Microarray and Proteomics Analysis on Neurotransmitter (Nicotinic Acetylcholine Cys Loop Receptor) By using Bioinformatics Tools

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Abstract: Excessive inflammation and tumor-necrosis factor (TNF) synthesis causes morbidity and mortality in diverse human diseases including endotoxaemia, sepisis, rheumatoid arthritis and inflammatory bowel diseases. Highly conserved, endogenous mechanisms normally regulate the magnitude of innate responses and prevent excessive inflammation. The release of system, through the vagus nerve, can inhibit significantly and rapidly the release of macrophage TNF, and attenuate systemic inflammatory responses. This physiological mechanism, termed the 'cholinergic anti-inflammatory pathway' has major implications in immunology and in therapeutics; however, the identity of the essential macrophage acetylcholine mediated (cholinergic) receptor that responds to vagus nerve signals was previously unknown. Nicotinic acetylcholine receptors, or nAChRs, are ionotropic receptors that form ligand-gated ion channels in cells' plasma membranes. Like the other type of acetylcholine receptors, muscarinic acetylcholine receptors (mAChRs), their opening is triggered by the neurotransmitter acetylcholine (ACh), but they are also opened by nicotine. Also in contrast to muscarinic ACh receptors, nicotinic acetylcholine cys loop receptors do not operate with a second messenger, but open themselves forming an ion channel. Their action is inhibited by curare. Nicotinic acetylcholine receptors are present in many tissues in the body. The neuronal receptors are found in the central nervous system and the peripheral nervous system. The neuromuscular receptors are found in the neuromuscular junctions of somatic muscles; stimulation of these receptors causes muscular contraction. Here we report that the nicotinic acetylcholine receptor alpha-7 subunit is required for acetylcholine inhibition of macrophage TNF release. Electrical stimulation of the vagus nerve inhibits TNF synthesis in wide- type mice, but fails to inhibit TNF synthesis in 7 deficient cytokine syntheses by the cholinergic anti-inflammation pathway. Modeller 9v2 was used to design the receptor nicotinic acetylcholine receptor alpha-7 subunit. Genemaths XT was used for microarray analysis of the receptor. Molecular docking of nicotinic acetylcholine cys loop receptor (alpha-7 subunit) with the ligand 1sq3 by Autodock 4.0 to obtain biomolecules for the control of neural diseases.
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1. Introduction

Recent advances in experimental genomics and proteomics, coupled with the wealth of sequence information available for a variety of organisms, have tremendous implications for how biomedical research is performed. Genomic techniques, such as complementary DNA (cDNA) microarrays, currently allow researchers to quickly and accurately quantify vast numbers of potential gene expression changes simultaneously (Date and Marcotte *et al.*, 2003). Modern proteomic techniques allow for the detection and elucidation of protein-protein interactions on a scale and at a speed never before possible. Although hurdles remain together, these tools open the possibility of enormous change in our ability to analyze and interpret complex biological processes. The field of neuroscience is particularly well suited to analysis with these new techniques, given the complexity of neuronal signaling and the diversity of cellular responses. Neurotransmitters are the chemicals which allow the transmission of signals from one neuron to the next across synapses. They are also found at the axon endings of motor neurons, where they stimulate the muscle fibers. And they and their close relatives are produced by some glands such as the pituitary and the adrenal glands. The deficiency of neurotransmitter causes Prolonged Emotional or physical stress, aging, weight loss dieting, abnormal sleep, certain medications, neurotoxins, hormone imbalances, genetic predisposition. Nicotinic receptors also are distributed to pre terminal, axonal, dendritic, and somatic locations (Lena *et al.,* 1993 and Zarei *et al.,* 1999). Nicotinic acetylcholine receptors (nAChRs) mediate fast excitatory neurotransmission in neurons and muscles. To identify nAChR accessory proteins, which may regulate their expression or function, we performed tandem affinity purification of the levamisole-sensitive nAChR from *Caenorhabditis elegans*, mass spectrometry of associated component

and RNAi-based screening for effects on *in vivo* nicotine sensitivity.

