The neurotransmitter receptor triad: Nicotinic acetylcholine receptor, γ-aminobutyric acid receptor (type A) and inhibitory glycine receptor

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Many different neuroactive substances known as neurotransmitters, which are involved in signal transduction, bind to specific membrane receptors in the vertebrate central nervous system. Of particular interest are the similarities shared by the nicotinic acetylcholine receptor, γ-aminobutyric acid receptor and inhibitory glycine receptor with respect to their pentameric structure, their neurotransmitter binding pockets, and colocalization with gephyrin. All three receptors undergo phosphorylation catalysed by protein kinases A and C. The biochemical defects in relation to these receptors result in the clinical manifestation of certain human disorders.

RECENT studies have indicated that the agonist binding pockets of the excitatory nicotinic acetylcholine receptor (nAChR), the inhibitory γ-amino butyric acid receptor subtype A ($GABA_AR$) and the inhibitory neuronal glycine receptor (GlyR) are formed from conserved amino acid positions in the extracellular domains of the ligand-binding subunits. Alignment of the subunit sequences of these three receptors shows that a motif of aromatic side-chains equivalent to positions 159–161 of GlyR α1 subunit is highly conserved at the homologous positions of the ligand-binding $GABA_AR$ and nAChR proteins (Figure 1).

It has been demonstrated that the exchange of the aromatic residues at positions 159 and 161 of GlyR generates receptors that are preferentially gated by agonists with longer carbon chains, such as β-alanine, taurine or γ-amino butyric acid (GABA). Moreover, it was shown that the replacement of phenyl alanine at position 159 by a tyrosine residue was sufficient to convert the GlyR into GABA-responsive protein[1].

Another feature indicating a close relationship between nAChR, $GABA_AR$ and GlyR is the colocalization of gephyrin, a peripheral membrane protein, with GlyR-, nAChR- and $GABA_AR$-containing areas of brain. Further, experimental results indicate that determinants of subunit assembly are in part located at homologous positions in these three ligand-gated ion channel proteins[2–4].

The main objective of the following sections is to discuss the biochemistry of the ligand-gated ion channels (with emphasis laid on nAChR, $GABA_AR$ and GlyR), their modulation by phosphorylation and the defects underlying specific human disorders in relation to these receptors.

The biochemistry of ligand-gated ion channels

Ligand-gated ion channels constitute one of the two major classes of neurotransmitter receptors[5]. These are multimeric protein complexes; they serve to transduce the extracellular signals to the cell interior by acting, as their name suggests, as ion channels. The binding of their respective ligands induces the opening of these channels, which are then rendered permeable to specific ions. They are then said to convert the chemical signal (ligand binding) released from one cell into an electrical signal (hyperpolarization or depolarization) that propagates along the target cell membrane. Thus, they provide for rapid dialogue between cells of the central nervous system. (This class is exemplified by the excitatory nAChR, the inhibitory GlyR, the inhibitory $GABA_AR$, etc.).

To date six families of ligand-gated ion channel receptors that mediate information in brain and neuromuscular junction have been characterized at the level of their amino acid sequences[6,7]. They are: (i) the excitatory nAChRs of neuromuscular and neuronal origin, which conduct cations; (ii) the excitatory neuronal kainate-type glutamate-activated channels, which conduct cations;

Receptor	Subunit	Position		Boxed	
GlyR	α1	154	M Q L E S	F G Y	T M N D L
$GABA_AR$	α1	155	L K F G S	Y A Y	T R A E V
$GABA_AR$	β1	152	L E I E S	Y G Y	T T D D I
nAChR	β1	144	M K L G T	W T Y	D G S V V

Figure 1. Alignment of partial GlyR, $GABA_AR$ and nAChR subunit sequences The regions homologous to amino acids 154–166 of the GlyR1 subunit are shown; amino acids homologous to residues 159–161 of the GlyRα subunit are boxed. (Based on ref. 1)

