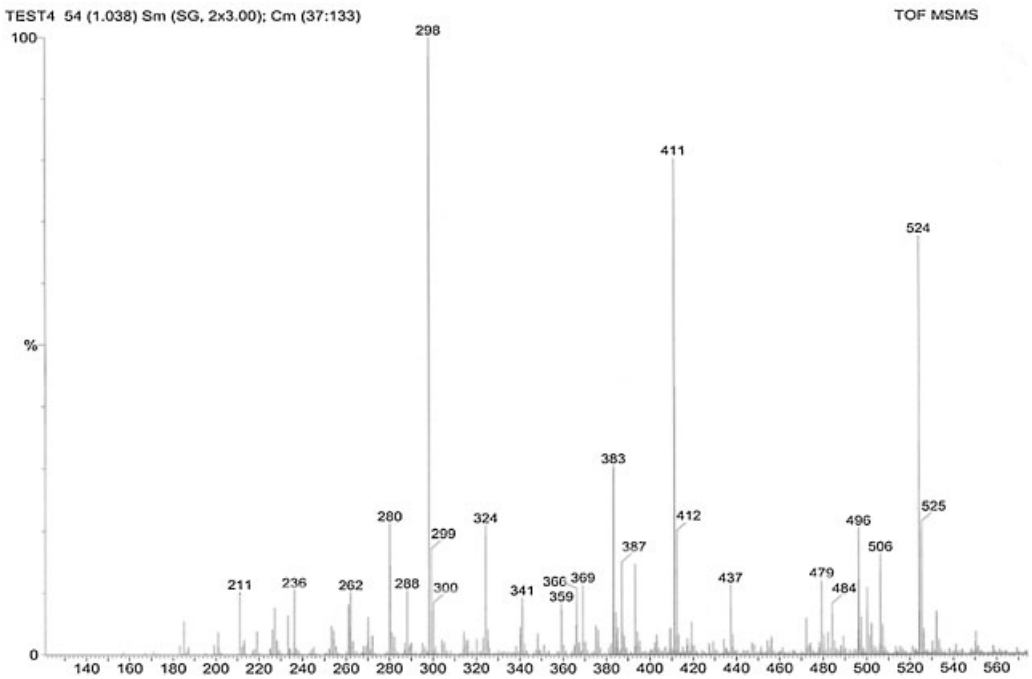

Figure 7. Tandem LC/MS experiments were performed on a micromass Q-TOF Micro mass spectrometer. Trypsin-digested purified mouse V2Rx receptor was introduced into the system and ions of interest were selected for tandem mass spectrometry using the data-dependent switching feature within MassLynx software. Fragmentation energy was similarly automatically selected using this feature. Product ion spectra were coupled and demonstrated using MaxEnt 3 software, within the charge state parameter.

RESULTS AND DISCUSSION

An extracellular protein localized in the 109 kDa region in 10% SDS-PAGE was subjected to anion-exchange chromatography (Vydac QAE column) which excluded the V2Rx receptor from other protein constituents. The ion-exchange chromatogram (Fig. 1) clearly demonstrated that the V2Rx receptor was eluted between the 4th and 5th minutes of retention time. In addition, some minor peaks were also noticed. The minor peaks were observed at the retention times of 5.1, 7, 7.9, 9.9, 11, 12 and 14 min, respectively. The relative abundance of the major peak was found to be 30–35%, whereas all minor peaks were found to be less than 10%. The purity of the protein in the eluate of the major peak was checked through 10% SDS-PAGE, which showed a single band of full length protein and 1-300 V2Rx receptor with molecular weights of 109 kDa, respectively, in Coomassie Brilliant Blue staining (Fig. 2). In addition, the purity and molecular weight were further confirmed with liquid chromatography linked to mass spectrometry. The data (Fig. 3) clearly demonstrated a larger peak at approximately *m/z* 110 and minor peaks at *m/z* 85, 111, 113 and 120. The percentage of relative abundance is directly proportional to peak length. Subsequently, OBPs were identified in a variety of species, including pig, rabbit, mouse and rat.[21] Two rat OBPs have already been cloned and the cDNA sequence is known.[22] Different subtypes of OBPs were discovered first in rat[25] and subsequently also in other species.[23]

Peptide micro-sequencing was carried out using a peptide sequencer and sequencing was carried out as per the standard protocol given in the instrument manual for 36 cycles to study the character of the N-terminal sequence. This analytical study clearly showed the sequential arrangement of amino acids present in the N-terminal region of the V2Rx receptor (Fig. 4(a)). The obtained peptide sequence was subjected to BLAST-P search which revealed the rest of the sequence information like the total number of amino acids (912aa), sequence similarity (99.5%) with the vomeronasal receptor protein, etc. The bioinformatic analysis was further extended to discover the lipocallin signature, which authentically proved that the V2Rx receptor possessed five conserved lipocallin motifs (CXXXC) in its full length sequence. In addition, it also showed sequence similarity with members of the superfamily of the G-protein coupled receptor, namely, glutamate receptor, putative ligand protein, etc. (Fig. 4(b) and Table 1).

