Published in final edited form as:

Neuroscientist. 2004 August ; 10(4): 325–336.

Signaling Cascades Regulating NMDA Receptor Sensitivity to Ethanol

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Abstract

One of the major targets for ethanol (alcohol) in the brain is the *N*-methyl-D-aspartate (NMDA) receptor, a glutamate-gated ion channel. Intriguingly, the effects of ethanol on the NMDA receptor are not homogeneous throughout the brain. This review focuses on recent studies revealing molecular mechanisms that mediate the actions of ethanol on the NMDA receptor in different brain regions via changes in NMDA receptor phosphorylation and compartmentalization. Specifically, the role of the scaffolding protein RACK1 and the regulatory protein DARPP-32 in mediating the distinct effects of ethanol is presented.

Keywords

N-methyl-D-aspartate (NMDA); Ethanol; Phosphorylation; RACK1; DARPP-32

Alcoholism is a devastating disease that affects 14 million people in the United States alone and costs society \$165 billion a year. Alcoholism is defined as uncontrolled consumption of alcohol (ethanol) despite the negative consequences. The disease manifests itself or results from phenotypes such as tolerance, dependence, and withdrawal and/or craving. Considerable effort has been focused on elucidating the mechanisms that underlie the development and maintenance of alcohol addiction. Interestingly, although ethanol is a small diffusible molecule (Fig. 1), only a defined number of targets have been identified that are altered as the result of acute or chronic exposure of neurons to ethanol (Fig. 1). Even more intriguing is the fact that ethanol affects the same targets differently in different brain regions. In this review, I will present examples of how signaling events that regulate the phosphorylation state and compartmentalization of proteins determine the activities of ethanol on the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR).

Posttranslation Modification: Phosphorylation/Dephosphorylation

The most common posttranslation modification is the addition of a phosphate group to the serine, threonine, or tyrosine residues of a protein substrate. This reaction, termed *phosphorylation*, is achieved via activation of protein kinases, which are divided into three groups: serine/threonine kinases, tyrosine kinases, and dual-specificity kinases, which are able to phosphorylate all three residues. The opposite reaction, in which a phosphate group is removed from a protein (dephosphorylation), is performed by either serine/threonine or tyrosine phosphatases. Receptor kinases and phosphatases are integral parts of the plasma membrane, whereas nonreceptor kinases and phosphatases reside intracellularly, with their subcellular compartmentalization determined in large part by protein-lipid or protein-protein interactions, as will be discussed in sections 3 and 6. Kinases and phosphatases are specifically

activated by signals that are usually transduced from outside to inside the cell. For example, activation of membranal receptors results in the generation of second messengers. Second messengers in turn directly or indirectly initiate a signaling cascade that includes the activation of kinases and/or phosphatases. The phosphorylation state of a protein is therefore a balance between phosphorylation and dephosphorylation events, with phosphorylation usually being the “on” signal and dephosphorylation the “off” signal.

Other posttranslation modifications, which will not be discussed in this review, include myristoylation (Farazi and others 2001), palmitoylation, and the more recently discovered ubiquitination and sumoylation (Schwartz and Hochstrasser 2003).

Compartmentalization

Approximately 500 kinases and 150 phosphatases have been identified in the human genome. Each kinase or phosphatase has approximately 20 and 60 substrates, respectively (Cohen 1999). A fundamental question in signal transduction is how specificity is achieved. In addition, signaling cascades are complex and must therefore be tightly regulated with precision and speed. In the past decade, it has become increasingly clear that scaffolding proteins play a major role in controlling specificity of signal transduction cascades (Bauman and Scott 2002). Scaffolding proteins interact with kinases and phosphatases away from, or in close proximity to, their corresponding substrates and can also serve as a platform to assemble multisignaling protein complexes of the same or different signaling cascades, allowing tightly orchestrated events to occur. Thus, scaffolding proteins provide both spatial and temporal organization of signaling cascades.