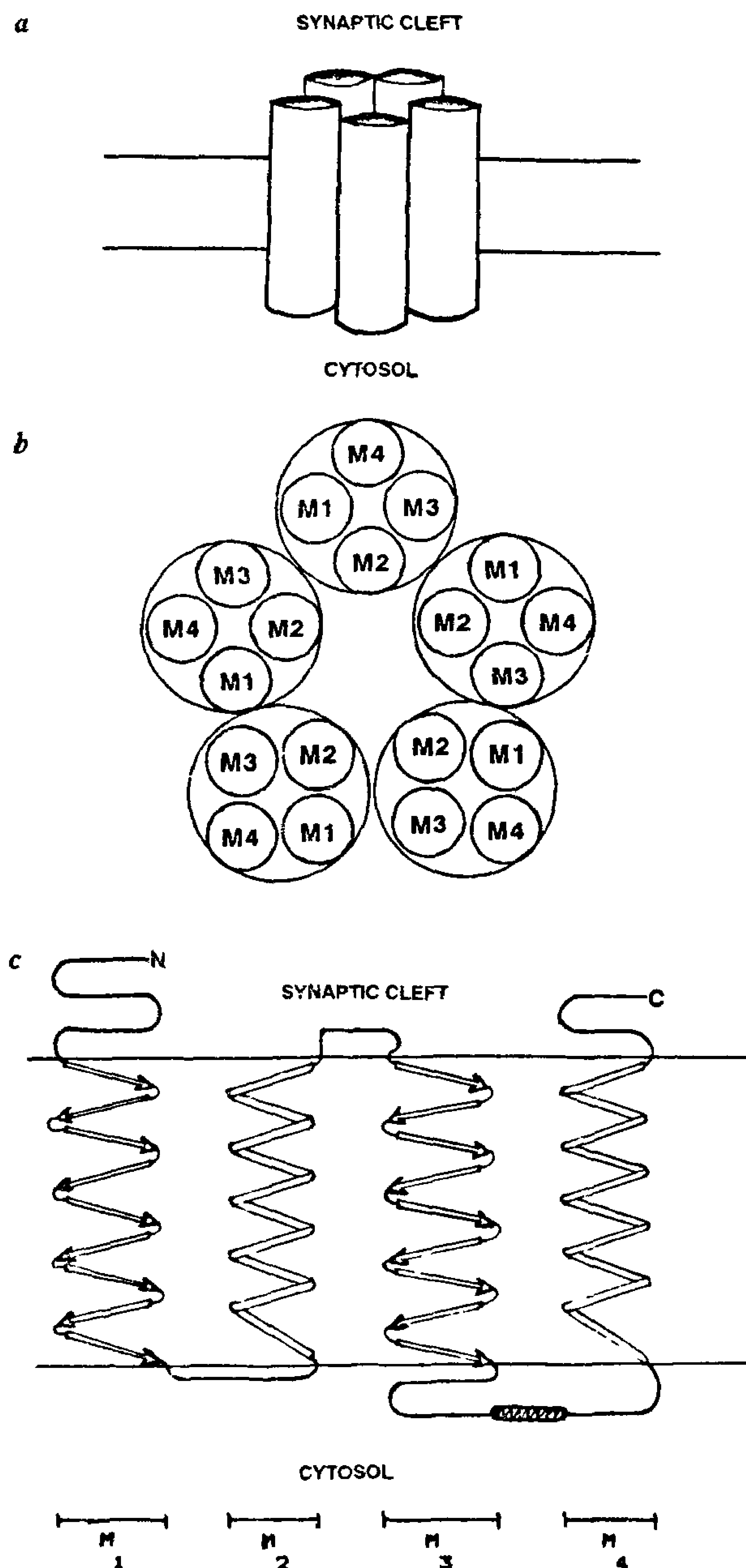

Figure 2. Schematic model of the topology of an idealized ligand-gated ion channel *a*, Pentameric subunit structure viewed through an angle to the plane of the membrane. *b*, Schematic model representing the arrangement of the transmembrane domains, namely M_1, M_2, M_3 and M_4 (depicted as smaller circles) within each subunit (the larger circles) around the central ion channel (based on ref. 8) *c*, Membrane topology of an individual subunit containing transmembrane domains $M_1 - M_4$, viewed through the plane of the membrane The hatched rectangle indicates the region of the subunit that contains the phosphorylation sites for various protein kinases (Based on ref 56)

(iii) the excitatory neuronal serotonin receptor (5-HT_3 receptor), which also conducts cations; (iv) the inhibitory neuronal GlyR, which conducts chloride; (v) the inhibitory neuronal $GABA_AR$, which also conducts chloride; and (vi) the sarcoplasmic reticulum ryanodine receptor, which conducts calcium.