FTICR-MS reveals the relative abundance of the amino acid composition of the V2Rx receptor (Fig. 5). The Ion Spray mass spectrum for proteolysed and fractionated N-terminal region of the V2Rx receptor expressing in HEK cells is shown in Fig. 6. The mass spectrometer was operated in TOF-only mode and raw data were generated between *m/z* 200 and

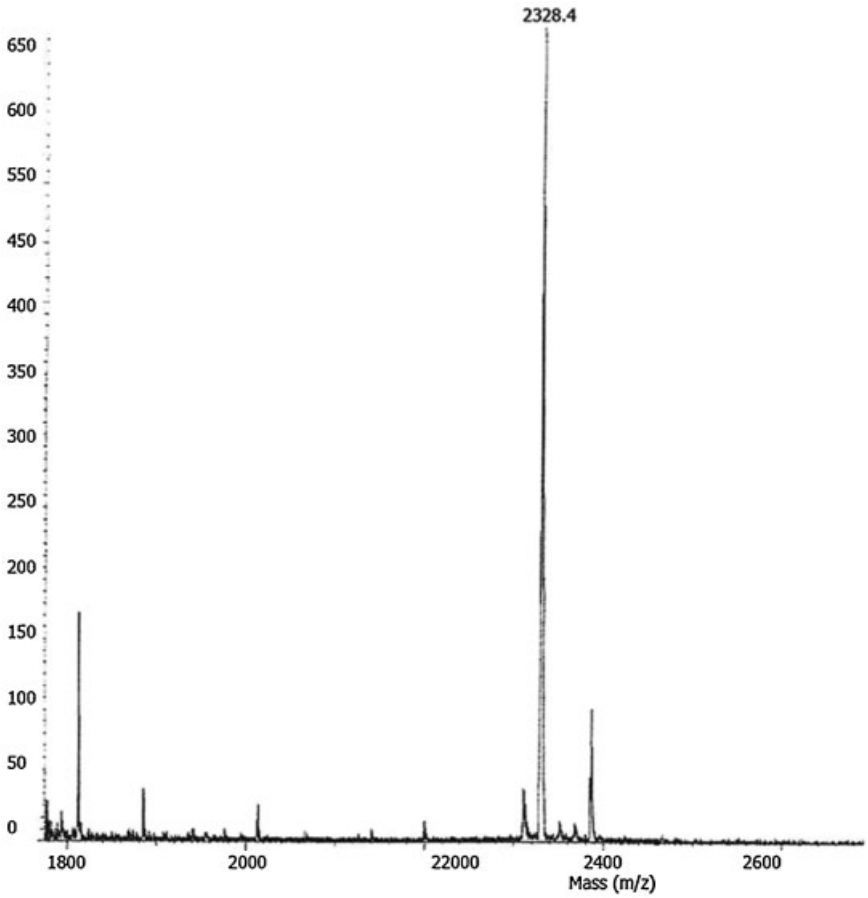

Figure 8. LC/MS peptide analysis revealed the purity of V2Rx N terminus of the receptor protein expressing in vomeronasal neurons. The mass of the resulting peptide was determined using MALDI-TOF-MS.

900 with a scan and interscan time of 2.4/0.1 s. Finally, raw data were deconvoluted and transformed to a true mass scale using MasEnt1 software. LC/MS peptide analysis revealed the purity of the V2Rx N-terminus of the receptor protein expressing in vomeronasal neurons (Figs. 7 and 8). These findings unanimously support the findings of Pevsner *et al*.[24] They rightly pointed out that the OBP binds with several odorant molecules including amines. In addition, results given in Fig. 5 describe the multiple sequence alignment and showed sequence similarity of V2Rx with lipocalin members belonging to the superfamily of G-protein coupled receptors. Our result gains scientific support from Dulac and Torello.[25] All these reports confirm the occurrence of odorant and pheromone binding proteins and their sequence similarity with lipocalin and G-protein coupled receptors.[26]