A topical example of a scaffolding protein is RACK1. RACK1 is a 36-kDa ubiquitously expressed protein that is highly expressed in the brain. RACK1 was originally cloned and identified as a protein kinase C (PKC) anchoring protein (Ron and others 1994) and was later found to interact with the activated form of the β IIPKC isozyme, to shuttle β IIPKC to the appropriate substrate site, and to enhance substrate phosphorylation by PKC (Ron and others 1994, 1995, 1999). In recent years, the spectrum of RACK1 functions has broadened, and numerous additional proteins have been identified as RACK1 binding partners (for a review, see McCahill and others 2002). These proteins include signaling proteins such as the phosphodiesterase PDE4D5 (Yarwood and others 1999), the tyrosine phosphatase PTP μ (Mourton and others 2001), kinases such as Fyn (Yaka and others 2002), and intracellular tails of different receptors such as β integrin (Liliental and Chang 1998), GABA $_A$ (Brandon and others 1999), the insulin-like growth factor receptor (Kiely and others 2002), and the NMDAR (Yaka and others 2002). RACK1 belongs to the WD40 family of proteins (for review, see Smith and others 1999). WD40 repeats mediate protein-protein interactions, and RACK1 contains seven WD40 repeats (Ron and others 1994). Crystal structure analysis of another seven WD40 repeat-containing protein, the β subunit of G protein (G β), revealed that the repeats form a seven-blade propeller structure, allowing the interaction with several proteins via the different repeats (Garcia-Higuera and others 1996; Sondek and others 1996). The interaction of RACK1 with proteins allows the compartmentalization of signaling proteins and the regulation of diverse cellular activities (for review, see McCahill and others 2002). A unique feature of RACK1 is its ability to translocate to different intracellular compartments upon activation of specific signal transduction cascades. For example, activation of the PKC signaling pathway induces RACK1 to translocate together with β IIPKC to its substrate site (Ron and others 1999), whereas activation of the cAMP/protein kinase A (PKA) pathway induces RACK1 translocation to the nucleus (Ron and others 2000; He and others 2002; Yaka, He, and others 2003). Nuclear RACK1 in turn alters the expression of several genes (He and others 2002; Yaka, He, and others 2003). More recently, RACK1 has been found to be an important regulator of ribosome assembly and activation via its interaction with β IIPKC (Ceci

and others 2003). Finally, RACK1 is an important regulator of the phosphorylation state and function of the NMDAR (Yaka and others 2002; Yaka, He, and others 2003) as described in section 6.

N-methyl-D-aspartate Receptor

Approximately half of the synapses in the brain are excitatory synapses that use glutamate as their neurotransmitter. When glutamate is released from the presynaptic terminal, it binds two groups of postsynaptic receptors, metabotropic (mGluR) and ionotropic (iGluR) receptors. Whereas mGluRs act by the formation of second messengers, the iGluRs are ion channels that allow the influx of positive ions to the postsynaptic neurons. There are three different iGluRs: AMPA, kainite, and NMDA. Binding of glutamate to its receptors results in a conformational change and opening of the channel to allow the entry of sodium ions, resulting in the depolarization of the postsynaptic neuron (for review, see Sucher and others 1996). The NMDAR channel has several unique features. The channel is highly permeable to calcium and at the resting membrane potential is blocked in a voltage-dependent manner by magnesium (for review, see Yamakura and Shimoji 1999). The activity of the channel is modulated by endogenous ligands such as glycine, zinc, polyamines, and neurosteroids (for review, see Monaghan and others 1998). The NMDARs are important mediators of neuronal plasticity and are required for most forms of long-term potentiation (LTP), a process thought to be the principal cellular mechanism underlying learning and memory (see Nicoll and Malenka 1995 for review). Drugs that modulate glutamate neurotransmission, specifically the NMDAR, are increasingly gaining interest for the treatment of a wide range of disorders such as schizophrenia, depression, anxiety, addiction, pain, epilepsy, stroke, and neurodegenerative diseases, such as Alzheimer's and amyotrophic lateral sclerosis (for reviews, see Heresco-Levy and Javitt 1998; Cull-Candy and others 2001).

