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The Function of GluR1 and GluR2 in Cerebellar and Hippocampal LTP and LTD is Regulated by Interplay of Phosphorylation and *O*-GlcNAc Modification

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ABSTRACT

Long-term potentiation (LTP) and long-term depression (LTD) are the current models of synaptic plasticity and widely believed to explain how different kinds of memory are stored in different brain regions. Induction of LTP and LTD in different regions of brain undoubtedly involve trafficking of AMPA receptor to and from synapses. Hippocampal LTP involves phosphorylation of GluR1 subunit of AMPA receptor and its delivery to synapse whereas; LTD is the result of dephosphorylation and endocytosis of GluR1 containing AMPA receptor. Conversely the cerebellar LTD is maintained by the phosphorylation of GluR2 which promotes receptor endocytosis while dephosphorylation of GluR2 triggers receptor expression at the cell surface and results in LTP. The interplay of phosphorylation and *O*-GlcNAc modification is known as functional switch in many neuronal proteins. In this study it is hypothesized that a same phenomenon underlies as LTD and LTP switching, by predicting the potential of different Ser/Thr residues for phosphorylation, *O*-GlcNAc modification and their possible interplay. We suggest the involvement of *O*-GlcNAc modification of dephosphorylated GluR1 in maintaining the hippocampal LTD and that of dephosphorylated GluR2 in cerebral LTP. J. Cell. Biochem. 109: 585–597, 2010.

KEY WORDS: LEARNING AND MEMORY; INTERPLAY OF PHOSPHORYLATION AND *O*-GlcNAc MODIFICATION; GLUTAMATE RECEPTOR; AMPA RECEPTOR; LONG-TERM POTENTIATION; LONG-TERM DEPRESSION

The process of learning induces formation of new memory. The main mechanisms of memory formation in the hippocampus and many other brain regions are long-term potentiation (LTP) and long-term depression (LTD), which induces an activity-dependent and persistent change in synaptic plasticity [Hebb, 1949; Eccles, 1964; Alkon and Nelson, 1990; Kandel, 1997]. The involvement of multifunctional proteins in learning and memory has been documented [Whitlock et al., 2006]. Functional switch of a protein is often induced by dynamic posttranslational modifications (PTMs). These PTMs occur dynamically in different proteins, resulting in reversible structural/conformational changes, thereby inducing a functional switch. Such dynamic PTMs, including phosphorylation of the alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor (AMPAR) have been reported in regulating learning and memory formation [Whitlock et al., 2006] for instance switching of the resting state receptor into LTP or LTD and vice versa.

The AMPAR is a subclass of glutamate receptor [Dingledine et al., 1999] composed of four subunits GluR1-4. These four subunits combine differently to form homo- and heterotetrameric AMPA receptors [Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994; Rosenmund et al., 1998; Collingridge et al., 2004]. Different brain regions of mammals including the cerebral cortex, basal ganglia, thalamus, hypothalamus, hippocampus, cerebellum, and

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spinal cord, show higher degrees of expression of this receptor [Blackstone et al., 1992; Dure and Young, 1995].

The AMPA receptors composed of GluR1/GluR2 and GluR2/GluR3 subunits are usually dominant in the hippocampus [Wenthold et al., 1996]. AMPA receptors regulate most of the excitatory synaptic transmission in mammalian central nervous system [Lee et al., 2003]. Bidirectional trafficking of AMPA receptor to and away from synapses has been shown to play an important role in synaptic plasticity [Jiang et al., 2007]. The trafficking process is regulated by different PTMs of the receptor and its associated proteins [Barry and Ziff, 2002; Bredt and Nicoll, 2003]. On receiving an action potential, the presynaptic neuron releases glutamate into the synapse and glutamate binds to the AMPA receptor on postsynaptic neuron. Binding of glutamate opens an ion channel of the AMPA receptor, leading to influx of Na^+. This Na^+ influx depolarizes the postsynaptic neuron sufficiently to remove the Mg^{2+} blockade of *N*-methyl-D-aspartic acid (NMDA) receptor and allow Ca^{2+} to flow inside. This increase in Ca^{2+} level activates different protein kinases such as protein kinase C (PKC), calcium/calmodulin binding phosphate kinase II (CaMKII) and protein kinase A (PKA) [Mammen et al., 1997; Roche et al., 1996; Boehm et al., 2006]. These kinases phosphorylate AMPA receptors at different Ser and Thr residues and the phosphorylated receptors exert their regulatory functions.

The two processes LTP and LTD have been described as functional mirror images. The biochemical changes that underlie both processes are therefore also considered to be the opposite of one another [Arnold et al., 2000]. Phosphorylation of GluR1 subunit of the AMPA receptor by different kinases, including phosphorylation of Ser 818 by PKC [Boehm et al., 2006], Ser 831 by PKC and CaMK-II [Roche et al., 1996; Mammen et al., 1997], and Ser 845 by PKA [Roche et al., 1996], occurs during hippocampal LTP. On the contrary dephosphorylation of Ser 856 (Ser 880 in precursor) of GluR2 has been reported during cerebellar LTP [Seidenman et al., 2003]. Whereas, the dephosphorylation of Ser 818, 831, and 845 of GluR1 during hippocampal LTD and phosphorylation of Ser 856 of GluR2 by PKC during cerebellar LTD have been reported [Seidenman et al., 2003].

Besides phosphorylation, *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification is also an important PTM for protein regulations. This refers to addition of a single sugar (*O*-GlcNAc) residue catalyzed by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGNase), in turn is responsible for the removal of *O*-GlcNAc from Ser/Thr residues [Kreppel et al., 1997; Gao et al., 2001], making the *O*-GlcNAc modification as dynamic as phosphorylation. *O*-GlcNAc, OGT, and *O*-GlcNAcase are found abundantly in different brain regions, especially in the hippocampus [Kreppel et al., 1997; Gao et al., 2001; Okuyama and Marshall, 2003; Liu et al., 2004]. The presence of OGT and *O*-GlcNAc has also been reported in synaptosomes, where they regulate different synaptic proteins [Cole and Hart, 2001]. *O*-GlcNAc modification has a complex behavior in transcription regulation, usually acting as a transcription repressor but sometimes also as transcription activator [Kreppel et al., 1997; Lubas et al., 1997; Gewinner et al., 2004].

Alternative phosphorylation and *O*-GlcNAc modification on the same or neighboring Ser/Thr residues is well documented in several nuclear and cytosolic proteins [Wells et al., 2003]. The phenomenon is known as the Yin Yang hypothesis and the Ser/Thr residues involved in this complex interplay of phosphorylation and *O*-GlcNAc modification are named as Yin Yang site. In most of the cases such complex interplay of *O*-GlcNAc modification and phosphorylation results in a functional switch of protein. An interruption of this interplay process in different proteins may cause the protein to become permanently on or off, as it occurs in many neurodegenerative diseases [Arnold et al., 1996] and diabetes [Baron et al., 1995]. Such blocks may be seen both at the transcriptional and translational levels [Datta et al., 1989; Chou et al., 1995]. Assessing such functional switches regulated by the interplay of PTMs designing specific experimental protocols are of much time-consuming, expensive with a high risk of obtaining uncertain results. Computational tools are helpful for experimentalists to define the scope of directed studies, thus minimizing utilization of resources and reducing uncertainty in the results obtained.

