PREDICTION OF TRANSMEMBRANE SEGMENTS FOR THE MONOMERIC SUBUNITS OF THE IONOTROPIC GLUTAMATE RECEPTOR ACTIVATED BY N-METHYL-D-ASPARTATE

by

Leonardo R. Lareo[1] & Carlos Corredor[2]

Abstract

Lareo, L. R. & C. Corredor: Prediction of transmembrane segments for the monomeric subunits of the ionotropic glutamate receptor activated by N-methyl-D-aspartate. Rev. Acad. Colomb. Cienc. **32**(122): 5-13, 2008. ISSN 0370-3908.

The elucidation of transmembrane spanning domain structure is still a challenging experimental problem in protein chemistry. Several important proteins belong to the membrane protein group from which we have chosen the ionotropic glutamate receptor activated by N-methyl-D-aspartate as the subject of our work. This heteromeric protein has a broad range of important physiological functions and seems to be involved in pathological entities. A better knowledge of its sequence and possible structure may help understand its different physiological roles and might be useful for the design of drugs to treat disorders in which it is involved. The present work predicts the sequence of the transmembrane spanning segments of the different receptor subunits using different available algorithms. It compares the predictions obtained with the few published experimentally determined segments, finding good agreement between them.

Key words: transmembrane domains prediction, ionotropic glutamate receptor sensible to N-Methyl-D-aspartate, protein structure prediction, receptor.

Resumen

La determinación de los dominios proteicos transmembranales sigue siendo un problema no completamente resuelto dentro de la química de las proteínas. Al grupo de proteínas de membrana pertenecen importantes estructuras entre las que se encuentra el receptor ionotrópico de glutamato

1 Pontificia Universidad Javeriana. School of Sciences. Department of Nutrition and Biochemistry. Computational and Structural Biochemistry and Bioinformatics. Correo electrónico: l.lareo@javeriana.edu.co Bogotá, Colombia, S. A.

2 Pontificia Universidad Javeriana. School of Sciences. Department of Nutrition and Biochemistry. Computational and Structural Biochemistry and Bioinformatics. Correo electrónico: ccorredo@javeriana.edu.co

activado por N-metil-aspartato, tópico del presente trabajo. Esta proteína multiheteromérica se encuentra involucrada en importantes eventos fisiológicos, así como en muchas entidades patológicas. Una mejor comprensión de las propiedades de su secuencia y de su estructura ayudarán para una mejor comprensión de sus distintos roles y para unas mejores aproximaciones en el diseño de drogas para tratar los desordenes en los que está involucrado. En el presente trabajo se predicen las secuencias que hacen parte de todas las regiones transmembranales de las diferentes subunidades constituyentes del receptor por medio de diversos algoritmos para finalmente generar una predicción de consenso. Se comparan las predicciones con los pocos datos experimentales previamente publicados encontrándose una alta calidad de las predicciones.

Palabras clave: predicción, dominios transmembranales, receptor ionotrópico de glutamato activado por N-metil-D-aspartato, estructura protéica, receptores.

Introduction

Most functional membrane proteins have one or more transmembrane segments whose function ranges from simply anchoring the protein to the membrane, as in Class 1 proteins, to forming ion channels that may open or close upon electrical potential changes. The ionotropic glutamate receptor activated by N-methyl-D-aspartate (iGluR-NMDA) is such a membrane protein.

iGluR-NMDA belongs to the ionotropic receptors family that has three functionally different types: a-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPA), high-affinity kainate receptors and N-Methyl-D-Aspartate (iGluR-NMDA) receptors.

iGluR-NMDA is a heteromeric subunits assembly (**Hawkins *et al*.,** 1999; **Kashiwagi *et al*.,** 1997), but it is still unclear whether it is tetrameric or pentameric (**Laube *et al*.,** 1998; **Rosemund *et al*.,** 1998; **Ferrer-Montiel & Montal**, 1996). It is differentially distributed throughout the CNS (**Nusser**, 2000) and it has been shown to mediate the fast synaptic action of the major excitatory neurotransmitter, L-glutamate (**Cochilla & Alfors**, 1999). These receptors are multimodulated. Glycine (**Hirai *et al*.,** 1996), polyamines (spermine and spermidine) (**Kashiwagi *et al*.,** 1997), histamine and, under some conditions, cations (**McBain & Mayer**, 1994; **Paoletti *et al*.,** 1997) can act as positive modulators. NMDA receptors are coupled to high conductance cationic channels permeable to Ca^{+2}, K^{+} and Na^{+} ions. (**Cushing *et al*.,** 1999).

