Membrane glycoprotein M6a, which belongs to the tetraspan proteolipid protein family, promotes structural plasticity in neurons and cell lines by unknown mechanisms. This glycoprotein is encoded by Gpm6a, a stress-regulated gene. The hippocampus of animals chronically stressed by either psychosocial or physical stressors shows decreased M6a expression. Stressed Gpm6a-null mice develop a claustrophobia-like phenotype. In humans, de novo duplication of GPM6A results in learning/behavioral abnormalities, and two single-nucleotide polymorphisms (SNPs) in the non-coding region are linked to mood disorders. Here, we studied M6a dimerization in neuronal membranes and its functional relevance. We showed that the self-interaction of M6a transmembrane domains (TMDs) might be driving M6a dimerization, which is required to induce filopodia formation. Glycine mutants located in TMD2 and TMD4 of M6a affected its dimerization, thus preventing M6a-induced filopodia formation in neurons. In silico analysis of three non-synonymous SNPs located in the coding region of TMDs suggested that these mutations induce protein instability. Indeed, these SNPs prevented M6a from being functional in neurons, owing to decreased stability, dimerization or improper folding. Interestingly, SNP3 (W141R), which caused endoplasmic reticulum retention, is equivalent to that mutated in PLP1, W161L, which causes demyelinating Pelizaeus–Merzbacher disease.

Keywords: neuronal plasticity, oligomer, proteolipid protein family, SNPs, TOXCAT system, transmembrane domains.


M6a is a membrane glycoprotein encoded by the gene GPM6A. M6a, together with M6b, proteolipid protein (PLP) and DM20, belongs to the PLP family, and has been related to neuronal plasticity in different experimental models (Lagenaur et al. 1992; Alfonso et al. 2005; Michibata et al. 2009; Huang et al. 2011; Sato et al. 2011a; Zappia et al. 2012). M6a positively contributes to neuritogenesis, filopodia outgrowth and synapse formation in M6a-over-expressing neurons, but its mechanism of action remains unknown (Alfonso et al. 2005; Fuchsova et al. 2009; Formoso et al. 2015). In mice and treeshrews, we identified Gpm6a as a chronic stress-regulated gene (Alfonso et al. 2006). Recently, El-Kordi et al. demonstrated an association between claustrophobia susceptibility and Gpm6a-null mutant mice (El-Kordi et al. 2013). Besides, knockout mice for Gpm6a and Gpm6b show decreased axon elongation and a thinner corpus callosum (Mita et al. 2014). M6a has been associated with various disorders (Table 1). Regarding mental health, de novo duplication of GPM6A has been reported in a patient with learning/behavioral abnormalities (Gregor et al. 2014), and single-nucleotide polymorphisms (SNPs) in the non-coding region have been associated with...
schizophrenia, claustrophobia and bipolar disorders (Boks et al. 2008; Greenwood et al. 2012; El-Kordi et al. 2013).

PLP family members have four transmembrane domains (TMDs), one internal and two external loops, and the N- and C-tails facing the cytoplasm (Fig. 1a). Since M6a is an integral membrane protein, its function should be influenced by its protein–protein and protein–lipid interactions taking place on the cell surface. Many of these interactions are driven by their membrane-spanning helix in TMDs (Levy and Shoham 2005a,b; Berger et al. 2010), through Van der Waals forces and hydrogen bonds (Engelman et al. 2003; Engelman 2005). Interactions between TMDs can be strengthened or debilitated by the presence of certain amino acids. For instance, glycines aid in these interactions by allowing the formation of hydrogen bonds, whereas positively charged amino acids disrupt adequate protein folding, preventing TMDs interactions (Fink et al. 2012). Thus, changes in one amino acid can lead to disruption of these interactions and normal activity of M6a. Disease-associated mutations in TMDs involving changes of non-polar to polar residues have been reported in several transmembrane proteins (Partridge et al. 2004). Concerning PLP1, missense mutations located in TMDs have been associated with different forms of varying severity of Pelizaeus–Merzbacher disease (PMD) (Roboti et al. 2009; White and Kramer-Albers 2014).

Here, we examined the contribution of the self-interaction of the TMDs of M6a to its oligomerization and function. On the cell surface, M6a was mainly in the dimeric form, which might be partially explained by the high interaction between each TMD. Particularly, certain glycines present in TMD2 and TMD4 were critical to induce filopodia. We also studied three non-synonymous SNPs located in the coding region of the TMDs of GPM6A reported in the dbSNP of the national center for biotechnology information. All of them affected M6a-induced filopodia formation by impairing its stability, dimerization and/or folding. Interestingly, the SNP located in TMD3 (W141R), which induced M6a endoplasmic reticulum (ER) retention, correlated with a missense mutation reported for PLP1, which causes PMD.

Table 1 Glycoprotein M6a: Genomic variants and expression related disorders

<table>
<thead>
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<th>Abnormality</th>
<th>Reported changes</th>
<th>Organism</th>
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<td>Mental disorders</td>
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<td>Down-regulated expression in the hippocampus</td>
<td>Treeshrew (Tupaia belangeri)</td>
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<td>Mouse (Mus musculus)</td>
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<td>Colorectal cancer</td>
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<td>Knockout of both Gpm6a and Gpm6b</td>
<td>Mouse (Mus musculus)</td>
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Materials and methods

Animals
Sprague–Dawley female rats maintained at the Facultad de Ciencias Veterinarias of the University of Buenos Aires (Buenos Aires,
Argentina) were used. All animal procedures were carried out according to the guidelines of the National Institutes of Health (publications No. 80-23) and the Committee for the Care and Use of Laboratory Animals of the Universidad de San Martín (CICUAE-UNSAM No. 03/2011) (Buenos Aires, Argentina).