This study was intended to investigate the potential interplay sites of phosphorylation and *O*-GlcNAc modification in GluR1 and GluR2, their possible involvement in ligand and or agonist binding, receptor assembly and functional switches of AMPA receptor implicated in regulating the hippocampal and cereberal LTP and LTD. Association of phosphorylated GluR1 in hippocampal LTP and that of GluR2 in cerebellar LTD is well known. It is proposed that the dephosphorylated GluR1 and GluR2 are most likely to undergo *O*-GlcNAc modification. Involvement of *O*-GlcNAc modification of the former is proposed to be related with hippocampal LTD and that of later in cerebellar LTP.

MATERIALS AND METHODS

The Fasta sequences of human GluR1 and GluR2 were taken from Swiss-Prot sequence database with entry names GRIA1_HUMAN and GRIA2_HUMAN, respectively [Boeckmann et al., 2003]. Homology search for the retrieved sequences was performed utilizing BLAST at NCBI with default parameters [Altschul et al., 1997]. A total of 99 hits were searched for GluR1, all having zero E-value and high bit score ranging from 1,888 to 1,271. Out of these 99, seven orthologs were selected, representing each a major mammalian or vertebrate group. The selected seven sequences were with accession numbers B7U3W4, Q90855, Q38PU8.1, P23818, P19490, AAK52501.1, and B3DGS8 from *Columba livia*, *Gallus gallus*, *Macaca fascicularis*, *Mus musculus*, *Rattus norvegicus*, *Oreochromis mossambicus*, and *Danio rerio*, respectively. For GluR2 there were total 100 hits, all with E-value zero and 1,828 as highest and 1,347 as lowest bit score. Seven sequences, other than human GluR2, were selected including NP_001070157.1 from *Taeniopygia guttata*, CAA82799.1 from *C. livia*, P23819.3 from *M. musculus*, P19491.2 from *R. norvegicus*, Q38PU7.1 from *M. fascicularis*, Q5R4M0.1 from *Sumatran orangutan*, and NP_571970.2 from *D. rerio*. Selected sequences were multiply aligned utilizing ClustalW [Thompson et al., 1994] separately for GluR1 and GluR2.

Human and other vertebrate GluR1 and GluR2 contain signal peptide. Before running prediction on human sequence and multiple alignments of all the sequences the signal peptides were removed

from each of the sequences. The details of signal peptides removed were taken from the relevant database annotations for all mammalian and avian sequences. But no detail of signal peptide for two vertebrate (*O. mossambicus* and *D. rerio*) sequences of GluR1 was found in the annotations. SignalP 3.0 [Bendtsen et al., 2004] was utilized to predict signal peptide in the two sequences and the predicted region of the two sequences were removed. Similarly, the signal peptide for each selected sequence of GluR2 was removed according to the detail provided in the relevant database annotation except for that of *D. rerio*, whose signal peptide detail was missing in the annotation. Signal peptide length for GluR2 sequence was again predicted by SignalP 3.0 [Bendtsen et al., 2004] and the peptide was removed from this sequence.

PTMs PREDICTION TOOLS

NetPhos 2.0 was used for predicting the potential phosphorylation sites on both proteins (GluR1 and GluR2) [Blom et al., 1999]. This program predicts the phosphorylation potential for each Ser, Thr, and Tyr residues, and calculates the score of phosphorylation potential for each Ser/Thr/Tyr residues. NetPhos 2.0 utilizes a threshold value 0.5 for any Ser/Thr/Tyr to be predicted as potential phosphorylation sites. YingOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang) (unpublished) server was used to predict *O*-GlcNAc modification potential sites in GluR1 and GluR2. This program calculates the *O*-glycosylation potential for all Ser, Thr, and Tyr residues in a protein sequence and also crosschecks these sites against potential phosphorylation sites to determine the possible Yin Yang sites, the sites that have high potential for both modifications. The threshold value for this program is highly variable. It is adjusted according to the surface accessibility of amino acids.

Kinase-specific predictions for potential phosphorylation sites were also performed with NetPhosK 1.0 [Blom et al., 2004] and KinasePhos 2.0 [Huang et al., 2005], both of which predict phosphorylation potential at each Ser, Thr, and Tyr residues catalyzed by a specific kinase, Predphospho [Kim et al., 2004] is another kinase-specific phosphorylation prediction tool developed employing support vector machine (SVM) for prediction.

All PTMs predicting methods used above are based on one of the machine learning techniques including artificial neural networks (ANNs), SVM, etc. These machine learning techniques utilized experimentally verified data for the process of learning to search/find/predict-specific pattern(s) and function elucidation on complex data sets which are either difficult or impossible to determine empirically.

STRUCTURE MODELING OF GluR1

The three-dimensional (3D) structure of GluR1, including S1 and S2 domains, was modeled with the help of the automated homology modeling server SWISSMODEL [Kiefer et al., 2009]. Another recently reported tool for comparative modeling of protein–ligand complex named @TOME-2 was also utilized [Pons and Labesse, 2009]. It takes an input of proteins sequence and performs all steps of homology modeling of protein utilizing modeler and then docks the protein with the ligand molecules (taken from different complexes in PDB, by AutoDock). The fold recognition process is performed by four tools: two of which are sequence–sequence comparison methods (Psi-BLAST and HH search) and the other two, sequence–structure comparison methods (FUGU and SP3) that search suitable templates. This search resulted in many protein structures/complexes with and without glutamate as ligand molecule. From this search result all those entries with 30% or more sequence or structure identity and covering both S1 and S2 regions in query to subject alignments, were selected as templates. A total of 18 entries, after removing the isoforms, were found with an identity of 30% or more but after dropping the entries which were not producing the alignments covering both S1 and S2 regions of GluR1, resulted in a final selection of 10 PDB entries as templates including 1YAE (GluR6, Rat), 2RC8 (NMDAR3, Rat), 2A5S (NMDAR2, Rat), 1PB7 (NMDAR1, Rat), 1MQI (GluR2 in complex with kainate, Rat), 1FTK (GluR2 in complex with Fluoro-Willardiine, Rat), 3C31 (GluR5, Rat), 2V3U (GluRdelta-2, Rat), 2PVU (RKH binding protein, *Geobacillus stearothermophilus*), and 2PYY (GluR0, *Nostoc*). The ATOME-2 can extract the ligands from the complexes of PDB entries. In the ligand selection step, glutamate was selected as ligand molecule from PDB entries including 3DP6 (a complex of GluR3 with glutamate, Rat), 1FTJ (a complex of GluR2 with glutamate, Rat), 2A5S (a complex of NMDAR2 with glutamate, Rat), 1II5 (a complex of GluR0 with glutamate, Synechocystis), 2PYY (a complex of GluR0 and glutamate, *Nostoc*), 2VHA (a complex of periplasmic binding transporter protein with glutamate, *Shigella*).

In both approaches, modeling the GluR1 receptor with glutamate, the S1 and S2 (binding regions 1 and 2) domains with linker region containing the sequence patch of GluR1 (from amino acid number 388–799) was taken for modeling and glutamate binding studies with the receptor. No template for whole receptor sequence can be found in PDB. Thus a sequence patch, containing all amino acids involved in binding with glutamate and their flanking sequences that can affect its binding region, was taken.