These macromolecular functional receptor complexes in CNS are constituted by three major subunit families: NR1, NR2, and NR3 (**Blahos & Wenthold**, 1996; **Hawkins *et al*.,** 1999). Alternative splicing generates eight isoforms for the NR1 subfamily that have been identified in rat brain. Three of them have also been identified in human neural tissue (**Nakanishi *et al*.,** 1992). The NR2 subfamily consists of four individual subunits, NR2A to NR2D (**Briecombe *et al*.,** 1997). The NR3 subfamily consists of two individual subunits, NR3A and NR3D (**Andersson *et al*.,** 2001).

Heteromeric NMDA receptor channels made up of a combination of NR1 and NR2/NR3 subunits are known to differ in physiological and pharmacological properties (**Arden *et al*.,** 1998; **Honer *et al*.,** 1998).

This work shows an *in silico* prediction for the transmembrane spanning segments for all fourteen monomeric subunits that can assemble in three to seven subunit groups to build the heteromeric iGluR-NMDA identified in rat brain.

Determination of membrane proteins structures is still at the edge of structural biology. Fully resolved three-dimensional structures at high resolution are available for a few – just about 50 - independent integral membrane protein from a total of about 19000 structures reported in the data bank. This is a mere 0.3% (**Berman *et al*.,** 2000). Given the scarcity of tertiary structure information, other experimental methods have been used to determine the membrane domains topology (**Jennings** 1989) which includes analyses of gene fusion proteins and studies of biochemically modified membrane proteins (**Traxler *et al*.,** 1993). These methods have only added to the list another few resolved membrane protein structures (**Arora & Tamm** 2001).

Theoretical prediction algorithms which take primary structure alone into account have been shown to be important in detecting membrane-spanning segments. Early work in this field primarily addressed the identification of transmembrane segments in the chain. Later work attempted not only to identify transmembrane domains within the sequence but to determine what is their orientation and topology with respect to the membrane they transverse.

By definition, transmembrane domains are protein segments which span a cytoplasmic membrane and are therefore exposed to the hydrophobic and low dielectric constant membrane interior. This means that the segment

must itself be hydrophobic in order to have an energetically favorable conformation. The first transmembrane domain prediction methods still widely used exploit Kyte's & Doolittle's (**Kyte & Doolittle** 1982) hydropathy index. Eisenberg *et al*. (**Eisenberg *et al*.,** 1982) later introduced the helical hydrophobic moment concept that expanded Kyte's & Doolittle's hydrophaty summation to include a directional coefficient, thereby adding orientation of the amino acid within a helix to the measure of hydrophaty. A similar approach has been taken by Rao & Argos (**Rao & Argos** 1986), who consider residues that break the transmembrane helices to improve the reliability of the predictions. In the beginning of 1990's, von Hiejne (**von Hiejne** 1992) described a conserved region of positively charged amino acids found on the cytoplasmic side of transmembrane domains that provide the basis for a new predictive method that integrated hydrophaty analysis with information assessment of the positive inside rule to locate transmembrane domains and assign an orientation or topology to these domains.

Complex, data trained, software algorithms are the basis for most of the more recent transmembrane domain prediction methods. Rost & Sander (**Rost & Sander** 1994) created a neural network system which was trained to recognize both the positive right hand rule and regions of high hydrophobicity and, from that data, make transmembrane domain prediction. This generates a group of so-called PHDhtm programs to produce the final transmembrane predictions (**Rost**, 1996).

Cserzo ***et al*.** (**Cserzo *et al*.,** 1997) developed the dense alignment surface method (DAS) to improve weaknesses of the PHDhtm program. The DAS profile averages individual cumulative score profiles, calculated from pair comparisons of non-redundant, transmembrane proteins with known topology.

Another approach is the use of Hidden Markov Models. The advantage of this type of method is that it predicts both the domain regions and their topology. (**Krogh *et al*.,** 2001; **Tusnady & Simon** 1998).

Two recently published independent evaluations have examined a large amount of transmembrane prediction methods. Both papers agree that currently used prediction methods provide good quality results with up to 85% success in transmembrane helices predicted correctly (**Moller *et al*.,** 2001; **Chen & Rost** 2002).