These ligand-gated ion channel neurotransmitter receptors are pentameric complexes of subunits which are integral membrane proteins arranged around a central aqueous pore (Figure 2*a*, *b*)[8]. In a given receptor, these subunits reveal a high degree of sequence homology[9] The sequence identity extends to other classes of receptors too. The hydropathy profiles displayed by the subunits of different families of these receptors are similar, suggesting that all possess a common transmembrane folding[9-14]. They are, therefore, said to form a superfamily of ligand-gated ion channels.

The minimal model for transmembrane organization of the subunits, which was proposed for Torpedo nAChR subunits[15,16], has been extended to all members of the superfamily[8]. It consists of

(a) a hydrophilic N-terminal domain oriented towards the synaptic cleft and carrying the glycosylated moieties,

(b) a compact hydrophobic region subdivided into three uncharged segments long enough to span the membranes and referred to as M_1, M_2 and M_3,

(c) a hydrophilic domain oriented towards the cytosol, which carries the consensus site for phosphorylation, and

(d) a short carboxy terminal hydrophobic transmembrane segment denoted as M_4

In this minimal model both the N- and C-terminals are oriented towards the synaptic cleft (Figure 2*c*)[9]. However, this view has recently been challenged for glutamate receptor[17].

The extracellular domain of each of the receptor subunits forms a cylindrical vestibule which extends above the membrane plane. The agonist binding domains are located on the N-terminal domain and the cylindrical vestibule forms the ion channel, which lies along the axis of pseudosymmetry of the molecule[6]. Accordingly, the agonist site and the ion channel are topographically distinct. Hence, indirect or allosteric interactions account for the opening of the ion channels by their respective ligands[18].

Directed mutagenesis of the receptor subunits coupled with their transient expression in Xenopus oocytes has suggested that among the four hydrophobic putative membrane-spanning regions, namely M_1, M_2, M_3 and M_4, primarily M_2 and then M_1 and M_3 are most critical, and M_4 is the least required for ion conductance[5]. Therefore, each of the subunits contributes its M_2 region to line the inner wall of the ion channel[6,9]. Superimposed rings of charged residues bordering the M_2 regions of