Nicotinic acetylcholine receptors, or nAChRs, are cholinergic receptors that directly linked to an ion channel and do not make use of a second messenger as metabotropic receptors do. Nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of ligand-gated ion channels that includes GABA$_A$, glycine, and 5-HT3 serotonin receptors (McGehee and Role, 1995 and Hogg *et al.*, 2003). A wide variety of subtypes of nAChRs arise from combinations of subunits that compose the channel-receptor complex. Although these subtypes display a range of different functional and pharmacological properties, they share basic features. They occupy three main functional st ates in response to agonist: closed at rest, open pore, and closed desensitized. Brief exposure to high concentrations of the neurotransmitter, acetylcholine (ACh), causes opening of the water-filled, cation-selective pore. After a couple of milliseconds, the receptor closes to a no nconducting state. Prolonged exposure to low concentrations of nicotine, as obtained from tobacco use, produces significant desensitization, which stabilizes the receptor in a closed state that is unresponsive to agonist (Dani   *et al.*, 2000 and Giniatullin *et al.*, 2005).

A protein microarray is a piece of glass on which different molecules of protein have been affixed at separate locations in an ordered manner thus forming a microscopic array. These are used to identify protein-protein interactions, to identify the substrates of protein kinases, or to identify the targets of biologically active small molecules. The most common protein microarray is the antibody microarray, where antibodies are spotted onto the protein chip and are used as *capture molecules* to detect proteins from cell lysate solutions. It is widely believed that thousands of genes and their products (i-e., RNA and proteins) in a given liven living organism function in a complicated and orchestrated way that creates the mystery of life. However, traditional methods in molecular biology generally work on a "one gene in one experiment" basis, which means that the throughput is very limited and the "whole picture" of gene function is hard to obtain. In the past several years, a new technology, called DNA microarray has attracted tremendous interests among biologists. This technology promises to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously (Rang et *al.*, 2003).

Drug design is an iterative process which begins when a chemist identifies a compound that displays an interesting biological profile and ends when both the activity profile and the chemical synthesis of the new chemical entity are optimized. Traditional approaches to drug discovery rely on a step-wise synthesis and screening program for large numbers of compounds to optimize activity profiles. Over the past ten to twenty years, scientists have used computer models of new chemical entities to help define activity profiles, geometries and reactivities. Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs (Kitchen *et al.*, 2004).

The main objective of this present work is to study the comparative modeling of nicotinic acetylcholine cys loop receptor by using different bioinformatics tools and software and also to find out the protein and ligand interaction using Autodock 4.0 which will be very much helpful for future analysis on the drug designing against the neural diseases.

2. Materials and Methods
2.1. Materials

nAChR (alpha-7subunit), 1sq3, Ligand, Clustal W, T-Coffee, Modeller-9v2, Q-sitefinder, I-moltalk, Genemaths-XT, Autodock-4.0, NCBI, PDB.

2.2. Methods
2.2.1. Modeling

In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Lengauer and Rarey, 1996). Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using for example scoring functions. There are two types of modeling the first one is modeling and another is advance modeling. Model was prepared by identifying the related structures of the molecule and selection of the template followed by alignment of target sequences with template structures. Then a model was built for the target using information from template structures.

2.2.2. Microarray analysis

Microarrays are a very powerful tool for quantifying the amount of RNA in samples; however, their ability to query essentially every gene in a genome, which can number in the tens of thousands, presents analytical and interpretative problems. As a result, a variety of software and web-based tools have been developed to help with these issues. This article highlights and reviews some of the tools for the first steps in the analysis of a microarray study. The

analysis of massive data sets produced by high density gene arrays (microarrays) and gene chip requires special tools capable of processing and analyzing many thousands of data arrays that originates from a collection of different sample or that were monitored during different biological processes. Typically the analysis of microarray involves 3 steps. The first one is Preprocessing and normalization of the raw materials. Then filter out those genes which are significantly over expressed or under expressed from the masses of invariant genes. Detecting groups of genes or associating certain cluster of genes of arrays though unsupervised learning techniques (Tseng *et al*., 2001).