Materials and methods

Selection of rat iGluR-NMDA sequences

The following sequences were selected from more than 70 sequences deposited in the Gene Bank at NCBI. Nomenclature used is an adaptation of Hollman's (**Hollman** 1999) proposal for nomenclature. For isoform NR1-1a, **GI:475554**; for NR1-1b, **GI:475558**; for NR1-2a, **GI:475556**; for NR1-2b, **GI:475560**; for NR1-3a, **GI:475562**; for NR1-3b, **GI:475564**; for NR1-4a, **GI:475566**; for NR1-4b, **GI:475568**; for NR2A, **GI:2155310**; for NR2B, **GI:205739**; for NR2C, **GI:205735**; for NR2D, **GI475552**; for NR3A, **GI:5305435**, and for NR3B, **GI:20376816**. Criteria for selection were: minimal inconsistencies, complete reports and complete annotated registration.

Transmembrane spanning segment prediction algorithms

The following eight programs were selected from a broad range of transmembrane prediction algorithms on the basis of published quality evaluations (**Moller *et al*.,** 2001; **Chen & Rost** 2002): DAS from Stockholm University (**Cserzo *et al*.,** 1997); HMMTOP from the Hungarian Academy of Sciences (**Tusnady & Simon** 2001); SOSUI from Tokyo University of Agriculture & Technology (**Mitaku *et al*.,** 2002); TMHMM from the Center for Biological Sequence Analysis in Denmark (**Krogh** *et al*., 2001); TMPred from EMBnet (**Hoffmann & Stoffel** 1993); TopPredII from Stockholm University (**Claros & von Heijne** 1994); SMART from EMBL (**Letunic *et al*.,** 2002) and SAPS from ISREC (**Brendel *et al*.,** 1992). The number of transmembrane segments was predicted with each of the eight programs selected. The topology was determined by knowledge of the total receptor orientation (**Whitehorn *et al*.,** 1999; **Sprengel *et al*.,** 1998). There was close agreement about the number, but not so, about the length of each of the predicted segments. To overcome this difficulty, we aligned the eight predicted segments using the program ClustalW from EBI (**Higgins *et al*.,** 1994) to obtain a consensus sequence. The results were confirmed using DIALING from University of Bielefeld (**Abdeddaïm & Morgenstern** 2001). This process was repeated for each segment of every subunit so as to obtain complete predictions for the 14 subunits selected.

Results

Number of predicted transmembrane spanning segments

The total number of transmembrane segments predicted was fifty. Three transmembrane segments were predicted for all eight isoforms of NR1 and for the two NR3 subunits. For NR2 subunit types, five transmembrane segments were identified. Analysis of the eight NR1 transmembrane segment sequences (TMSS) showed that they could be grouped in two clusters. The three TMSS were identical

within the four isoforms in each of the two clusters. For this reason, in the rest of the paper we will consider only two TMSS, 1a and 1b, for the NR1 subunits.

In Table 1 data obtained for the predicted TMSS is shown. The topology is surmised from the well documented fact that the N-terminal is extracellular and the C-terminal is intracellular.

Comparison of predicted transmembrane segments

When TMSS of the same relative location within the protein are compared among themselves they show strong similarities. We only found some minor amino acid substitutions in the five transmembrane segments of the NR2 subunits with some semi-conservative changes. The same is true for the three TMSS of NR3 subunits. However, if we align the five NR2, the three NR3 or the three NR1 TMSS, no homology between them is found, as should be expected if they represent different segments which contribute to the final protein structure.

Even though there is little homology between TMSS of the same subunit, there seems to be some conservative sequences in the segments which might have to do with its lipid environment. This conserved sequence is made up of 9-10 neutral or aromatic amino acids which seem to lie in the very middle of the overall TMSS amino acid sequence.

Location of the transmembrane segment into the total subunit sequence

When the complete subunit sequences are aligned, a pattern emerges suggesting that the relative location of all TMSS within the overall chain seems to be highly conserved. This might imply a possible common origin for all subunits and it allows for the possibility of building three dimensional assemblies to form channels using different possible subunits with the right geometry for assembly.

Three dimensional ensembles of the TMSS to form channels depend on, at least, some physicochemical properties such as molecular weight, difference in surface charge, capacity to bind water molecules and differences between charged and nonpolar residues around the axis of the segment. These properties were calculated and they are shown in table 2.

Discussion

Quality of transmembrane segment prediction

There are few published experimental data on TMSS. Radistch ***et al.*** (Radistch ***et al.,*** 1993) extracted and sequenced the second transmembrane segments of NR2A and NR2C subunits obtained from rat brain. We predict here the TMSS for the second segments of NR2A and NR2C subunits. The predicted TMSS show a similarity score with the experimental ones higher than 95% as shown in Table 3 where results of ClustalW alignments are presented. These findings allow us to suggest that similar scores might be found for other TMSS predicted here.

