ON THE MECHANISM OF AGONIST BINDING AND CHANNEL GATING IN NICOTINIC ACETYLCHOLINE RECEPTORS

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by

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CERTIFICATE

This is to certify that the thesis titled "On the mechanism of agonist binding and channel gating in the nicotinic acetylcholine receptors" submitted by Ms. Nadira Khatoon, Kusuma School of Biological Sciences to the Indian Institute of Technology Delhi, New Delhi for the award of the degree of Doctor of Philosophy in the faculty of Sciences is a *bonafide* record of original research work carried out by the candidate. The results contained in the thesis have not been submitted, in part or full, to any other university or institute for the award of any degree.

I further certify that Ms. Nadira Khatoon has fulfilled all the requirements for the submission of the thesis.

Certified by

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Abstract

Nicotinic acetylcholine receptors (AChRs) expressed in the central and peripheral nervous system belong to a superfamily of pentameric *ligand-gated* ion channels (LGICs). They mediate fast excitatory signal transmission at neuronal and *neuromuscular* synapses. AChRs are typical allosteric proteins that exist in multiple conformational states, including resting closed (C), active open (O), and desensitized (D) states, which differ in their affinities for agonists. Neurotransmitter molecules bind to the orthosteric transmitter-binding sites (TBS) located in the extracellular domain (ECD) of the receptor, triggering local conformational changes that are followed by global conformational rearrangements (gating) leading to channel opening, ion flux, and membrane depolarization. Agonists bind with greater affinity to O than to C, thereby driving the equilibrium toward the active state. These processes are energetically linked through a thermodynamic cycle connecting agonist binding, channel gating, and ion permeation.

The neuronal nicotinic AChRs are critical for nervous system functions including cognition, attention, memory formation and auditory responses. They are strongly implicated in mood disorders, nicotine addiction, analgesia and are targets for the treatment of Alzheimer's (AD), Parkinson's (PD), depression, epilepsy, and schizophrenia. The AChRs expressed at the neuromuscular junction (NMJ) synapses are indispensable for muscle contraction, and they are implicated in several disorders including congenital myasthenia (CMS), Escobar, and lethal multiple pterygium syndromes.

The neuronal nicotinic α3β4 and α3β2 AChRs, expressed in the medial habenular-interpeduncular (mHb-IPN) circuit, have been strongly implicated in nicotine addiction and withdrawal. Although the single-channel kinetics of α3-containing receptors have been characterized, the energetics of constitutive and liganded gating remain poorly understood. In

chapter 3, systematic dissection of the binding and gating behavior of the neuronal α3β4 AChRs was done by using high resolution single channel patch-clamp recordings, protein engineering and thermodynamic analysis. Three independent methods were used to estimate the intrinsic gating energy (ΔG_0 = +5.62 kcal/mol) of the α3β4 receptors which was found to be less than the neuromuscular AChRs. Further the efficacy (En) and affinity (K_d) of the α3β4 AChRs were determined by the β4 dosage. $(\alpha3)_2(\beta4)_3$ exhibits a higher diliganded gating equilibrium constant (E_2 = 5.0) vs the $(\alpha3)_3(\beta4)_2$ (E_2 = 1.5) stoichiometry.

The neuromuscular AChR was then used as a model system to investigate the molecular determinants of low-affinity nicotine binding. In Chapter 4, alanine-scanning mutagenesis, free-energy measurements, and molecular dynamics simulations demonstrated that conserved aromatic residues within the transmitter-binding site contribute unequally to agonist binding energy, with αY190 acting as a critical determinant of nicotine binding. Structural rearrangements within the binding pocket correlated with the measured energetic effects of the mutations.