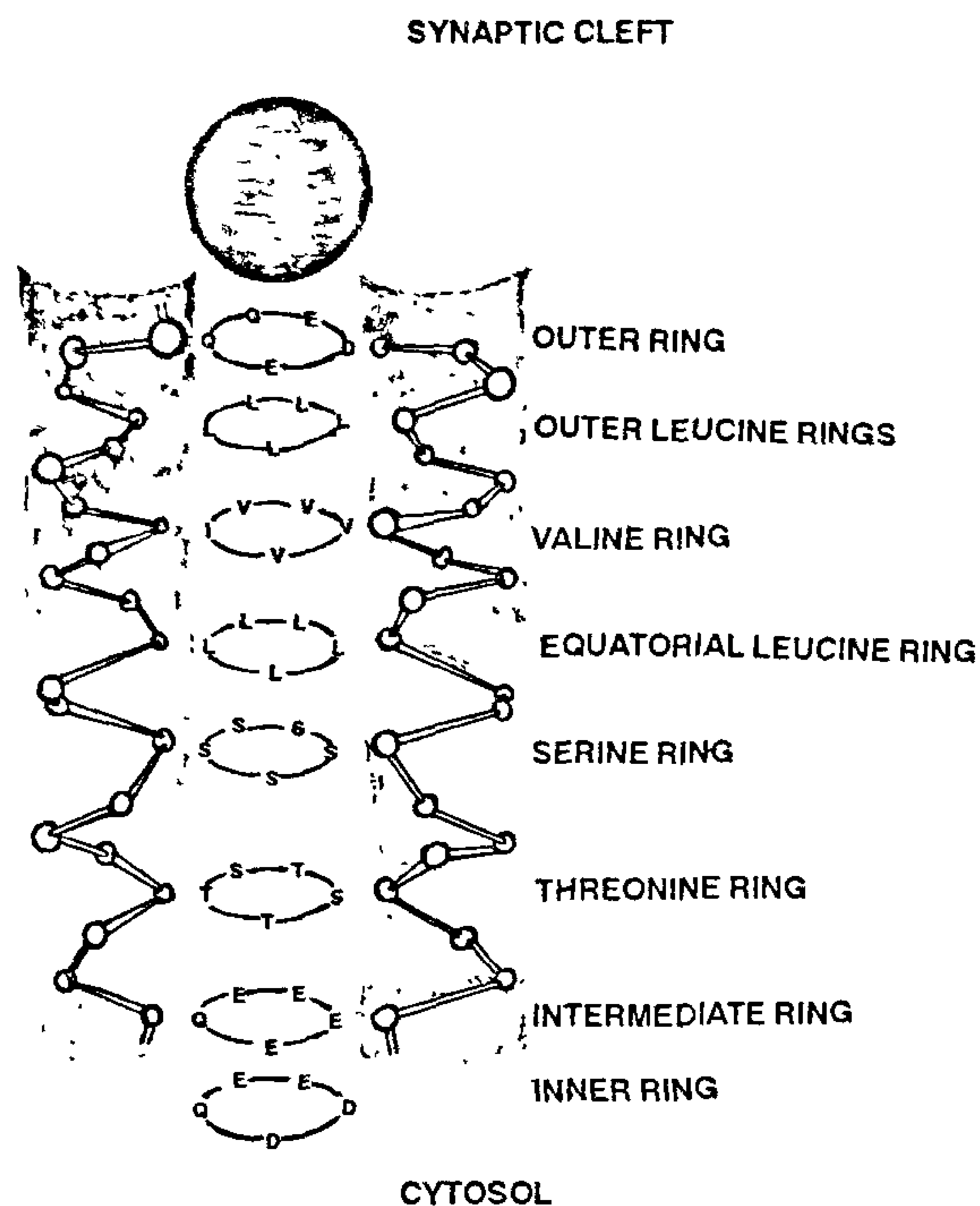

Figure 3. The arrangement of the M_2 segments of two subunits as transmembrane helices, wherein the superimposed rings of amino acids line the lumen of the ion channel. The channel is framed by rings of negatively charged amino acids at both the ends; these are called the inner ring and the intermediate ring at the cytosolic end, and the outer ring at the synaptic cleft end At a low divalent ion concentration, removal of negative charges at these positions reduces both the inward and the outward currents. The other rings (from N- to C-terminal end) of the M_2 segments are· threonine ring, serine ring, equatorial leucine ring, valine ring and outer leucine ring. (Based on ref. 9)

the receptor have been shown to determine the conductivity of the ion channel (Figure 3). In all the subunits, charged amino acids are present at both ends of M_2.

In fact, high channel conductance of nAChR for cations is due in part to the negatively charged vestibule. The expected net negative charge within the vestibule is in excess of 10 negative charges, comprised of 31 negative and 21 positive groups. This excess negative charge density results in the repulsion of anions and concentration of cations in the channel mouth[19].

Significant in this regard is the fact that, $GABA_AR$ and GlyR, which conduct chloride ions (anions), have excess positive charge of about the same amount in their vestibular region. This suggests that charged vestibules may be a general mechanism of enhancing cation or anion selectivity[6].

The nAChR has a subunit stoichiometry of $\alpha_2\beta\gamma\delta$ in the neuromuscular junction[20], whereas it is just $\alpha_2\beta_3$ in the central nervous system and autonomic ganglia[21].

The subunit stoichiometry of GlyR which mediates synaptic inhibition in brain stem and spinal cord areas of the central nervous system (CNS) has been established[10] to be $\alpha_3\beta_2$, but which of the six α, four β, three γ, one δ and one ρ subunits comprise the $GABA_AR$ which mediates synaptic inhibition in other areas of the CNS is unclear as yet[22].

GlyR (ref. 23) and $GABA_AR$ (ref. 24) have been purified, and the amino acid sequence of their subunits and their functional expression studied[10–12]. Their subunits show sequence homology with each other to such an extent that the mutation of only two amino acids of the subunit of the GlyR renders it responsive to GABA[1,25] These subunits have significant homology with the nAChR subunits also, further stressing the fact that all three receptors have a close relationship[1,7,9–12].