Fig. 1 M6a forms dimers in cell membranes. (a) Topological prediction of M6a structure. M6a possesses four transmembrane domains (TMDs), one minor (EC1) and one major (EC2) extracellular loop, one intracellular (IC) loop and the N- and C-terminal regions facing the cytoplasm. Highlighted are the different mutants assayed. The white circles indicate the relative positions of the glycine residues replaced by alanine, and the dark circles represent the three single-nucleotide polymorphism (SNP)s located in GPM6A TMDs identified in the dbSNP database. (b) Western blot analysis of the oligomeric states of endogenous M6a in plasma membranes from cultured hippocampal neurons (left) and adult hippocampus (right). The samples treated or not with chemical crosslinkers [DSP or dimethyl pimelimidate·2 HCl (DMP)] were subjected to SDS-PAGE and immunoblotted with a polyclonal antibody that recognizes the C-terminal domain of M6a. The expected monomeric band (35 kD) and a dimeric band (70 kD) are indicated. (c) TOXCAT in vitro assay for TMDs self-interaction, in which the DNA coding for each of the four TMDs was cloned into the plasmid pccKAN fused in frame with an N-terminal ToxR’ transcriptional activation domain and in the C-terminal with the periplasmic maltose-binding domain of the maltose-binding protein (MBP), generating a chimeric protein (ToxR-TMD-MBP). ToxR protein from Vibrio cholerae is a specific transcriptional activator that requires dimerization to induce expression of any protein downstream of the ctx promoter. Self-interaction mediated by the TMDs in the Escherichia coli periplasmic membrane leads to the dimerization of the ToxR cytoplasmic domains, which results in the activation of the reporter gene Chloramphenicol acetyltransferase (CAT). CAT expression can be used as a measure of the extent of TMDs self-association. (d) Quantitative assay of M6a TMDs self-interaction. Each TMD of M6a was studied separately, jointly with the TMD of glycophorin A (GPA) and pccKAN as positive and negative controls, respectively. The graph shows CAT activity of the chimeras containing M6a TMDs relative to GPA in at least three independent experiments. Results are expressed as the mean ± SEM. Significant differences were determined by Student’s t test. **pccKAN versus TMD4 (0.0694 ± 0.1274; t6 = 4.470; p = 0.0042) and ***pccKAN versus TMD1 (0.1058 ± 0.0096; t6 = 9.053; p = 0.0001), TMD2 (0.4510 ± 0.0770; t7 = 5.056; p = 0.0007), and TMD3 (0.3448 ± 0.0731; t6 = 4.585; p = 0.0006). Lysates from E. coli expressing the corresponding M6a-TMD chimera were subjected to SDS-PAGE and immunoblotted with an anti-MBP monoclonal antibody (mAb) to determine the levels of chimera expressed.

Reagents and antibodies
Actin filaments were stained with rhodamine-conjugated phalloidin (1/1000, Molecular Probes, Eugene, OR, USA). Primary antibodies were: monoclonal anti-M6a rat IgG (1/250) (Medical and Biological Laboratories, Nagoya, Japan), polyclonal antibody anti-calnexin
and then cloned into the GPM6A of ose-binding protein (MBP) chimeras containing TMDs 1, 2, 3 and 4 obtained as described before (Scorticati et al. 2011). Secondary antibodies were as follows: Alexa flour 568 goat anti-rabbit IgG (1/1000), rhodamine red-conjugated goat anti-rat IgG (1/1000) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and swine anti-rabbit IgG conjugated to horse-radish peroxidase (1/16000) (Dako, Glostrup, Denmark).

Other reagents were Dithiobis[succinimidylpropionate] (DSP), which is a lipophilic membrane-permeable cleavable cross-linker (Pierce, Rockford, IL, USA), and dimethyl pimelimidate (1/16000) (DAKO, Glostrup, Denmark).

Plasmids

The sequence of the TMDs of human GPM6A (1/2/3/4) was determined with UniprotKB (TMD1: LGGIPYASLIATILLYAGV ALP; TMD2: TMDIFKYYVIGAAFFYGGYLLMEVG; TMD3: AWFIMLTYFLMLAWLGVTAFTSLPV; TMD4: HLFIVALAGA GAAVIMVHYLMVLS). Plasmids encoding ToxR(TMD) maltose-binding protein (MBP) chimeras containing TMDs 1, 2, 3 and 4 of GPM6A were generated with two overlapping oligonucleotides and then cloned into the Nhel/BamHI sites of the pccKAN vector kindly provided by Prof. Dirk Schneider (Albert-Ludwigs-Universität, Germany).

For green fluorescent protein (GFP)-tagged proteins, a plasmid encoding for GFP (EGFP-C1; Clontech Laboratories, Palo Alto, CA, USA) fused in frame with the sequence encoding M6a (Alfonso et al. 2005) was used. For independent expression of M6a and EGFP, plasmid pRES2-EGFP was used (Clontech). The coding sequences of M6a and the mutants were cloned between the Apal and KpnI sites of the plasmid.

The mutants G23/24A, G23/24/39A, G87A, G87/96/103A, G220/222A, and G220/222A were generated in pccKAN, whereas the mutants G23/24A, G39A, G23/24/39A, G87/96/103A, G220/222A, SNP1 (F93C, rs11545190), SNP2 (I97S, rs11729990), and SNP3 (W141R, rs11545193) were generated in M6a:EGFP-C1 and/or pRES2-EGFP vectors by standard PCR mutagenesis techniques using BD Advantage 2 Polymerase Mix (BD Biosciences Clontech, Heidelberg, Germany). Two overlapping oligonucleotides containing the target mutation (Macrogen, Seoul, Korea) were used to amplify the template DNA. DpnI endonuclease (New England Biolabs, Ipswich, MA, USA) was used to digest the parental DNA template and select for mutations containing novel synthesized DNA. The identity of all constructs was verified by DNA sequencing.

