UNDERSTANDING THE NEUROBIOLOGICAL MECHANISMS OF LEARNING AND MEMORY: CELLULAR, MOLECULAR AND GENE REGULATION IMPLICATED IN SYNAPTIC PLASTICITY AND LONG-TERM POTENTIATION. PART IV C

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SUMMARY

Extensive cellular and behavioral studies have led to the postulation that memories are encoded by changes in synaptic strength between neurons, as demonstrated by the correlation between the long-term changes in animal's behavior and long-term changes in neuronal connections underlying a specific behavior in invertebrate animals, or even in vertebrate animals, where cellular models of synaptic plasticity using genetic approaches, such as *Long-Term Potentiation* (LTP) and *Long-Term Depression* (LTD), have been shown to depend on long-term changes in synaptic activity implicated in behavioral learning and memory. Long-term memory (LTM) is crucial for animal's survival and represents a mechanism that underlies fundamental neurobiological events in the Nervous System of vertebrate and non-vertebrate species including the human. Long-term changes in synaptic connectivity as well as long-term behavioral changes (both activities that underlie several properties of LTM and are used as a parameter to explain the long-lasting enhancement of neuronal function after a stimulus) have been demonstrated to rely on signals that initially occur in the cell body. LTP is a form of synaptic plasticity widely accepted as a cellular model for stabilization of synapses in neurobiological phenomena, such as development and learning and memory. Much of the experimental work concerning LTP in learning has been focused on the NMDA receptor-dependent forms of LTP. But several questions have arisen regarding the issue if LTP equals memory. If LTP has a real role in memory, a more appropriate hypothesis should be stated by postulating that activity-dependent synaptic plasticity and multiple forms of memory known to exist, share a common core; that is, the synaptic plasticity and memory hypothesis states that *activity dependent synaptic plasticity is induced at appropriate synapses during memory formation*. Synaptic plasticity is a physiological phenomenon that induces specific patterns of neural activity sustained by chemical and molecular mechanisms, that give rise to changes in synaptic efficacy and neural excitability that long outlast the events that trigger them. Based on the several properties of synaptic plasticity discovered, LTP may be proposed as a suitable neuronal mechanism for the development of several memory systems, including initial encoding and storage of memory traces and initial phases of trace consolidation over time. Such memory processing made up by LTP or LTD most probably occur as a network specific process, making LTP a universal mechanism for encoding and storting memory traces and what gets encoded is part of a network property rather than mechanisms working at individual synapses. For example, the type of information processed at the hippocampus is quite different from the information processed by the amygdala, and such information should remain if the mechanisms of plasticity operating in each brain area are conserved. Decades of research have demonstrated that LTP in the hippocampus is induced by synaptic activity and that cytoplasmic membrane-bound molecule(s) are required to transduce extracellular signals mediated by receptor-activation into activation of intracellular signaling processes. Most of these processes depend on intracellular calcium activity, and thereby on calcium-dependent mechanisms that are recruited for LTP induction and expression. For instance, NMDA receptors have been shown to be essential for initiation of LTP, but expression of this phenomenon in brought primarily by AMPA receptors. Induction of LTP in CA1 hippocampal region has been shown to depend on increases of intracellular calcium and activation of specific calcium-dependent molecules, such as the calcium/calmodulin-dependent protein kinase (CaMKII), whose cell expression is confined predominantly at postsynaptic densities. Moreover, long-term expression of LTP requires protein synthesis, where transient signals will be linked to activation of specific genes that ultimately will determine growth and remodeling of potential active synapses. Different types of synapses may express and use a different set of molecules mediating activation of intracellular signaling pathways for initiating and maintaining

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synaptic plasticity. Several studies have demonstrated that neuronal modifications of neurotransmitter receptors or membrane-receptor subunits at postsynaptic densities represent one of the neuronal mechanisms by which neurons regulate their synaptic strength. For instance, it has been demonstrated that neuronal dendrites are able to regulate their own transmembrane receptor synthesis in response to external stimuli (i.e., GluR2 subunit of AMPA receptor) and such molecular mechanisms pose important implications in the understanding of how individual synapses are selectively strengthened. In addition, recent experiments have demonstrated that specific intracellular signaling molecules (i.e., neuronal Synaptic GTPase-activating protein or SynGAP) is selectively expressed and enriched at excitatory synapses. Interesting enough, are the evidences that demostrate that different subsets of protein kinases (MAPKs, SAPKs, MAPKAKs, p38MAPK, etc.) and intracellular signaling pathways activate transcription factors (AP-1 complex, CREB) that regulate the expression of different immediate early genes (IEG) that are crucial for neuronal development, glutamate receptor trafficking to specific synapses and for LTP induction. Much of the neurochemical and molecular changes that occurred in synaptic plasticity, may be well associated with dynamic morphological changes in spine synapses as suggested to participate in the development and consolidation of LTP. In addition, glial cells, known to participate in the excitatory neurotransmission in the CNS besides their conceptualized cellular function, as elements for structural support and homeostasis; may play an important role in synaptic plasticity and thereby may regulate the information processed in the brain. As hippocampal LTP, has been the target of intensive molecular genetic analysis, several studies have demonstrated that LTP is altered when particular single genes are knocked out or overexpressed in null mutant mice or transgenic mice. Such studies have led to the amazing observation that variations in LTP exist within natural inbred mouse strains.

Key words: G proteins, GTPase, Exchange factors, RAS, Signaling pathways, Ca^{2+}-CaMKII, LTP, Nuclear Transcription Factors.

RESUMEN

Extensos estudios celulares y conductuales han llevado a la postulación de que la memoria es codificada por cambios en la fuerza sináptica entre las neuronas, como se ha demostrado por medio de la correlación entre los cambios a largo plazo en la conducta de los animales y en las conexiones neuronales que generan una conducta específica en animales invertebrados o vertebrados; en donde modelos celulares de plasticidad sináptica, usando aproximaciones genéticas como el fenómeno de potenciación de largo plazo (LTP) o el fenómeno de la depresión de largo plazo (LTD), han demostrado que dependen de cambios a largo plazo en la actividad sináptica implicada en las conductas de aprendizaje y memoria. La memoria de largo plazo (LTM) es crucial para la sobrevivencia de los animales y representa un mecanismo fundamental para los eventos neurobiológicos en el Sistema Nervioso de las especies de vertebrados e invertebrados, incluyendo la humana. Los cambios a largo plazo en la conectividad sináptica, así como los cambios conductuales de largo plazo (ambas actividades son responsables de varias propiedades que caracterizan el fenómeno de LTM y se usan como parámetros funcionales para explicar el aumento de la actividad neuronal dependiente de estímulos) han demostrado que las señales ocurren inicialmente en el cuerpo celular. El fenómeno biológico de LTP es una forma de plasticidad sináptica ampliamente aceptada como un modelo celular que promueve la estabilización de sinapsis activas y que participa en eventos neurobiológicos como el desarrollo, el aprendizaje y la memoria. Una gran mayoría de los trabajos experimentales concerniente al fenómeno biológico del LTP en el aprendizaje ha sido enfocada a la actividad funcional de los receptores glutamatérgicos, tipo NMDA. Si bien muchas preguntas han surgido con respecto a si el fenómeno de LTP es equivalente a la función de memoria, esto es, si el fenómeno de LTP juega un papel real y preponderante en la función de memoria, entonces, una hipótesis apropiada debería postular que tanto el fenómeno de LTP como la actividad dependiente de los eventos de plasticidad sináptica y de múltiples formas de memoria que existen, compartan un denominador común, lo que permite postular la hipótesis que sugiere que la actividad dependiente de la plasticidad sináptica es inducida en sinapsis particulares y específicas durante la formación de aprendizaje y consolidación de la memoria. La plasticidad sináptica es un fenómeno fisiológico que induce patrones específicos de actividad neuronal, sostenidos por mecanismos químicos y moleculares, que dan origen a cambios en la eficiencia sináptica y en la excitabilidad neuronal que perdura por más tiempo que los eventos que las originan. Con base en algunas propiedades de plasticidad sináptica recientemente estudiadas y documentadas, el fenómeno de LTP puede ser propuesto como un mecanismo neuronal para el desarrollo de algunos sistemas de memoria, que incluyen la codificación inicial, el almacenamiento de la memoria y las primeras fases de la consolidación de la misma. Si el procesamiento funcional de la memoria es mediado por el fenómeno de LTP o LTD, muy probablemente ocurre como un proceso específico dentro de una red de circuitos neuronales, situando al fenómeno de LTP como un mecanismo universal para la codificación y almacenaje de la memoria. Asimismo, la codificación sería parte de una propiedad de red neuronal más que de un mecanismo neuronal de contactos sinápticos individuales. Por ejemplo, el tipo de información procesada en el hipocampo es muy diferente de la información procesada por la amígdala y esta información puede permanecer si el mecanismo de plasticidad que opera en cada región del cerebro se conserva con el tiempo. Décadas de investigación han demostrado que el fenómeno de LTP en el hipocampo es inducido por la actividad sináptica y por moléculas citoplasmáticas unidas a la membrana que son requeridas para traducir las señales extracelulares mediadas por la activación del receptor dentro de la activación de procesos de señalización intracelular. La mayoría de estos procesos depende de los movimientos del calcio intracelular, y de este modo los mecanismos dependientes del calcio son necesarios para la inducción y la expresión de este fenómeno celular. En este contexto, se ha demostrado que los receptores glutamatérgicos, tipo NMDA, son esenciales para la iniciación del fenómeno de LTP; sin embargo, la expresión de este fenómeno requiere la participación de los subtipos de receptores glutamatégicos, AMPA. Más aún, se ha demostrado que la inducción del fenómeno de LTP en la región hipocampal CA1 depende de los aumentos intracelulares de calcio, así como de la subsecuente activación de moléculas proteicas-calcio-dependientes, tal como lo representa la proteína kinasa dependiente de calcio, calmodulina (CaMKII). La expresión de esta proteína kinasa-dependiente de calcio en la neurona ha sido ampliamente demostrada en las densidades postsinápticas (PSD). Por otra parte, la expresión a largo plazo del fenómeno de LTP requiere la síntesis de proteínas, en donde las señales transitorias pueden

estar ligadas a la activación de genes específicos que determinarán en última instancia el crecimiento y la remodelación de sinapsis potencialmente activas. Diversos tipos de sinapsis pueden expresar y hacer uso de distintos grupos de moléculas proteicas que participan en la activación de diferentes vías de señalamiento intracelular y que por igual son responsables de las fases iniciales y de sostenimiento de los eventos de plasticidad sináptica. Varios estudios han demostrado que las modificaciones neuronales de los receptores específicos de unión de alta afinidad de diferentes neurotransmisores o de las subunidades proteicas que componen estos receptores membranales en las densidades postsinápticas (PSD) representan uno de los mecanismos celulares por los cuales las neuronas regulan su actividad de reforzamiento sináptico. Por ejemplo, se ha demostrado que las dendritas neuronales pueden regular su propia síntesis de receptores proteicos membranales en respuesta a estímulos externos (por ejemplo, la subunidad GluR2 del receptor glutamatérgico, AMPA) y tales mecanismos moleculares implican importantes planteamientos en la comprensión de cómo las sinapsis individuales se consolidan selectivamente. Más aún, recientes experimentos han demostrado que moléculas que participan en vías de señalamiento intracelular (v.g., la proteína sináptica neuronal con actividad de GTPasa, denominada como SynGAP) están selectivamente expresadas y enriquecidas en neuronas que median respuestas sinápticas excitatorias. De forma interesante, estos estudios han demostrado que diversos subgrupos de proteínas kinasas (v.g., MAPKs, SAPKs, MAPKAKs, p38MAPK, etc.) implicadas en la activación de diversas vías de señalamiento intracelular son reponsables de la actividad funcional de distintos factores de trascripción (v.g., complejo AP-1, C-Fos, Jun, CREB, etc.) que a su vez regulan la expresión de múltiples genes de expresión temprana [intermediate early genes (IEG), por sus siglas en inglés] que son cruciales para el desarrollo neuronal, para la regulación del transporte vesicular de receptores glutamatérgicos a sinapsis específicas, así como para la inducción del fenómeno de LTP. Muchos de los cambios neuroquímicos y moleculares que ocurren en los eventos de plasticidad sináptica se pueden asociar con cambios morfo-celulares dinámicos en las espinas sinápticas, tal como diversos estudios lo han demostrado durante el desarrollo y la consolidación del fenómeno de LTP. Si bien diversos trabajos experimentales han demostrado la participación de las células gliales en la neurotransmisión excitatoria en el SNC, estas células, además de ejercer una función celular ampliamente conceptualizada como elementos de soporte estructural y de homeostasis, tienen un papel crucial en los eventos de plasticidad sináptica de tal forma que también regulan la información procesada en el cerebro de los mamíferos, incluyendo los sistemas neuronales de especies de invertebrados. No obstante, el fenómeno de LTP en el hipocampo ha sido el blanco de mayor intensidad de estudio y en particular del análisis genético molecular, donde a este respecto varios estudios han demostrado que el fenómeno de LTP está alterado cuando ciertos genes particulares son inhabilitados permanentemente *(knockout)* o temporalmente *(knockdown)* en su expresión funcional y/o sobre-expresados en ratones mutantes nulos o en ratones transgénicos. Estos estudios han llevado a observaciones interesantes que demuestran que dentro de las diferentes cepas naturales del ratón existen variaciones naturales en la expresión del fenómeno de LTP.

Palabras clave: Proteínas G, factores de intercambio GTPasa, vías de señalamiento intracelular tipo Ras, Ca^{2+}-CaM KII, LTP, factores de transcripción nuclear.