These observations might explain the discrepancy of the PBP affinity constants for odourants found to be varied.[13] For instance, Flowers *et al*.[27] found that the binding curves for 1,8-ANS (1-Anilinonaphthalene-8-sulfonic acid) on OBPs could be obtained by titration of 2 μM protein with increasing concentrations of chromophore. The following experiment was designed as described by Paolinio *et al*.[28] for porcine V2Rx 1-AMA and proved to be a ligand for the mouse pheromones. In the presence of protein, the emission spectrum showed high frequency at 485 nm corresponding to specific 1-AMA fluorescence. Based on this previous report, data were collected (given in Fig. 9) showing the binding of 1-AMA as followed by fluorescence, the intensity of which is saturable. Assuming that the protein was saturable by 1-AMA, as strongly suggested by titration curves, we read the fluorescence intensity at 485 nm to calculate the relative amount of bound 1-AMA. Data presented in Fig. 10 shows that the fluorescence titration curve was greatly modified when methanol was used as solvent for 1-AMA. Consequently, 10% methanol was used to determine the binding parameters with a 10 pM V2Rx solution to ensure that the V2Rx molecule bound to one 1-AMA molecule (1:1 ratio). Consequently, dissolving 1-AMA in 10% methanol was used preferably for further experiments. The fluorescence binding assay has been used in experiments aimed to test a classical odourant, 2 octanamine (methyl heptyl), as shown in Fig. 11. The pheromonal compound was found to be able to displace 1-AMA quite

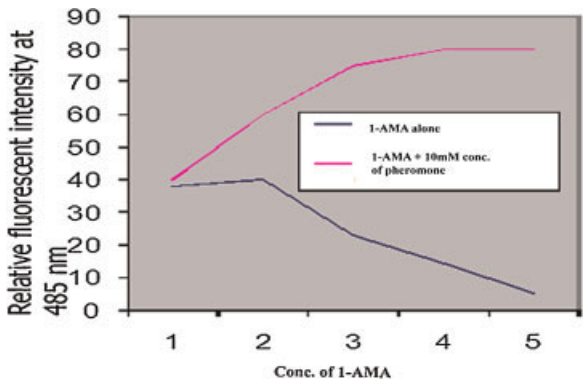

Figure 9. Molecular spectroscopic results for the pheromone displacement with the 1-AMA probe during a competitive binding assay.

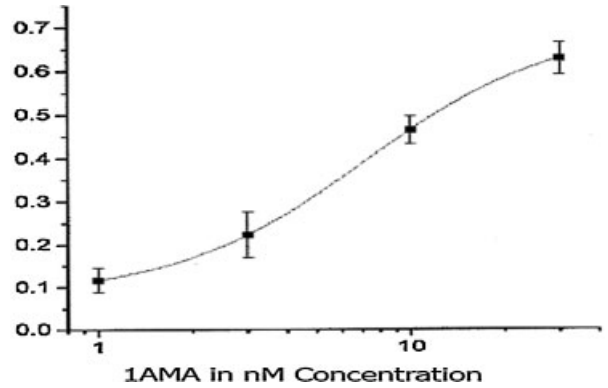

Figure 10. Level of fluorescence was found to increase with an increase in the concentration of 1-AMA, even up to 15 nM concentration.

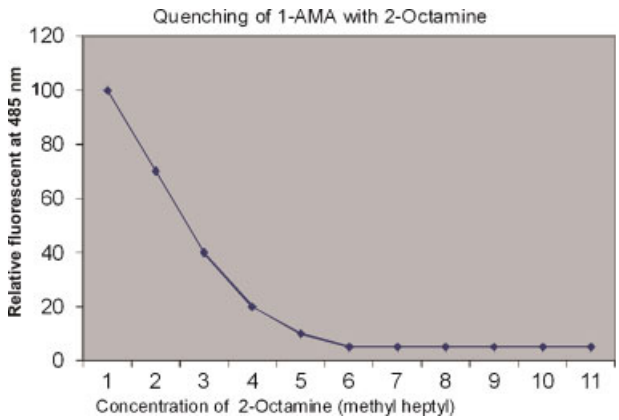

Figure 11. Binding assay for 1-AMA with the identified sex pheromone compound, 2-octanamine (methyl heptyl).

efficiently. It was able to displace half of the 1-AMA molecules from V2Rx at 0.3 μM, which is far less than ethanol (45 mM) and dimethyl sulfoxide at the concentration of 50 mM (Fig. 11). Data retrieved in fluorescence experiments and a biometric assay proved that the V2Rx receptor contains a single binding site at the N-terminal region. A similar result was observed in the case of bovine OBP,[29] but proved different from porcine OBP-1 which has been shown to bind 1 AMA with an equimolar stoichiometry.[20]

CONCLUSIONS

In rodents, sexual advertisement and gender recognition are mostly mediated by chemo-signals. Specifically, there is ample evidence indicating that female mice are 'innately' attracted by male sexual pheromones that have critical non-volatile components and are detected by the vomeronasal organ. These pheromones can only get access to the vomeronasal organ by active pumping mechanisms that require close contact with the source of the stimulus (e.g. scent marking) during chemo-investigation. Thus our findings also demonstrate that V2Rx performs its function as a sex pheromone receptor. The present data reveal a specific

interaction of the pheromone receptor, V2Rx, with 2-octanamine, the identified sex pheromone of mouse.

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