TOXCAT Assay

For the TOXCAT assay, plasmids encoding ToxR’(TMD1/2/3/4) MBP chimera and the corresponding mutants were transformed into Escherichia coli MM39 cells (malE deletion strain), and plated on Luria Bertani (LB) plates (with agar 1.5%, 100 μg/mL ampicillin and 25 μg/mL streptomycin); colonies were inoculated into LB medium (with 100 μg/mL ampicillin) and stored as glycerol stocks at -70°C. To grow all the mutants at the same time, LB cultures and/or pIRES2-EGFP vectors by standard PCR mutagenesis techniques using BD Advantage 2 Polymerase Mix (BD Biosciences Clontech, Heidelberg, Germany). Two overlapping oligonucleotides containing the target mutation (Macrogen, Seoul, Korea) were used to amplify the template DNA. DpnI endonuclease (New England Biolabs, Ipswich, MA, USA) was used to digest the parental DNA template and select for mutations containing novel synthesized DNA. The identity of all constructs was verified by DNA sequencing.

Chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay (ELISA)

Chloramphenicol Acetyltransferase (CAT) concentration was measured with the CAT ELISA (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The chimeras containing the TMD of glycophrin A (GPA) and the plasmid alone (pccKAN) were used as positive and negative controls, respectively. CAT ELISA results were normalized to the amount of CAT induced by GPA expression or by each TMD wt (Li et al. 2004). The amount of CAT in each assay is considered equivalent to CAT activity.

Hippocampal cultures and plasmid transfection

Dissociated neuronal cultures were prepared from rat hippocampi of embryonic day 19, as previously described (Formoso et al. 2014). Briefly, tissues were treated with 0.25% trypsin in Hanks’ solution at 37°C for 15 min. A single-cell solution was prepared in Neurobasal medium (NB, Invitrogen, Leiden, the Netherlands) containing 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (NBIX) with 10% (v/v) horse serum. Cells were seeded on coverslips coated with 0.1 mg/mL poly-L-lysine hydrobromide (Sigma) and 20 mg/mL laminin (Invitrogen) at a density of 35,000 cells/cm². After 2 h, the medium was changed to NB/N2 (NBIX 1 g/L ovalbumin; N2 and B27 serum-free supplements from Invitrogen). Based on morphological characteristics, we estimated that more than 90% of the cells in the culture were neurons.

At 4 days in vitro (DIV), neurons were transiently transfected with 3 μg of DNA mixed with 1 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunocytochemistry

After 1 day of transfection, cells were fixed in 4% paraformaldehyde, 4% sucrose in phosphate buffered saline (PBS) at 4°C for 10 min. Fixation was followed or not by permeabilization with 0.1% Triton X-100 in PBS for 2 min. Fixed cells were blocked with 3% bovine serum albumin in PBS for 1–2 h followed by incubation with the primary antibody in 3% bovine serum albumin for 16 h at 4°C and with secondary antibodies for 1 h at 25°C. F-actin was stained with phallolidin for 1 h at 25°C. Coverslips were mounted in FluorSave Reagent (Calbiochem, San Diego, CA, USA). To quantify filopodial protrusions, fluorescent images were acquired with a Nikon E600 microscope equipped with epifluorescence illumination (Nikon, Tokyo, Japan). Filopodium density was quantified as previously described (Scorticati et al. 2011). Briefly, filopodium density (number of protrusions stained with phallolidin per 20 μm of dendrite length measured within 50 μm from the soma) was quantified in 40–60 dendrites per group.

Subcellular localization studies were performed using a confocal laser microscope Olympus FV1000 attached to an inverted microscope Olympus IX81 (Melville, NY, USA). Confocal images were acquired in sequential mode with the Fluoview (version 3.3, Olympus, Melville, NY, USA). Images were processed in Adobe Photoshop (version 8.0.1; Adobe Systems, San Jose, CA, USA).

Immunoblotting

Crosslinking samples

Hippocampal tissues from adult rats were disaggregated with 0.25% trypsin at 37°C for 30 min, stopped with fetal bovine serum and

washed twice with cold PBS. The homogenates were divided into three groups: treated with DSP (spacer arm length 12 Å), treated with DMP (spacer arm length 9 Å), and non-treated. DSP was dissolved in dimethyl sulfoxide to a 20-mM stock concentration prior to addition to the sample at a final concentration of 2 mM according to the manufacturer’s instructions. DMP was dissolved in PBS at a final concentration of 20 mM and added directly to the sample. The samples were incubated with the crosslinkers for 30 min at 25°C. The reaction was stopped with Tris-HCl (pH 7.6) to a final concentration of 20 mM.

Hippocampal neurons (10 × 10⁶ cells) were seeded on a 55-cm² culture dish as explained above. At 4 DIV, the cells were divided into three groups: treated with DSP, treated with DMP, and non-treated, as described for the hippocampus.

All the samples, including hippocampal tissue and cultured hippocampal neurons, were then treated with lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) for 30 min at 4°C. An aliquot for each condition was taken to measure protein concentration.

Murine neuroblastoma N2a cells were cultured in Dulbecco’s modified Eagle’s medium with penicillin, streptomycin, and 20% fetal bovine serum. Cells growing in 6-well plates were transiently transfected with 10 μg of DNA mixed with 15 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Afterwards, for each condition, N2a cells were divided into two, washed twice with cold PBS and treated or not with DSP, as explained above. An aliquot for each condition was taken to determine the percentage of transfected cells by using a cell FlowMax cytometer PASIII (Partec, Munster, Germany). The samples were then treated with lysis buffer for 30 min at 4°C.

Western blotting

For samples from rat hippocampus or hippocampal neurons, lysates containing an equal amount of protein were subjected to Western blotting. In the case of N2a transfected cells, lysates containing an equal number of transfected cells were used, by considering both the number of cells and the percentage of transfected cells for each condition obtained by cell flow cytometry (events). Samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and proteins transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA, USA) in a tank blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in PBS containing 5% of non-fat dried milk for 1 h at 25°C and incubated with primary antibody at 4°C overnight. The blots were washed with Tris-buffered saline supplemented with 0.2% Tween and incubated with horse radish peroxidase-conjugated secondary antibodies at 25°C for 2 h. Antigen-antibody complexes were detected according to a standard Enhanced Chemiluminescence Western blotting protocol using Super Signal Chemiluminescent Substrate (Pierce) and X-OMAT films (Kodak).