While the mechanism of agonist binding to the low-affinity C state has been extensively studied, agonist association with the high-affinity open state remains poorly understood. In Chapter 5, engineered constitutively active AChRs, single-channel recordings, and molecular dynamics simulations were used to investigate agonist binding to O. Our results indicate that: 1. At the αγ-binding site, for a series of agonists, the O state agonist association rates (j_{on}) approached the upper limit of diffusion-controlled reactions, and the agonist affinity was 2-orders of magnitude higher than the αδ- and αε-binding sites. 2. Electrostatic surface charge density due to 4 negatively charged residues (γE57, γD113, γD174, and γE180) stabilizes capped loop C, compact TBS, and bound agonist in the AO state. 3. Mutating these residues in combination reversed the jon and the binding affinity of the αγ- to values comparable to the αδ- and αε -binding sites. The charge neutralization mutations show additive effects on the

affinity of agonist binding to the O state. The results were discussed in reference to the role of 'outer-zone' charged residues of the TBS in determining agonist binding to the high-affinity state. Further, the significance of high vs low affinity agonist association in shaping the synaptic response at the NMJ, especially in the presence of disease-causing mutations was discussed.

Finally, Chapter 6 addressed how the pore gate opens to permit ion conduction. The 'gate' in the pore of the neuromuscular AChR is a complex, semi-independent, macromolecular structural element that regulates ion conduction. Mutational analysis, thermodynamic measurements, coupled interaction studies, and molecular dynamics simulations demonstrated that the closed pore presents a substantial energetic barrier (+22.5 kcal mol^{-1}) to ion permeation. Strong correlations between pore hydrophobicity and gating energetics provided evidence for hydrophobic wetting↔dewetting transitions during channel opening. Interaction energy measurements and simulations further suggested that channel opening involves coordinated rearrangements of the pore-lining M2 helices, asymmetric movement of gate residues, and hydration of the gate region.

Taken together, the findings of this thesis establish a thermodynamic framework for AChR activation. Chapter 3 defines the energetic basis of constitutive and liganded gating, while Chapters 4 and 5 demonstrate how state-dependent agonist affinity is determined by binding-site architecture, conserved aromatic residues, and electrostatic steering. Chapter 6 shows that ion permeation is controlled by hydrophobic wetting↔dewetting transitions at the pore gate. Collectively, these results link agonist binding, allosteric gating, and pore opening within a common energetic framework, providing new insight into the molecular mechanisms of pentameric ligand-gated ion channels and the basis of disease-associated receptor dysfunction.

सार

निकोटिनिक एसिटाइलकोलाइन रिसेप्टर्स (एसीएचआर) पेंटामेरिक लिगैंड-गेटेड आयन चैनलों (एलजीआईसी) के एक सुपर फैमिली से संबंधित हैं। वे न्यूरोनल और न्यूरोमस्कुलर सिनेप्स में तेजी से उत्तेजक संकेत संचरण में मध्यस्थता करते हैं। ए. सी. एच. आर. विशिष्ट एलोस्टेरिक प्रोटीन हैं जो कई संरचनात्मक अवस्थाओं में मौजूद होते हैं, जिनमें विश्राम बंद (सी) सक्रिय खुले (ओ) और असंवेदनशील (डी) अवस्थाएं शामिल हैं, जो एगोनिस्टों के लिए अपनी आत्मीयताओं में भिन्न होते हैं। न्यूरोट्रांसमीटर अणु रिसेप्टर के बाह्य कोशिकीय क्षेत्र (ई. सी. डी.) में स्थित ऑर्थोस्टेरिक ट्रांसमीटर-बाइंडिंग साइट (टी. बी. एस.) से जुड़ते हैं, जो स्थानीय संरचनात्मक परिवर्तनों को ट्रिगर करते हैं जिनके बाद वैश्विक संरचनात्मक पुनर्व्यवस्था (गेटिंग) होती है जिससे चैनल ओपनिंग, आयन फ्लक्स और झिल्ली विध्रुवीकरण होता है। एगोनिस्ट सी की तुलना में ओ के साथ अधिक आत्मीयता के साथ जुड़ते हैं, जिससे संतुलन सक्रिय अवस्था की ओर बढ़ जाता है। ये प्रक्रियाएँ एगोनिस्ट बाइंडिंग, चैनल गेटिंग और आयन पारगमन को जोड़ने वाले थर्मोडायनामिक चक्र के माध्यम से ऊर्जावान रूप से जुड़ी हुई हैं।