VII. Intracellular signaling pathways implicated in expression of LTP

LTP is induced and maintained following Ca^{2+} influx, through either ion channel associated with NMDA glutamate receptor activation or directly through VGCCs (Orban et al., 1999). The Ca^{2+} influx is believed to produce potentiated responses through the concerted action of the protein kinase cascade, that involves protein kinase A (PKA) and C (PKC), tyrosine kinase of the Src family and the Ca^{2+}/calmodulin dependent kinases (CaMKII and CaMKIV)(Ghosh, Greenberg, 1995; Chen, Tonegawa, 1997; Silva et al., 1997). All these intracellular signaling pathways convey molecular messages that lead to gene transcription in neurons, where the cAMP-response-element–binding protein (CREB) is crucial in modulating gene transcription (Orban et al., 1999). Interfering with these signaling molecules causes significant effects on LTP and long-term memory formation (Silva, Murphy, 1999)(see below). One of the most significant intracellular pathways as supported from several evidences is the Ras-MAPK pathway. This intracellular signaling pathway has been shown to be implicated in cell proliferation and differentiation, and also implicated in neuronal plasticity events and behavior (Orban et al., 1999).

a) Heterotrimeric G proteins and Small G proteins. G proteins comprise distinct families of heterotrimeric protein subunits of large molecular weights (38-52kD), mostly composed by at least different isoforms of α subunits (which contain an intrinsic GTPase activity) sharing common β and χ subunits. For G proteins to interact with other intracellular proteins and plasma membrane they need to be modified by incorporation of long-chain fatty acids, mainly by palmitoylation and mystiroylation [e.g., myristoyl groups (C14) are added via amide links to N-terminal glycine; palmitoyl groups (C16) are added to cysteine residues within a specific amino acid sequence consensus; isoprenyl groups (C20) are added to cysteine residues of specific C-terminal motifs in small G proteins, see below] (Nestler, Duman, 1999). Overall, these protein subunits comprising most of the G protein superfamily couple activation of plasma membrane receptors to a myriad of intracellular functions and play a crucial role in signal transduction in different cellular processes, such as vesicle transport, cytoskeletal assembly, cell growth and protein synthesis (Nestler, Duman, 1999). These proteins have a different cellular distribution and therefore they show distinct functional properties. Over 35 heterotrimeric G proteins subunits have been identified and characterized in the CNS of mammals, using a combination of biochemical and molecular cloning techniques. Two

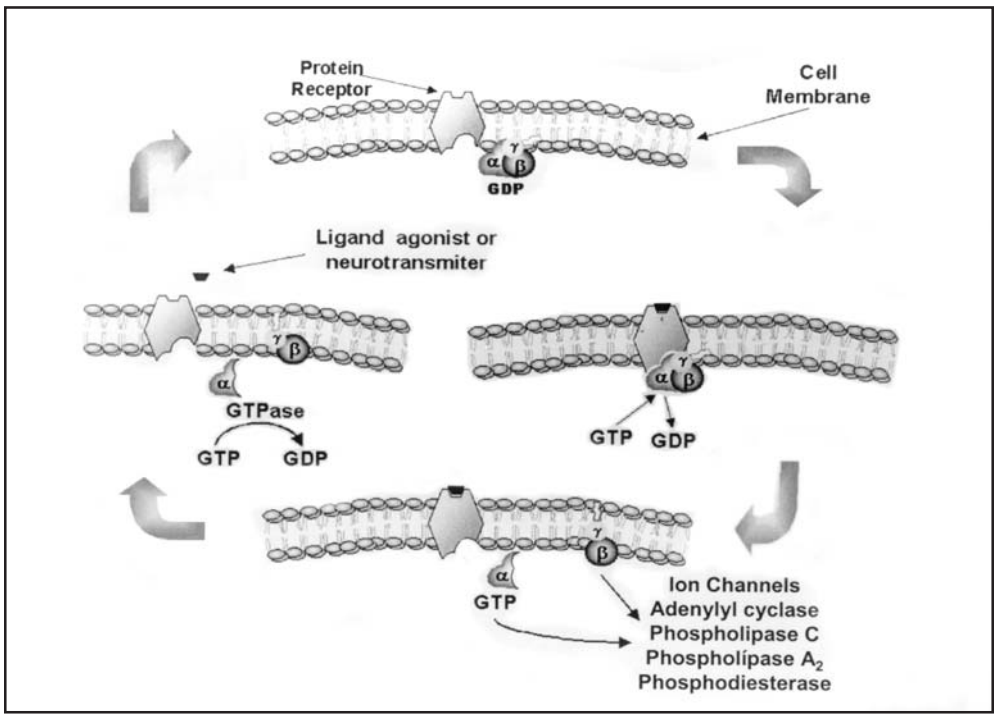

Fig. 1A. Schematic representation of the functional activation of heterotrimeric G protein cycle. Most cells in the nervous system that respond to extracellular signals (peptide and neurotransmitters) express in their membranes a family of heterotrimeric G proteins that are involved mainly in transmembrane signaling, and couple the activation of several membrane receptors (G protein coupled receptor, GPCRs) to a variety of intracellular signaling pathways or processes. Under resting conditions, G proteins are expressed in the cell membranes as heterotrimers of three protein subunits, defined as α,β,χ; that are loosely associated with membrane receptors. Activation of membrane receptor by its cognate-ligand, leads to the physical association of the heterotrimer via the α subunit, producing the dissociation of GDP from the subunit and the replacement for GTP. Association of GTP induces the dissociation of the heterodimer βχ from the complex, producing a free α subunit dissociated from its receptor. The conformation induced by the free GTP-α subunit and the free βχ protein subunits makes them functionally bioactive components that regulate different effector proteins and thereby, a variety of biological responses depending on the type of subunit and cell implicated in the functional response. As the α subunit contains an intrinsic GTPase activity, GTP is hydrolyzed to GDP; an action that leads to the re-association of free α-subunits with βχ subunit complex, restoring the original heterotrimeric conformation of the G protein complex (see text for complete description) (figure and text were adapted from Nestler and Duman, 1999 with modifications by the principal author of the present series).

identified types of heterotrimeric proteins, referred to as Gs and Gi, were found to couple activation of plasma membrane receptors, either to stimulate or inhibit the adenylyl cyclase system, an enzyme that catalyses the synthesis of cAMP (Nestler, Duman, 1999)(figure 1A). G proteins may couple some neurotransmitters receptors to ion channels, where G protein subunits released from the G protein-receptor interaction can couple the activation of specific membrane receptors in the brain to the functional activation of specific ion channels (Wickman, Clapham, 1995; Schneider et al., 1997). For instance, G-protein coupling mechanisms have been studied for the opioid, D2-dopaminergic, α2-adrenergic, muscarinic cholinergic, 5HT1A-serotonin, and GABA-B receptors, to which they activate the inward rectifying K^+ channel (GIRK) via the Go and/or Gi protein subtypes or by interaction to L-type, voltage-gated Ca^{2+}-channels, mediating an inhibitory effect on these ion channels (Nestler, Duman, 1999). Moreover, G proteins regulate the intracellular concentration of second messengers (i.e., cAMP), via the activation or inhibition of the adenylyl cyclase. Activation results from the uncoupling of the Gαs protein subunit from the activated membrane receptor, whereby the free βχ heterodimer subunit complex activates other adenylyl cyclase subtypes. Conversely, inhibition of the synthesis of cAMP is thoroughly mediated by the release of the Gαi from activated protein receptors, thus inhibiting other forms of adenylyl cyclases, whereas free βχ subunits dissociated from the heterotrimeric G protein may inhibit the activity of different subtypes of adenylyl cyclase molecules (Nestler, Duman, 1999). In addition, G proteins, via the Gαq protein subunit, may couple receptor stimulation of phosphoinositide second messenger pathway, through the activation of the PI-PLC system, which catalyses the hydrolysis of PIP2 and the subsequent synthesis of the second messenger inositol triphosphate (IP3) and diacyl-glycerol (DAG) (Nestler, Duman, 1999). Several studies have

demonstrated not only that the membrane-bound βχ subunits released from the heterotrimeric G-protein complex are able to bind to the C-terminal domain of specific protein kinases (referred as to G protein receptor kinases or GRks), which induce the phosphorylation of G protein-coupled receptors (while bound by their ligand agonists) an activate the intracellular signal for receptor desensitization (Inglese et al., 1995); including the regulation of important effector pathway for growth factor receptors, referred to as the mitogen-activated protein kinase (MAP-kinase) pathway (Lopez-Ilasca et al., 1997; Nestler, Duman, 1999). As the Gα subunits contain two specific domains, one identified as the GTPase activity, implicated in the exchange of GTP for GDP, and the other, identified as the GTP-binding site, which regulate the binding of the βχ subunits and several effector proteins (Nestler, Duman, 1999); it has been demonstrated that certain protein modulators, found initially in yeast and subsequently in the mammalian tissues, defined as GTPase- activating proteins (GAPs, see below) are able to stimulate the GTPase activity of the Gα subunits (Nestler, Duman, 1999). Several of these protein modulators, termed as regulators of G protein-signaling proteins or RGS proteins, have been found to be expressed in the several regions of CNS of mammals in a highly specific pattern. Although the exact function of RGS in the brain is poorly understood, one clear property of such proteins is the hasten hydrolysis of GTP to GDP, mediated through the GTPase activity of Gα subunits, thus, restoring the inactive heterotrimeric G protein complex. In such context, different subtypes of RGS proteins may modulate the activity of several forms of Gα subunits with the net result that RGS inhibit the biological activity of G proteins. Alterations in RGS protein activity, as a result of their expression and phosphorylation may modulate G protein activity and therefore, the sensitivity of specific G-protein coupled receptors (Nestler, Duman, 1999) (figure 1B).

In addition to the well-characterized heterotrimeric G proteins, other forms of G proteins play significant roles in cell function. These proteins belong to a large family of "small G proteins", termed this way a based on their low molecular weight (20K-35K)(McCormick, 1995; Marshall, 1996). These

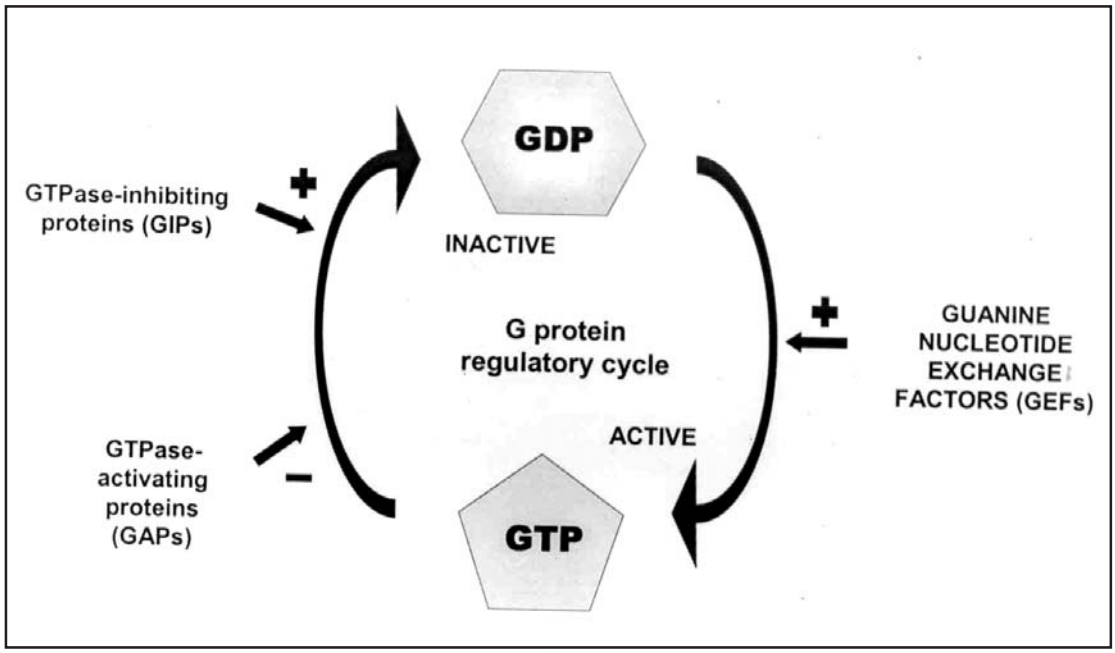

Fig. 1B. Schematic representation of the functional cycle regulation of the G protein-binding of GDP and GTP by protein regulators. The binding of GDP/GTP is regulated by cycles that drive the functional activity of G proteins mediated by different regulatory proteins. In this context, some proteins facilitate the release of GDP from the α-subunit of the G protein complex, enhancing the functional activity of G proteins via the GTP-bound form, as depicted in the figure. These proteins, defined as *nucleotide exchange factors or GEFs,* have specific domains to bind to heterotrimeric G proteins, and include also a large family of several *small G proteins.* Another subset of proteins, represented by *GTPase-activating proteins or GAPs,* comprise distinct *regulators of G-protein signaling proteins or RGS,* including several a number of *GAPs* that regulate various forms of small G proteins. Basically, when these proteins activate the intrinsic GTPase-activity of G proteins, facilitating the formation of the GDP-bound form of the G protein complex, they enhance the inhibition of the functional activity of the heterotrimeric protein complex. Conversely, *GTPase-inhibiting proteins or GIPs* produce the opposite effects (see text for more details) (figure and text were adapted from Nestler and Duman, 1999, with modifications by the principal author of the present series).