Statistical analysis

Statistical analysis was performed using Student’s t Test. Calculations were performed with GraphPad Prism 5.0 (San Diego, CA, USA) and data are expressed as mean ± SEM.

Results

M6a forms dimers in membranes

The presence of M6a oligomers in plasma membranes from adult rat hippocampus and cultured hippocampal neurons was analysed using two membrane permeable chemical crosslinkers, DSP (spacer arm length 12 Å) and DMP (spacer arm length 9 Å) (Fig. 1b). Western blots under control conditions showed the expected band of approximately 35 kD corresponding to endogenous monomeric M6a. Samples treated with both crosslinkers showed a second band of approximately 70 kD consistent with M6a dimers. The hippocampal samples showed bands that could represent other forms of multimers. The M6a monomeric form was detectable under DMP treatment, but not under DSP treatment. Because of this distinctive behavior, in the following assays we decided to use DSP-crosslinking only.

Many proteins that possess TMDs form oligomers through homotypic helix-helix interactions. To determine whether this is the case of M6a, we measured the relative TMD self-association strength in the in vitro TOXCAT assay (Fig. 1c) (Russ and Engelman 1999). Since the secondary structure of M6a is unknown, we determined the amino acid residues comprising each of the four TMDs in human M6a by using the protein knowledge database UniProtKB (Fig. 2a). Each of the four TMD was fused in frame with the periplasmic domain of MBP and with the cytoplasmic ToxR’ domain into the plasmid pccKAN, and analysed independently in the TOXCAT assay (see Materials and methods). Fig. 1d shows the strength of self-interaction measured as the CAT levels for each construct normalized to GPA (reference protein). The graphic shows that all M6a transmembrane domains can self-interact and that TMD2 shows higher interaction strength (Fig. 1d). Western blots using anti-MBP antibody were performed to certify that the difference in CAT levels of each TMD is not due to variations in the total amount of the fusion protein expressed. Extracts from cells expressing each TMD showed bands of equivalent intensity of approximately 75 kD (Fig. 1d). These results show that M6a forms dimers in neuronal membranes probably through the self-interaction of its TMDs.

Glycine residues present in TMD2 and TMD4 of M6a are important for their self-interaction

Glycine residues are widely studied as key amino acids that drive the interaction between TMDs (Mueller et al. 2014). Using TOXCAT, we evaluated the self-interaction of a panel of mutants constructed by replacing glycine residues by alanine (Figs 1a and 2a) in each TMD. Fig. 2 shows the CAT activity for each TMD and its mutant version. None of the mutants for TMD1 or TMD3 assayed showed significant differences in the interaction compared to their wt forms (Fig. 2b). In the case of TMD2, only the triple mutant G87/96/103A showed a significant reduction in the interaction
compared with TMD2 wt-expressing cells. The double mutant in TMD4 (G220/222A) displayed the most dramatic decrease in CAT activity, showing only 15% left compared to its wild-type form. The lysates from E. coli cultures expressing each mutant that were subjected to Western blot exhibited similar levels of chimeric proteins in all the mutants assessed (Fig. 2b, lower panel). These results indicate that certain glycine residues present in TMD2 and TMD4 are important for M6a self-interaction and could partially explain M6a dimerization in hippocampal neuronal membranes.

The self-interaction within TMD2 and TMD4 is important for M6a function in primary hippocampal neurons

Mutants G87/96/103A and G220/222A, which had reduced self-interaction, were generated and expressed fused to GFP. All GFP-tagged protein-expressing cells showed equal amounts of the protein (Figure S1A). To determine whether these mutants are affected in their folding, two immunostaining analyses were developed. Monoclonal antibodies were used to recognize M6a major external loop EC2 (M6a monoclonal antibody, mAb) and calnexin, an ER chaperone resident protein. Fig. 3a shows images of each construct (in green) labeled with M6a mAb (in magenta) that recognizes surface expression of M6a and each mutant (magnification in white). Neither mutant G87/96/103A nor mutant G220/222A colocalized with calnexin (Fig. 3b, in magenta). The scatter plot analyses of the colocalization of both immunostainings are shown in Figures S2 and S3. These findings indicate that the glycine mutants do not affect the subcellular localization of M6a on the cell surface.

To test whether M6a self-association is involved in the signaling pathway leading to filopodia formation, we assessed the ability of M6a-TMDs mutants to induce filopodia in neurons. Fig. 4a shows representative inverted images of each construct and their magnification. The average number of filopodia in neurons expressing M6a wt was, as previously reported, significantly higher than that in the control group. The glycine mutants, by contrast, did not induce filopodia formation and showed levels similar to GFP-expressing neurons used as control (Fig. 4b). We also assayed TMD1 glycine mutants, which did not affect self-interaction in the TOXCAT assay, for filopodia formation (Figure S4) and found that none of these constructs impaired M6a function. This result supports the idea that the effect found in mutants G87/96/103A and G220/222A is due to a decreased self-interaction.

To visualize whether dimerization is affected by the presence of mutants G87/96/103A or G220/222A, we used the murine neuroblastoma N2a cells that do not express M6a. The construct used expresses M6a and GFP independently (pIRE52-EGFP). Transfected cells were treated or not (control) with DSP. All pIRE52-EGFP protein-expressing cells showed equal amounts of the protein and we measured the percentage of cells transfected as loading control (Figure S1B). As expected, untreated samples displayed the M6a monomeric band (35 kD), whereas all crosslinked samples displayed the band at the predictable size of a dimer (70 kD). However, in the lanes corresponding to both mutant forms of the protein, the monomeric band was also present (Fig. 4c). These results show that the glycine residues present in TMD2 and TMD4 partially contribute to M6a dimer formation in the cell membrane but are critical for M6a filopodia induction.