NMDARs are heteromers composed of an obligatory NR1 subunit and combinations of different modulatory NR2 (A–D) subunits (for reviews, see Sucher and others 1996; Cull-Candy and others 2001) and NR3 (A–B) (Das and others 1998; Matsuda and others 2003). Eight isoforms of the NR1 subunit exist due to alternative splicing of a single NR1 gene. The alternatively spliced forms of NR1 are derived from either an insertion of a region into the extracellular N-terminal of the subunit (N1) and/or the insertion of one or both domains (C1 and C2) into the intracellular tail of NR1 (for review, see Cull-Candy and others 2001) (Fig. 2). The NR1 splice variants and the NR2 subunits are expressed at different levels during development and have different patterns of distribution in the brain (for review, see Cull-Candy and others 2001). The functional properties of the channel such as conductance, open probability, clustering, and trafficking depend on subunit composition of the channel (for review, see Monaghan and others 1998). For example, channels that contain the NR2B subunit have longer open probability as compared with those that contain the NR2A subunits. The intracellular tails of the NMDAR subunits are highly important for the proper function of the channel. Mice lacking the NR1 or NR2B subunit or mice expressing a mutant form of the NR2B that lack the intracellular tail of the channel die shortly after birth (Forrest and others 1994; Kutsuwada and others 1996; Sprengel and others 1998). NR2A deletion mutant mice exhibit deficits in LTP as well as spatial learning and memory and have reduced tissue injury after brain ischemia (Sakimura and others 1995; Wang and others 2003). Transgenic mice overexpressing the NR2B subunit in the forebrain show enhanced channel activity, enhanced LTP, and enhanced learning and memory (Tang and others 1999). Recently, NR2B-containing NMDARs were found to be important for the NMDAR-dependent activation of the mitogen-activated protein kinase pathway (Krapivinsky and others 2003), a signaling pathway important for synaptic plasticity. The cytoplasmic tails for the NMDARs serve as a platform for phosphorylation and protein assembly events that are important for the regulation of the NMDAR as described in sections 5 and 6.

Regulation of NMDAR Function by Phosphorylation/Dephosphorylation

To understand how ethanol modulates the function of the NMDAR, it is necessary to understand the signaling mechanisms involved in the regulation of the channel.

One of the most important means of regulating the NMDAR function is phosphorylation. The NMDAR subunits NR2A and NR2B are phosphorylated on serine and tyrosine residues, and the alternatively spliced isoforms of the NR1 subunits are phosphorylated on serine residues (Fig. 2). The highly homologous tyrosine kinases Fyn and Src have been implicated thus far in phosphorylating the NR2 subunits on tyrosine residues on sites depicted in Figure 2 (Suzuki and Okumura-Noji 1995; Kohr and Seeburg 1996; Tezuka and others 1999; Nakazawa and others 2001; Yaka, He, and others 2003; Thornton and others 2003). It is unclear, however, whether both kinases phosphorylate both subunits under physiological conditions, which sites are phosphorylated by which kinase, and whether different signaling pathways activate the two kinases. Many NMDAR functions have been linked to tyrosine phosphorylation of the NR2 subunits. Phosphorylation results in the enhancement of channel function because tyrosine kinase inhibitors inhibit whereas application of Src kinase enhances NMDAR-mediated currents (Wang and Salter 1994; Chen and Leonard 1996; Yu and others 1997; Yu and Salter 1999). Both Fyn and Src have been implicated in playing an important role in LTP. NR2B phosphorylation is enhanced after the induction of LTP in the CA1 region of the hippocampus (Kojima and others 1997; Nakazawa and others 2001), whereas PTK-specific inhibitors of the Src family prevent it (Lu and others 1998). In addition, LTP is blunted in mice in which the fyn gene was deleted (Grant and others 1992). Tyrosine phosphorylation of NR2 subunits has also been found to regulate the trafficking of the subunits in striatal neurons (Dunah and Standaert 2001) and is elevated in different in vitro and in vivo paradigms. For example, tyrosine phosphorylation of the NR2 subunits is observed in taste aversion learning (Rosenblum and others 1997), during transient global ischemia (Takagi and others 1997), and after acute injection of hypnotic doses of ethanol as described in section 8 (Miyakawa and others 1997; Yaka, Phamluong, and others 2003).

The serine/threonine kinases, cAMP-dependent PKA, PKC, calcium-/calmodulin-dependent protein kinase II (CaM kinase II) and cyclin-dependent protein kinase 5 (CDK5), also phosphorylate the NMDAR subunits at different sites (Fig. 2). PKA phosphorylates the NR1 subunit on residue 897 (Leonard and Hell 1997; Tingley and others 1997), and this phosphorylation has recently been found to be essential for dopamine D1 receptor (D1R)-mediated phosphorylation of the transcription factor CREB (Dudman and others 2003). In addition, activation of postsynaptic PKA has been reported to enhance channel activity (Raman and others 1996) and to be important for the expression of LTP in the CA1 region of the hippocampus (Duffy and Nguyen 2003).