न्यूरोनल निकोटिनिक एसीएचआर तंत्रिका तंत्र के कार्यों के लिए महत्वपूर्ण हैं जिनमें अनुभूति, ध्यान, स्मृति निर्माण और श्रवण प्रतिक्रियाएं शामिल हैं। वे मनोदशा विकारों, निकोटीन की लत, एनाल्जेसिया में दृढ़ता से शामिल हैं और अल्जाइमर (एडी) पार्किंसंस (पीडी) अवसाद, मिर्गी और सिज़ोफ्रेनिया के उपचार के लिए लक्ष्य हैं। न्यूरोमस्कुलर जंक्शन (एन. एम. जे.) सिनेप्स पर व्यक्त ए. सी. एच. आर. मांसपेशियों के संकुचन के लिए अपरिहार्य हैं, और वे जन्मजात मायस्थेनिया (सी. एम. एस.) एस्कोबार, और घातक मल्टीपल पैटरीजियम सिंड्रोम सहित कई विकारों में शामिल हैं।

न्यूरोनल निकोटिनिक $\alpha3\beta4$ और $\alpha3\beta2$ AChRs, जो मध्य हैबेनुलर-इंटरपेडुन्कुलर (mHb-IPN) सर्किट में व्यक्त किए गए हैं, को निकोटीन की लत और वापसी में दृढ़ता से फंसाया गया है। हालाँकि $\alpha3$-युक्त रिसेप्टर्स के एकल-चैनल कैनेटीक्स की विशेषताएँ दी गई हैं, लेकिन संरचनात्मक और लिगैंडेड गेटिंग के

एनर्जेटिक्स को बहुत कम समझा गया है। अध्याय 3 में, न्यूरोनल α3β4 AChR के बाइंडिंग और गेटिंग व्यवहार का व्यवस्थित विच्छेदन उच्च रिज़ॉल्यूशन एकल चैनल पैच-क्लैंप रिकॉर्डिंग, प्रोटीन इंजीनियरिंग और थर्मोडायनामिक विश्लेषण का उपयोग करके किया गया था। तीन स्वतंत्र विधियों का उपयोग α3β4 रिसेप्टर्स की आंतरिक गेटिंग ऊर्जा (ΔG_0 = +5.62 kcal/mol) का अनुमान लगाने के लिए किया गया था जो न्यूरोमस्कुलर AChR से कम पाया गया था। इसके अलावा α3β4 AChR की प्रभावकारिता (E_n) और आत्मीयता (K_d) को β4 खुराक द्वारा निर्धारित किया गया था। $(\alpha3)_2(\beta4)_3$ एक उच्च परिश्रमशील गेटिंग संतुलन स्थिरांक (E_2 = 5.0) बनाम $(\alpha3)_3(\beta4)_2$ (E_2 = 1.5) स्टोइकोमेट्री प्रदर्शित करता है।

न्यूरोमस्कुलर एसीएचआर का उपयोग तब कम आकर्षण वाले निकोटीन बाइंडिंग के आणविक निर्धारकों की जांच करने के लिए एक मॉडल प्रणाली के रूप में किया गया था। अध्याय 4 में, एलेनिन-स्कैनिंग उत्परिवर्तन, मुक्त-ऊर्जा माप, और आणविक गतिकी सिमुलेशन ने प्रदर्शित किया कि ट्रांसमीटर-बाइंडिंग साइट के भीतर संरक्षित सुगंधित अवशेष एगोनिस्ट बाइंडिंग ऊर्जा में असमान रूप से योगदान करते हैं, जिसमें αY190 निकोटीन बाइंडिंग के महत्वपूर्ण निर्धारक के रूप में कार्य करता है। बाइंडिंग पॉकेट के भीतर संरचनात्मक पुनर्व्यवस्था उत्परिवर्तन के मापा ऊर्जावान प्रभावों के साथ सहसंबद्ध है।