SNP1, SNP2, and SNP3 impair the normal function of M6a

Analysis of the national center for biotechnology information database for SNPs in the coding region of human M6a TMDs showed three SNPs (Table 2). SNP1 (rs11545190, F93C) and SNP2 (rs11729990, I97S) are located in TMD2. In both cases, the non-synonymous SNPs result in a non-polar amino acid replaced by a polar one. SNP2 was validated by both the HapMap Project and Cluster. Regarding SNP3 (rs11545193, W141R), in the coding region for the TMD3 of M6a, a non-polar amino acid is changed for a basic one with a positive charge. In silico analysis with the functional annotation tool SNPnexus predicted that these SNPs might be potentially damaging (MacKenzie and Fleming 2008; Dayem Ullah et al. 2012). The risk score prediction of the functional effect of non-synonymous SNPs using the Function Analysis and Selection Tool for Single Nucleotide Polymorphisms (FastSNP) web wrapper indicated a high-risk score (range 0–5) for the three TMD-SNPs. We also used I-Mutant 2.0 to predict the automatic estimation of protein stability change upon single amino acid substitution. The amino acid sequence of human M6a was submitted to the I-mutant 2.0 software (Bologna Biocomputing Group, University of Bologna, Bologna, Italy) as an input file. The free energy change values (DDG, Kcal/mol) of the three SNPs were negative, meaning decreased protein stability (Kramer-Albers et al. 2006; Miyauchi et al. 2006). Finally, the alignment of M6a with PLP protein (Fig. 5a) showed that the amino acids at the positions where SNP1 and SNP3 are located were conserved in both proteins. Interestingly, the amino acid affected in SNP3 corresponds with the PLP1 mutation (W162L), which impair protein folding and causes a mild form of PMD (Roboti et al. 2009). The results obtained by in silico analysis predicted that these SNPs have high risk of damage and destabilization. We therefore studied their effect on M6a function.

GPM6A genetic variants were introduced independently in the EGFP-C1 vector that codes for M6a fused to GFP and were transfected in neurons at 4 DIV. As in the case of glycine mutants, SNP1- and SNP2-expressing cells showed equal amounts of the protein, whereas SNP3-expressing cells showed a decreased (~7 fold) protein expression compared with M6a-expressing cells (Figure S1A). Fig. 5b shows...
inverted images of each transfected neuron expressing the indicated proteins. The quantitative analysis of the number of processes showed that none of the SNPs-expressing neurons were able to induce filopodia compared with M6a wt-expressing cells. In fact, the number of filopodia induced by all SNPs was significantly lower than that in M6a-expressing cells. These results confirm the in silico prediction of the deleterious functional effect of the missense mutations in GPM6A.

Microscopic observation of the neurons expressing the TMD mutants showed that SNP3 appeared to be retained within the cells rather than inserted in the cell membrane. To confirm this hypothesis, two immunostainings were developed (Fig. 3). In Fig. 3a, the left panel, shows cells expressing each of the mutants (green) labeled with anti-M6a mAb (magenta) and the corresponding magnification. SNP1- and SNP2-expressing cells were recognized by the

The functional role of M6a-TMD associations

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<td>22- LAAIPYASLIATILLYAGVALF-43</td>
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<tr>
<td>TMD2-WT</td>
<td>G87A</td>
<td>G87/96/103A</td>
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<tr>
<td>TMD3-WT</td>
<td>G143A</td>
<td>G87/96/103A</td>
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<tr>
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<tr>
<td>TMD4-WT</td>
<td>G220A</td>
<td>G220/222A</td>
</tr>
<tr>
<td>212- HLFIVALAAGAAVIMVHYLMVLS-236</td>
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Fig. 2 Glycine residues present in transmembrane domain (TMD)2 and TMD4 of M6a are important for their self-association. (a) Amino acid sequence of predicted human M6a TMDs and the mutants assayed where the glycine residues (in gray) were replaced by alanine. (b) The TOXCAT assay was performed for each mutant of M6a TMDs and their chloramphenicol acetyltransferase (CAT) activity was measured and normalized to their wt form. As explained in Fig. 1D, the levels of chimera expression were determined by immunoblotting, using the anti-maltose-binding protein (MBP) antibody. G23/24A, G23/24/39A, G87A, G143A, and G220A showed no significant differences compared to their wt form, whereas TMD2 triple mutant G87/96/103A and TMD4 double mutant G220/222A showed a significant decrease compared with their wt form. Results are expressed as the mean ± SEM and significant differences were determined by Student’s t-test. TMD1 versus G23/24A (0.8170 ± 0.0510; t6 = 1.307; p = 0.2612) and G23/24/39A (1.0370 ± 0.1147; t6 = 0.2566; p = 0.8077). TMD2 versus G87A (1.0330 ± 0.1612; t6 = 0.13; p = 0.4504). TMD3 versus G143A (1.339 ± 0.0141; t6 = 1.270; p = 0.2510). TMD4 versus G220A (1.1610 ± 0.0934; t6 = 0.7818; p = 0.4641). *TMD2 versus G87/96/103A (0.5483 ± 0.0355; t6 = 1.971; p = 0.0481), and ***TMD4 versus G220/222A (0.1424 ± 0.0167; t6 = 6.835; p < 0.0001). At least three independent experiments were analysed.
M6a mAb. The overlapping colors were observed all along the cell surface. SNP3-expressing cells showed no signal on the cell membrane (absence of green color) as with GFP-transfected cells (Fig. 3). In the latter, endogenous M6a is observed as magenta dots in the neuronal membranes. This led us to conclude that the presence of SNP3 prevented the protein from reaching the cell surface. This could be due to improper folding of the protein and its consequent retention in the ER. The right panel shows representative images of cells transfected with each construct subjected to staining for calnexin under permeabilization conditions. Neither SNP1 nor SNP2 colocalized with calnexin (magenta). Conversely, SNP3 (green) showed complete colocalization with the ER resident chaperone protein (merged image in white). Figure S2–3 shows the scatter plot analyses of the colocalization of both immunostainings. These results demonstrate that the presence of SNP3 affects M6a proper folding and subcellular localization, and might consequently affect M6a function.