The combination of ion channels expressed by a neuron is responsible for its unique electrical properties like excitability and ability to propagate action potentials. The permeability of these ion channels to their respective ions, in addition to being regulated by membrane potential (in voltage-gated ion channels) and neurotransmitters (in ligand-gated ion channels), is also altered by protein phosphorylation[26].

Protein phosphorylation

Four decades since the first report implicating it in glycogen metabolism, protein phosphorylation has now come about to be acknowledged as the most common posttranslational modification involved in the regulation of protein functions.

Protein phosphorylation is regarded as the fundamental event in most cellular processes, and especially so in signal transduction[27,28]. Signals impinging on cells have their effects amplified and disseminated by a network of protein phosphorylation and dephosphorylation systems; the enzymes catalysing these reactions are further subject to control via cascades of phosphorylation and dephosphorylation reactions. Phosphorylation has also been shown to mediate many actions of the second messengers. Most extracellular signals including hormones, growth factors, neurotransmitters, electric potential and components of extracellular matrix alter the state of phosphorylation of intracellular proteins. These extracellular signals modify the activities of kinases and/or phosphatases either directly (e.g. receptors with kinase activity) or via cascades of enzymatic reactions (e.g. receptors–G-proteins–enzyme–second-messenger–protein-kinase) (Figure 4)[26]. Such cascades involving second messenger and protein kinase systems display a number of properties which allow amplification, integration, convergence and modulation of multiple and simultaneous internal and external signals.