2.2.3. Docking

Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Lengauer and Rarey, 1996). Here the nicotinic acetylcholine receptor docked with 1SQ3 by using the bioinformatics software "Autodock 4.0". It is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Flexible residue file, retrieval of receptor from PDB, Macromolecule and Grid Parameter File were prepared. Then AutoGrid4 was started. After reading the Docking Log the visualizing Docked was confirmed. Then 3-D optimization was performed.

3.   Results And Discussion

We have outlined the various steps involved in the process of homology modeling and methods of protein modeling as well as microarray analysis. Homology modeling has been applied to nicotinic acetylcholine cys loop receptor alpha-7 subunit. Out of those 10 targets (models) the best one was chosen according to the highest score obtained by verify 3D with that target energy minimization had done. Docking was also performed with the same target and four different ligands. After docking we can find the ligand (Fig-1) for the receptor which may effective for designing the drugs for different neural diseases like: Parkinson disease, short term memory loss, endotoxaemia, sepisis, rheumatoid arthritis and inflammatory bowel diseases. cDNA microarray and proteomic findings of relevance to schizophrenia and Alzheimer's disease (AD) as 2 representative areas of neuroscience research. The potential for these techniques to help unravel the underlying pathology of complex neurological and neuropsychiatric conditions is considerable and warrants continued investigation (Date and Marcotte *et al*., 2003).
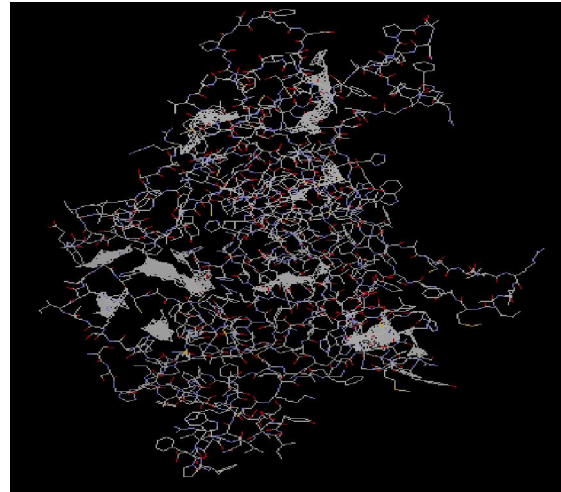

Fig-1: This is the 3D structure of alpha-7 sub unit of nicotinic acetylcholine receptor shown by Q-siteFinder

The more effective and functional model was prepared by molecular modeling by using Modeller9v2, which contains more number of alpha helices and beta strands. The expression level of the receptor was analyzed by microarray analysis by using bioinformatics software "Genemaths- XT". Nicotinic acetylcholine was docked into the active pocket of this homology-modeled 1sq3 and the most probable configuration of the enzyme-inhibitor complex was assessed by molecular dynamics studies. Our nAChRs model included 24 dipeptide hydroxamic acid derivatives, using the conformation of structural ligands based on the nAChRs -1SQ3 complex as obtained through homology modeling, docking and finally molecular dynamics.

The multiple sequence alignment among 10 different molecules of alpha-7 subunit of nicotinic acetylcholine by "Clustal W" shows more number of stars which indicates more number of identities (Fig-2). In this figure we can easily identify that there were more number of stars in the multiple sequence alignment. That means more similarities among the sequences of 10 different alpha-7 subunits.
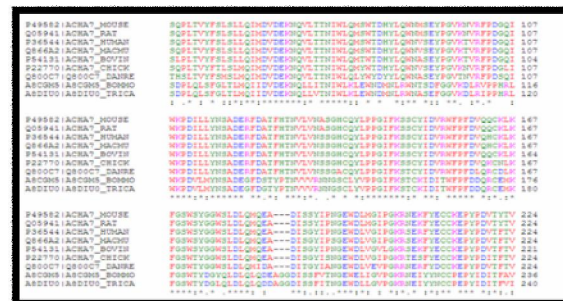

Fig-2: The multiple sequence alignment among10 different sequences of alpha-7 by Clustal-W.