FIG. 3 Both M6a transmembrane domain (TMD) mutants assemble and fold correctly within the neuronal membrane. Primary hippocampal neurons at 4 days in vitro (DIV) were transfected with green fluorescent protein (GFP) alone (control), M6a wt, or the mutants G87/96/103A, and G220/222A cloned into the EGFP-C1 plasmid (in green). (a) The day after transfection, the non-permeabilized cells were fixed and labeled for M6a with the anti-M6a mAb that specifically recognizes M6a EC2 (major external loop) (in magenta). Cells were analysed with a confocal microscope with a 60 × oil-immersion objective. The white color in the merged image and the higher magnification, 20 μm, indicates the recognition by the anti-M6a mAb in all transfected cells. No colocalization was found in GFP-expressing cells with M6a mAb. Scale bar: 20 μm. (b) The day after transfection, cells were permeabilized, fixed, and labeled for the endoplasmic reticulum resident chaperone calnexin with the anti-calnexin mAb (in magenta). The white color in the merged image and magnifications of GFP-expressing cells shows partial colocalization with calnexin. The absence of white color in the merged image of M6a wt and the TMD mutants demonstrates the lack of colocalization with calnexin. Scale bar: 20 μm.

We next analysed how SNP1 and SNP2 affected M6a membrane dimerization in N2a cells (Fig. 6b). Cells were transfected with M6a wt, SNP1, SNP2 or pIRES2-EGFP alone. The percentage of transfected cells in each condition was quantified and the samples were divided in two to be treated or not with DSP (Figure S1B). Under control conditions, the samples of transfected cells showed bands of similar intensity corresponding to the 35-kD M6a monomer. The lanes corresponding to the crosslinked (DSP) samples showed an intense band that corresponds to M6a dimers at 70 kD, whereas the lanes corresponding to SNP1- and SNP2-expressing cells showed a band corresponding to the monomer. This result shows that the presence of SNP1 or SNP2 in GPM6A interferes with M6a dimerization on the cell surface.

Discussion

In this work, we showed that M6a might be forming dimers in the cell membrane through its transmembrane helix-helix
interactions. In TMD2 and TMD4, these α-helix interactions are driven by certain glycines that are also critical to induce filopodia formation in neurons. Besides, three non-synonymous SNPs located in the TMDs of the human GPM6A gene were analysed regarding their stability, subcellular localization, dimerization, and function. Our results demonstrate that these nsSNPs impair M6a function and are a potential risk due to reduced protein stability, decreased dimer formation or inappropriate protein folding.

According to topology predictions, M6a and PLP family members share structural similarity with the tetraspanin protein family (Fig. 1a). Through protein-protein (homo- and heterotypic) and protein–lipid interactions, tetranspins coordinate different signaling pathways, forming the well-known tetranspin-enriched microdomains (TEMs) (Mazzocca et al. 2014). Homotypic-specific interactions between TMDs are largely driven and stabilized by defined packing between two helices and electrostatic forces. The specific tetraspanin functions among different cell types depend on the heterotypic associations within the TEMs with different proteins and/or lipids. Thus, TEMs change their composition and localization in response to internal or external stimuli. We have previously found that M6a is partially distributed in specific lipid microdomains, a location that is critical to

Fig. 4 The self-interaction of transmembrane domain (TMD)2 and TMD4 is important for M6a function in primary hippocampal neurons. (a) Representative inverted images and their magnifications, 20 μm, of hippocampal neurons at 5 days in vitro (DIV) transfected with M6a wt, G87/96/103A, G220/222A, or green fluorescent protein (GFP) alone. Scale bar: 20 μm and 10 μm for magnifications. (b) The number of processes in 20 μm of neurite length was quantified. M6a wt-expressing neurons showed a higher number of processes than GFP-expressing cells. G87/96/103A- and G220/222A-expressing cells showed levels similar to those of GFP-expressing ones. Results are expressed as mean ± SEM of at least 45–65 neurites in three independent experiments. Significant differences were determined by Student’s t-test. ***M6a versus GFP (0.9565 ± 0.2628; \( t_{65} = 7.2320; \ p < 0.0001 \)); ***M6a versus mutants G87/96/103A (1.5350 ± 0.2498; \( t_{65} = 7.8110; \ p < 0.0001 \)) and G220/222A (1.9660 ± 0.2956; \( t_{65} = 6.1720; \ p < 0.0001 \)). (c) Neuroblastoma N2a cells were transfected with M6a wt and mutants G87/96/103A and G220/222A cloned into the plasmid pIRES2-EGFP. Lysates containing an equal percentage of transfected cells for each condition treated or not with dithiobis [succinimidylpropionate] (DSP) were subjected to SDS-PAGE and immunoblotted with an anti-M6a polyclonal antibody. Under control conditions (left panel), a monomeric band of M6a (35 kD) was detected in all samples. Under DSP treatment (right panel), only one dimeric form of M6a (70 kD) was found in M6a-expressing N2a cells. The double and triple mutants showed both the dimeric and the monomeric forms of M6a. GFP-expressing N2a cells were used as a negative control.
induce filopodia formation (Scorticati et al. 2011). We also found that M6a colocalizes with the lipid raft resident family of Src kinases in different cell types and that M6a phosphorylated at Y251 induces neuritogenesis by recruitment and activation of Src and Akt kinases to the cell surface (Formoso et al. 2014).