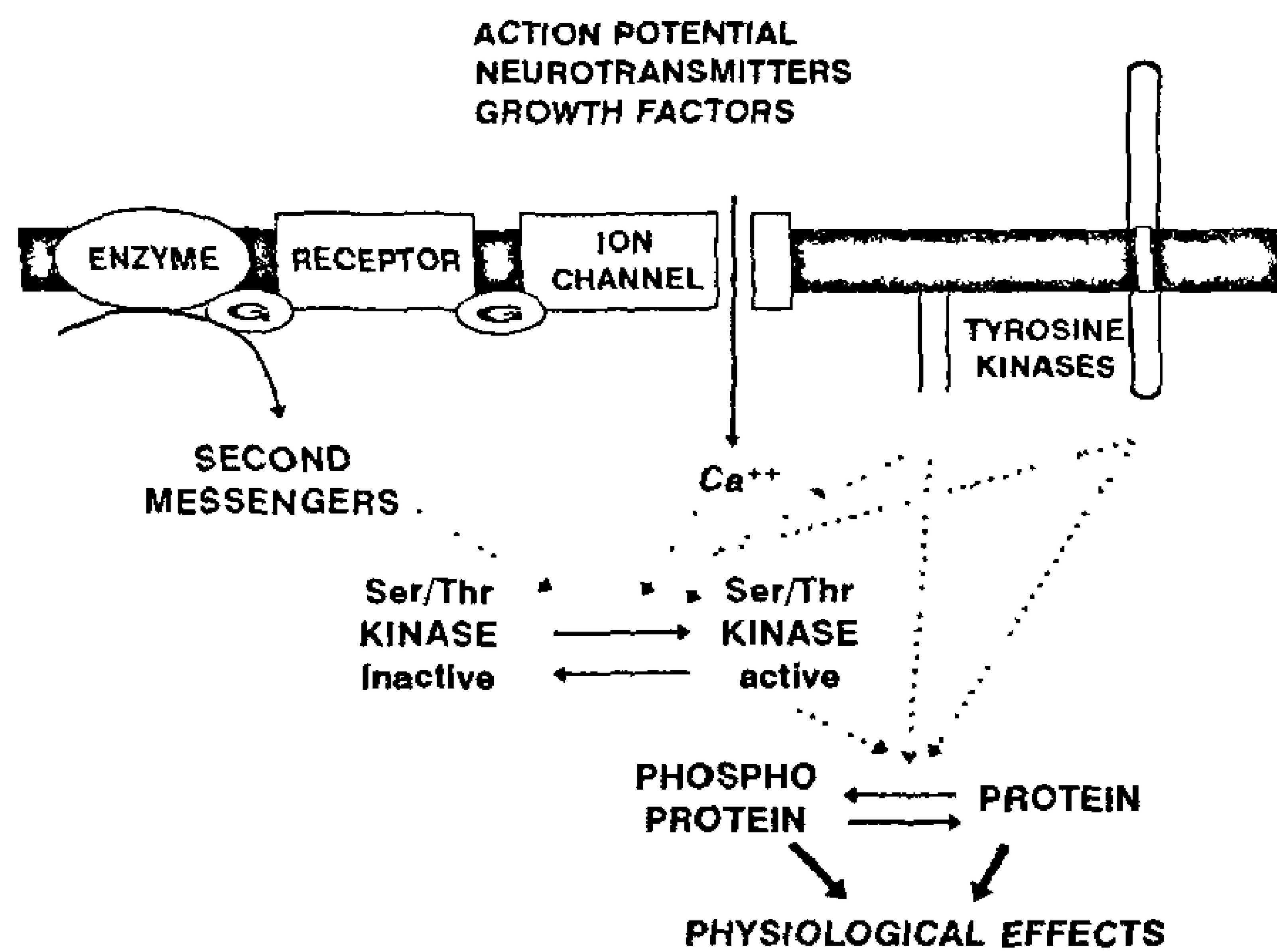

Figure 4. Schematic representation of some pathways by which extracellular signals alter protein phosphorylation in neurons. G in the figure indicates G (guanine nucleotide binding) protein.

Moreover, since the properties of many enzymatic components of signal transduction pathways are themselves altered by phosphorylation, the responsiveness of a cell to extracellular signal depends in part on the extent to which its proteins are phosphorylated. In other words, the cell keeps track of its recent history, encoded in its pattern of protein phosphorylation. Thus, protein phosphorylation allows not only the transduction of extracellular signals into the biochemical reactions inside the cells, but also the computation of several extracellular signals and their integration over time. It is, therefore, not surprising that most of the protein kinases and phosphatases are highly expressed in neurons, which are cells specialized in information processing[26].

Many proteins that are involved in synaptic function have been shown to be phosphorylated. These include proteins which play a role in neurotransmitter synthesis and release, voltage-gated ion channels, ligand-gated ion channels and G-protein-linked neurotransmitter receptors. The ion channels are a particularly appropriate target for the modulation of synaptic transmission by protein phosphorylation, since they are central to the process of signal transduction across the postsynaptic membrane[5,29].

Phosphorylation of the ligand-gated ion channels

Nicotinic acetylcholine receptor phosphorylation

Phosphorylation of nAChR has been extensively studied and documented[30,31]. One of the characteristics recorded for nAChR is its desensitization. In the continued presence of the agonist, the receptor rapidly fluctuates between conducting and nonconducting states for several hundred milliseconds and then enters a nonconducting, no-longer responsive desensitized state.

A comparison of the properties of the phosphorylated and nonphosphorylated nAChR shows that the rate of desensitization is enhanced in phosphorylated nAChR[32], whereas the channel conductance and the mean channel open time are not altered. Protein kinase activators like cyclic adenosine monophosphate (cAMP) analogues and phorbol esters are also recorded to enhance the rate of desensitization of nAChRs in different cell lines[33-36]

Tyrosine phosphorylation of nAChR is known to occur subsequent to innervation of muscle during development and is abolished by denervation. These findings establish the role of the neuron in regulating nAChR tyrosine phosphorylation[8].

Extracellular signals that are potential regulators of protein tyrosine phosphorylation have been investigated for their effect on the phosphorylation of nAChR. Agrin and basic fibroblast growth factor (bFGF) are the two well-documented examples. Agrin is known to induce tyrosine phosphorylation of nAChR in cultured myotubes[38] and the bFGF receptor is known to be a tyrosine kinase[37]. Whereas agrin mediates the nerve-induced aggregation of nAChR beneath the nerve terminals[8], bFGF bound to latex beads induces nAChR cluster formation in the Xenopus myoblast, at the bead myoblast contact point.

Drugs which inhibit tyrosine phosphorylation block both agrin-induced and bFGF-induced nAChR clustering[38]. Thus, tyrosine phosphorylation of nAChR most probably alters its interaction with the cytoskeleton in such a way as to cause the receptor immobilization, which results in accumulation of the receptor aggregates at the synaptic sites.