Senes *et al*. (**Senes *et al.,*** 2000) and Liu *et al*. (**Liu *et al.,*** 2002) recently reported a large statistical analysis of transmembrane segments of nearly 13,000 sequences. They found a pattern in the distribution of amino acid residues in the segments considered. In Figure 1 we show the amino acid distribution in our predicted segments. Even though our sample is small in comparison, it shows similar pattern

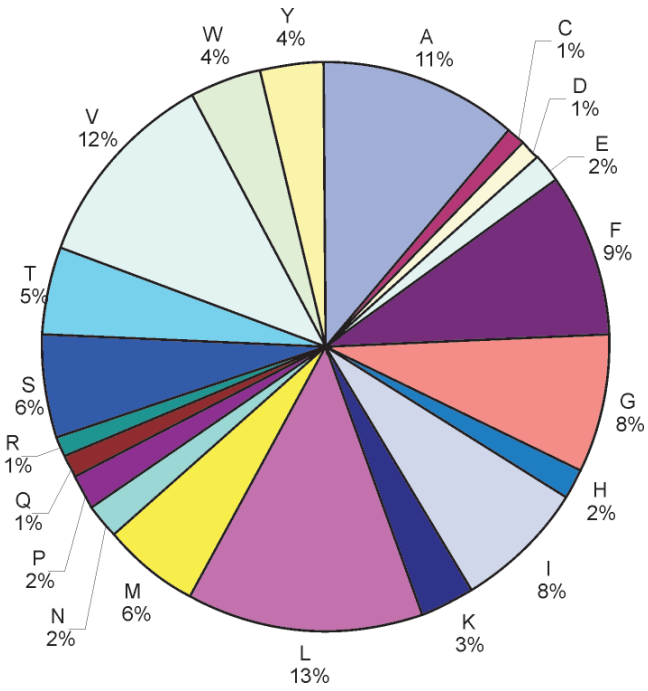


Figure 1. Amino acid composition of predicted TMSS.

distribution as the one found by Senes (**Senes *et al.,*** 2000) and Liu (**Liu *et al.,*** 2002).

The following patterns are apparent from the figure. Only four amino acids (L, V, A, F) represent close to one half of the transmembrane residues, while only six amino acids (L, V, A, F, G, I) correspond to two-thirds of the total. The most abundant 2-tuples are AX, VX, LX, IX which represent 15.0%; 14.5%; 10.0% and 6.0% respectively of the total 400 possibilities. VF and LV are close to a third of all VX and LX (5.8%), while IF is half of the IX (3.5%) and AA and AV are each a fifth of AX

Table 1. Sequences, length, topology and localization into the total precursor monomeric subunit sequence for the transmembrane segments predicted.