TABLE 1
Description of some of the cellular processes mediated by small G proteins

Small G protein Subtypes	*Cellular functions*
Ras	Signal transduction-mediated by growth factor and MAP-kinase pathways
Rac	Signal transduction in the control of cellular stress responses and MAP-kinase pathways (defined also as CDC42)
Rab	(Ras related protein to the brain) are mostly localized to synaptic vesicles, regulating vesicle trafficking, fusion, and exocytosis.
Rho	Assembly of actin microfilaments
ARF	(ADP-ribosylation factor) ADP- ribosylation of Gαs induced by cholera toxin
EFTU	(Eukaryotic elongation factor) Regulate protein synthesis at ribosomes
Ran	Nuclear-cytoplasmic trafficking of RNA and protein

(Text and table adapted from original from Nestler and Duman, 1999, and modified by author for the publication of the present review).

proteins bind to guanine nucleotides and have intrinsic GTPase activity, to which they cycle through GDP-GTP bound forms (figure 1A). One interesting aspect of the several forms of G proteins is that binding of GTP dramatically changes the affinity of the protein for any other target molecules, through a specific conformational change (Nestler, Duman, 1999). Small G proteins function as molecular switches that control several cellular processes as shown in table 1.

b)Biochemical characterization of the Ras-MAPK signaling pathway. Ras was the first member of the small GTP-binding protein subfamily to be discovered. Characterization of the functional role of this protein subfamily implicated in cellular events (Lowry, Willumsen, 1993), has been shown, these proteins (e.g., $p21^{Ras}$) are abundantly expressed in the inner face of the membrane where they attach to, by means of the prenylation of the C-terminus domain. These submembrane proteins cycles between inactive conformation when bound to GDP and to an active conformation when bound to GTP. Change of both states depends on the activity of distinct regulatory molecules; namely, guanine-nucleotide exchange factors (GEFS) which catalyze the exchange of GDP-bound form for the GTP-bound form (Orban et al., 1999) (figures 1A and 1B). Moreover, other signaling molecules included within a large subfamily of small GTPase molecules are the GTPase activating proteins or GAPs, which are involved in stimulating the weak intrinsic GTPase activity contained within the Ras molecules (Feig, 1994). As it occurs for many of the intracellular enzymes, their activity is highly regulated at multilevel steps (Orban et al., 1999) and similarly activation of GEFs (GEFs for Ras-signaling pathway include the SOS1/2, Ras-GRF1/2) occurs by several organized mechanisms. One mechanism depends on ligand binding and activation of receptor tyrosine kinases (RTKs), such as the neurotrophin-Trk receptor family (figure 2A). Binding of neurotrophins (see below) or any ligands to RTKs induces a conformational change allowing the receptor to autophosphorylate. Such phosphorylation at tyrosine residues provides docking sites for proteins to attach to the phosphorylated receptor, as is the case for adapter protein Grb-2 or different intermediate factors (e.g., IRS-1,IRS-2 Shc, FRS-2, Gab-1) which include proteins that themselves undergo tyrosine phosphorylation and are able to bind Grb-2 after RTK activation. Binding of Grb-2 to these proteins and/or activated RTKs induces the accumulation of protein complexes formed by Grb-2/SOS1-2 at localized sites of the cell membrane. Such complex, via SOS (the GEFs of the Ras family) activates $p21^{Ras}$ (Pawson et al., 1995; Kouhara et al., 1997). Interestingly enough, is that activation of the Ras signaling pathway (via $p21^{Ras}$ activation) results from the tyrosine phosphorylation of the RTKs. This highly regulated process is mediated through the activation of either cell adhesion molecules, such as the integrins molecules, and /or activation of cytokine receptors induced by the binding of their specific peptide ligands, referred as cytokines hormones (Orban et al., 1999). Activation of RTKs, implicating the receptor phosphorylation at the tyrosine residue, activates a subgroup of structurally related intracellular protein kinases, referred to as focal adhesion kinases or FAKs and the Janus kinases or JAKs, respectively (Orban et al., 1999). Several studies have shown that G-protein coupled receptors or GCPRs, which represent the sort of receptors for most of the peptide hormones identified in the CNS of mammals, including classical neurotransmitters; have been shown to activate the Ras-signaling pathway, including both ionotropic receptors, such as glutamate NMDA receptors, and some of the large family of voltage-dependent calcium channels. Thus, activation of the Ras-signaling pathway mediated through the activation of either membrane receptors or calcium-channels is quite complex, and the activation depends on the regulated activity of different guanine-nucleotide- exchange factors (GEFs) as explained above. Moreover, three different mechanisms have been shown to activate the Ras-signaling pathway, which can act independently or in concert (figure 2B). One mediated, via Ca^{2+} influx through L-type voltage-sensitive calcium channels (VSCCs); and another, via Gi or Gq-coupled receptors. In both instances, the result is the activation of the intracellular tyrosine kinases,

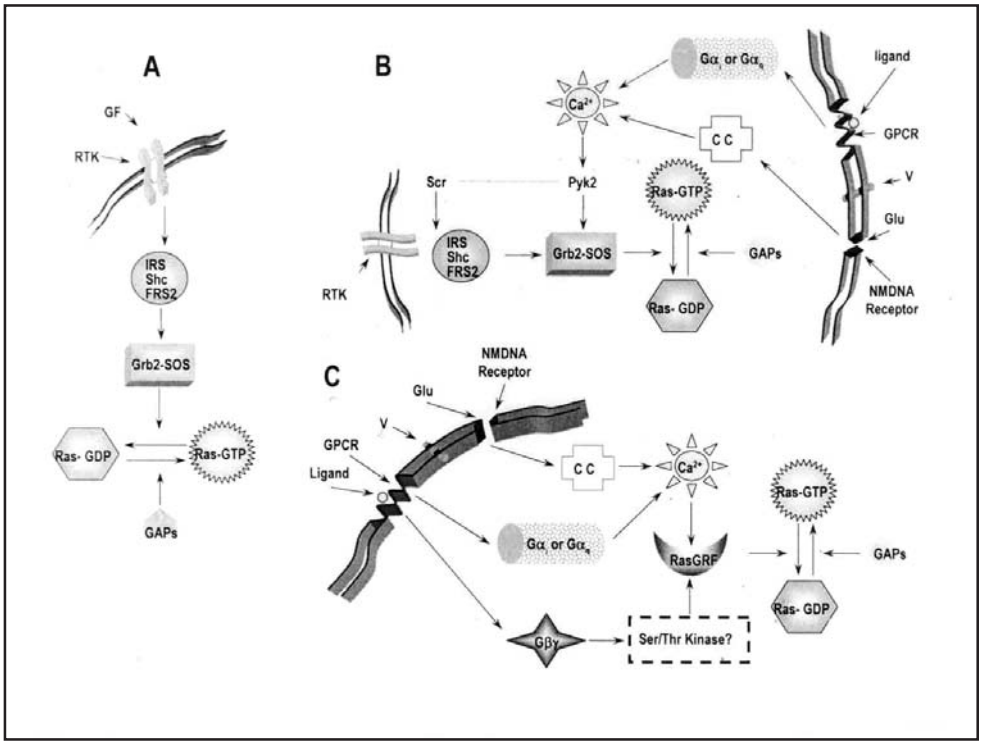

Fig. 2. Schematic representation of the functional activation of Ras-signaling pathways by extracellular signals. **(A)** Depicts the best-described pathway throughout activation receptor tyrosine kinase (RTK) that characterized the large *Trk* family of receptors of neurotrophins, cytokines, and growth factors. Receptor activation leads to recruitment of tyrosine-phosphorylated intermediate factors; IRS1-2, Shc, FRS2(see text) which depending on the activated-receptor might result in the recruitment of Grb-2 to which they bind, producing the subsequent recruitment of Grb-2/SOS complexes in a submembrane domain. **(B)** Depicts the Ca2+ mediating activation of the Ras signaling pathway. Ligand activation of G-protein-coupled receptors (GCPRs) or NMDA-receptor activation by glutamate (Glu) or voltage-activated (V) coupled to L-type-voltage-dependent calcium-channels (CC) activation and Ca2+ influx, may result in the activation of non-receptor tyrosine kinases (Pyk2, Src) creating phosphotyrosine docking sites for the Grb-2/SOS complex (SOS » GEF) with the subsequent activation RAS signaling pathway. Activation of Src kinase may activate RTKs (i.e,EGFRs) causing their binding to intermediate factors, and the recruitment of the Grb-2/SOS complex. **(C)** Depicts the signaling pathways that lead to Ras activation through Ras-GRF activation. Ras-GRF may be activated by Ca2+-influx through the CC that comprise an (IQ) motif that replaces the GRB2 binding domain found in SOS, allowing the binding of Ca2+/CaM complexes and the subsequent activation of the Ca2+-dependent activation of exchange factor, Ras-GRF. Same activationand phosphorylation of Ras-GRF is brought through the release of G-protein bc subunits after GPCRs activation and the activation of Ser/Thr kinases (see text for more details)(figures and text adapted from Orban et al., 1999 with specific modifications performed by author for the present publication of this review).

referred to as Pyk-2 and Src (Orban et al., 1999). Upon generation of phosphotyrosine residues (docking sites) on the intermediary protein factors (see above) serves as a molecular signal to recruit the Grb2-SOS protein complex, to be transported to the membrane. Activation of RTKs, such as the epidermal growth factor receptors (EGFRs) via the phosphorylation of tyrosine residues through the Src-kinase, might induce the recruitment of protein factors, such as Sch proteins and these ones mediating the binding of the Grb2-SOS protein complex (figure 2B). A third mechanism is mediated through the release of G-protein $\beta\chi$ protein subunits, after activation of G-protein coupled receptors, inducing the phosphorylation of Ras-GRF by Ser/Thr specific kinases and the subsequent activation of the Ras-GDP-GTP exchange activity (figure 2C) (Orban et al., 1999).

Ras signaling pathway may be activated by novel identified factors, defined as Ras-specific-GEF, Ras-GRP, factors that are known to be activated by diacylglycerol (DAG) agonists or activators of PKC and shown to bind Ca^{2+} (Ebinu et al., 1998). Other new factors, shown to be localized at specific synapses and associated with the scaffold protein PSD95 and NMDA receptors, include the Ras-Gap and SynGAP (Orban et al., 1999).

Ras effectors (immediate downstream signal transducers of Ras) represent proteins that mainly bind to GTP-bound form of Ras and are implicated in different Ras-dependent signal-transduction pathways (Marshall, 1995). One well-described pathway involved the Raf family proteins (e.g., Raf1, A-Raf and B-Raf) where activation of Raf proteins by activated Ras, after its phosphorylation at Ser/Thr residues, enhances their

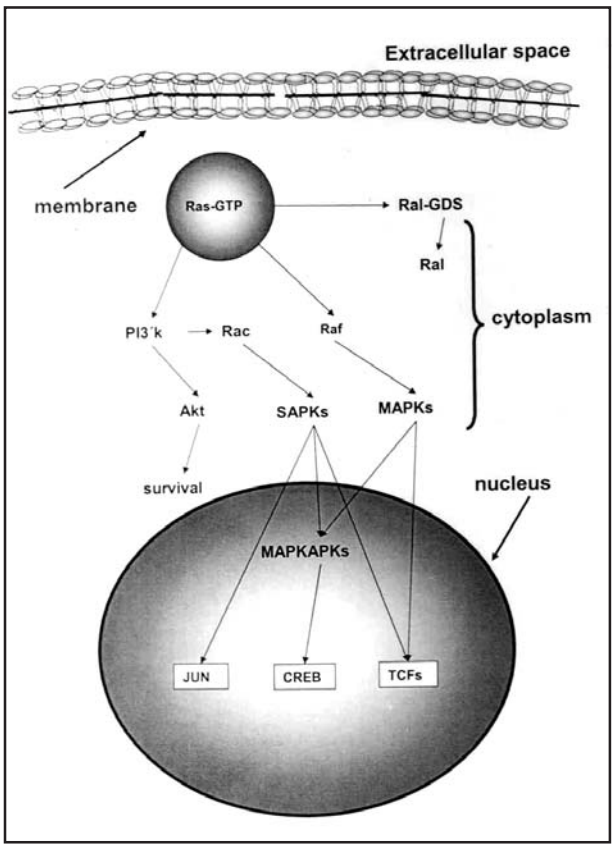

Fig. 3A. Schematic illustration of some of the RAS-signaling pathways downstream to activation of nuclear transcription factors. The immediate downstream signal transducers of Ras, or Ras protein effectors, are known to be proteins that bind Ras proteins adopting the GTP-bound form. These signal transducers are depicted in the illustration where arrows show the direct or indirect activation of these protein effectors downstream to the activation/regulation of several nuclear transcription factors (see text for more details) (figures and text were adapted from Orban et al., 1999 with specific modifications performed by principal author of the present series).

relocation of these proteins to the cell membrane with the further activation of mitogen-activated protein kinase kinases (MKKs/MEKs), that in turn phosphorylate MAPKs on both Thr and Tyr residues, due to the dual-specificity kinase activity of these phophorylating proteins, activating MAPKs, which enhance the further phosphorylation, activation and translocation to the nucleus of a large range of cytoplasmic protein substrates (figure 3A) (Orban et al., 1999). Substrates of MAPKs include the mitogen-activated protein-kinase activated kinases of MAPKAPKs (which phosphorylate the nuclear transcription factor, CREB) and specific MAPKs localized in the nucleus that phosphorylate a subfamily of nuclear transcription factors, such as the ternary complex factor or TCF of ElK-1 and Sap-1 (chapter X and figures 3A, 3B) (Orban et al., 1999).

In addition, recent experiments have demonstrated that a RasGAP (Ras GTPase-activating protein) defined as the neuronal Synaptic GTPase-activating protein or SynGAP, is selectively expressed in the brain and specifically enriched at excitatory synapses, and negatively regulates Ras activity and its downstream signaling pathways. Mutant mice lacking the SynGAP protein exhibit postnatal lethality, thereby indicating the crucial role of this protein during neuronal development. Moreover, molecular experiments performed in neuronal cultures from same homozygous mutant mice have demonstrated that neurons express more synaptic AMPA receptor clusters, suggesting that SynGAP regulate glutamate receptor synaptic targeting and heterozygous mutant mice express a defect in hippocampal LTP. Thus, these set of results posit that Ras signaling pathway mediated by SynGAP is crucial for proper neuronal development, glutamate receptor trafficking to specific synapses, and for LTP induction (Kim et al., 2003).

c) Protein Kinase Pathways of Ras-Raf-MAPKs. Several studies have acknowledge that MAPKs can be activated by Ras independent-cAMP-dependent mechanisms, which involve the activity of GTPase-Rap-1 and the B-Raf kinase as shown for the activation of TrkA receptors by NGF (Vossler et al., 1997; York et al., 1998). Another significant protein kinase pathway related to the Ras-Raf-MAPK pathways begins with the activation of the lipid kinase, the phosphatidyl inositol 3´kinase (PI3´K) induced by Ras, demonstrating that the p110 catalytic subunit of this kinase is able to associate with $p21^{Ras}$ (figure 3A) (Orban et al., 1999). Several studies have demonstrated that this PI3´K may activate a complex kinase cascade, initiating with the activation protein kinase B (a Ser/Thr kinase) implicated in the prevention of apoptosis in distinct cell lines and primary neurons (Dudek et al., 1997; Franke et al., 1997). Interestingly enough is the demonstration that this lipid kinase may link the Ras pathway to a subset of Rho subfamily of small GTPases, implicated in the rearrangement regulation of the actin cytoskeleton (Hall, 1998). Moreover, activation of different protein kinase cascade by PI3´K, mediated through the activation of a subset of different protein-exchange factors, may lead to the phosphorylation of specific nuclear transcription factor kinases (e.g., c-Jun N-terminal kinases or JNKs) and p38MAPKs, which represent a subset of proteins of the stress-activated protein kinase (SAPK) family, activated by stimuli such as UV irradiation, heat shock, hyperosmolarity and the immune-mediated hormones, cytokines (Orban et al., 1999).