COMPARISON OF MODELED GluR1 STRUCTURE WITH THAT OF GluR2 AND OTHER PARALOGS

The structure for synthetic construct of S1–S2 core of GluR2 and other paralogs has been resolved experimentally by many groups in different complex forms including complexes of GluR2 and other paralogs, including GluR3, GluR4, GluR5, GluR6, GluR0, NMDAR1, NMDAR2, and NMDAR3, with glutamate and/or its agonists. Different complexes of GluR2 and other paralogs with glutamate were downloaded from PDB to compare the glutamate binding region with that of the GluR1 modeled by SWISSMODEL and Atome2.0. Different structure visualization softwares were utilized for this comparison.

RESULTS

GluR1

Different potentially phosphorylatable Ser/Thr/Tyr residues were predicted in GluR1 (Table I) of *Homo sapiens* by NetPhos 2.0. A total of 56 phosphorylated sites were predicted. Amongst these 56 potential phosphorylation sites, 30 were Ser, 13 were Thr, and 13 were Tyr (Fig. 1a).

TABLE I. GluR1 Prediction Results by NetPhos 2.0 and YinOYang 1.2

	Ser	Thr	Tyr
Phosphorylation sites	18, 49, 117, 138, 288, 297, 435, 506, 512, 543, 549, 561, 564, 588, 591, 593, 627, 631, 650, 658, 692, 776, 781, 784, 814, 818, 845, 851, 854, 862	53, 75, 99, 161, 162, 395, 559, 560, 681, 682, 716, 732, 840	65, 167, 270, 276, 363, 401, 417, 446, 465, 545, 669, 696, 707
O-GlcNAc sites	18, 255, 627, 845 271, 375, 453, 560, 780, 838	–	
Yin Yang sites	18, 627, 845	560	–
False negative Yin Yang sites	818, 831	–	–

YingOYang 1.2 predicted 10 potential sites for *O*-GlcNAc modification including Ser 18, 255, 627, and 845; and Thr 271, 375, 453, 560, 780, and 838 (Fig. 1b). Out of these 10 potential sites 4 were predicted as Yin Yang sites. Conservation status of Ser 18 and 627 (predicted Yin Yang site) showed a complete conservation in mammals, aves and pisces, while Ser 845 is only conserved in mammals and pisces (Fig. 2). Thr 560 is only conserved in mammals, whereas, it is a conserved substitution in form of Ser in pisces group (Fig. 2) and alanine in aves (Fig. 2). Additionally, Ser 831 has experimentally been proven to be phosphorylated in human embryonic kidney cells (HEK cells) by PKC [Roche et al., 1996] but this site was not predicted to be potential both by NetPhos 2.0 and YingOYang 1.2 servers. Ser 831 is also conserved in all selected mammals, pisces, and aves sequences. Similarly, phosphorylation of Ser 818 by PKC has been documented in vitro [Boehm et al., 2006] but none of the prediction method for

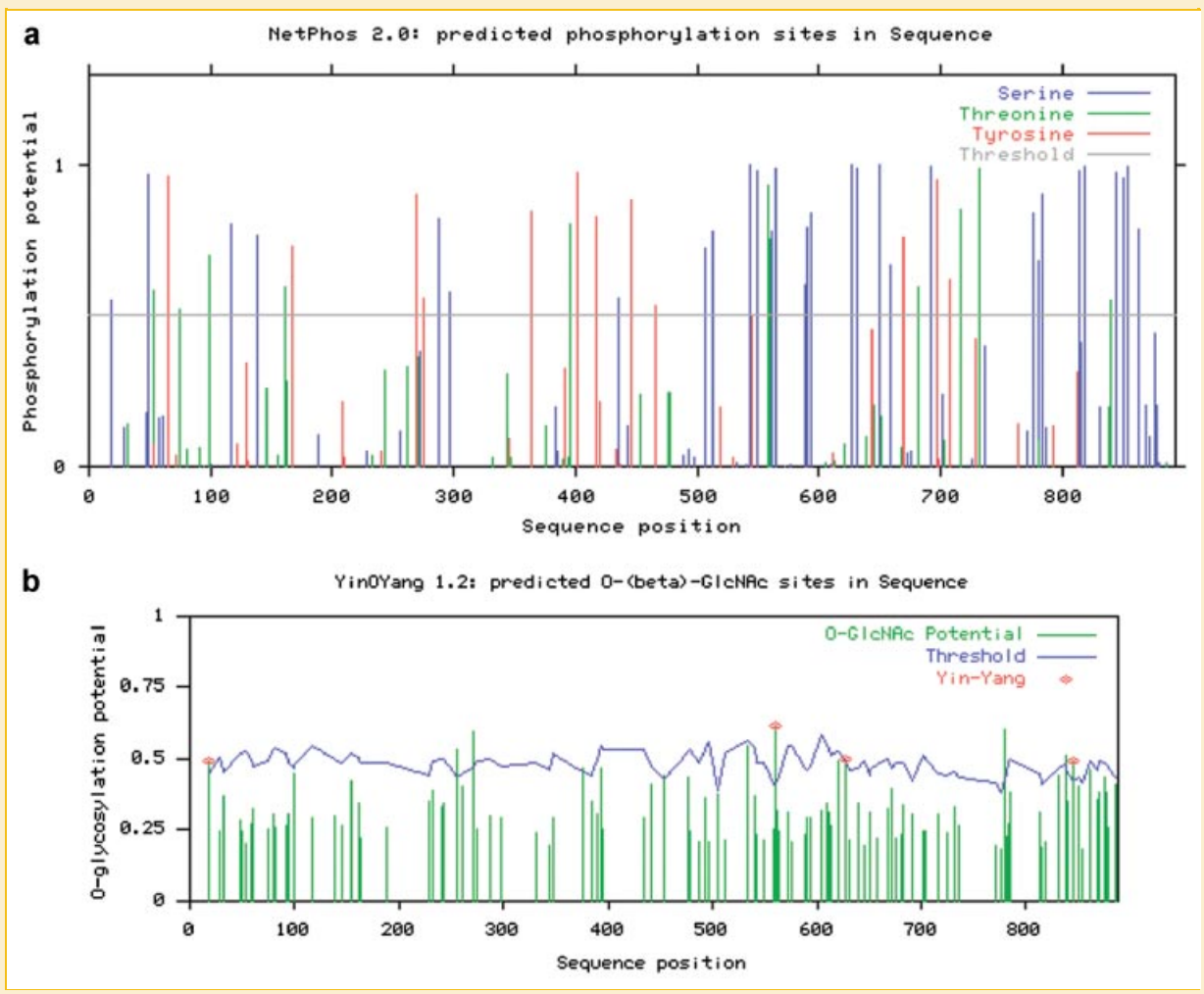

Fig. 1. Graphic representation of the potential of amino acid residues (Ser, Thr, and Tyr) for phosphorylation (a) and potential for *O*-GlcNAc modification (b) in GluR1 of human. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