Phosphorylation of nAChR by calcium-diacylglycerol-regulated protein kinase or protein kinase C (PKC) in cultured chick myotubes results in the dispersal of nAChR aggregates and dephosphorylation promotes aggregation of the receptor. Diagonally opposite results are observed when phosphorylation due to cAMP-dependent protein kinase or protein kinase A (PKA) is activated[39].

All these facts suggest that phosphorylation, apart from enhancing the rate of desensitization of nAChR, also plays a vital role in its assembly and aggregation during synaptogenesis.

Recently, the autoregulation of phosphorylation of nAChR has been reported. This reveals a novel signal transduction pathway whereby the nAChR may regulate its own functional activity through phosphorylation[40].

GABA receptor phosphorylation

The evidence for direct modulation of GABA receptor function by phosphorylation is compelling. A basal-level

phosphorylation appears necessary to maintain $GABA_AR$ function[41]. Further, kinases activated in response to intracellular second messengers have been shown to regulate $GABA_AR$, resulting in a variety of functional effects, sometimes contradictory[42–44].

In different systems, for instance, PKA activation can potentiate, inhibit or have no effect on the amplitude of the GABA-elicited currents[45].

Phosphorylation due to PKC has been shown to decrease the current amplitude of some, but not all, GABA receptors.

These differential effects may be due to indirect effects of phosphorylation of other regulatory proteins; of equal importance is the fact that the functional properties of a given GABA receptor depend on the subunit composition. Different combinations of these subunits render the receptor responsive to different agonists and to a varied extent to the same agonist/antagonist[6,22].

Ethanol, man's most widely used and oldest psychoactive drug, acts by inducing the opening of $GABA_AR$ chloride ion channel. Recently, it has been reported that the alternatively spliced intracellular segment of the γ_2 subunit (namely, γ_2L, which has an eight amino acid insert), is required for the ethanol sensitivity in some GABA receptors. It is interesting to note that this intracellular segment contains a consensus PKC phosphorylation site[46]. Hence, it is possible that phosphorylation is involved in regulating receptor modulation at the ethanol-sensitive site.

Thus, in addition to their direct effects, intracellular phosphorylation events may also regulate GABA receptor more indirectly through effects on other modulatory sites.

Glycine receptor phosphorylation

GlyR can be phosphorylated both *in vitro* and *in vivo*, in response to PKA or PKC activators[47]. In Xenopus oocytes PKC activators decrease glycine-induced currents while PKA modulators increase the response to glycine. Although such data do not provide evidence for the actual phosphorylation of glycine receptor channels in the oocytes, the results obtained in intact spinal cord cells are consistent with a direct effect of phosphorylation in receptor function.

Given the variety of neurotransmitters, neuropeptides and growth factors known to regulate the intracellular activities of these two protein kinases, the fact that they modulate GlyR gives rise to intriguing possibilities, especially in the glycinergic neurotransmission in the spinal cord[48]. The dual mechanism of regulation by PKA and PKC modulators provides a sensitive instrument for the integrated modulation of the receptor by other extracellular messengers acting on the same neuron. This includes the previous, simultaneous or subsequent activation of a variety of G-protein-coupled receptors known to cause changes in cAMP or diacyl glycerol levels, which would further lead to changes in the subsequent neuronal response to glycine.

GlyR phosphorylation may prove to be a common mechanism by which the inhibitory action of glycine is regulated. This possibility is appealing, especially in case of pain transmission neurons. Two neurotransmitters, namely 5-hydroxytryptamine and noradrenaline, have important roles in the control of pain transmission at medulla and spinal levels. They are also known to alter the cAMP levels in target cells through their specific receptors. Thus, their observed effects may be mediated through the cAMP-dependent potentiation of inhibitory glycinergic transmission. Therefore, although further investigation is necessary, GlyR phosphorylation represents an attractive mechanism for the supraspinal regulation of some spinal cord processes. The integration of such cross-talking mechanisms of the neuronal membrane level accounts for transient and long-term changes in the efficacy of synaptic transmission, thus contributing to synaptic plasticity.