Both MAPks and SAPKs pathways phosphorylate and activate several transcription factors involved in

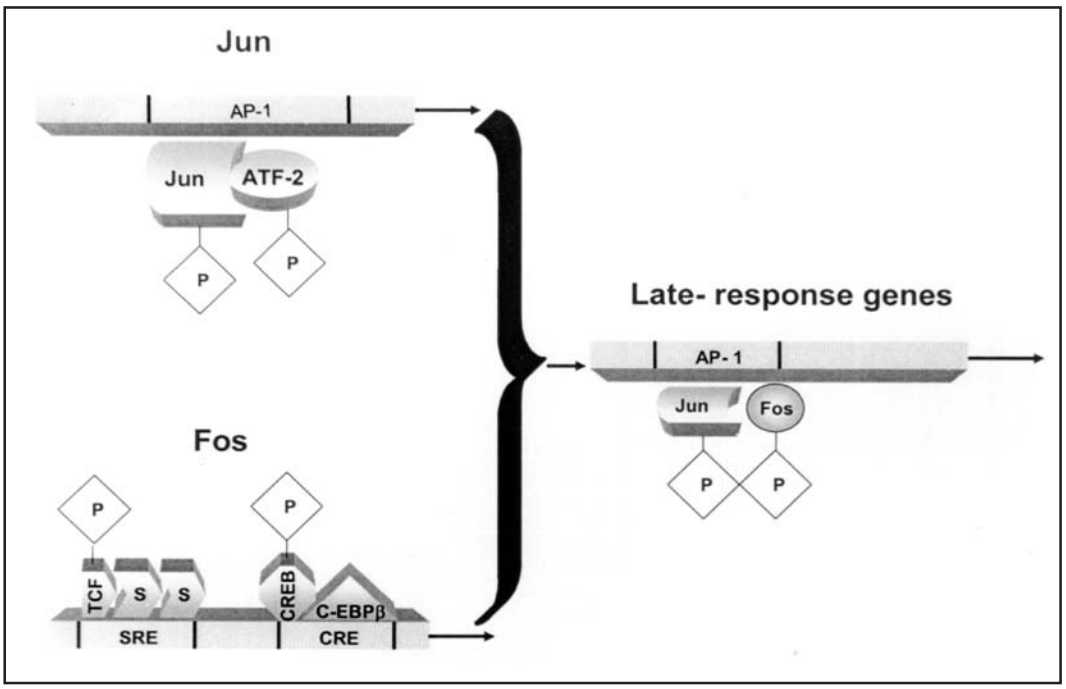

Fig. 3B. Schematic representation of activation of nuclear transcription factors subsequent to the activation of RAS-related signaling pathway. Activation of Ras-related signaling pathway induce the downstream-activation of a protein kinase cascade that leads to phosphorylation of MAPKs and SAKs that results in the activation, via phosphorylation (as indicated by P enclosed inside the rhomboid) of several transcription modulators, such as AP-1 complex; CREB transcription factor; SRE (serum response element) and TCF, (ternary-complex factor) as depicted in the illustration (see text and section X for more details). Transcription factors activated by Ras-signaling pathways are shown to bind to the *jun* and *fos* promoters. Thus, transcription of these immediate-early genes (IEGs) leads to further transcription of late-response genes, which are responsible for the development of long-term memory and neuronal-related plasticity events. Moreover, expression of latte-response genes can be successfully achieved in the presence of cooperative binding of transcription factors depicted in the figure. C-EPBb: CCAAT-enhancer binding protein b; CRE: cAMP- response element; CREB: cAMP-response element-binding protein (figures and text were adapted from Orban et al., 1999 with modifications performed by principal author of the present series).

the regulation of immediate early genes (IEG) response, such as the AP-1 transcription regulator, represented by a stable active dimer of the c-Jun-Fos heterodimer. Regarding the regulation of the transcription of genes, several studies have demonstrated a complex convergence on the activities of both MAPKs and SAPKs in activating several nuclear transcription factors (figures 3A, 3B) (Orban et al., 1999).

For instance, CREB transcription factor may be activated and thus phosphorylated via a cAMP independent-phosphorylation pathway at Ser 133, mediated by NGF via the RSK2, a MAPKAKs signal pathway, that fully depends on the activation of RAS-signaling pathway (Xing et al., 1996). In a similar fashion, the p38MAPK may also phosphorylate CREB and several other transcription factors that induce their transcription regulatory activity of heterodimers (e.g., CEBPβ, ATF-1, CREM) (See figure 3B, for a full description of activation of transcription factors and DNA response elements as a result of the activation of the Ras-signaling pathway)(Orban et al., 1999).

d) Functional implications of MAPKs in synaptic plasticity and hippocampal LTP. Behavioral studies of the learned defensive response in the marine mollusk Aplysia have shown that the molecular mechanisms that lead to short-term and long-term changes in synaptic plasticity of the learned behavior response, depends on the activity of serotonin transmission system, modulated by 5-HT (Pittenger, Kandel, 2003; Byrne, Kandel, 1996). In this context, several experiments have demonstrated that the mechanisms that favor long-term plasticity of the learned behavioral response, depend on the activation of the MAPK cascade that converge in the activation of a downstream signaling pathway, carried out from receptor activation by the neurotransmitter, via second messengers to nuclear modulators of gene transcription (figure 3C) (Kornhauser, Greenberg, 1997). These experiments have shown that while 5-HT induces the translocation of MAPK from the cytoplasm to the nucleus of presynaptic neurons, mediated through the activation of the cAMP dependent pathway, 5-HT does not alter the cytosolic location of MAPK in the motoneuron; as evidenced by the electrophysiological recordings obtained on the plasticity and learning responses in this marine specie (Kornhauser, Greenberg,

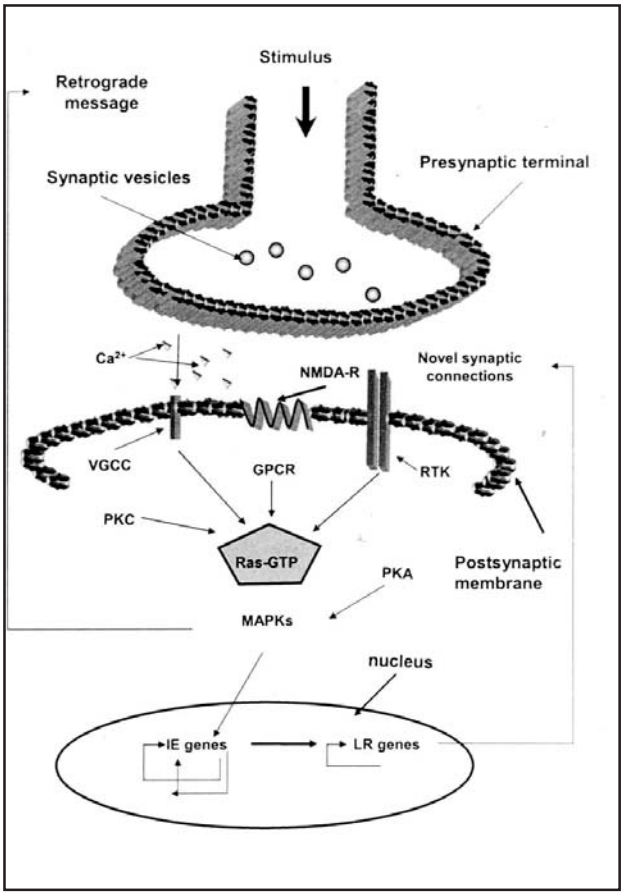

Fig. 3C. Signaling pathways involved in synaptic plasticity and long term mermory formation: the role of Ras and MAP family kinases. Different routes have been proposed for inducing the activation of Ras signaling pathway douwnstream to MAPK activation that leads to synaptic activation and long-term plasticity in brain areas such as hippocampus and amygdala, known to mediate long-term mediated plasticity events as well as long-term potentiation, particularly in the CA1 region of hippocampus (see text formore details). Influx of Ca^{2+} through voltage-gated Ca^{2+}- channels (VGCC) or through Ca^{2+}-channels coupled to the NMDA receptor mediate the qctivation of Ras. In same context, G-coupled receptors (GPCRs) and receptor-tyrosine kinases (RTK) including Protein kinase C (PKC) may modulate the activation of Ras signaling pathway. Thus, activation of activated form of Ras (Ras-GTP) may induce the subsequent activation of MAPKs cascade directly or indirectly by protein kinase A (PKA). This activation may lead to the generation of retrograde signals that sustain the synaptic plasticity mediating mechanisms that induce the activation of gene expression in the nucleus, activating immediate early-genes (IEG) as well as late-gene expression that may lead to the expression of proper functional proteins implicated in the formation of novel synaptic connections or or synaptic remodelling required for LTP expression and memory consolidation (text and figures adapted from Orban et al., 1999 and modified by principal author for the publication of this review).

1997; Orban et al., 1999). Besides these observations, implicating the activation of MAPK and its intracellular translocation associated with long-term synaptic plasticity, several studies have demonstrated that the abolishment of the MAPK signaling pathway, mediated by either injection of anti-MAPK antibodies or application of specific inhibitors of MEK (MAPK protein activator, see above), both inhibit long-term facilitation signaling process from sensory neurons to motoneurons, via synaptic input (Martin et al., 1997; Orban et al., 1999). Moreover, these studies have demonstrated that MAPK phosphorylates the transmembrane domain of the cell adhesion molecule, apCAM (an extracellular protein involved in the reorganization of the synaptic terminals in Aplysia) that leads to activation of IEGs through the phosphorylation of CREB (Bayley et al., 1997; Orban et al., 1999). These results posit that the activation of 5-HT receptors, via repeated application of 5-HT leads to activation of MAPK, leading to the tagging of the transmembrane domain of the CAM molecules, subsequent proteolysis and internalization of tagged peptide fragments (possibly mediated by the C-terminal hydrolase activity of ubiquitin molecules, see below), followed by the subsequent rearrangement of synapses, leading to observe the long-term synaptic plasticity events shown to occur in this invertebrate marine specie (Orban et al., 1999).

Furthermore, several experiments performed to understand the molecular mechanisms of synaptic plasticity in the CNS of mammals, have shown that MAPK might be relevant in the induction of long-lasting plasticity in the rodent hippocampus and thereby, for the expression of several forms of memory, including spatial memory (Orban et al., 1999). These studies demonstrated that induction of LTP in the hippocampus leads to the activation of MAPK (although transiently), showing that specific isoforms of MAPK (p42 MAPK but not p48 MAPK) could be activated by either high-frequency stimulation (HFS), PKC activation (via phorbol esters) or NMDA receptor activation in the hippocampal CA1 region, and prevented by NMDA receptor antagonists as well (English, Sweat, 1996; Orban et al., 1999). Moreover, pharmacological inhibition of MEK prevented MAPK activation induced by NMDA receptor activation or HFS used for the induction of LTP. Thus, these experiments demonstrated that MEK inhibitors could block the induction of LTP, with no altered effects on the expression of previously induced LTP (figure 3C) (English, Sweat, 1997; Orban et al., 1999).

Besides the large constellation of studies supporting the functional significance of the Ras-dependent signaling pathway in the development of synaptic plasticity events in the hippocampus, molecular studies demonstrated that heterozygous mutant mice carrying specific mutation on the Ras-GAP NF1 show specific impairment in spatial memory tasks (Silva et al., 1997), supporting evidences that clearly demonstrate the implication of this intracellular signaling pathway in

the physiology of the hippocampus (Orban et al., 1999). Overall, these studies provide strong evidence that MAPKs are crucial for long-term plasticity in the mammalian hippocampus as shown in Aplysia, showing that activation of the Ras signaling pathway modulates MAPK activation, which is crucial for synaptic plasticity events implicated in different forms of learning and memory, including emotional learning (Orban et al., 1999).

Different studies have demonstrated the required activation of the extracellular signal-related kinase-2 (ERK2) for the consolidation of associative memories induced by factors and neurotransmitter receptor stimulation (Giovannini et al., 2003). Such studies have shown that *in vitro* stimulation of either H2 or H3 histaminergic receptors (by infusion of histamine into dorsal hippocampus) in hippocampal CA3 pyramidal cells induces the activation of ERK2, which results in the improvement of memory consolidation after contextual fear conditioning. Administration of selective inhibitors of ERK kinase (i.e., U0126) prevents memory improvement induced by H2/H3 receptor agonists, without causing altered changes in the behavior (Giovannini et al., 2003). Moreover, early activation of hippocampal ERK2 does not seem to be required for the expression of long-term fear memories. This set of results demonstrate at least a direct correlation between active-dependent phosphorylation of ERK kinase and memory formation (Giovannini et al., 2003). Protein kinase cascade as part of the activation of membrane-receptor signaling pathways are known to be crucial for synaptic plasticity and memory. In this context, recent experiments have demonstrated that ERK kinase cascade, mediated through the phosphorylation of cytoplasmic and nuclear ERK, signaling downstream to the active phosphorylation of the nuclear transcription factor ELK-1 is involved in the NMDA-dependent long-term depression (LTD) expression at the commisural input into CA1 pyramidal cells in the adult rat hippocampus (Thiels et al., 2002). Moreover, pair-pulse stimulation inducing LTD, in the presence of ERK kinase inhibitors, such as SL327, failed to induce a long-lasting LTD expression, demonstrating thus that ERK activation with subsequent increased phosphorylation of ELK-1 (but not CREB) represents a crucial mechanism for the persistence of LTD (Thiels et al., 2003).