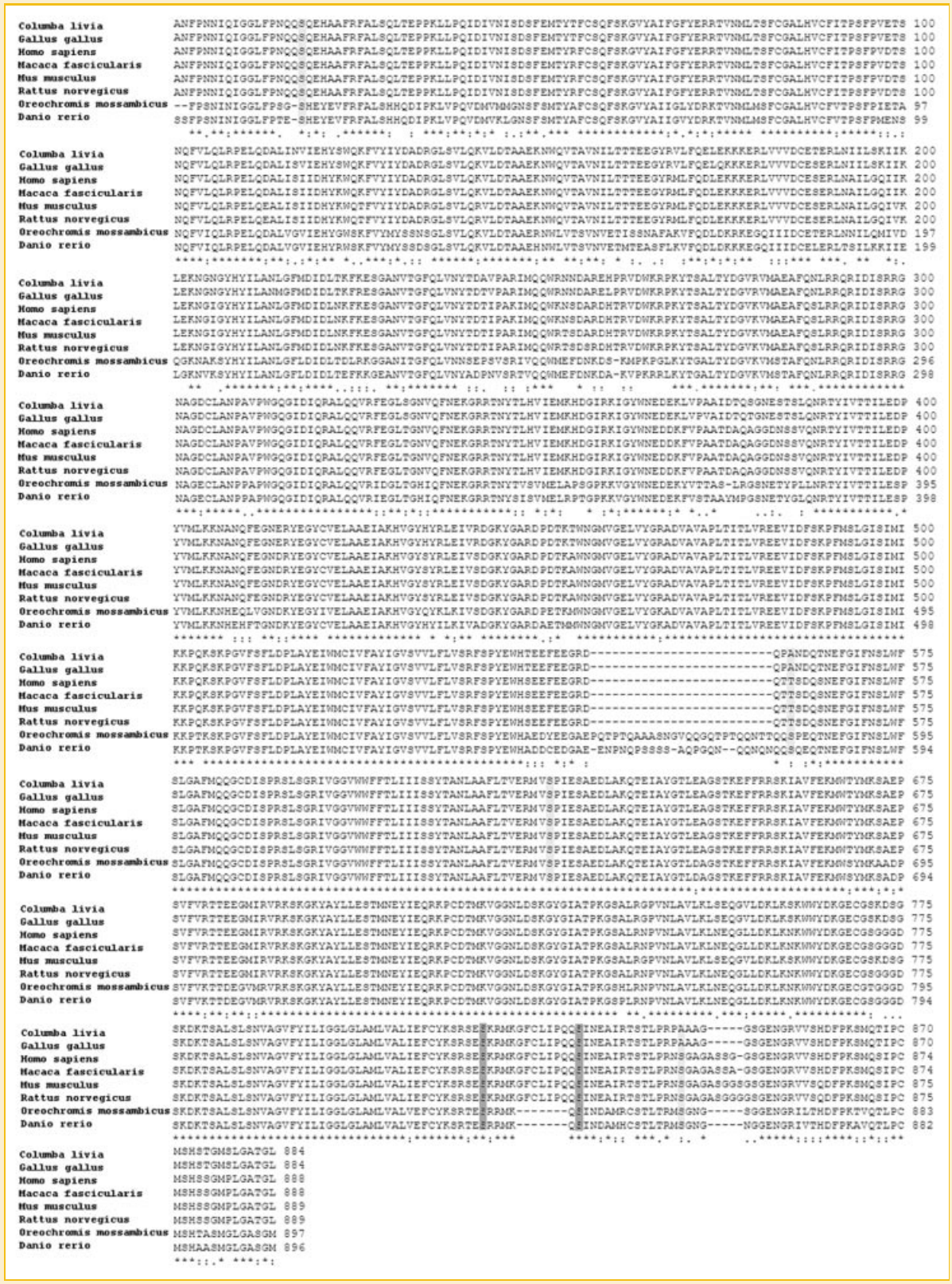

Fig. 2. Multiply aligned sequences of GluR1 in different mammals and other vertebrates show higher degree of conservation throughout the length. The amino acids residues those are potential for phosphorylation and/or for *O*-GlcNAc modification are highlighted.

phosphorylation and *O*-GlcNAc modification predicted this site as a potential site. This Ser 818 is also completely conserved in all mammalian and other vertebrate sequences (Fig. 3a). Moreover, it has also been well documented that PKC and OGT recognition sites are similar [Matthews et al., 2005]. Therefore it is suggested, in view of the fact that the Ser 818 and 831 are known for phosphorylation by PKC can also be modified by *O*-GlcNAc. Thus both of these sites are false negative Ying Yang site.

Kinase-specific phosphorylation prediction results have been summarized in Table II. The phosphorylation potential of Ser 831 was predicted to be negative both by NetPhosK 1.0 and Predphospho, consistent with those of NetPhos 2.0 and YingOYang 1.2. But KinasePhos 2.0 predicted 10 kinases for potentially phosphorylating Ser 831. This shows that Ser 831 is falsely predicted as nonphosphorylated site.

GluR2

GluR2 also showed a higher number of potentially phosphorylatable Ser/Thr/Tyr residues predicted by NetPhos 2.0 (Fig. 3a). A total of 52 sites showed phosphorylation potential, out of which 31 were Ser, 8 were Thr, and 13 were Tyr (Table III). The YingOYang 1.2 server predicted five potential amino acid residues for *O*-GlcNAc modification with Ser 628, 834, and 835, and Thr 272 and 781 (Fig. 3b). The only predicted Yin Yang site was Ser 628. Additionally, Ser 856 has been experimentally proven to be phosphorylated playing a role in cerebellar LTD and LTP [Seidenman et al., 2003]. But this site was predicted to have a very low (negative or lower than the prediction threshold score) potential for phosphorylation by NetPhos 2.0, and KinasePhos 2.0. Similarly, for *O*-GlcNAc modification, a low potential (negative or lower than the prediction threshold score) by YinOYang 1.2 was observed. On the other hand a higher potential (higher than the threshold value) for phosphorylation was predicted by NetPhosK 1.0 and Predphospho (Table IV) catalyzed by PKC. Therefore, Ser 856, with a high potential for phosphorylation and conserved in all mammalian and other vertebrate groups (Fig. 4), is a strong candidate for kinase action. If PKC kinase can act on Ser 856, then Ser 856 has equal chances to be GlcNAc modified by OGT. Thus this Ser 856 (880 in the