Subunit	TMSS	Topology	Sequence-Localization	Length
NR1-1a	**1**	o → i	T^{561}LWLLVGLSVHVVAVMLYLLD^{581}	21
	2	i → o	L^{632}GMVWAGFAMIIVASYTANLAAFLVL^{657}	26
	3	o → i	M^{813}AGVFMLVAGGIVAGIFLIFIEIAY^{837}	25
NR1-1b	**1**	o → i	T^{582}LWLLVGLSVHVVAMLYLLDR^{603}	21
	2	i → o	L^{653}GMVWAGFAMIIVASYTANLAAFLVL^{678}	26
	3	o → i	M^{834}AGVFMLVAGGIVAGIFLIFIEIAY^{858}	25
NR2A	**1**	o → i	S^{554}ASVWVMMFVMLLIVSAIAVFVFEYF^{579}	26
	2	i → o	P^{594}HGPSFTIGKAIWLLWGLVFNNSVPVQ^{620}	27
	3	o → i	S^{627}KIMVSVWAFFAVIFLASYTANLAAFMIQ^{655}	29
	4	i → o	K^{746}LVTIGSGYIFATTGYGIALQKGSPWKRQID^{776}	31
	5	o → i	M^{817}AGVFYMLAAAMALSLITFIWEHLFYWKLR^{836}	30
NR2B	**1**	o → i	A^{555}ADVWVMMFVMLLIVSAVAVFVFEYF^{580}	26
	2	i → o	P^{595}GGPSFTIGKAIWLLWGLVFNNSVPVQ^{621}	27
	3	o → i	S^{628}KIMVSVWAFFAVIFLASYTANLAAFMIQ^{656}	29
	4	i → o	K^{747}LVTIGSGKVFASTGYGIAIQKDSGWKRQVD^{777}	31
	5	o → i	A^{819}GVFYMLGAAMALSLITFICEHLFYWQFRH^{848}	30
NR2C	**1**	o → i	S^{552}PAVWVMMFVMCLTVVAITVFMFEYF^{577}	26
	2	i → o	P^{592}GGPSFTIGKSVWLLWALVFNNSVPIE^{618}	27
	3	o → i	S^{625}KIMVLVWAFFAVIFLASYTANLAAFMIQ^{653}	29
	4	i → o	K^{744}LVTIGSGKVFATTGYGIAMQKDSHWKRAID^{774}	31
	5	o → i	A^{816}GVFYMLLVAMGLALLVFAWEHLVYWKLRH^{845}	30
NR2D	**1**	o → i	S^{579}PAVWVMMFVMCLTVVAVTVFIFEYL^{604}	26
	2	i → o	P^{619}GGSTFTIGKSIWLLWGLVFNNSVPVE^{644}	27
	3	o → i	S^{652}KIMVLVWAFFAVIFLASYTANLAAFMIQ^{680}	29
	4	i → o	K^{771}LVTIGSGKVFATTGYGIALHKGSRWKRPID^{801}	31
	5	o → i	A^{843}GVFYMLLVAMGLSLLVFAWEHLVYWRLRH^{872}	30
NR3A	**1**	o → i	W^{674}TMWLGIFVALHITAIFLTLY^{695}	21
	2	i → o	F^{744}LMNLWAIFCMFCLSTYTANLAAVMVGE^{771}	28
	3	o → i	F^{931}SGLFVLLCIGFGLSILTTIGEHIV^{955}	25
NR3B	**1**	o → i	W^{575}SMWVGVFAALHLTALFLTLY^{595}	21
	2	i → o	F^{644}LMNLWAIFCLVLSSYTANLAAVMVGD^{671}	27
	3	o → i	F^{831}SGLFVLLCLGLGSALLTSLGEHVF^{855}	25

(3.3%). Notice that X in these cases are all hydrophobic residues. LLX and LVX are the most abundant 3-tuples found with LLV and LVF predominant. Finally, TIGX, AFXX, LVXX, VFXX were the greatest 4-tuples patterns found. All these data closely agree with the statistical reports cited above.

Conserved transmembrane spanning segments in the iGluR-NMDA subunits

From Tables 2 and 3 is evident that TMSS are highly conserved. In the case of the NR2 TM1's there is at least 70% identity and 80% positivity among them. Similar values can be found for the TM1's of the NR3 subunits. On the other hand, if all TMSS of a given subunit are considered, the similarity value is lower but it is still significant. This might imply that some topological arrangements are preferred in order to ensure the right pore conformation. The internal part of the pore is highly conserved as shown by the fact that nearly half of the amino acids in the TMSS which probably make up the pore have a high level of similarity. However, this does not apply to the amino acids at the pore ends. This might explain why the different environments just outside the TMSS might control cation flux even though the pore's core has the same environment independent of the ion that goes trough it.

This view is supported by the data presented in Table 2, where some characteristics of the predicted pore sequence are analyzed. Considering that values larger than 0.5 in the mean hydropathy GRAVY scale are good indicators of transmembrane topology, the data show that predicted TMSS comply with this condition. A few of them present lower values which can be explained by the higher content of the hydrophilic residues conserved among the hydrophobic residues. This agrees with the values reported for the amphypatic moment which is a measure of the distribution asymmetry of hydrophobic and hydrophilic residues along its axes. A value near zero indicates relatively

Table 2. Physico-chemical data for the transmembrane spanning segments predicted for the iGluR-NMDA subunits.