VIII. Postsynaptic protein phosphorylation and LTP: NMDA and AMPA glutamate receptors.

Rapid excitatory synaptic transmission in the mammalian forebrain is mediated by the activation of two types of ionotropic receptors, AMPA and NMDA receptors, localized at the postsynaptic density (PSD) coupled to several signaling molecules that modulate synaptic transmission (Soderling, Derkach, 2000; Malenka, 1994). Activated AMPA receptors mediate the postsynaptic depolarization by Na^+ influx, whereas NMDA receptors are mostly non-responsive due to their voltage-dependent block by Mg^{2+}. This voltage-dependent receptor blocking by Mg^{2+} ions, can be overcome by the intense postsynaptic depolarization caused by the strong afferent stimulation, resulting in the activation in NMDA receptor which mediate Na^+ and Ca^{2+} influx (Malenka, 1994). The efficiency of transmission mediated by these glutamatergic synapses can result in a weakened LTD induced by prolonged low frequency stimulation by brief, high frequency stimulation of afferent pathways (Malenka, 1994). Experimental paradigms that use different afferent stimulation protocols to induce LTP in CA1 region of the hippocampus have demonstrated the increased Ca^{2+} levels in dendritic spines (see section II) largely mediated through NMDA receptor-activation. This process is important to LTP initiation, as shown to be prevented by intracellular Ca^{2+} chelators and selective antagonists and/or blockers of the NMDA receptors. Although NMDA receptors are essential for initiation of LTP, expression of this phenomenon is brought about primarily by AMPA receptors (Soderling, Derkach, 2000; Malenka, 1994). As explained above (see section III), several controversial issues, still on dispute, are being discussed concerning whether LTP in areas of the hippocampus is driven mostly by presynaptic or by postsynaptic mechanisms (e.g., increase AMPA receptor response). Recent observations have demonstrated that E-LTP expression is predominantly driven by postsynaptic mechanisms at postsynaptic sites at Schaffer-collateral-commisural synapses in the CA1 region (Nayak et al., 1996; Soderling, Derkach, 2000). NMDA receptors are basically represented by heteromeric channels that consist of NR1 and NR2A-D subunits, which are integrated at the PSD with several other proteins (PSD95, Ca^{2+}-calmodulin, SAP102, α-actinin, and the tyrosin-kinase family members) (Soderling, Derkach, 2000; Kennedy, 1998). Both NR2A and NR2B subunits are known to be phosphorylated at C-terminal tyrosine residues by intracellular tyrosine kinases, that potentiate ion channel current through recombinant channels of NR1-NR2A and/or NR1-NR2B, thus relieving the basal zinc inhibition of NMDA receptors (Zheng et al., 1998).

Interesting observations have demonstrated that induction of LTP produces an activation of SRC within 1-5min (Lu et al., 1998) with the resultant tyrosine-

residue phosphorylation of NR2B (Rostas et al., 1996; Rosenblum et al., 1996). SRC infusion (as LTP does) produces a significant enhancement of AMPA-receptor-mediated current that is dependent on the activation of NMDA-receptor channel and Ca^{2+} influx and, conversely, application of SRC-specific inhibitors prior to HFS, prevents LTP induction (Lu et al., 1998). As SRC has been demonstrated to be localized to the PSD and shown to co-precipitate with NMDA receptor, it seems plausible to posit that potentiation of AMPA-receptor mediated current is an indirect effect caused by SRC-mediated enhancement of NMDA receptor function (Yu et al., 1997). Moreover, several studies have shown that not only tyrosine kinase FYN potentiates NMDA-receptor-mediated current, but that FYN interaction with the NR2A receptor subunit is mediated by the scaffold protein PSD95 (Tezuka et al., 1999). Thus, this set of observations (as explained in section VII; see Ras-signaling pathway and Ras-MAPK kinase system) suggest that LTP induction produces a rapid activation of SRC-family kinase, that enhance NMDA-receptor-mediated through tyrosine-

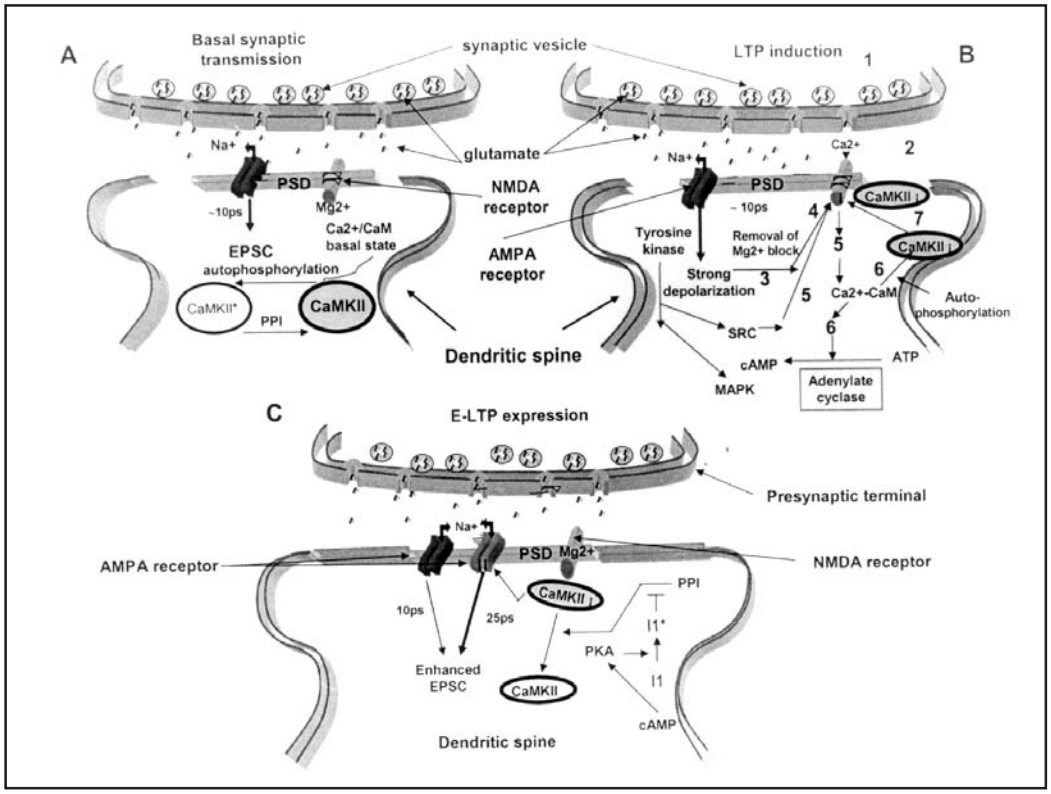

Fig. 4. Protein phosphorylation mechanisms at postsynaptic neurons during LTP formation in the hippocampal CA1 region. **(A) D**uring basal synaptic transmission induced by weak afferent stimulation, glutamate (triangles) release from synaptic vesicles (exocytosis) at presynapsis leads to activation of AMPA receptor inducing a low-conductance state (10pS) resulting in an EPSC. During this condition, NMDA receptors result to be inactivated due to the voltage-dependent block of their Ca^{2+}-channel by Mg^{2+} ions. Moreover, most of the Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) results to be inactivated, with very few molecules of same enzyme, resulting in the phosphorylated-active state (CaMKII*). This situation is basically due to the low levels of intracellular Ca^{2+}-calmodulin (Ca^{2+}-CaM) and high activity of protein phosphatase 1 (PP1). **(B)** Tetanic stimulation of afferent hippocampal fibers used to induce LTP (1) leads to an enhance vesicle-exocytosis of glutamate at the presynaptic terminal (2) and thereby, a strong postsynaptic depolarization mediated throught the activation of AMPA receptors (3) which enhance the Mg^{2+} block of ionotropic glutamate-NMDA receptors(4). Moreover, activation of AMPA receptors may induce the activation of different subset of the tyrosine kinases family (i.e., associated SRS-tyrosine kinases, SRS in the figure) that are involved in the phosphorylation (*) of the NMDA receptor channel, resulting in the induced-pemeability of Ca^{2+}, and thereby, inducing an enhance conductance state of the ion channel (5). The increase concentration levels of intracellular Ca^{2+}, leads to the increase levels of Ca^{2+}-calmodulin (Ca^{2+}-CaM) complex which elicits the stimualtion and activation of adenylate cyclase as well as, the autphosphorylation activity of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII*) (6). The active form of CaMKII (CaMKII*) favors its translocation to the postsynaptic density (PSD) favoring its interaction with the NMDA receptor (7). **(C)** Expression of the early phase of LTP (E-LTP expression) is mediated at leat in part, through the CaMKII-dependent phosphorylation (CaMKII*) of the GluR1 subunit of the AMPA receptor (*) which leads to a higher conductance state (from @ 10 pS to @ 25 pS) of the ionotropic channel-receptor. The expression of the constitutive autophosphorylated form od CamKII (CaMKII*) is sustained mainly to the protein kinase A (PKA)-mediated phosphorylation of protein inhibitor 1(I1), which inhibits the dephosphorylation activity of PP1 on CaMKII and GluR1 protein subunit. Such mechanisms favors the activity of molecules in the specific pathways shown in the figure (see text for full explanation) (text and figures adapted from Soderling & Derkach, 2000; and modified by principal author for the publication of this review).

residue phosphorylation (Salter, 1998), which in turn increases Ca^{2+} influx into the dendritic spine. Such mechanisms then trigger the intracellular signaling pathways that lead to the potentiation of AMPA receptors (see figures 4 A and B) (Soderling, Derkach, 2000; Salter, 1998). Although this hypothesis seems to be quite convincing, several studies have shown on one hand, that activation of NMDA receptor through tyrosine–residue phosphorylation by tyrosine kinase activity might be mediated through protein kinase C (PKC), while others, showed that AMPA receptors might be activated independently of Na^{+} or Ca^{2+} influx, through the association of this receptor with the SRC-family member protein kinase, LYN, as a result of the activation of the mitogen-activated protein kinase system (MAPK) (figure 4C) (Lu et al., 1999; Soderling, Derkach, 2000).

Moreover, the functional activity of MAPK family (which includes the subfamily of extracellular signal–regulated protein kinase or ERKs; Jun N-terminal kinase of JNK; and p38 MAPK) has been shown to be implicated in the development of synaptic plasticity underlying learning and memory (Kornhausser, Greenberg, 1997; Impey et al., 1999; Orban et al., 1999). Mitogen-activated-protein kinases, ERK and p38 MAPK, have been shown to be expressed abundantly in the CNS and specifically in areas implicated in learning and memory, such as the hippocampus and cerebellum (Ortiz et al., 1995; Zhen et al., 1999; Lee et al., 2000). Activation of ERKs in the hippocampus has been demonstrated to be crucial in acquisition of long-term spatial memory in the rat (Blum et al., 1999); in memory consolidation for fear condition associated with activation of MAPK in the amygdala (Atkins et al., 1998; Cammarota et al., 2000), as well in the activation of the same subfamily of protein kinases and CREB in the cerebral cortex associated with taste-aversion learning in the mouse (Swank et al., 2000 b). Parallel experiments using pharmacological antagonisms of MAPKs have demonstrated the impairment of spatial learning memory (Blum et al., 1999), fear conditioning processing in the rat (Atkins et al., 1999; Schafe et al., 1999), as well as taste-aversion learning in the mouse (Swank et al., 2000a]. In a similar context, several works have evidenced that long-term memory may involve the activation of different subfamilies of MAPKs, as demonstrated by the activation of ERKs and JNK in the rat insular cortex after consolidation of long-term memory of a novel taste, but not for p38 MAPK (Berman et al., 1998). Thus, depending on the learning task being used, different brain areas may be involved in addition to the activation of a different subset of MAPKs in long-term learning and memory (Zhen et al., 2001). In such a context, it has been recently demonstrated that such situation may be occurring using the classical conditioned eye-blink response in the rabbit (Zhen et al., 2001). These experiments have demonstrated that eye-blink condition produced a significant, bilateral activation of ERK and p38 MAPK in the anterior cerebellar vermis, and specific activation of ERKs in the dorsal hippocampus with no change of p38 MAPK or JNK in these areas, including several other areas of the rabbit brain (Zhen et al., 2001). P38 MAPK was shown to be implicated specifically in the acquisition of the conditioned eye-blink reflex (CR), after showing that intraventricular injection of a selective p38 MAPK inhibitor retarded this CR acquisition, but not inhibitors of ERK activity. Thus, this specific MAPK kinase has been suggested to be activated during associative learning processing, playing its crucial role in the regulation of transcriptional events that lead to memory consolidation (Zhen et al., 2001). Moreover, several studies have shown that expression of LTP in the CA1 region of hippocampus requires activation of cAMP-dependent protein kinase (PKA) in postsynaptic cells. Such studies have demonstrated that postsynaptic infusion of specific PKA inhibitors caused a significant decay of EPSCs to pretetanization amplitudes (within 1.5 hr) in CA1 pyramidal neurons, when compared to the evoked-increase of EPSCs for up to 2 hr after high frequency stimulation (HFS) of the Schaffer collateral pathway (Duffy, Nguyen, 2003).

a) Regulation AMPA receptors through phosphorylation mechanisms. As induction of LTP in CA1 region has been shown to be blocked by either inhibitors of Ser/Thr protein kinases or by postsynaptic chelation of Ca^{2+}, thus abolishing the intracellular activity of Ca^{2+} dependent protein kinases (e.g., Ca^{2+}/calmodulin-depemdent protein kinase II or CaMKII and PKC) it seems reasonable to posit that LTP is mediated by the activity of such Ca^{2+} dependent protein kinases (Soderling, Derkach, 2000). Moreover, studies performed to inhibit protein kinase activity through the application of specific peptide inhibitors of these two kinases of calmidazolium (a CaM antagonist) into pyramidal cells resulted in the blocking of the induction of LTP, demonstrating thus the postsynaptic site of their actions (Wang, Kelly, 1995). In addition, other experiments performed by infusion of Ca^{2+} and CaM or activated CaMKII demonstrated that this protein potentiates synaptic current and obliterates subsequent induction of LTP (Wang et al., 1995; Lledo et al., 1995). Thus, such results point out that CaMKII potentiates synaptic current using the same mechanisms as LTP (Soderling, Derkach, 2000). Moreover, GluR2 subunits of AMPA receptors have been shown to contain a