The primed numbers related to the location relative to the position commonly considered as the first residue of M2. Amino acid sequence alignment of three subunits of cys loop receptor shows good conservations across species indicating functional in ion transport.

The confidence in the alignment used here for the model building was very good. The quality of the model was enough to use it as base model for the docking of 1SQ3. Among 35 models it was the best model because it has more number of alpha helices and beta strands and so this is more advanced and more effective than others (Fig-2). The active sites of the model can easily identify through "Q-siteFinder".

The active sites of the model were shown by the Ramachandran plot. In Ramachandran plot if the disallowed area is less than 2% then it indicates that the model is good and here the model contains 0.6% of disallowed area (Fig-3). That means this is a very good model having good structure. The percentage of core area and the allowed was 95.5% of the total area according to Phi/Psi angle.
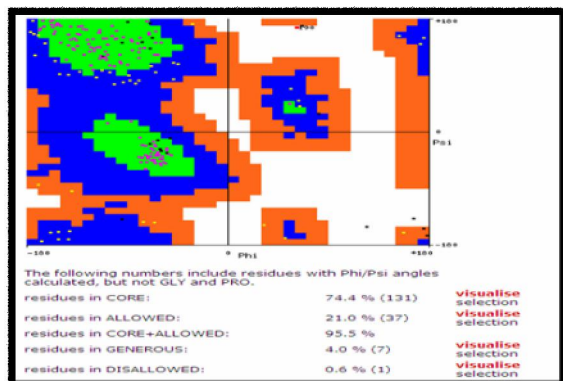

Fig-3: Ramachandran plot showing the percentage of disallowed area and the allowed area of the modeled protein of alpha-7.

cDNA microarray and proteomic findings of relevance to schizophrenia and Alzheimer's disease (AD) as 2 representative areas of neuroscience research. The potential for these techniques to help unravel the underlying pathology of complex neurological and neuropsychiatric conditions is considerable and warrants continued investigation (Marcotte *et al.*, 2003). Genes with similar gene expression can be identified using self-organizing maps (SOM) and hierarchical clustering (Quackenbush, 2001; Tamayo *et al.,* 1999 and Valafar, 2002). Genemaths-XT has the advantage that it provides an ordering of clusters, whereby each cluster consists of a group of genes with similar gene expression profiles. Grouping based on similarity in

expression behavior is also useful for functional interpretation of known genes.

The expression level of 10 random sets genes significantly distinguished between brains of control and mutatant mice. Among them the two most down regulated were the spg21 and pls genes both spg21 and pts genes are located on mouse chromosome 9. These genes were selected as the set of genes being related to the treatment. Analysis using public expression database demonstrated that their expression levels were determined by the mouse background strain and by the, nAChR deletion transport (Fig-4).

Among all 10 compositions of alpha-7 subunits after allowing them in a microarray, the relative percentage arises between 9.0 to 11.5%. The cumulative percentages arise between 11.5 to 100.0% (Table-1). That indicates it is a very good model having good structure. In this sense, it seems to be that the loop C, which contains highly conserved residues, plays not only a predominant binding role, but also may be determinant in the activator or inhibitory effects of 1SQ3 on the alpha-7 sub unit of nAChR.