Subunit/ Transmembrane segment	Molecular weight (Da)	pI	Mean hydropathy (GRAVY)	Amphypathy moment
NR1-TM1	2355	3.43	1.93	0.01
NR1-TM2	2743	3.12	1.88	0.03
NR1-TM3	2616	3.10	2.23	0.04
NR2A-TM1	3001	3.10	2.15	0.03
NR2A-TM2	2979	3.77	0.47	0.05
NR2A-TM3	3241	3.50	1.49	0.03
NR2A-TM4	3370	4.13	-0.04	0.02
NR2A-TM5	3594	3.96	1.13	0.00
NR2B-TM1	2999	3.05	2.14	0.03
NR2B-TM2	2898	3.50	0.57	0.05
NR2B-TM3	3241	3.50	1.49	0.03
NR2B-TM4	3311	4.22	-0.19	0.01
NR2B-TM5	3537	3.90	0.98	0.00
NR2C-TM1	3063	3.10	1.88	0.02
NR2C-TM2	2929	3.47	0.56	0.04
NR2C-TM3	3267	3.50	1.65	0.04
NR2C-TM4	3380	4.46	-0.18	0.02
NR2C-TM5	3548	4.19	1.22	0.02
NR2D-TM1	2997	3.10	2.01	0.03
NR2D-TM2	2919	3.47	0.51	0.05
NR2D-TM3	3267	3.50	1.65	0.04
NR2D-TM4	3350	5.65	-0.16	0.02
NR2D-TM5	3592	4.19	1.11	0.01
NR3A-TM1	2510	3.50	1.68	0.04
NR3A-TM2	3161	3.10	1.40	0.01
NR3A-TM3	2650	3.47	1.80	0.06
NR3B-TM1	2440	3.50	1.50	0.03
NR3BTM2	2964	3.07	1.47	0.01
NR3B-TM3	2594	3.47	1.60	0.04

Table 3. Alignments of reported TM2 segments and predicted TMSS.

TM2NR2A-Predicted	PGGPSFTIGKAIWLLWGLVFNNSVPVQ-
gi|385459|gb|AAB27000.1|	——SFTIGKAIWLLWGLVFQNSVPVQN
	**********:******
TM2NR2C-Predicted	PGGPSFTIGKSVWLLWALVFNNSVPIE-
gi|385458|gb|AAB26999.1|	——SFTIGKSVWLLWALVFQNSVPIEN
	**********:******

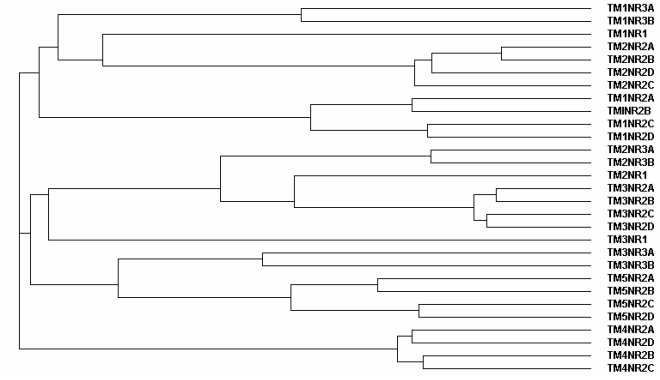
homogeneous distribution while increasing values point to ever larger asymmetries. Segments with low hydropathy present higher values of amphypatic moment that imply that the asymmetry is due to a higher presence of hydrophilic residues in the segment. The molecular weights reported are very close as was expected from sequence similarities among them. It should be noted the low values of pI, which imply an unexpected acidic membrane environment.

Grouping of predicted TMSS

Taking into account the similarities between subunits discussed above, we did a clustering of the different subunits using the normal criteria for the ClustalW program and obtained figure 2.

When all the 29 transmembrane spanning segments were aligned with Clustal, the clustering obtained is similar to the grouping obtained by considering the physical and chemical characteristics only as shown in the Table 6. Notice that NR1 and NR3 subunit types tend to cluster together while NR2's form another cluster. The transmembrane segments appeared to show some interchangeability as a result of these properties.

Figure 2. Dendrograme of the transmembrane sequences of all the subunits showing their clustering in terms of global similarities.



This fact agrees with the proposal of Franciolini and Petris (**Franciolini & Petris** 1989) that calcium channels were first to appear during evolution due to the importance of this cation as a messenger and modulator for most cellular signaling processes. This can also be the reason for the presence of iGluR-NMDA subunits in many peripheral non excitable tissues where calcium is important for function as was described in the introduction to the present work.

Acknowledgments

Financial support for this work was provided by grant No. 1428 from Pontificia Universidad Javeriana.

References

Abdeddaïm, S. & B. Morgenstern. 2001. Speeding up the DIALIGN multiple alignment program by using the 'Greedy Alignment of BIOlogical Sequences LIBrary' GABIOS-LIB. Lecture Notes in Computer Science **2066**: 1-11.

Andersson, **O., A. Stenqvist, A. Attersand & G. von Euler.** 2001. Nucleotide sequence, genomic organization, and chromosomal localization of genes encoding the human NMDA receptor subunits NR3A and NR3B. Genomics **78**: 178-184.