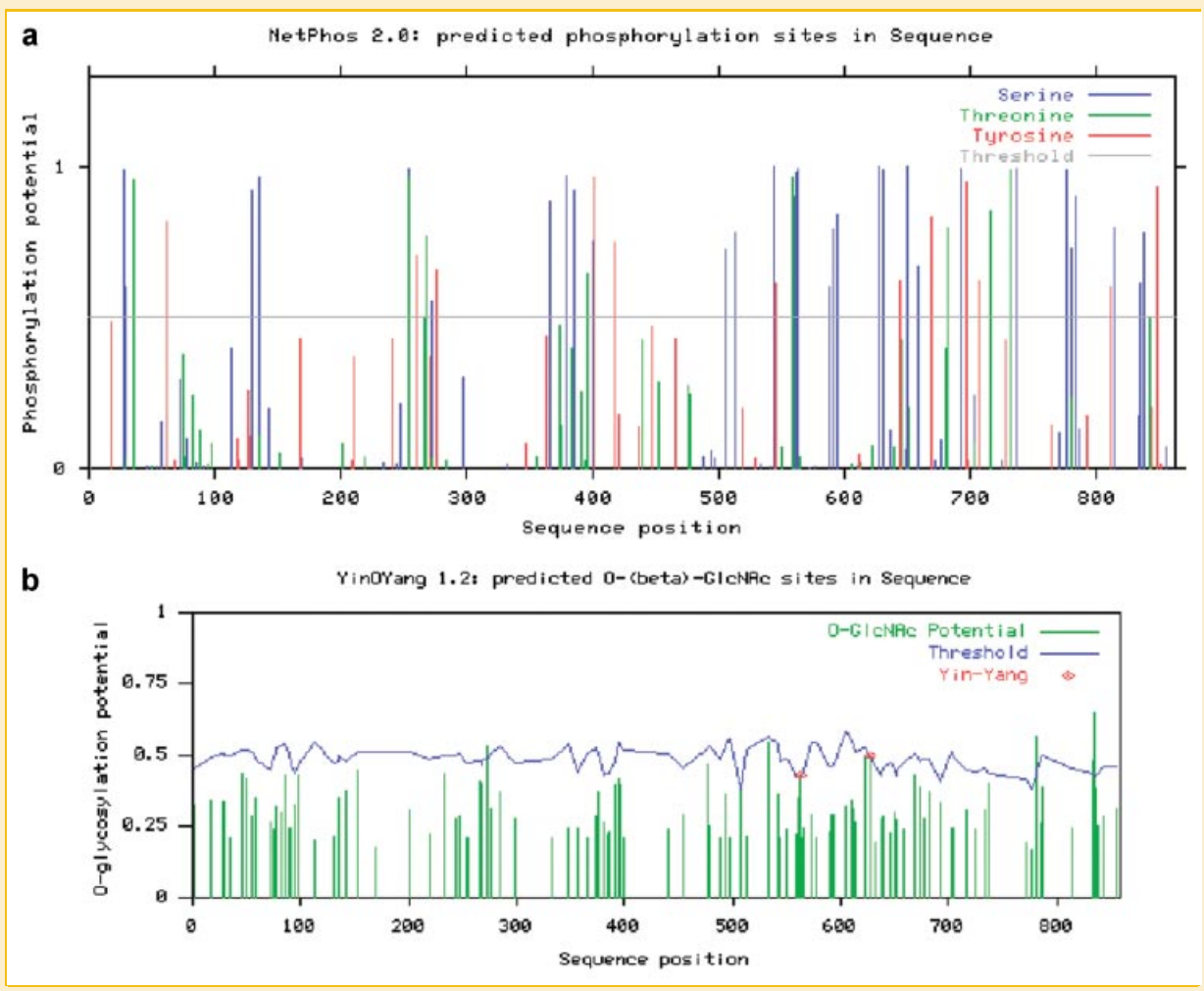

Fig. 3. Graphs showing the potential of different amino acids for phosphorylation (a) and for *O*-GlcNAc modification (b) in GluR2 of human predicted by NetPhos 2.0 and YinOYang 1.2, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Prediction Results of GluR1 by NetPhosK 1.0, Predphospho, and KinasePhos 2.0

Site	NetPhosK 1.0		Predphospho		KinasePhos 2.0	
Ser 18	DNAPK	0.62	–	–	AKT1	0.508
					PKB	0.727
					ATM	0.987
					PKC	0.728
					IKK	0.656
					PKG	0.507
					RSK	0.536
					STK4	0.512
					CHK1	0.512
					PDK	0.5
Ser 627	–	–	PKA	0.571	AKT1	0.5
			CDK	0.905	PKB	0.523
					ATM	0.952
					PKG	0.5
					CAM	0.898
					RSK	0.513
					STK4	0.513
					CHK1	0.513
					CK1	0.524
					CK2	0.783
Ser 831 (False-negative)	–	–	–	–	ATK1	0.508
					ATM	0.957
					PKC	0.892
					IKK	0.522
					PKG	0.507
					RSK	0.54
					STK4	0.512
					CHK1	0.512
					CK1	0.792
					PDK	0.5
					CK2	0.647
Ser 845	–	–	PKA	0.751	AKT1	0.5
					ATM	0.906
					Aurora	0.961
					IKK	0.616
					PKG	0.5
					RSK	0.534
					STK4	0.512
					CHK1	0.512
					CK1	0.815
Thr 560	CKII	0.59	–	–	GRK	0.542
					PKB	0.519

precursor) was given as a false negatively predicted Yin Yang site (Table III).

STRUCTURAL CHARACTERISTICS OF GluR1 COMPARED WITH OTHER PARALOGS

The modeling results provided by SWISSMODEL included three different models. The first model was developed utilizing a template 2rcbA (NR3, a member of NMDA receptor), whereas the second and third models utilized 3bfuC (GluR2 ligand binding core), and 1mm7B (GluR2 ligand binding core) as templates, respectively.

TABLE IV. Kinase-Specific Phosphorylation Prediction Results of GluR2

Amino acids	NetPhosK 1.0		Predphospho		KinasePhos 2.0	
Ser 628	–	–	PKA	0.566	AKT1	0.5
			CDK	0.874	PKB	0.523
					ATM	0.952
					PKG	0.5
					CAM	0.898
					RSK	0.531
					STK4	0.531
					CHK1	0.531
					CK1	0.524
					CK2	0.783
Ser 856	PKC	0.72	PKC	0.623	–	–

Evaluation and visualization of these three models revealed that the only model built utilizing 2rcbA as template was with the all essential amino acids of the S1 and S2 binding core, while the other two structures only contained fragments of amino acids either of S1 region or of S2 regions. The binding sites and binding regions in the model built with the template 2rcbA were found to be conserved (Fig. 5). Comparative modeling of the S1 and S2 binding core of human GluR1 by @TOME-2.0 generated many models of the S1 and S2 core with glutamate in the binding cleft. In most of the complexes generated by @TOME-2.0, the binding cleft was conserved structurally, as it was the in template, but orientation and fitting of glutamate in the ligand binding cleft of GluR1 was different in different complexes (Fig. 6). A complex of the structural model of GluR1 developed with a template of 1YAE (GluR6) showed the best fitting of glutamate of 2A5S (a complex of NMDAR2 with glutamate) origin (Fig. 7).

DISCUSSION

Learning, acquiring and, retaining memory are neurological functions considered to be regulated by changes in synaptic plasticity [Hebb, 1949; Eccles, 1964; Alkon and Nelson, 1990; Kandel, 1997]. LTP and LTD are the cellular and molecular models of memory storage in response to learning. The changes in synaptic plasticity are often induced by PTMs of different neuronal proteins [Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973; Miller and Mayford, 1999; Braunewell and Manahan-Vaughan, 2001]. Properties of LTP are very similar to that of memory, as both exist in two phases of short- and long-term duration. Therefore, both of these processes are associated and can last for hours and days

TABLE III. GluR2 Prediction Results by NetPhos 2.0 and YinOYang 1.2

	Ser	Thr	Tyr
Phosphorylation sites	28, 30, 130, 135, 254, 273, 366, 380, 385, 400, 507, 513, 544, 561, 562, 564, 589, 592, 594, 628, 632, 651, 659, 693, 738, 777, 782, 785, 815, 836, 839	35, 255, 268, 396, 554, 683, 717, 733	62, 261, 287, 402, 418, 546, 644, 670, 697, 708, 813, 849
O-GlcNAc sites	562, 628, 834, 835	272, 781	–
Yin Yang sites	562, 628	–	–
False negative Yin Yang sites	856	–	–