Grid Map	Atom type	Minimum Energy (Kcal/mol)	Maximum Energy (Kcal/mol)
1	A	-0.88	2.01e+05
2	C	-0.97	2.01e+05
3	OA	-1.02	2.00e +05
4	N	-0.88	2.00e+05
5	SA	-1.17	2.01e+05
6	HD	-0.57	1.07e+05
7	e	-15.99	3.60e+01
8	d	0.00	1.72e+00

Table-1: The percentages of the relative and the cumulative molecules in the microarray of alpha-7 molecule.

The protein model of alpha-7 was docked with ligand 1SQ3 by the "Autodock 4.0". At this stage, preliminary simulations (work in progress) showed that the free energy of binding between the fragment 1SQ3 of the whole peptide and the loop C from the alpha7 nAChR oscillates between 0.00 kcal/mol and 15.00kcal/mol (Table-2), and easily reaching binding constants of the order of femtomolar concentrations.
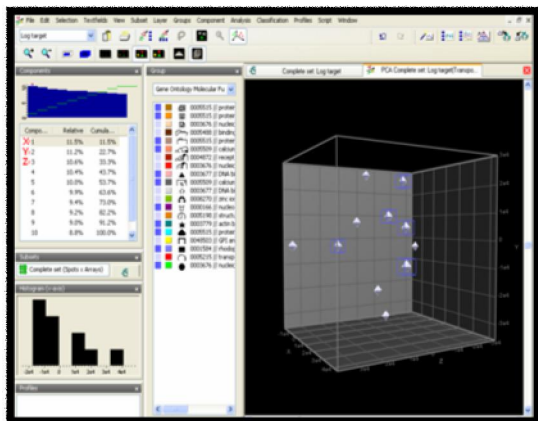
Fig-4: The expression level of randomly collected
nicotinic acetylcholine

Table-2: The minimum and the maximum energy
level of the array in Genemaths-XT

Composition	Relatives	Cumulative
1	11.5%	11.5%
2	11.2%	22.7%
3	10.6%	33.3%
4	10.4%	43.7%
5	10.0%	53.7%
6	9.9%	63.6%
7	9.4%	73.0%
8	9.2%	82.2%
9	9.0%	91.2%
10	8.8%	100.0%

In this docking model, it was noted that the 1SQ3 covers many functional faces of the principal and complementary faces of the receptor alpha-7 sub unit, but has major interactions with the loop C of the receptor, located near the ligand binding site and at the interface of two monomeric forms of the receptor, and constituted by 1SQ3.

l   -5.0 Guaranteed wrong structure. Bad structure or poor model
l   -3.0 Probably bad structure or unrefined model. Doubtful structure or model
l   -2.0 Structure OK or good model. Good structures
l   0.0 Good structures.
l   2.0 Good structures. Unusually Good structures.
l   4.0 Probably a strange model of a perfect helix.

The maximum energy level was calculated between 1.07e+05 to 3.60e+0 (Table-2). It indicates that it can be easily reaching binding constants of the order of femtomolar concentrations. After docking the complex nAChR-1SQ3 the Z-score was 0.00, calculated by the most common bioinformatics software"Modeller9v2".that means it is a very good structure. Default parameters given by the program were used to calculate the hydrogen: donor–acceptor heavy atom distance $<3.9A^0$, a distance 62.5 A^0 between the hydrogen atom donor and the acceptor, and a donor-hydrogen acceptor angle $>90^0$. The output file reported a total of 495 hydrogen bonds, none of them observed at the interface of the alpha-7 nAChR–1SQ3 complex.

We have outlined the various steps involved in the process of homology modeling and methods of protein modeling as well as microarray analysis. Homology modeling has been applied to nicotinic acetylcholine cys loop receptor alpha-7 subunit. Out of those 10 targets (models) the best one was chosen according to the highest score obtained by verify 3D with that target energy minimization had done. Docking was also performed with the same target and four different ligands. After docking we can find the ligand (Fig-5) for the receptor which may effective for designing the drugs for different neural diseases like: Parkinson disease, short term memory loss, Endotoxaemia, Sepisis, rheumatoid arthritis and inflammatory bowel diseases.