Arden, **S. R., J. R., Sinor, W. K. Potthoff, & E. Aizenman.** 1998. Subunit-specificiy interactions of cyanide with the N-Methyl-D-Aspartate receptor. J. Biol. Chem., **273**: 21505-21511.

Arora, **A. & L. K. Tamm.** 2001. Biophysical approaches to membrane protein structure determination. Curr. Opin. Struct. Boil. **11**: 540-547.

Berman, **H. M., J., Westbrook, Z., Feng, G., Gilliland, T. N., Bhat, H., Weissig, I. N. Shindyalov & P. E. Bourne.** 2000. The Protein Data Bank. Nucleic Acids Research, **28**: 235-242.

Blahos, **J. & R. J. Wenthold**. 1996. Relationship between N-methyl-D-Aspartate receptor NR1 splice variants and NR2 subunits. J. Biol. Chem. **271**: 15669-15674.

Brendel, V., P., Bucher, I. R., Nourbakhsh, B. E. Blaisdell, S. Karlin. 1992. Methods and Algorithms for Statistical Analysis of Protein Sequences. Proc. Natl. Acad. Sci. USA, 89: 2002-2006.

Briecombe, **J. C., F. A. Boeckman & E. Aizenman**. 1997. Functional consequences of NR2 subunit composition in single recombinant N-Methyl-D-Aspartate receptors. Proc. Natl. Acad. Sci. USA, **94**: 11019-11024.

Chen, **C. & B. Rost.** 2002. State-of-the-art membrane protein prediction. Applied Bioinformatics **1**: 21-35.

Claros, **M. G., & G. von Heijne.** 1994. TopPred II: An Improved Software For Membrane Protein Structure Predictions. CABIOS **10:** 685-686.

Cochilla, **A. J. & S. Alford.** 1999. NMDA receptor-mediated control of presynaptic calcium and neurotransmitter release. J. Neuroscience **19**: 193-205.

Cserzo, **M., E., Wallin, I., Simon, G. von Heijne & A. Elofsson.** 1997. Prediction of transmembrane alpha-helices in procariotic membrane proteins: the Dense Alignment Surface method. Prot. Eng. **10**: 673-676.

Cushing, **A., M. J., Price-Jones, R., Graves, A. J., Harris, K. T., Hughes, D. Bleakman & D. Lodge.** 1999. Measurement of calcium flux through ionotropic glutamate receptors using Cytostar-T scintillating microplates. J. Neurosc. Meth. **90**: 33-36

Eisenberg, **D., Weiss, R. M. & Trwilliger, T. C.** 1982. The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature **299**: 371-374.

Ferrer-Montiel, **A. V. & Montal, M.** 1996. Pentameric subunit stoichiometry of a neuronal glutamate receptor. Proc. Natl. Acad. Sci. USA, **93**: 2741-2744.

Franciolini, **F. & A. Petris,** 1989. Evolution of ionic channels of biological membranes. Mol. Biol. Evol. **6**: 503-513.

Hawkins, **L. H., P. L. Chazot & F. A. Stephenson.** 1999. Biochemical evidence for the co-association of three N-methyl-D-aspartate NMDA. R2 subunits in recombinant NMDA receptors. J. Biol. Chem. **274**: 27211-27218.

Higgins, **D., J. Thompson, T. Gibson, J. D. Thompson, D.G. Higgins & T. J. Gibson** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673-4680.

Hirai, **H., J. Kirsch, B. Laube, H. Betz & J. Kuhse.** 1996. The glycine binding site of the N-methyl-D-aspartate receptor subunit NR1, identification of novel determinants of co-agonist potentiation in the extracellular M3-M4 loop region. Proc. Nat. Acad. Sci. USA, **93**: 6031-6036.

Hoffmann, K. & W. Stoffel. 1993. TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler 374: 166-168.

Hollman, **M.** 1999. Structure of ionotropic glutamate receptors. *In*: P. Jonas & Monyer, H., Eds. Ionotropic glutamate receptors in the CNS. Berlin: Springer-Verlag, pp. 3-98.

Honer, **M., D. Benke, B.Laube, J. Kuhse, R. Heckendorn, H. Allgeiers, C. Angst, H. Monyer, P. T. Seeburg, H. Betz & H. Mohler.** 1998. Differentiation of glycine antagonist sites of N-methyl-D-Aspartate receptor subtypes. J. Biol. Chem. **273**: 11158-11163.

Jennings, **M. L.** 1989. Topography of membrane proteins. Annu. Rev. Biochem. **58**: 999-1027.