PDZ (postsynaptic density 95/Discs large/zona occludens 1) ligand domain that once phosphorylated at the serine residue 880 (S880) disrupts the interaction of the GluR2 with the glutamate receptor-interacting protein/AMPA receptor-binding protein, or GRIP/ABP complex. Point mutation performed on GluR2 protein subunit in order to mimic or prevent phosphorylation of the protein (GluR2 S880) resulted in the exclusion of receptors from synapses; depressed synaptic transmission and partial occlusion of LTD in the induced-phosphorylation state of the protein. Conversely, prevention of the phosphorylating state in mutant GluR2 resulted in reduction of LTD. Such studies demonstrated that disruption of GluR2 with GRIP/ABP protein complex by S880 phosphorylation may enhance the removal of synaptic AMPA receptors from membrane and modulate the expression of activity-dependent synaptic depression (Seidenman et al., 2003).

b) Regulation of intracellular CaMKII activity. CaMKII is an oligomeric protein that consists of 10-12 subunits. This calcium binding protein is a major constituent of the PSD, and based on its physicochemical properties, makes it a unique transducer of Ca^{2+} signals (Braun, Schulman, 1995). CaMKII is inactive in its basal state due to the presence of an auto-inhibitory domain that effects a steric block on the substrate binding domain. Binding of Ca^{2+} to an adjacent domain, in close proximity to the auto- inhibitory domain, alters the structural conformation of the protein complex, disrupting its inhibitory interaction, and thereby inducing activation of the kinase activity within the protein (Soderling, Derkach, 2000). The activated kinase undergoes a rapid intersubunit phosphorylation at threonine residues (Thr286), located within the auto-inhibitory domain, before it can phosphorylate exogenous substrates. This phosphorylation activity within the auto-inhibitory domain is crucial for it regulates three main functions: i) allows the association of CaMKII with the PSD, facilitating its interaction with NMDA receptor (Leonard et al., 1999); ii) it decreases the dissociation rate of Ca^{2+}-CaM by three orders of magnitude, and iii) after Ca^{2+}-CaM dissociation, the autophosphorylated kinase retains its full kinase activity to modify exogenous substrates (Braun, Schulman, 1995). The rapid autophosphorylation activity of CaMKII allows the generation of a constitutively active CaMKII that besides phosphorylates exogenous substrates more slowly, catalyzes additioning autophosphorylation on different sites within the protein subunits. Such mechanisms implie that transient elevation of Ca^{2+} concentration in dendritic spines is actually translated into prolonged kinase activity that endures even in the absence of the increased Ca^{2+} levels, until protein phosphatase dephosphorylate Thr residues (at Thr 286) (Soderling, Derkach, 2000). This protein (protein phosphatase 1, PP1), which has been shown to be expressed at the PSD, might be responsible for the dephosphorylation at Thr residue 286, with the further inactivation of the PSD-associated CaMKII (Strack et al.,1997). Stimulation of NMDA receptor in cultured hippocampal neurons has shown that Ca^{2+} influx leads to the autophosphorylation activity of CaMKII at Thr 286, as well as to the generation of its Ca^{2+} –independent activity. Other studies have shown that induction of LTP in hippocampal slices results in activation of CaMKII within a minute, and this activity endures at least 1 h (Fukunaga et al., 1993; Soderling, Derkach, 2000). Moreover, in mutant transgenic mice, where Thr 286 in CaMKII has been changed for Ala, the animals expressed normal basal synaptic transmission but do no exhibit any form of LTP (Giese et al., 1999; Soderling, Derkach, 2000).

A vast number of protein substrates have been demonstrated to be phosphorylated by CaMKII, and several of them have been shown to be activated during LTP (Soderling, Derkach, 2000). One of these protein substrates is the AMPA receptor, which becomes potentiated during LTP. In the CA1 hippocampal region, the heterotrimeric AMPA receptor consists mainly of GluR1 and GluR2 subunits, and several experiments using cultured hippocampal neurons have demonstrated that after NMDA-receptor stimulation with the subsequent Ca^{2+} influx, CaMKII phosphorylates the native AMPA receptor (Tan et al., 1994) or at the GluR1 subunit at Ser 381(Barria et al., 1997 a; Mammen et al, 1997), a receptor shown to be associated to the PSD (McGlade-McCulloh et al., 1993). Most important is that this receptor results to be phosphorylated, via the GluR1 subunit, after the induction of LTP in the CA1 hippocampal area (Barria et al., 1997 b), and thereby demonstrates the crucial role of GluR1 subunit for CaMKII-mediated LTP (Soderling, Derkach, 2000). Although LTP has been shown to be enhanced in knockout mice deficient of the GluR2-subunit, due perhaps to the increased Ca^{2+} permeability (Jia et al., 1996), the adult GluR1-subunit knockout mouse shows normal basal synaptic transmission, but a complete absence of LTP in the CA1 region (Zamanillo et al., 1999). Thus, these results not only demonstrate the important role and requirement of GluR1 subunit in LTP in the CA1 region, and its CaMKII-dependent activity, but also the postsynaptic locus of LTP expression (Soderling, Derkach, 2000). Moreover, this transgenic mouse shows no impairment of spatial

learning task (in the water-maze paradigm), while CA1-region LTP was completely prevented, data that show a dichotomy between LTP in the CA1 field, and the acquisition of spatial learning (Zamanillo et al., 1999; Soderling, Derkach, 2000). Phosphorylation of AMPA receptors by CaMKII (either on the native receptor in cultured hippocampal neurons or CA1 pyramidal neurons or HEK cells expressing the GluR1 receptor subunit) (Barria et al., 1997 a; McGlade-McCulloh, et al., 1993; Lledo et al., 1995) results in slow potentiation of whole-cell AMPA-receptor–mediated current (15-30 min), which correlates with the slow-rate of AMPA receptor phosphorylation (Soderling, Derkach, 2000). In addition, several experimental works have demonstrated that induction of LTP produces an enhancement of AMPA-receptor responsiveness that develops over a 30 min period, which correlates with the detected rate of AMPA-receptor phosphorylation and potentiation by CaMKII (Davies et al., 1989; Soderling, Derkach, 2000). In the same context, induction of LTP leads to a rapid and sustained generation of Ca^{2+} -independent CaMKII activity, which correlates with the autophosphorylation activity of the protein at Thr286, as demonstrated and confirmed by phosphospecific antibody for Thr286 (Barr et al., 1997 b), thus showing that LTP increases phosphorylation of AMPA receptors catalyzed via a Ca^{2+} -independent form of CaMKII (Soderling, Derkach, 2000; Barria et al., 1997 b).

Such experiments have led to postulate the hypothesis which explains that CaMKII is activated within the first minutes of LTP induction, due to its autophosphorylation activity at Thr286, which endure for as long as 1h, thus facilitating the translocation of CaMKII to the PSD (figure 4B). The Ca^{2+}-independent activity of CaMKII slowly phosphorylates the GluR1 subunit of AMPA receptor, producing a potentiation of the AMPA-receptor-mediated current, as a result of an increase in single-channel conductance (Soderling, Derkach, 2000)(figure 4C).

This interpretation proposed by these authors has been confirmed by several experimental works that demonstrate that induction of LTP increases AMPA-receptor channel unitary conductance in 60% of the potentiated cells (Benke et al., 1998). Moreover, not only these regulatory mechanisms seem to be present at synapses in mammalian species (rat and mouse) but also in non-mammalian species, where the activity-dependent enhancement of glutamate-mediated transmission has been shown in the goldfish Mauthner-cell synapse, which seems to require activation of postsynaptic CaMKII (Pereda et al., 1998). Several other experiments have demonstrated that GluR1 can be phosphorylated by other protein kinases such as PKC, showing that a long-lasting activation of this enzyme occurs after induction of LTP (Klan et al., 1993; Sactor et al., 1993). Moreover, PKC regulates AMPA-receptor activity by phosphorylating the GluR1 protein subunit at Ser831 (Mammen et al., 1997). PKA has been shown also to phosphorylate GluR1 at other serine residues such as Ser845, which causes a potentiation of AMPA-receptor-mediated current (Roche et al., 1996). Although it has been demonstrated that GluR1 phosphorylation at Ser845, occurs only in basal conditions and its dephosphorylation occurs in LTD (Kameyama et al., 1998), phosphorylation of PKA under basal conditions might result from the translocation of PKA to PSD, mediated through its anchoring protein, AKAP (kinase A anchoring protein) by which it may interact with the ionotropic glutamate NMDA receptor subtype, within the PSD (Westphal et al., 1999; Soderling, Derkach, 2000). Recent studies have demostrated that the *glr-1*, an homolog of the mammalian non-NMDA glutamate receptors (AMPA receptors) is crucial for the expression of long-term habituation in the worm C. Elegans. Thus, long-term-memory in these species seems to be dependent on the activity of this glutamate receptor subtype, including in the expression and localization changes of glutamate receptors (Rose et al., 2003).

c) NMDA receptor phosphorylation. The ionotropic glutamate NMDA receptor subtype consist of a heteromer comprised of NR1 and at least one of the four NR2 subunits defined as NR2A-D (Yakamura, Shimoji, 1999). One crucial characteristic of the NR2 subunits is the long intracellular C-terminal domain (tail) required for channel function (Sprengel et al., 1998) and as by themselves they cannot form a functional NMDA receptor, they need to be coexpressed with NR1 subunits (Monyer et al., 1994). Both NR2A and B are differentially tyrosine phosphorylated, where the NR2B subunit represents one of the most prominent phophorylated proteins in the PSD (Yaka et al., 2001; Moon et al., 1994). In such context, application of tyrosine kinase inhibitors causes a progressive decrease in NMDA receptor-mediated currents, and conversely, inhibition of protein tyrosine phosphatases results in an increase in NMDA receptor-mediated currents (Yaka et al., 2001; Wang, Slater, 1995). As described above, the Src family of protein tyrosine kinases (PTKs) has been identified as the enzymes responsible for the phosphorylation of NR2 protein receptor subunit, regulating the NMDA receptor activity (Yaka et al., 2001; Yu et al., 1997; Wang, Slater, 1995). Thereby, tyrosine phosphorylation of the NMDA receptor is increased in LTP as it has been demonstrated that both Src and FYN are necessary for the induction of

LTP(Kojima et al., 1997; Salter, 1998). Moreover, several studies have recently demonstrated that protein kinases are associated and compartamentalized with scaffolding proteins at the PSD (Pawson, Scott, 1997) and many of these scaffolding proteins result to associate with the NMDA receptor complex within the PSD (Kennedy, 1997; Walikinis et al., 2000; Husi et al., 2000). One of these proteins defined as RACK1 (originally identified as protein kinase C-binding protein) has been identified to be associated with the NR2B and FYN and to interact with several signaling proteins (i.e., kinases, phosphatases, phosphodiesterase as well as cell surface receptors) (Ron et al., 1994; Chang et al., 1998; Yarwood et al., 1999; Mouton et al., 2001; Brandon et al., 1999; Geijsen et al., 1999; Yaka et al., 2001). In such a context, recent experiments have demonstrated that the regulatory mechanism that drives the phosphorylation state and thereby the functional activity of the NMDA receptor is through the inhibitory scaffolding protein, RACK1, as shown to bind both NR2B subunit of the NMDA receptor and the nonreceptor protein tyrosine kinase, FYN. RACK1 inhibits FYN phosphorylation of NR2B receptor subunit and thereby decreases NMDA receptor-mediated currents in CA1 hippocampal slices. Thus, RACK1 may represent one of the protein regulators of NMDA receptor function and thus mediate important activities in synaptic plasticity, including learning and memory processing events (Yaka et al., 2001).

d) Phosphatases implicated in LTP (E-LTP). Similar to protein kinases, neurons synthesized several protein kinases, such as calcineurin (PP2B), protein phosphatases 1 or PP1, phosphatase 2A or PP2A, which dephosphorylate Thr286 in CaMKII and thus reverse the constitutive activity of the enzyme to basal levels (Soderling, Derkach, 2000). As PP1 and PP2A are highly expressed in PSD, and several evidences point out that PP1 is mainly responsible of dephosphorylating PSD-associated CaMKII (Strack et al., 1997).

In order for LTP to supress the activity of PP1, and thus to avoid the rapid dephosphorylation of CaMKII and of GluR1 as well, once the concentration of Ca^{2+} concentration reaches basal values; regulatory mechanisms are switched on and that allows for the inactivation of phosphatases activity. In this context, the catalytic subunit of PP1 can interact with several proteins (some of them serve to localize PP1 to subcellular organelles and others act as inhibitory subunits) (Soderling, Derkach, 2000). One of these proteins, defined as protein inhibitor 1 (I1) needs to be phosphorylated by PKA to inhibit PP1, and dephosphorylation of I1, catalyze by PP2B (protein anchored to the PSD through AKAB) will thereby produce the reverse reaction (Cohen, 1989; Westphal et al., 1999). Thus, regulation of PP1 activity via phosphorylation of I1 mediated by PKA has been demonstrated to occur during LTP (Blitzer et al., 1998). In such a context, during induction of the early phase of LTP (E-LTP) a rapid transient activation of PKA, as a subsequent event of activation of adenylyl cyclase I has been detected (Roberson, Sweat, 1996), indicating the requirement of cAMP levels and PKA activity in E-LTP. This requirement can be substituted by the postsynaptic injection of the phosphorylated inhibitor I1, and several experiments have demonstrated, on one hand, that LTP induction results in I1 phosphorylation and thereby in the inhibition of PP1 activity (Blitzer et al., 1998) and on the other, that inhibition of CaMKII activation by KN62 prevented LTP induction, just after injection of the phosphorylated stable form of I1 (figure 4C). These results connote that one of the regulatory mechanisms produced by PP1 inhibition by I1 is to prolong the activation of CaMKII, and therefore the maintenance of the phosphorylation state of GluR1 (Soderling, Derkach, 2000). Interestingly enough, is the observation that postsynaptic injection of a CaMKII inhibitor peptide can induce an inhibition of chemically-induced transient synaptic potentiation in hippocampal slices (40 min duration) and conversely be converted into a prolonged NMDA-receptor dependent potentiation by pretreatment of slices with PKA activators (e.g., forskolin) or inhibitors of PP1 and PP2A (e.g., calyculin A), producing a significant increase on the level of CaMKII THr286 autophosphorylation activity (Makhinson et al., 1999; Soderling, Derkach, 2000). These results are consistent with the hypothesis postulated which remarks that stable synaptic potentiation is associated with long CaMKII activation, requiring the inhibition of protein phosphatases (probably mediated throughout I1 phosphorylation mediated by PKA) (Soderling, Derkach, 2000).

e) Silent synapses implicated in LTP. Only 60% of potentiated CA1 neurons have been shown to produce an increased in AMPA-receptor channel unitary conductance (Benke et al., 1998), implying that other mechanisms might be acting, besides phosphorylation of AMPA receptors by CaMKII (Soderling, Derkach, 2000). This situation, based on the *silent synapse hypothesis* (Isaac et al., 1995) posits that prior to LTP induction some synapses might not express functional AMPA receptors, which occurs after induction of LTP (AMPA-receptor-mediated currents), probably mediated by recruitment of AMPA receptors to active synapses, from an intracellular pool; a parallel situation similar to what occurs with rapid insertion of

phosphorylated glucose transporters into cell membranes of adipocytes induced by insulin (Pessin et al., 1999). Moreover, several experiments have demonstrated that besides the detected complex interaction between NSF, SNAP and GluR2 (specific for this AMPA-receptor subunit and not for GluR1 subunit), whose disruption causes a rundown of AMPA-receptor mediated synaptic current (Nishime et al., 1998; Song et al., 1998). The postsynaptic infusion of membrane-fusion pathway inhibitors (e.g., inhibitors of NSF or SNAP proteins) significantly disrupts LTP, without changes on the NMDA-receptor mediated baseline synaptic transmission (as previously demonstrated that GluR2-subunit knockout mouse do not express altered changes in basal synaptic transmission and LTP) (Jia et al., 1996). In addition, infusion of the membrane-fusion protein, SNAP, enhances synaptic transmission, which is blocked by prior induction of LTP (Lledo et al., 1998). Supporting the *silent synapse hypothesis* is the observation that LTP induction produces a redistribution of epitope-tagged of transiently expressed GluR1 (within the first 30 min) from intracellular sites in dendritic shaft to dendritic spines of apical dendrites in hippocampal slices (Shi et al., 1999). Thus, these results, based on the silent synapse hypothesis, posit that functional AMPA receptors operate after their translocation to active potentiated synapses (an hypothesis that still needsfurther demonstration)(Soderling, Derkach, 2000).