जबकि निम्न-आत्मीयता सी अवस्था के लिए एगोनिस्ट बंधन के तंत्र का व्यापक रूप से अध्ययन किया गया है, उच्च-आत्मीयता खुली अवस्था के साथ एगोनिस्ट संबंध को बहुत कम समझा गया है। अध्याय 5 में, ओ के साथ एगोनिस्ट बाइंडिंग की जांच करने के लिए इंजीनियर संवैधानिक रूप से सक्रिय एसीएचआर, एकल-चैनल रिकॉर्डिंग और आणविक गतिशीलता सिमुलेशन का उपयोग किया गया था। हमारे परिणाम बताते हैं किः 1. αγ-बाइंडिंग साइट पर, एगोनिस्टों की एक श्रृंखला के लिए, ओ स्टेट एगोनिस्ट एसोसिएशन रेट्स (जोन) प्रसार-नियंत्रित प्रतिक्रियाओं की ऊपरी सीमा तक पहुंच गई, और एगोनिस्ट आत्मीयता αδ- और αε-बाइंडिंग साइटों की तुलना में परिमाण के 2-ऑर्डर अधिक थी। 2. 4 ऋणात्मक आवेशित अवशेषों (γE57, γD113, γD174, और γE180) के कारण इलेक्ट्रोस्टैटिक सतह आवेश घनत्व AO अवस्था में कैप्ड

लूप C, कॉम्पैक्ट TBS, और बाउंड एगोनिस्ट को स्थिर करता है। 3. इन अवशेषों को संयोजन में उत्परिवर्तित करने से αδ-और αε-बाध्यकारी साइटों के तुलनीय मूल्यों के लिए αγ-की जोन और बाध्यकारी आत्मीयता उलट गई। चार्ज न्यूट्रलाइजेशन उत्परिवर्तन ओ अवस्था के लिए एगोनिस्ट बाइंडिंग की आत्मीयता पर योगात्मक प्रभाव दिखाते हैं। टी. बी. एस. के 'बाहरी-क्षेत्र' आवेशित अवशेषों की भूमिका के संदर्भ में उच्च-आत्मीयता अवस्था के लिए एगोनिस्ट बंधन निर्धारित करने में परिणामों पर चर्चा की गई थी। इसके अलावा, एनएमजे में सिनैप्टिक प्रतिक्रिया को आकार देने में उच्च बनाम निम्न आत्मीयता एगोनिस्ट एसोसिएशन के महत्व पर चर्चा की गई, विशेष रूप से रोग पैदा करने वाले उत्परिवर्तन की उपस्थिति में।

अंत में, अध्याय 6 में बताया गया कि आयन चालन की अनुमति देने के लिए छिद्र द्वार कैसे खुलता है। न्यूरोमस्कुलर एसीएचआर के छिद्र में 'गेट' एक जटिल, अर्ध-स्वतंत्र, मैक्रोमोलेक्युलर संरचनात्मक तत्व है जो आयन चालन को नियंत्रित करता है। उत्परिवर्तन विश्लेषण, थर्मोडायनामिक माप, युग्मित अंतःक्रिया अध्ययन, और आणविक गतिकी सिमुलेशन ने प्रदर्शित किया कि बंद छिद्र आयन पारगमन के लिए एक पर्याप्त ऊर्जावान बाधा (+22.5 kcal mol^{-1}) प्रस्तुत करता है। छिद्र हाइड्रोफोबिसिटी और गेटिंग एनर्जेटिक्स के बीच मजबूत सहसंबंधों ने चैनल खोलने के दौरान हाइड्रोफोबिक गीलेपन के लिए सबूत प्रदान किए। अंतःक्रिया ऊर्जा मापों और अनुकरणों ने आगे सुझाव दिया कि चैनल खोलने में छिद्र-अस्तर M2 हेलिक्स की समन्वित पुनर्व्यवस्था, गेट अवशेषों की असममित गति और गेट क्षेत्र का जलयोजन शामिल है।

एक साथ लिए जाने पर, इस थीसिस के निष्कर्ष एसीएचआर सक्रियण के लिए एक थर्मोडायनामिक ढांचा स्थापित करते हैं। अध्याय 3 संरचनात्मक और लिगैंडेड गेटिंग के ऊर्जावान आधार को परिभाषित करता है, जबकि अध्याय 4 और 5 प्रदर्शित करते हैं कि कैसे राज्य-निर्भर एगोनिस्ट आत्मीयता को बाइंडिंग-साइट आर्किटेक्चर, संरक्षित सुगंधित अवशेषों और इलेक्ट्रोस्टैटिक स्टीयरिंग द्वारा निर्धारित किया जाता