The defects underlying human disorders related to the neuroreceptors nAChR, $GABA_AR$ and GlyR

nAChR

Myasthenia gravis (MG) is a neuromuscular disorder characterized by weakness and fatigability of skeletal muscles. The underlying defect is a decrease in the number of available nAChRs at the neuromuscular junctions, due to an antibody-mediated autoimmune attack. Anti-nAChR antibodies cause accelerated destruction and the functional impairment of nAChR and failure of neuromuscular transmission. These antibodies act by three distinct mechanisms:

1. nAChR may be degraded at an accelerated rate by a mechanism involving crosslinking of the receptors.
2. The site in nAChR that normally binds acetyl choline may be blocked by the antibodies.
3. The postsynaptic muscle membrane may be damaged by the antibody in collaboration with the complement.

Thymus plays a role in the process of manifestation of autoimmunity. Muscle-like cells within the thymus, called myoid cells, bear nAChR-like protein on their surface. This acetylcholine-receptor-like protein is a glycoprotein, binds α-bungarotoxin irreversibly and has strong structural similarities with muscle nAChR. Its molecular weight, isoelectric point and amino acid composition are all very similar to those of nAChR found in mammalian muscle and the fish electroplax. This protein

most probably serves as the source of autoantigen and triggers the autoimmune reaction within the thymus gland[49–51].

$GABA_AR$

Epilepsies are a group of disorders characterized by chronic, recurrent paroxysmal changes in the neurological function caused by abnormalities in the electrical activity in the brain. Each episode of neurological dysfunction is called a seizure. Seizures may be convulsive when they are accompanied by motor manifestations or other changes in neurological function[52].

Current thinking awards GABAergic mechanisms a central role in the pathogenesis and treatment of epilepsy. The strongest evidence for a role of GABA in epilepsy is the fact that the pharmacological enhancement of GABAergic function produces antiepileptic effect. Several anticonvulsant drugs, including barbiturates and benzodiazepines, enhance GABA action at its primary sites, namely the GABA-benzodiazepine chloride ion channel complex[53].

GABA agonists are predominantly inhibitory and antiepileptic, whereas GABA antagonists produce seizures. Experimental agents that inhibit the breakdown of GABA are also predominantly antiepileptic.

Additional confirmation of the importance of GABA in epilepsy comes from analysis of neurotransmitter markers from animal models of acquired and genetic epilepsy. Pathological analysis of brain tissue from epileptic patients using positron emission tomography indicates either abnormalities in or the loss of GABA/benzodiazepine receptors[53].

GlyR

Reduced glycinergic inhibition caused by subconvulsive strychnine poisoning results in muscular hypertonia, heightened reflex excitability and an exaggerated response to sensory stimuli. These symptoms are similar to those of the autosomal dominant neurological disorder called startle disease, also known as hyperekplexia or kok's disease. This disorder is characterized by a marked muscular hypertonia in infancy, and an exaggerated startle response to unexpected sensory stimuli persistent in adulthood. Sudden jerking of limbs, or occasionally of trunk muscles, is observed; sudden noise or touch may cause the subject to jump or to fling an extremity.

More recently, point mutations in the gene encoding the subunit of the glycine receptor have been identified in startle disease pedigrees. These mutations map to arginine 271 located at the extracellular margin of the channel-lining M_2 region. In startle pedigrees the arginine 271 is replaced by an uncharged amino acid leucine or glutamine[54].

Site-directed mutagenesis of the GlyR cDNA coupled with their expression in mammalian 293 cells and their further studies using patch clamp technique have led to the discovery that the startle disease mutations cause no major alterations in ion permeation through GlyR, but rather dramatically reduce the receptor's agonist sensitivity. This finding, in addition to explaining the startle disease phenotype, identifies an unexpected structural determinant of the GlyR function.

This apart, analysis of other startle disease mutations may provide further important insights into the mechanisms of activation of the ligand-gated ion channel receptor superfamily.

Interestingly, startle response (abnormally enhanced startle response to acoustic stimuli) is also observed as one of the manifestations in the calcium calmodulin kinase II (CaMkII) mutant mice developed by Silva *et al.*[55]. The startle response is not observed in the wild-type mice, which have intact CaMKII. Whether there is any relationship between the startle response due to defective GlyR and that due to the absence of CaMKII, and if yes, the question as to what is its nature, has to be addressed in future.

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