Kashiwagi, **K., A. J. Pahk, T. Masuko, K. Igarashi & K. Williams.** 1997. Block and modulation of N-methyl-D-aspartate receptors by polyamines and protons, role of amino acid residues in the transmembrane and pore-forming regions of NR1 and NR2 subunits. Mol. Pharm. **52**: 701-713.

Krogh, **A., B. Larsson, G. von Heijne & E. Sonnhammer.** 2001. Prediction of transmembrane protein topology with a Hidden Markov Model: Application to complete genomes. J. Mol. Biol. **305**: 567-580.

Kyte, J. & R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**: 105-132.

Laube, B., J. Kuhse, & H. Betz. 1998. Evidence for a tetrameric structure of recombinant NMDA receptor. J. Neuroscience **18**: 2954-2961.

Letunic, I., L. Goodstadt, N. J. Dickens, J. Doerks, J. Schultz, R. Mott, F. Ciccarelli, R. R. Copley, C. P. Ponting & P. Bork. 2002. Recent improvements to the SMART domain-based sequence annotation resource. Nucleic Acids Research 30: 242-244.

Liu, **Y., D. M. Engelmann & M. Gerstein.** 2002. Genomic analysis of membrane protein families: abundance and conserved motifs. Genome Biol. **3**: research0054.

Mitaku, **S., T. Hirokawa, & T. Tsuji.** 2002. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. Bioinformatics **18**: 608-616.

McBain, **C. J. & M. L. Mayer.** 1994. N-Methyl-D-Aspartartic acid receptor structure and function. Physiol. Rev. **74**: 723-760.

Moller, **S., M. Croning, & R. Apweiler.** 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17**: 646-653.

Nakanishi, **N., R. Axel, & N. A. Shneider.** 1992. Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. Proc. Natl. Acad. Sci. USA, **89**: 8552-8556.

Nusser, Z. 2000. AMPA and NMDA receptors, similarities and differences in their synaptic distribution. Curr. Opin. Neurobiol. **10**: 337-341.

Paoletti, P., P. Ascher, & J. Neyton. 1997. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J. Neurosci. **17**: 5711-5725.

Raditsch, M., J. P. Ruppersberg, T. Kuner, W. Gunther, R. Schoepfer, P. H. Seeburg, W. Jahn & V. Witzemann. 1993. Subunit-specific block of cloned NMDA receptors by argiotoxin636. FEBS *Lett.* **324**: 63-66.

Rao, J. K. M. & P. Argos. 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biophys. Biochem. Acta**, 869**: 197-214.

Rosenmund, C., Y. Stern-Bach, & C. F. Stevens. 1998. The tetrameric structure of a glutamate receptor channel. Science **280**: 1596-1599.

Rost, B. & C. Sander. 1994. Combinig evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19**: 521-533.

Rost, B. 1996. PHD: predicting 1D protein structure by profile based neural networks. Meth. Enzymol. **266**: 525-539.

Senes, A., M. Gerstein & D. M. Engelmann. 2000. Statistical analysis of amino acid patterns in transmembrane helices: The GxxxG motif occurs frequently and in association with b-branched residues at neighboring positions. J. Mol. Biol. **296**: 921-936.

Sprengel, R., B. Suchanek, C. Amico, R. Brusa, N. Burnashev, A. Rozov, O. Hvalby, V. Jensen, O. Paulsen, P. Andersen, J. J. Kim, R. F. Thompson, W. Sun, L. C. Webster, S. G. N. Grant, J. A. Eilers, J. Konnerth, Li, J. O. McNamara & P. H. Seeburg. 1998. Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell **92:** 279-289.

Traxler, B., D. Boyd, & J. Beckwith. 1993. The topological analysis of integral cytoplasmatic membrane proteins. J. Membrane Biol. **132**: 1-11.

Tusnady, G. & I. Simon. 1998. Principles governing amino acid composition of integral membrane proteins: Application to topology prediction. J. Mol. Biol. **283**: 489-506.

Tusnady, G. E. & I. Simon. 2001. The HMMTOP transmembrane topology prediction server. Bioinformatics **17**: 849-850.

von Heijne, G. 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive inside rule. J. Mol. Biol. **225**: 487-494.

Whitehorn, E. A., W. J. Dower, & M. Li. 1999. Expression of extracellular N-terminal domain of NMDA receptor in mammalian cells. *In*: Li, M.Ed. NMDA receptor protocols. Humana Press, Totowa, New Jersey. pp. 61-72.

Recibido: abril 15 de 2008

Aceptado para su publicación: mayo 8 de 2008