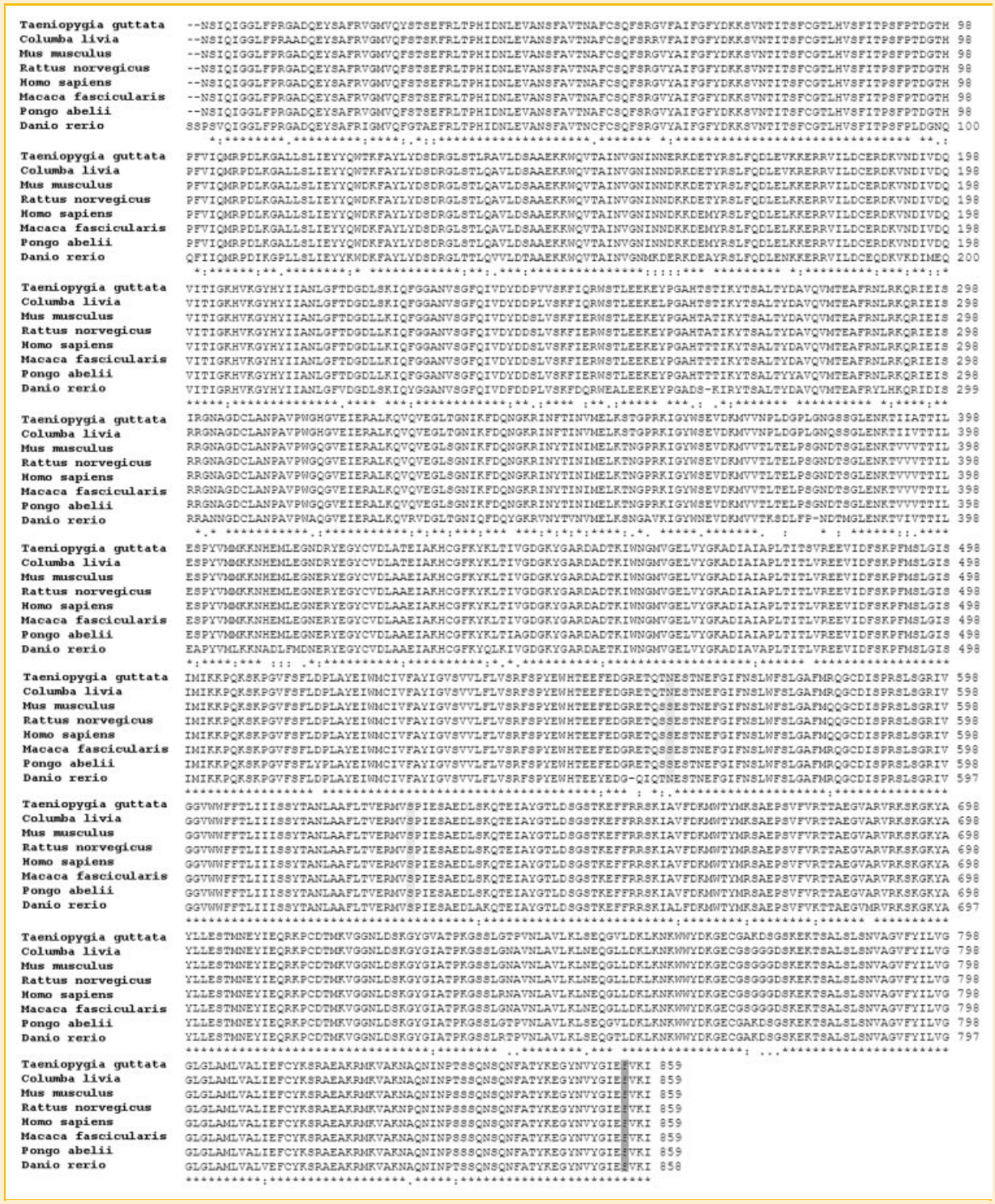

Fig. 4. Multiple alignments of different mammalian and other vertebrate sequences of GluR2 show a thorough conservation of the sequence at vertebrate level. Different amino acids that were found potential for phosphorylation and *O*-GlcNAc modification are also highlighted.

[Lynch, 2004]. LTP is associated with the transportation (exocytosis) of AMPA receptor to the synapses and LTD with the transportation (endocytosis) of AMPA receptor away from synapses [Heynen et al., 2000].

Mechanism of signaling involved in inducing hipocampal or cerebellar LTP or LTD is not fully understood, but one consensus for signaling processes of glutamate receptors is that different PTMs throughout are involved. Phosphorylation is the key PTM for

Fig. 5. A homology model of human GluR1 through SWISSMODEL server utilizing automated modeling option. Through this option three models were received from the server utilizing three different templates namely: 2rcbA, 3bfuC, and 1mm7B. Among the three one that covered all amino acids of S1 and S2 core amino acids was with 2rcbA and other two were with either N- or C-terminal parts of the S1 and S2 core. This model with a template 2rcbA shows that the S1 and S2 core have been folded correctly. The binding cleft contains the amino acids Arg and Glu acid almost in the same position as it is present in other GluRs but the glutamate binding cleft is compact as it was modeled with out ligand. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

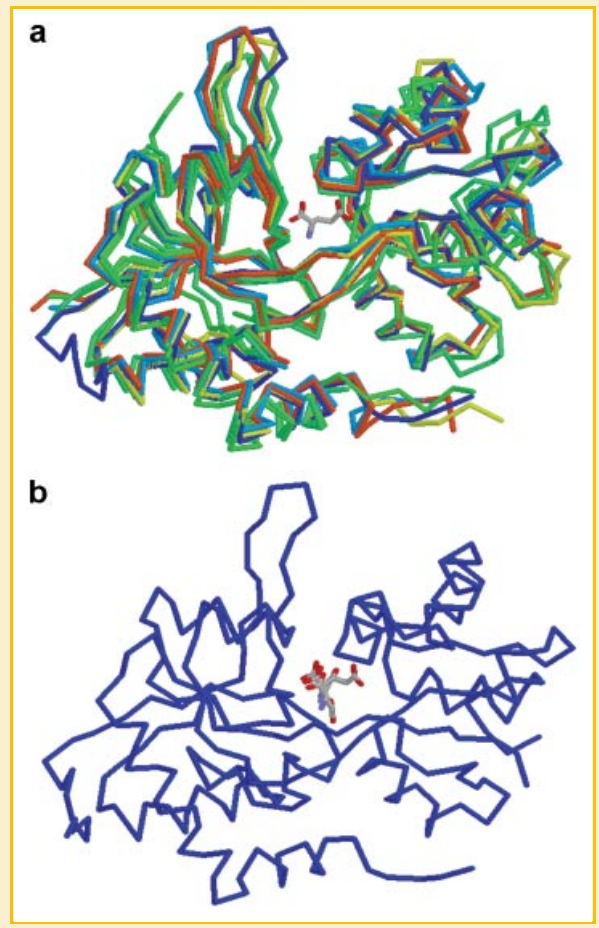

Fig. 6. The 3D models of GluR1 with glutamate were also developed utilizing the Atome server. Through this server 3D structures of different GluRs and related proteins were utilized as templates and the glutamate complexed with these AMPA or other receptors. Different complexes were developed at 3D level by docking the 3D models of S1 and S2 core of GluR1 (utilizing different templates including 2V3U, 1MQI, 1PB7, 3C31, 2A5S, 2PVU, 2PYY, 2RC8, 1FTK ANF 1YAE) with the glutamate ligand (coordinates extracted from 1FTJ, 2A5S, 1II5, and 2PYY). Superimposing of all structural models showed that the binding cleft is almost conserved in all structures (a) but the difference lies in orientation of glutamate in the ligand binding cleft and those of the amino acids that are involved in binding with the ligand (b). Thus different complexes of GluR1 models with glutamate of different origin showed that the fitting of glutamate in ligand binding cleft was with different orientations of the ligand and receptor binding elements. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