IX. Cell-regulation of synaptic glutamate receptors involved in LTP: AMPA receptors

Plastic changes in the CNS have been demonstrated to be stored partially through changes in the strength of synaptic connections. Several works have shown that changes in the number of glutamate receptors clustered at the active zones of synaptic sites play a crucial role in different forms of long-lasting activity-dependent synaptic plasticity, including LTP, LTD and synaptic scaling (Turrigiano, 2002; Malinow, Malenka, 2002; Hiyashi et al., 2000; O´Brien et al., 1998). These studies have been focused in understanding the mechanisms that regulate the process by which glutamate receptors at specific synapses, particularly the ionotropic AMPA receptors, move into and out of the postsynaptic membrane (Turrigiano, 2002; Malinow, Malenka, 2002). Basically, synaptic receptors embedded into cell membranes turnover in the membrane with a half-life ($t^{1/2}$) determined by the rate of exocytosis and endocytosis of its receptor-containing vesicles (Turrigiano, 2002) (for a comprehensive review of the several mechanisms mediated by protein phosphorylation implicated in the regulation of synaptic membrane traffic, membrane fusion of

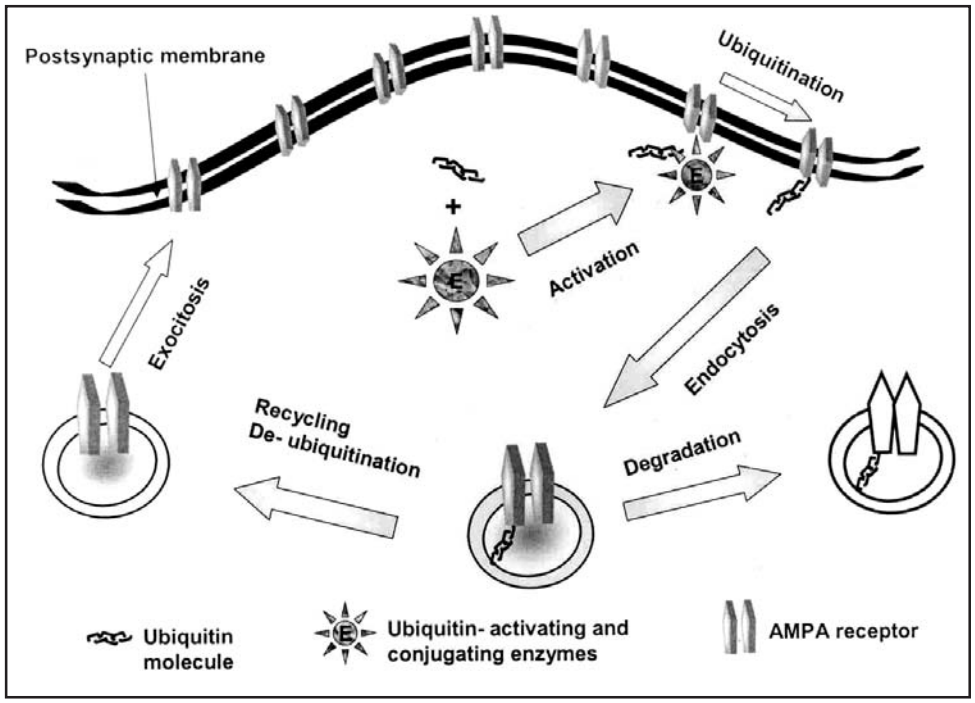

Fig. 5. Mecular mechanisms undelying the ubiquitination processing of AMPA receptor and the induce-receptor endocytosis. Endocytosis and exocytosis mechanisms elicit the constinously cycling of the AMPA receptors into and out of the membrane. Ubiquitination induced by ubiquitin-activating and ubiquitin- conjugating enzymes (E) elicits the endocytosis of AMPA receptors, and subsequently routed two separate intracellular pathways; either to degradation or to de-ubiquitination process that allows the recycling of the receptor back to the synaptic membrane (see text for full explanation) (text and figures adapted from Turrigiano, 2002; and modified by principal author for the publication of this review).

synaptic vesicles and recycling of synaptic vesicles (see Turner et al., 1999) (figure 5). For instance, AMPA receptors turnover quite rapidly, in minutes to hours (Malinow, Malenka, 2002). So, one possible mechanism offered by which activity could induce an accumulation of AMPA receptors at synaptic sites, would be due to alter the rates of exocytosis and endocytosis, or even, to induce the time-regulated insertion or removal of receptors (Hiyashi et al., 2000). Plasticity mechanisms that involve a loss of synaptic AMPA receptors, such as LTD, have shown that the same endocytic machinery involved in the regulation of presynaptic receptor/ vesicle endocytosis, is implicated in AMPA receptor endocytosis (Man et al., 2000; Wang et al., 2000), an intracellular mechanism that relies on the assembly clathrin-proteins that coat the membrane domain receptors, which induce the slowly pinch-off of the vesicles from the surrounding membrane (Turrigiano, 2002). LTD involves modest reductions of synaptic strength (20-30%), which suggest that not all synaptic receptors are subjected to regulated removal from synaptic membrane. As constitutive endocytosis removes only a small fraction of the total expressed membrane receptors per unit time, AMPA receptors can be tagged for removal by constitutive cycling mechanisms or in response to signals that mediate regulated endocytosis (Turrigiano, 2002). One general mechanism for targeting macromolecules to the intracellular trafficking of proteins is ubiquitination, which implies the addition of specific ubiquitin molecules to lysine groups into the target protein (Hochtrasser, 1996). In such a context, intracellular proteins can be mono-ubiquitinated or poly-ubiquitinated, depending on the intracellular route undertaken. For instance, proteins enrouted for degradation are commonly poly-ubiquitinated (Hochtrasser, 1996). In such a context, several experimental works have demonstrated that ubiquitination is the most important intracellular mechanism that regulates the targeting of AMPA receptors and their removal from synaptic membrane (Turrigiano, 2002). Using genetic manipulations in the nematode Caernohabditis Elegans (C. Elegans), several works demonstrated that the induced-increase and surface expression of one AMPA receptor subtype, GLR-1, in ventral cord interneurons, is specifically regulated by clathrin-mediated endocytosis after inducing a genetic mutation at the clathrin-adaptor protein, AP180 (Burbea et al., 2002). These results confirmed previous observations that suggested that the amount of synaptic AMPA receptors in the CNS in mammals is regulated by chlatrin-dependent endocytosis (Turrigiano, 2002). Furthermore, these genetic experiments showed that the accumulation of GLR-1 receptors at synaptic sites are regulated by ubiquitination of the AMPA receptor subtype, showing that enzyme-dependent activation of ubiquitin molecules and their addition to the target protein (after inducing, by genetic manipulation, the over expression of the intracellular pool of ubiquitin) significantly reduces the abundance of the synaptic GLR-1 receptor (Burbea et al., 2002). In addition, these experiments demonstrated not only that AP180 mutation prevented receptor endocytosis, but GLR-1 mutation prevented its specific ubiquitination, with the resultant convergence in that both processes increased the surface expression of GLR-1 at synapses. Moreover, AP180 mutation prevented the decrease in GLR-1 abundance after inducing an over-production of ubiquitin pool, suggesting that ubiquitination might precede endocytosis and thus ubiquitination of GLR-1 leads to clathrin-mediated endocytosis as authors proposed (Turrigiano, 2002). Several questions arise on whether AMPA receptors are mono- or poly-ubiquitinated, and if receptors once pinched off from the membrane, depending on its ubiquitination process, are enrouted to protein degradation or just recycled back to the synaptic membranes (Turrigiano, 2002). As ubiquitination seems to be an important regulatory mechanism for receptor-trafficking, several studies need to be performed in order to demonstrate what sort of signals and processes (i.e receptor agonists, exposure to hormones, altered levels of neuron activity) shown previously to modify surface expression or density of AMPA or NMDA receptors, are mediated by ubiquitination. Moreover, questions arise whether such molecular processes are significantly important in specific forms of synaptic plasticity such as LTP or LTD (Turrigiano, 2002). Overall, this set of results demonstrate that cellular expression of the AMPA-GLR-1 receptor subtype at synapses is regulated by two specific intracellular processes: one, the clathrin-mediated endocytosis and an other which is mediated by ubiquitination (Turrigiano, 2002). Recent experiments have shown that ZNRF proteins constitute a family of presynaptic E3 ubiquitin ligases, where ZNRF1 and ZNRF2 are proteins that contain an E# ubiquitin ligase activity that is highly expressed in the CNS of mammals, particularly active during development (Araki, Milbrandt, 2003). These proteins have been shown to be located in different compartments within presynaptic terminals, where ZNRF1 is associated with synaptic vesicle membranes, whereas ZNRF2 is highly expressed in presynaptic membranes (Araki, Milbrandt, 2003). Experiments inducing mutant protein ZNRF species, disrupting the C-terminal domain which contains a RINGER finger structure necessary for the normal functioning of E3

ubiquitin ligase, have demonstrated to inhibit the Ca^{2+} dependent exocytotic mechanisms in PC12 cells. These data have led to the proposition that ZNRF proteins may play a crucial role in the maintenance of neuronal transmission and synaptic plasticity through ubiquitination mechanisms (Araki, Milbrandt, 2003).

X. Regulation of genes in synaptic plasticity and memory formation

The molecular mechanisms that regulate gene expression are driven mainly by the regulated transcription of DNA, the genetic blueprint that contains the molecular information encoded in all living organisms. DNA transmits the required stored information from generation to generation that guides an organism's development and interaction with the environment. This information is stored in a deoxirybose-phosphate backbone bridged by paired purine [adenine (A) and guanine (G)]/pyrimidine [cytosine (C) and thymine (T)] nucleotide bases that finally structure the complex DNA double helix (Nestler, Hyman, Malenka, 2001). Since DNA is a linear polimer, it serves as a template for the synthesis of other macromolecules and therefore represents the ideal molecular engine for transfering information. The processes by which information is transfered with high fidelity from a template strand to a new molecule of DNA (i.e., DNA replication) or to RNA molecule (i.e., DNA-dependent RNA transcription) is basically performed by complementary base pairing. Transcription represents the first step in gene expression, where DNA serves as a template to synthesize a complementary single stranded RNA molecule. The RNA molecule subsequently dissociates from the DNA, allowing the original double helix to reanneal. Basically, the information contained within the DNA molecule is expressed through an intermediary mRNA molecule, which will be used further on for synthesis of proteins (i.e., RNA-dependent translation processing) (for more details of the mechanisms governing DNA replication, see Nestler, Hyman, Malenka, 2001). Under such a context, only a small percentage of chromosomal DNA in the human genome (≅ 4%) is responsible for encoding around 40,000 genes that encode RNA strands (Nestler, Hyman, Malenka, 2001). Most of the cell RNA is produced as mRNA that serves as an intermediary between DNA and protein synthesis. DNA contained within chromosomes comprises genes and extensive intergenic regions (which consist in long stretches of tandemly repeated unique sequences known as satellite DNA) that may be far from uniform; for instance, some whole chromosomes may indeed be gene rich or gene-poor (Nestler, Hyman, Malenka, 2001). Gene expression when regulated by the nucleotide sequence of a DNA molecule itself is known as *cys-regulation*, because regulatory and transcribed domains occur in the same DNA molecule. Thus, the nucleotide sequences implicated in cys-regulation serve as binding domains for several different regulatory proteins (that are not encoded by the stretches of DNA to which they bind) and are referred as *trans-acting factors*, and as they regulate transcription of DNA, they have been defined as *transcription factors* (Nestler, Hyman, Malenka, 2001). Transcription occurs in specific DNA domains in the open chromatin, which requires the recruitment of transcriptional activator proteins (i.e., histone acetylases) that enhance the displacement of the nucleosomes, which allows then for the binding of transcription factors to DNA at different core promoters and thus occurs the recruitment specific DNA-dependent-RNA polimerases (i.e. Polymerase I, II, III) in addition to other protein activators. The molecular events and steps implicated in the regulation of *transcription initiation* (which includes the construction of the protein complex at the transcription start site where synthesis of the phosphodiester bond between nucleotides takes place), the *elongation phase* (where RNA polimerase successfully transcribes the appropriate length of RNA without undergoing premature termination) and *regulated termination process* (the final mechanism that regulates the expression of some genes) that regulates the exact and correct termination of RNA transcription (for precise details see Nestler, Hyman, Malenka, 2001). After transcription of nuclear RNA, the first transcript product or *heteronuclear RNA* (hnRNA), is subjected to post-translational splicing mechanisms (mediated by a RNA-protein complex referred as *spliceosomes*) that are in charge of removing intervening DNA segments known as *introns* (stretches of DNA sequences that remain in the nucleus) that interrupt *exons* (the DNA sequences that code the required information into a specific segment of a mRNA in eukaryotes and which is exported from the nucleus), allowing for the formation of a mature mRNA. The mature mRNA is comprised of untranslated regions (UTRs) at their 5´and 3´ends where cys-regulatory elements (i.e., 5´-end capping referred to as 7mGpppN; poly(A) tail, polyadenylation sequence represented by AAUAAA, stem-loops), affect mRNA stability (half-life) and translability (rate at which mRNA is translated into protein) (Nestler, Hyman, Malenka, 2001). Several reports have estimated that many genes expressed in the brain of mammals and exposed to alternative splicing may result in an average of five splice variants/ gene (Nestler, Hyman, Malenka, 2001).

a) Regulation of gene expression by transcription factors.