है। अध्याय 6 से पता चलता है कि आयन पारगमन को छिद्र द्वार पर हाइड्रोफोबिक गीलेपन द्वारा नियंत्रित किया जाता है। सामूहिक रूप से, ये परिणाम एक सामान्य ऊर्जावान ढांचे के भीतर एगोनिस्ट बाइंडिंग, एलोस्टेरिक गेटिंग और छिद्र उद्घाटन को जोड़ते हैं, जो पेंटामेरिक लिगैंड-गेटेड आयन चैनलों के आणविक तंत्र और रोग से जुड़े रिसेप्टर डिसफंक्शन के आधार में नई अंतर्दृष्टि प्रदान करते हैं।

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List of abbreviations

ACh	Acetylcholine
AChBP	Acetylcholine binding protein
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
ADCs	Analog to digital converters
ADT	AutoDock Tools
AHP	Afterhyperpolarization
AIC	Akaike's information criterion
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ana	Anabasine
APBS	Adaptive Poisson-Boltzman Solver
ATP	Adenosine triphosphate
BIC	Bayesian information criterion
Btx	Bungatoxin
CaFE	Calculation of free energy
cAMP	Cyclic adenosine monohosphate
CCh	Carbamylcholine
Cho	Choline
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic guanosine monophosphate
CRC	Concentration response curve
CTxMI	Conotoxin
Cyt	Cytisine
DIC	Differential interference contrast

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECD	Extracellular domain
ELIC	*Erwinia* ligand-gated ion channel
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
FP	Forward primer
G_0	Unliganded gating energy
G_2	Diliganded gating energy
GABAA	γ-aminobutyric acid type A receptor
G_B	Binding energy
GHK	Goldman-Hodgkin-Katz
GLIC	*Gloeobacter violaceus* ligand-gated ion channel
GluCl	Glutamate-gated chloride channel
GlyR	Glycine receptor
GOF	Gain-of-function
HA	High-affinity
HBS	HEPES-buffered saline
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HH	Hodgkin-Huxley
HMM	Hidden Markov model
HT3R	Serotonin type 3 receptor

IP3	Inositol 1,4,5-trisphosphate
IPN	Interpeduncular nucleus
IR-DIC	Infrared differential interference contrast
J_d	High-affinity equilibrium dissociation constant
K_d	Low-affinity equilibrium dissociation constant
KNF	Koshland–Némethy–Filmer
LA	Low-affinity
LBD	Ligand binding domain
LFER	Linear free energy relationship
LGICs	Ligand-gated ion channels
LL	Log-likelihood
MCA	Mutant cycle analysis
MD	Molecular dynamics
mEPCs	Miniature endplate currents
MHb-IPN	Medial habenula-interpeduncular nucleus
MIL	Maximum Interval Likelihood
MM/PBSA	Molecular Mechanics/Poisson-Boltzmann Surface Area
MuSK	Muscle-specific kinase
MWC model	Model given by Monod, Wyman, and Changeux
NAc	Nucleus accumbens
NAMD	Nanoscale molecular dynamics
Nic	Nicotine
Nic-N	Ammonium group of pyrrolidine ring of Nic
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction

NRT	Nicotine replacement therapy
PBS	Phosphate buffered Saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PDL	Poly-D-lysine
POPC	Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
REFER	Rate-equilibrium free-energy relationship
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RP	Reverse primer
SKM	Segmental K-Means
TBD	Transmembrane domain
TBS	Transmitter binding site
TryE	Trypsin-EDTA
Var	Varenicline
VGICs	Voltage-gated ion channels
V_c	Command voltage
V_{hold}	Holding voltage
V_{in}	Voltage inside the membrane
V_{out}	Voltage outside the membrane
V_m	Membrane voltage
E_{rev}	Reversal potential
VMD	Visual molecular dynamics
VTA	Ventral tegmental area