signaling. Phosphorylation of glutamate receptors is usually the result of the activation of kinases by Ca^{2+}. Activation of kinases such as PKA, PKC, CAMKII by an elevated Ca^{2+} influx through the opened ion channel of tetrameric GluRs is preceded by glutamate binding to the receptors (GluR1-4). Glutamate binding to GluR1 and GluR2 is mediated by the basic Arg facing the acidic Glu in the binding cleft (Fig. 7) as it has been described earlier for GluR2 (Ligplot of ligand substrate interaction in PDBsum of 1ftj). The other two binding regions involved in glutamate binding in all glutamate receptors include two motifs; PLT/PLS/PLA and ST/AT almost facing each other. This shows that a conservation of the glutamate binding cleft in glutamate receptors determines glutamate binding, but a few deviations to this rule have been seen in different types of glutamate receptors. A 3D structural model of S1 and S2 core of GluR1 utilizing the template 2rcbA showed a conserved binding cleft without ligand glutamate (Fig. 5). Similarly, the model with 1YAE also showed binding of glutamate of 2A5S in it best orientation with in the cleft (Fig. 8) favoring the formation of all possible binding interactions as described earlier (Ligplot of ligand substrate interaction in PDBsum of 1ftj). Additionally, the ligand binding cleft in the model without glutamate is compact (Fig. 5) whereas the cleft is extended in the receptor–ligand binding models (Fig. 7). It is clear by comparison of the two structures that not only the binding cleft but also the neighboring structural regions also become extended following ligand binding to induce ion channel formation.

The structural models of S1 and S2 core of GluR1 with and without ligand shows that glutamate binding to the receptor results in expansion of the ligand binding cleft and its surrounding regions. When the surrounding regions assume a tetrameric form, they may help opening the ion channel, resulting in initiation of the signaling cascade, with activation of kinases, phosphorylation of cytoplasmic amino acid residues and finally induction of LTP and/or LTD.

Phosphorylation has been studied in many signaling processes [reviewed in de la Fuente van Bentem et al., 2008]. The role of phosphorylation on C-terminal domain of GluR1 and GluR2 is very important both in LTP and LTD conditions, consequently regulating the processes of memory and learning [Roche et al., 1996; Mammen et al., 1997; Seidenman et al., 2003; Boehm et al., 2006]. The phosphorylation in GluR1 is responsible for receptor exocytosis and

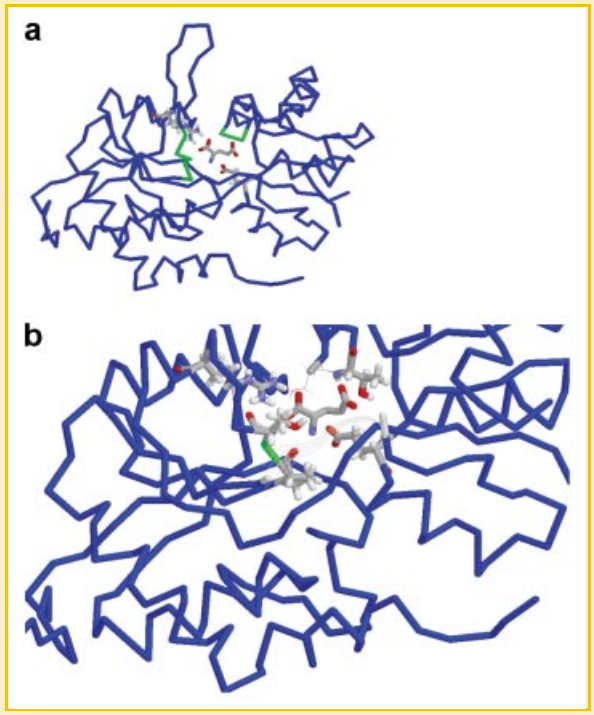

Fig. 7. (a) Comparative docking studies showed that one complex with a model of GluR1 originating from the template 1YAE and its complexed glutamate originating from 2A5S showed perfect orientation of glutamate in the ligand binding cleft of GluR1. In the ligand binding cleft Arg and Glu (in sticks style with CPK color mode). Similarly, other two binding regions PLT and ST with green color are also in favor of making all possible interactions as described earlier for GluR2 in 1FTJ (see Ligplot of PDBsum of 1FTJ). (b) The comparative docking results after evaluation and screening showed that one complex of S1 and S2 core of GluR1 modeled with template 1YAE with the glutamate of 2A5S showed perfect complex and can be compared to a typical receptor–ligand interaction of GluRs. Glutamate (in sticks style with CPK color mode) in the center of ligand binding cleft of GluR1 is surrounded by Arg and Glu (in sticks style with CPK color mode) from two sides and the two other binding regions PLT and ST. Here in PLT in lower position the P is in sticks style with CPK color mode L is in backbone style with green color and again T in sticks style with CPK color mode. Similarly, in ST in upper position the S is wire frame style with CPK color mode and T is in sticks style with CPK color mode. In this position all the possible interactions that are found in other GluRs are favoring (see Ligplot of PDBsum of 1FTJ). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

LTP, whereas in GluR2, it regulates receptor endocytocis and LTD [Roche et al., 1996; Mammen et al., 1997; Seidenman et al., 2003; Boehm et al., 2006]. Dephosphorylation of GluR1 triggers the endocytosis and LTD and that of GluR2 triggers exocytosis and LTP [Seidenman et al., 2003].