To obtain higher levels of transcription, a multiprotein

complex needs to be assembled, which requires the help of transcriptional activators that bind and thereby recognize cys-regulatory elements found in the gene (i.e., response elements) represented by specific recognition sites in the DNA that are localized upstream (5´-direction) or downstream (3´-direction) from the initiation translation site (Nestler, Hyman, Malenka, 2001). These regulatory elements conformed by DNA stretches of 7-15 base pairs in length, exert control either near (promoter elements) the core promoter where RNA polimerases (i.e., Polimerase II) bind to or at distance (enhancer elements). In such a context, both promoter and enhancer elements control the expression of genes in different cell types, including how and when these genes will be expressed, the levels of gene expression in basal and under stimulation conditions in response to either physiological and environmental stimuli or signals (Nestler, Hyman, Malenka, 2001). *Trancription factors (TFs)*, which bind to sequence-specific DNA segments and consist of physically different functional domains, include TFs referred to as *DNA-binding domain* (that bind to a transcription-activation domain that interacts with general transcription factors that comprise the multiprotein components of the Polimerase II complex so as to form an active transcription complex) and the multimerization domains (that allows the formation of multimers of same or different transcription factors). Thus, transcription factors may be functionally active only when they form dimers or form higher-order complexes, and may up-regulate DNA transcription (for instance, when the muscle-specific TF known as MyoD dimerizes with the muscle HLH protein E12/E47, activates a variety of genes in muscle cells that induce differentiation of the tissue) or down-regulate DNA transcription (for instance, in proliferating myoblast, muscle-genes are not actively transcribed due that these cells express a TF called Id, an analogous protein of MyoD, which lacks the basic DNA-binding domain that is commonly found in the N-terminal domain of HLH proteins that functionally act as transcription activators, despite of the high expression levels of MyoD and E12/E47 protein in these cells. In this context, MyoD-Id dimmers cannot bind DNA and thereby activate transcription) through the formation of homodimers or heterodimers of cell-specific transcription factors (Nestler, Hyman, Malenka, 2001). In different situations, negative regulation of transcription that occurs naturally in cells, may be brought out by the inactive dimerization of one protein between partners, or via the dimerization between an activator and its negative regulator, and although they may be capable of binding DNA, the negative regulator lacks an activation domain and therefore disrupts the formation of the multimeric transcription complex. Gene expression depends on the optimal regulation of heterodimers formation, as is the case of some members of the Fos family transcription factors (i.e., *c*-Fos) that have been characterized as strong activators when dimerized with a partner of the Jun family transcription factors (i.e., *c*-Jun). Several members of Fos-related proteins, defined as Fos-related antigen-1 or FRA-1, form heterodimers with either c-Jun producing a weaker activation of transcription when binded to DNA as compared to c-Fos, due perhaps to the preferential binding to a particular DNA domain containing the AP-1 site (figure 3C)(activator proteins known as AP-1 are transcription factors that bind as heterodimers or homodimers to a specific DNA sequence, defined as AP-1 sequence, and are essential in the regulation of gene expression in neurons by extracellular signals). Thus, the formation of heterodimers and several multimeric forms of such nuclear proteins increases the constellation of transcription factor complexes and thereby increases the enormous complexity of the molecular mechanisms that regulate the information on gene expression (Nestler, Hyman, Malenka, 2001). It is quite interesting that sequence-specific transcription factors (i.e., activator protein CREB) may interact with the basal transcription apparatus or core promoter (polimerase II complex) localized far distant via the looping of the DNA template, bringing both distant regions in contact and allowing the interaction of co-activators or adapter proteins (i.e., CREB-binding protein or CBP) bound to the polimerase II complex. Moreover, once activator proteins are phosphorylated, they become available for the assembly of the mature transcription apparatus, in response to physiological stimuli or extracellular signals (figures 3A-C). Thus, phosphorylation induces the ability of phosphoproteins to interact with different proteins, as is the case for CREB (cAMP response element binding protein). Transcription activation results after CREB is phosphorylated on specific serine residues, allowing its interaction with adapter proteins, such as CBP, which in turn activates the basal transcription apparatus (Nestler, Hyman, Malenka, 2001) (figure 6).

b) Regulation of gene expression via activation of transcription factors and signaling pathways. One crucial step by which extracellular signals (i.e., neurotransmitters, growth factors and cytokines) regulate the expression of genes is based on the transduction of signals from the cell membrane to the nucleus, whereby response elements (cys-regulatory sequences in genes that confer responsiveness to physiological stimuli) bind transcription factors whose activity or inhibition

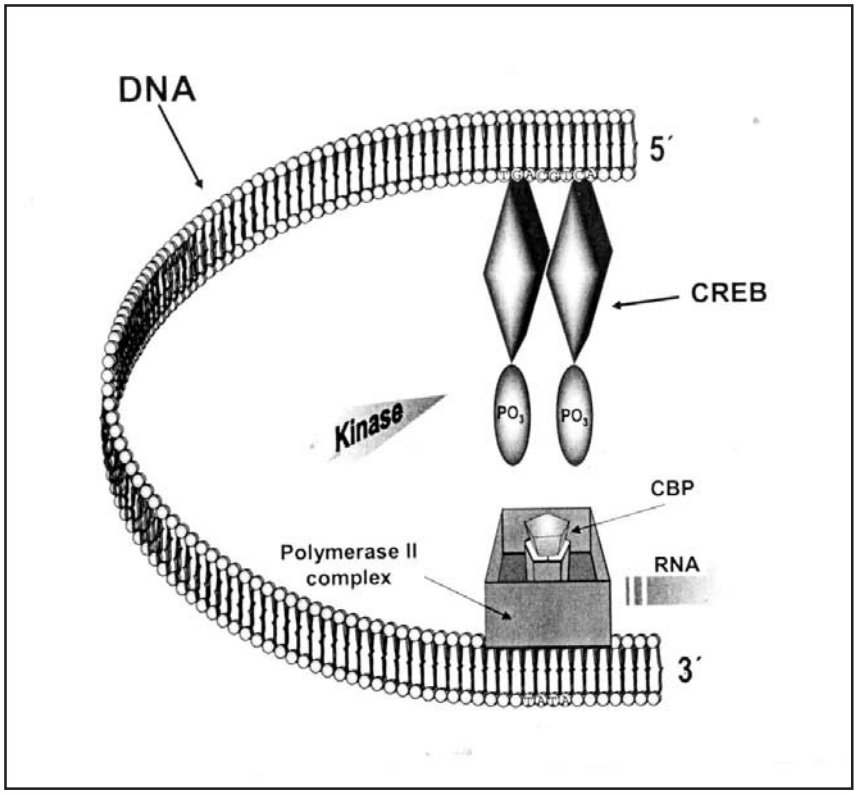

Fig. 6. Looping of the DNA template and interaction of basal transcription apparatus with transcription factors. Acitvators or repressors attached to the sequence-specific for transcription factors (CREB) (TGACGTCA) may interact with the basal transcription apparatus or core promoter represented by the Polimerase II complex attached to the TATA DNA sequence; which is localized far distant, via the looping of DNA template, bringing both distant regions in contact and allowing the interaction activators or adapter proteins such as, CREB-binding protein (CBP) bound to the Polimerase II complex. Kinase-dependent phosphorylation of activator protein CREB favors the interaction with CBP, and thereby for the assembly of the mature transcription apparatus in response to physiological stimuli or extracellular signals (see text for more details)(text and figure adapted form Nestler, Hyman and Malenka, 2001 and modified by principal author of the present review).

depends on the activation of specific physiological signals such as phosphorylation. Some transcription factors are translocated to the nucleus in response to their activation (i.e., steroid receptors, the cytoplasm transcription factor, NF-kB) (Nestler, Hyman, Malenka, 2001). Several transcription factors need to be phosphorylated or dephosphorylated before they can bind cys-regulatory sequences in the DNA. For instance, phosphorylation of signal transduction and activators of transcription or STATs by protein tyrosine kinases in the cytoplasm allows for their multimerization state and their proper DNA binding as a multimeric construction in the specific DNA binding site. In another situation, transcription factors are bound to their respective cys-regulatory elements in the nucleus basal conditions and once phosphorylated become active as transcriptional activators, as is the case for CREB [a phosphoprotein activated by protein kinase A or Ca^{2+}-Calmodulin that is properly bound to some response element defined as CREs, before cell stimulation](figure 3D, and sections in chapters, sections IV A and B). Several works have demonstrated that different signaling pathways converge in the activation of CREB in the CNS in mediating long-term adaptations, such as long- term memory, drug addiction and fear conditioning, neuronal survival during development, where all this neurobiological phenomena require the time-dependent expression of specific genes (Nestler, Hyman, Malenka, 2001; Finkbeiner, 2000). Interesting enough is that experiments that used DNA recombinant technology and showed the inactivation of CREB in Drosophila or partial inactivation of this TF in knockout mice, demonstrated deficit in long-term memory (Ahn et al., 1998; Bito et al., 1996; Nestler, Hyman, Malenka, 2001). Moreover, CREB knockout mice, used for the search of hippocampal LTP and long-term memory, have shown to exhibit a residual CREB activity, where identified CREB-like proteins (i.e., activating transcription factors or ATFs; and CRE modulators or CREM, among others) (De Cesare et al., 1999) may be able to partially compensate the knockout (Nestler, Hyman, Malenka, 2001). Other transcription factors, defined as AP-1 proteins, bind (see above) to specific DNA consensus sequence and not to CRE response elements, and thus AP-1 proteins influence which

transcription factors may regulate the expression of a specific group of genes (i.e., neurotensin, SP, D1R, NR1-NMDA glutamate receptor subunit, GluR2 AMPA glutamate receptor subunit, neurofilament proteins, tyrosine hydroxylase) induced by activation of specific signaling pathways in particular circuits of the brain (figure 3C) (Nestler, Hyman, Malenka, 2001). Furthermore, as it has been described previously, full CREB activation for stabilizing long-term learning and memory requires CREB phosphorylation mediated by the nuclear Ca^{2+}-CaMKIV in addition to activation of CBP (Spier et al., 2001). Several reports have demonstrated that Ca^{2+} entry following synaptic activation, allows for the Ca^{2+}-activated CaM to be translocated to the nucleus, influencing thus gene expression. But several reports have demonstrated recently that Ca^{2+} alone is sufficient to relay synaptic activation to the regulation of CREB in the nucleus with no recruitment of cytoplasmic signaling proteins to active transcription factor in the nucleus (Hardingham et al., 2001; Spier, 2001). These experiments demonstrated that Ca^{2+} influx through NMDA-receptors results in a Ca^{2+}-dependent- Ca^{2+} release from intracellular stores, whose concentration is enough to amplify and relay the transient Ca^{2+} elevation from the synapse to the nucleus (Hardingham et al., 2001; Spier, 2001). Furthermore, it was observed that nuclear Ca^{2+} concentration lasts longer and peaks just after dendritic Ca^{2+} increase in hippocampal neurons. Thus, hippocampal neurons induced with HFS, nuclear Ca^{2+} concentration, result to be higher enough to bring out a corresponding increase in CREB mediated transcription. This set of results demonstrates that synaptically-evoked Ca^{2+} increase in the nucleus is sufficient to activate CREB, and thereby making this cation a strong candidate in the activation of several neurobiological events, such as learning and memory and cognitive processes (Spier, 2001).

In such a context, recent experiments have demonstrated that a Ca^{2+} responsive E-box element or CARE-2 has been identified within the neurotrophic factor promoter site, namely, the BDNF promoter III that binds upstream stimulatory factors 1 and 2 (USF1/2) and these USFs have been shown to activate CARE-2-dependent transcription from the BDNF promoter III (Chen et al., 2003). Moreover, the experiments have demonstrated that Ca^{2+}-dependent signaling pathways in neurons regulate the transcriptional activity of USFs, which bind to promoters of several neuronal activated regulated genes in vivo, and thus such stimulatory factors may offer new functions in the regulation of activity-dependent transcription in neurons (Chen et al., 2003).

Furthermore, several studies have demonstrated that rat brain slices treated with glutamate induce a dramatic and progressive increase of the ERK and JNK/SAPK MAPK cascades (Vanhoutte et al., 1999). This effect leads to the increased phosphorylation of the Ser^{383} residue in ELK-1 and increase expression of *c-fos* mRNA. In addition, glutamate transiently increases the phosphorylation activity of CREB as compared to Elk-1 and *c-Fos*. Moreover, the ERK cascade has been shown to couple glutamate-signaling to the ternary complex factor (TCF), Elk-1 in the rat *striatum in vivo*, as demonstrated after application of MAPK kinase inhibitors (i.e., PD98059) (Vanhoutte et al., 1999). Such results have shown that ERK functions as a single molecular component that bridges two signaling pathways, *ERK-Elk-1* and *ERK-???-CREB* converging on the *c-fos* promoter in postmitotic neuronal cells, where CaMKs (calcium-calmodulin-kinase) activity regulates positively both pathways (Vanhoutte et al., 1999).

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