TMDs have a decisive role in the oligomerization of multi-spanning membrane proteins. Our results show that M6a might be forming mainly dimers in membranes from animal tissue and primary culture of hippocampal neurons, probably driven by the self-interaction of all TMDs (Fig. 1). However, we cannot discard the possibility of heterotypic interactions. The importance of glycine residues in the dimerization has been previously determined by scanning mutagenesis of the TMD of GPA, where it was found that the motif GxxxG aids in dimerization and that replacing only a glycine residue within this motif impairs GPA-TMD self-interaction (Brosig and Langosch 1998; Russ and Engelman 1999). Through subsequent substitution of single glycine residues that do not participate in a GxxxG motif, in each TMD, we determined that both triple and double mutants corresponding to TMD2 (G87/96/103A) and TMD4 (G220/222A) not only affect their

**Table 2 Non-synonymous Single Nucleotide Polymorphisms in human GPM6A: Prediction of their functional significance**

<table>
<thead>
<tr>
<th>SNPs symbol: GPM6A</th>
<th>Chr 4 pos</th>
<th>Region</th>
<th>AA</th>
<th>Prediction</th>
<th>DDG (Kcal/mol)</th>
<th>Risk</th>
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<td>176594940 (--)</td>
<td>Coding</td>
<td>F93C</td>
<td>Damaging</td>
<td>−1.66</td>
<td>3-4</td>
</tr>
<tr>
<td>nsSNP2 (rs11729990)</td>
<td>176594928 (+)</td>
<td>Coding</td>
<td>I97S</td>
<td>Damaging</td>
<td>−3.03</td>
<td>3-4</td>
</tr>
<tr>
<td>nsSNP3 (rs11545193)</td>
<td>176573105 (--)</td>
<td>Coding</td>
<td>W141R</td>
<td>Damaging</td>
<td>−1.57</td>
<td>3-4</td>
</tr>
</tbody>
</table>

*a*Validated by HapMap and cluster.

*b*SNPnexus.

*c*I-Mutant 2.0.

*d*FastSNP.

**Fig. 5** Single-nucleotide polymorphism (SNP)1, SNP2, and SNP3 impair the normal function of M6a. (a) Protein alignment of transmembrane domain (TMD)2 and TMD4 amino acid sequence corresponding to human proteolipid protein (PLP) and human M6a. Highlighted are the positions corresponding to SNP1, SNP2, and SNP3. Positions of amino acid identity are marked by an asterisk (*), while positions of chemical similarity are marked with colons ([;] Conserved and [;] Semi-conserved). (b) Representative inverted images of 20 µm of hippocampal neurons transfected at 4 days in vitro (DIV) with M6a wt or SNP1, SNP2, and SNP3 mutants, or green fluorescent protein (GFP) alone. Scale bar: 10 µm. (c) For each condition, the number of processes in 20 µm of neurite length was quantified. Neurons expressing M6a wt showed a significantly higher number of processes than control GFP-expressing neurons. SNP1-, SNP2- and SNP3-expressing neurons showed a smaller number of filopodia than neurons transfected with M6a wt. Results are expressed as mean ± SEM of at least 45–70 neurites in three independent experiments. Significant differences were determined by Student’s t-test.

***M6a versus GFP (1.00 ± 0.1683; t60 = 8.4230; p < 0.0001);***

***M6a versus SNP1 (1.6667 ± 0.2446; t48 = 5.4060; p < 0.0001);***

SNP2 (1.8370 ± 0.2090; t70 = 5.4190; p < 0.0001), and SNP3 (1.9660 ± 0.2956; t51 = 6.1720; p < 0.0001).
relative self-interaction but also disrupt M6a filopodia induction in neurons. Despite the decreased interaction strength in the glycine mutants (<50%), these mutants showed a feeble reduction of the M6a crosslinking pattern in the crosslinking assays. These results suggest that the glycine interactions that might occur in the lipid bilayer of the cell membrane participate in the signal cascade leading to filopodia induction. In agreement, disruption of self-association through TMDs has important functional consequences in other membrane proteins. For example, diminishing the homotypic interaction of the receptor tyrosine kinase attenuates the oncogenic activation in patients with type 2A multiple endocrine neoplasia (Kjaer et al. 2006). Abolishing TMD association of the integral lipid raft protein TM-agrin arrests filopodia induction activity in neurons and HEK293 cells (Ramseger et al. 2009; Porten et al. 2010). Within PLP family members, PLP, but neither DM20 nor M6b, is able to form oligomers, mainly dimers, in the cell membrane of oligodendrocytes (Dhaunchak and Nave 2007). Particularly, self-interaction was analysed only for the TMD4 of PLP,

Fig. 6 The presence of single-nucleotide polymorphism (SNP)3 prevents M6a from reaching the surface membrane. Primary hippocampal neurons at 4 days in vitro (DIV) were transfected with the mutants SNP1, SNP2 or SNP3 (in green). (a) Non-permeabilized cells were fixed and labeled for M6a with the anti-M6a mAb (in magenta). The white color in the merged image and the magnification, 20 μm, demonstrate the complete recognition by the anti-M6a mAb in cells transfected with SNP1 and SNP2 mutants (in white). The absence of white color in the merged image of the SNP3 mutant demonstrates the lack of recognition by the anti-M6a mAb. On the right panel, permeabilized cells were fixed and labeled for calnexin with the anti-calnexin mAb (in magenta). The white color in the merged image and the magnification demonstrates the colocalization of calnexin with SNP3. The absence of white color in the merged image corresponding to SNP1 and SNP2 demonstrates the lack of colocalization with calnexin. Scale bar: 20 μm. (b) N2a cells were transfected with M6a wt, SNP1, and SNP2 and cloned into plasmid pIRE2-EGFP. GFP-expressing cells were used as negative control. Cells were treated or not with the chemical crosslinker dithiobis [succinimidylpropionate] (DSP). Afterwards, lysates containing an equal percentage of transfected cells of each condition were subjected to SDS-PAGE and immunoblotted with an anti-M6a polyclonal antibody. Under control conditions (left), M6a wt, SNP1, and SNP2 showed a monomeric band (35 kD). Under DSP treatment (right), M6a-expressing N2a cells showed only the dimeric form of M6a (70 kD), whereas SNP1 and SNP2 mutants showed both the dimeric and the monomeric forms of M6a.
which is considered a site of various disease-causing mutations (A241P, A242V, A242E, G245A, G245E, A246T, A247E, A248P, and S252F) in patients with PMD (Stenson et al. 2009). The analysis of the above TMD4 mutants led to the conclusion that point mutations could strengthen self-association by causing aberrant folding of the protein in the ER or generating weak interactions among molecules on the cell surface (Swanton et al. 2005; Ng and Deber 2010).