Disparate findings in the literature indicate that nicotinic mechanisms significantly influence only particular forms of memory, and only under certain conditions. Nicotine or nicotinic receptors have been implicated in a wide range of neuronal dysfunctions and mental illness (Wonnacott, 1997; Le Novère and Changeux, 1995; Rang, 2000 and Page et al., 2006). Nicotinic mechanisms contribute to cognitive function, and the decline of nicotinic mechanisms or loss of nAChRs has been observed in AD, dementia with Lewy bodies, Down syndrome, autism, and Parkinson's disease (20, 140). Genetic evidence has linked nicotinic receptors to epilepsy and schizophrenia, and studies with mutant mice have implicated nAChRs in pain mechanisms, anxiety, and depression. In addition, nicotinic-based therapies have been proposed for Tourette's syndrome and attention deficit/hyperactivity disorder (ADHD) (Wonnacott, 1997). In (Fig-5) the location of the ligand binding site on a protein is identified which is of fundamental important for a range of applications including molecular docking , denovo drug design and structural identification and composition of fundamental sites.
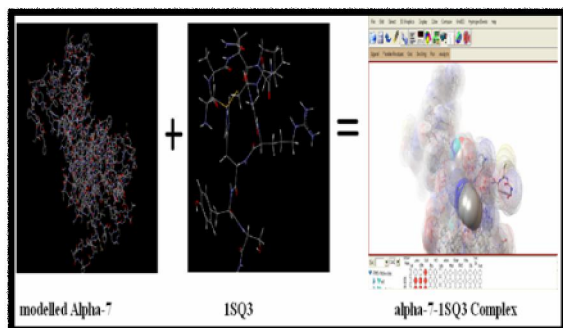
Fig-5: the model of alpha-7 subunit was docked with 1SQ3 and alpha-7-1SQ3 complex was formed

The protein model of alpha-7 was docked with ligand 1SQ3 by the bioinformatics software "Autodock-4.0". The subunit of the nicotinic acetylcholine receptor belongs to a multigene family (17 members in human) and the assembly of combinations of subunits results in a large number of different receptor. These receptors with highly variable kinetic, electrophysiological and pharmacological properties, respond different to nicotine, at very different effective concentrations. This function diversity allows them to take part in two major types of neurotransmissions. Classical synaptic transmission (wiring transmission) involves the release of high concentrations of neurotransmitter, acting on immediately neighboring receptor. In contrast paracrine transmission (volume transmission) involves neurotransmitters released by synaptic buttons or varicosities which varicosities, which then diffuse through the extra-cellular medium until they reach their receptor which may be distant.

4. Conclusion

Nicotinic acetylcholine receptors are widely expressed throughout the CNS, influencing electrical events in nearly every area of the mammalian brain. They enhance neurotransmitter release, modify circuit excitability, and influence synaptic plasticity. The synaptic physiological roles of nAChRs continue to be delineated, but important issues at the higher systems level have received less attention. This need is highlighted by nAChR participation in a diverse array of Neuronal pathologies, Including AD, Parkinson's disease, Schizophrenia, Epilepsy, and Addiction.

The complex systems-level nature of those diseases underscores nicotinic influences over local circuits and the long-range communication involved in attention and cognition. After docking we can find the ligand for the receptor which may effective for designing the drugs for different neural diseases like: Parkinson disease, short term memory loss,

Endotoxaemia, Sepisis, rheumatoid arthritis and inflammatory bowel diseases. However microarray analysis and proteomics analysis in experimental approach will authenticate for better analyzing about nicotinic acetylcholine receptor by using different bioinformatics software and tools which are more advanced. We suggest exploring several tools in an area and understanding the principles of the methods implemented before settling on one or a few to use regularly. By exploring several tools you will understand the potential of the various tools, how easy (or difficult) they are to use, and determine what you really want and need for your microarray analysis.

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