The phosphorylation of GluR1 at three sites is experimentally known and important in functional regulation of receptor. Phosphorylation of GluR1 at Ser 845 by PKA controls the conductance of the ion channel [Banke et al., 2000]. Additionally, phosphorylation of GluR1 was shown to be involved in adjusting the threshold for LTP induction [Blitzer et al., 1998], and contribute to the delivery of the receptor to synapses, as mutation at Ser 845 results in reduced LTP [Ehlers, 2000; Esteban et al., 2003]. However, phosphorylation of Ser 845 alone is not sufficient to transport the receptor to synapses [Ehlers, 2000; Esteban et al., 2003], but also requires the phosphorylation of other residues. Ser 845 shows potential for both phosphorylation and *O*-GlcNAc modification and has been predicted as a Yin Yang site by YinOYang 1.2 (Table I). The kinase CaMKII, present abundantly in synaptic cytoplasm [Kennedy et al., 1983; Kelly et al., 1984), phosphorylates Ser 831 increasing the conductance through the receptor, an important event to maintain LTP [Derkach et al., 1999; Luthi et al., 2004]. Maintaining the conductance processes by phosphorylation of Ser 831 of GluR1 by CaMKII is the result of autophosphorylation of the kinase which remains active for a long time even in the absence of Ca^{2+} movement, an essential process for LTP induction [Malenka et al., 1989; Malinow et al., 1989; Ocorr and Schulman, 1991; Silva et al., 1992; Fukunaga et al., 1993]. Phosphorylation of Ser 831 by CaMKII has no role in GluR1 trafficking to synapses but it triggers many signaling events that are essential to maintain LTP, as, for instance RasGAP signaling [Chen et al., 1998; Kim et al., 1998]. This site is predicted as false negative site for phosphorylation with very low prediction potential. This Ser 831 bears highly conserved status among different vertebrates (Fig. 4) and shows a prediction score near the threshold value for *O*-GlcNAc modification (Table I). Thus Ser 831 is most likely to be modified by *O*-GlcNAc as well. Phosphorylation of Ser 818 by PKC has been documented in vitro to play an essential role for GluR1 delivery to synapses leading to induction of LTP [Boehm et al., 2006]. This site was also placed among the false negatively predicted Yin Yang site on the basis that if PKC can catalyze the reaction of phosphorylation on this site then as described above earlier the OGT also has almost equal chances to catalyze *O*-GlcNAc modification in dephosphorylated state. Mutagenesis studies of phosphorylation sites (Ser 845, Ser 831) in mice GluR1 showed an impairment in spatial learning and also in LTD and LTP [Lee et al., 2000].

The only experimentally known phosphorylation site of GluR2 is Ser 856. Phosphorylation at this site blocks the interaction between GluR2 and glutamate receptor interacting protein 1 (GRIP1) and make synapses more vulnerable for PICKI, which triggers the receptor internalization and consequently results in LTD [Seidenman et al., 2003]. The dephosphorylation of this Ser 856 is achieved by PP1/2A [Lee et al., 2000] which can inhibit internalization of the receptor and LTD. The in silico phosphorylation (NetPhos 2.0) and *O*-GlcNAc modification (YinOYang 1.2) prediction methods do not predict the Ser 856 as a potential phosphorylation and *O*-GlcNAc modified site. But a phosphorylation potential of 0.72 for PKC by NetPhosK 1.0 and a potential of 0.623 for PKC by Predphospho was predicted (Tables III and IV). Additionally, this site is totally conserved in all vertebrate representatives (Fig. 4). Therefore, in the same scenario as described for Ser 818 and 831 of GluR1, this site (Ser 856 of GluR2) is also a false negative Yin Yang site and is most likely to be modified by *O*-GlcNAc in its dephosphorylated form promoting the inhibition of receptor internalization and consequently inhibiting cerebral LTD, favoring cerebral LTP.

O-GlcNAc is found almost everywhere in the brain but more abundantly in the hippocampus [Liu et al., 2004]. It plays an essential role in hippocampal learning and memory [Tallent et al., 2009]. *O*-GlcNAc modification is known to occur in neuronal

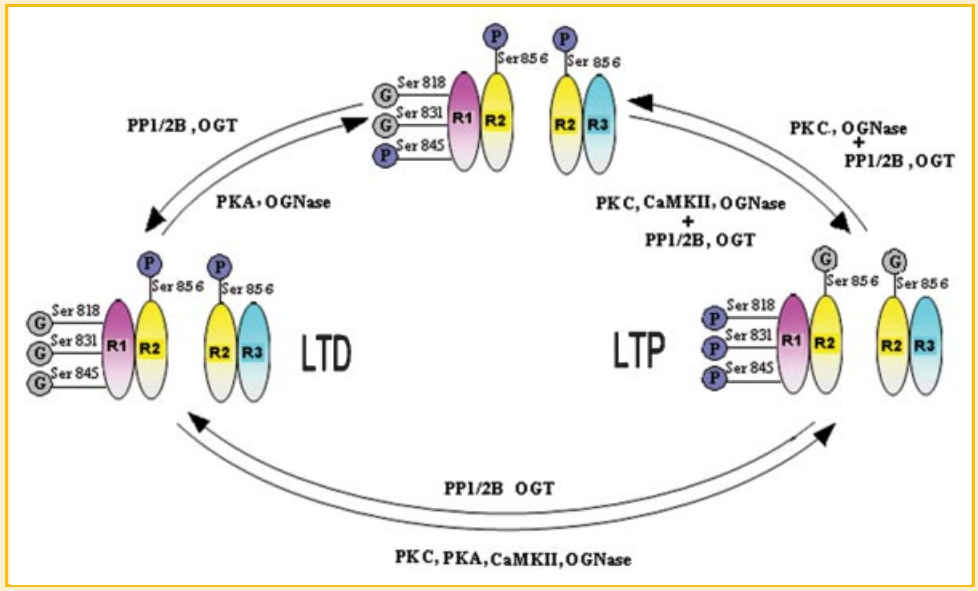

Fig. 8. A scheme showing the proposed mechanism of LTP and LTD induction and their interconversion by phosphorylation and GlcNAc interplay. The action of phosphorylation and that of *O*-GlcNAc modification on GluR1 and 2 is opposite. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteins [Querfurth and Selkoe, 1994; Dong et al., 1996; Haltiwanger et al., 1998]. The interplay between *O*-GlcNAc modification and phosphorylation in synapsins I and II has been documented in hippocampal LTP in presynaptic neurons [Tallent et al., 2009]. Interplay between OGT and kinases like PKC, PKA, and CAMKII for catalyzing the *O*-GlcNAc modification and phosphorylation on the same or neighboring Ser/Thr is well known [Griffith and Schmitz, 1999; Tallent et al., 2009]. The Ser 818, 831, and 845 have been predicted as Yin Yang sites in GluR1 as these sites are phosphorylated by PKA, PKC, and CaMKII, respectively, during LTP. It is most likely that these sites are postsynaptically modified by *O*-GlcNAc during hippocampal LTD. However, Glur2 that is phosphorylated during LTD in cerebellum by PKC in postsynaptic neuron may be *O*-GlcNAc modified during cerebellar LTP on Ser 856.

We suggest a mechanism involving interplay of phosphorylation and *O*-GlcNAc modifications of GluR1 and 2 signaling in the induction of LTP and/or LTD. Binding of glutamate to GluR1 and 2 results in expansion of their structures and leads to the opening of ion channels and Ca^{2+} influx allowing kinases to phosphorylate the Ser/Thr in C-terminal domains in the cytoplasmic regions. However, phosphorylation in C-terminus of GluR1 (Ser 818, 831, and 845) and 2 (Ser 865) results in the opposite effect by maintaining LTP in GluR1 and LTD in GluR2 (Fig. 8). Dephosphorylation of the two receptors also results in opposite affects as described earlier. We propose that involvement of *O*-GlcNAc modification on the same dephosphorylated Ser 818, 831, and 845 in GluR1 induces hippocampal LTD, whereas, the *O*-GlcNAc modification of GluR2 at dephosphorylated Ser 856 induces cerebellar LTP. A tetrameric form of the GluR1 dimer is phosphorylated on all Ser 818, 831, and 845, while the dimer of GluR2 is proposed to be *O*-GlcNAc modified during LTP (Fig. 8). In contrast, during LTD condition all Ser 818, 831, and 845 are proposed to be GlcNAc-modified and the dimer of GluR2 phosphorylated on Ser 856 (Fig. 8).

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