Interestingly, most of the PLP1 genetic variations found in PMD are gene duplications (60–70%). Of the remainder, only 20% constitute point mutations and are mostly located in TMD3 and TMD4, as previously described (Blanco-Barca et al. 2003; Combes et al. 2006; Bonnet-Dupeyron et al. 2008). In this work, we analysed three non-synonymous SNPs located in the coding region of the TMD2 and TMD3 of GPM6A (Table 2). The non-synonymous SNPs located in TMD2 (F93C and I97S) blocked M6a filopodia induction in hippocampal neurons, whereas the non-synonymous SNPs located in TMD3 (W141R) impaired M6a function through ER retention of the protein. Unfolded/misfolded proteins are known to be retained in the ER and induce their cytosolic degradation or cause ER stress (Roboti et al. 2009). We determined that SNP3 expression levels are lower, in agreement with what happens with the proteins that are retained in the ER and are consequently subjected to degradation. Interestingly, several neurodegenerative disorders are associated with misfolded proteins. For instance, mutations in the neuroserpin protein cause the autosomal dominant dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB) (Roussel et al. 2013). Miranda et al. found a correlation with the severity of the symptoms (phenotype), the accumulation of the polymers and the type of mutation in a fly model and that a mutant that induces rapid protein polymerization causes an early-onset disease (Miranda et al. 2008). In PLP-related disorders, recent studies suggest that missense mutations associated with ER retention and slow degradation (accumulation) (e.g., A242V) produce a severe form of PMD, whereas those associated with ER retention and fast protein degradation (e.g., W162L) produce a mild form of PMD (Roboti et al. 2009; White and Kramer-Albers 2014). Indeed, the amino acid mutant in the non-synonymous SNP located in TMD3 of M6a (W141) corresponds to W162 in PLP1 switched in the mild form of PMD (Fig. 5a). Gmp6a mutant mice and flies grow normally and lack evident behavioral affections, but mutant mice under mild stress develop claustraphobia (Zappa et al. 2012; El-Kordi et al. 2013). These results allow us to speculate that mental disorders associated with genetic variants in GPM6A might be linked to a gain-of-function (e.g., retention in the ER or duplications) rather than with an abolishment of gene expression.

We hypothesize that M6a receives an external stimulus through its EC2 and afterwards the potential specific interactions in the lipid membrane (homo/heterotypic protein–protein and lipid–protein) driven by certain residues stabilize the signal by forming TEMs and consequently regulate cascade pathways inside the cell. This hypothesis is based on our previous findings showing that blocking M6a-EC2 disrupts neurite/filopodia outgrowth and that rich-cholesterol clusters and Src and Akt kinases are functionally related with M6a in the cell membrane (Fuchs-ova et al. 2009; Scorticati et al. 2011; Formoso et al. 2014). In support of this hypothesis, in this work, we established the functional role of certain residues in M6a TMDs, which were seen to influence both self-interaction and stability. However, as in the case of glycine mutants, non-synonymous SNPs did not completely impair the crosslinking pattern, but totally arrested filopodia formation induced by M6a in neurons. We might consider at least three possibilities to explain this: (i) the switched amino acid could also be involved in the heterotypic interactions with other M6a TMDs or with other lipid raft resident proteins, (ii) mutants could induce unstable/weak interactions, and (iii) mutants might affect the quaternary structure of the protein. In all these cases, the evidence found suggests that the TMD mutants assayed are likely sufficient to affect the signaling cascade or to generate an improper signaling platform. In agreement, a missense mutation (e.g. I186T) that affects the binding of the protein to cholesterol and excludes PLP from the lipid raft microdomain in the cell surface causes Spastic Paraplegia Type 2 (Kramer-Albers et al. 2006).

The TMD1 and TMD3 of M6a are also able to self-interact. However, the glycine residues do not participate in this association nor in M6a filopodia induction. In the case of glycines, the interaction depends on the exposure of the glycine-rich interfaces to the respective transmembrane segment in the hydrophobic seal (Miyauuchi et al. 2010). Although glycines are ranked first in the list, other dimerization motifs that could be involved in TMD1 and TMD3 self-interaction that were not studied in this work have been reported. For instance, other ‘small residues’ as serines or threonines, leucines, and isoleucines are considered to be able to dimerize in vitro (Russ and Engelman 1999; Dawson et al. 2002). An extensive helix–helix interface mapping of these TMDs should be done to determine the exact dimerization pattern.

In conclusion, in the last 10 years, various cellular, anatomical, and pathological alterations have been associated with neuronal glycoprotein M6a, whose mechanism of action is still unknown. Sato et al. described the critical role of the TMD1 of M6a in inducing tubular processes in COS-7 cells (Sato et al. 2011b). This work provides the first evidence of the critical role of certain residues located on TMD2 and TMD4 of M6a in the mechanism of action and the potential risk of the SNPs reported here, particularly SNP3.

Acknowledgments and conflict of interest disclosure

We thank Prof. Marcela A. Brocco for kindly providing the M6a-SNPs-EGFP constructs and Silvia C. Billi for excellent technical assistance. KF and M.D.G are PhD students and CS and A.C.F. are researchers from the National Council for Research (CONICET). Contract grant sponsors: CAEN supplies, UNSAM and CONICET grants (to C.S) and Agencia Nacional de Promoción Científica y Tecnológica (to A.C.F.). The authors declare no competing interests.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Figure S1. M6a and its mutant’s expression levels are equal in both plasmids EGFP-C1 and pRES2-EGFP.

Figure S2. The presence of SNP3 prevents M6a from reaching the surface membrane.

Figure S3. SNP3 colocalizes with endoplasmic reticulum resident chaperone calnexin.

Figure S4. Glycine mutants present in TMD1 did not modify M6a induced filopodia formation.

References