Another kinase that specifically phosphorylates the NR1 subunit is PKC. PKC phosphorylates serine residues 890 and 896 on the C1 cassette of the NR1 subunit (Leonard and Hell 1997; Tingley and others 1997), and this regulates the export of the NR1 subunit from the endoplasmic reticulum and the corresponding forward trafficking of the subunit to the synapse (Scott and others 2001). PKC phosphorylation of NR2A on serine 1416 reduces CaM kinase II binding to NR2A (Gardoni and others 2001), and phosphorylation of NR2B on residues serine 1303 and 1323 has been implicated in the enhancement of channel function (Liao and others 2001). Finally, PKC activation indirectly induces the tyrosine phosphorylation of NR2A and NR2B (Lu and others 1999; Grosshans and Browning 2001) via the activation of the calcium-dependent tyrosine kinase Pyk2/Cak2 β (Huang and others 2001).

CaM kinase II also phosphorylates NR2B; its activation is directly linked to the activation of the NMDAR channel and the entry of calcium ions (Bayer and others 2001; Leonard and others

2002). This kinase directly interacts with NR2B (Bayer and others 2001) and phosphorylates the subunit on serine 1303, a site shared with PKC (Omkumar and others 1996). It is unclear, however, whether both kinases phosphorylate the same site in vivo and whether phosphorylation on this site leads to the same or different functional consequences. Finally, CDK5 has recently been shown to be activated on fore-brain ischemia resulting in the phosphorylation of NR2A on serine 1232, leading to CA1 hippocampal neuronal cell death (Wang and others 2003).

As mentioned above, the phosphorylation state of a protein is a balance between the activity of a kinase and a phosphatase, and thus phosphatases also play a critical role in regulating the activities of the NMDAR. The serine and threonine phosphatases, calcineurin (also known as PP2B) and PP1, are highly important for the regulation of the phosphorylation state and thus functions of NMDARs (for review, see Groth and others 2003). Calcineurin requires binding of calcium/calmodulin for its activation and in turn regulates the activity of PP1 via dopamine and cAMP-regulated phosphoprotein (molecular weight 32 kDa) (DARPP-32) as described in section 9.

Phospho-tyrosine residues of the NMDARs are dephosphorylated by protein tyrosine phosphatases (PTPs) (Wang and Salter 1994). PTP δ , for example, was found to negatively regulate LTP, suggesting that it inhibits NMDAR activity by dephosphorylating the channel (Uetani and others 2000). Finally, the striatal-enriched tyrosine phosphatase inhibits receptor activity, whereas inhibition of the phosphatase enhances NMDAR-mediated currents in the CA1 region of the hippocampus; this suggests a role in dephosphorylation of the NMDAR itself (Pelkey and others 2002).

Importantly, the balance between the phosphorylation and dephosphorylation state of the NMDAR subunit depends on highly regulated signaling events, and an example of such a signaling pathway is described in the study by Raman and others (1996). The researchers found that in hippocampal neurons, constitutively active PKA phosphorylates the channel during quiescent conditions. However, activation of the NMDAR and subsequent calcium entry activates calcineurin, which dephosphorylates serine residues on NMDAR leading to the reduction of NMDAR-mediated excitatory postsynaptic currents (EPSCs). Conversely, activation of the cAMP/PKA pathway via the Gas protein-coupled receptor (GasPCR) results in phosphorylation of the channel to a degree that overcomes calcineurin's activity, leading to enhancement of NMDAR-mediated EPSCs.

Regulation of NMDAR Function via Protein-Protein Interactions

The NMDAR is the core component of the postsynaptic density (PSD) structure (for reviews, see Kennedy 1997, 2000; Sheng and Kim 2002). The PSD is a well-organized structure within postsynaptic membranes of glutamatergic neurons that contains—in addition to the NMDAR, kinases, phosphatases, cytoskeletal proteins, and small G proteins—adhesion molecules and a large number of scaffolding/anchoring proteins. Scaffolding proteins are essential for the orchestration of activity and specificity of signaling proteins within the PSD, for the assembly of kinases and phosphatases in close proximity with the NMDAR, and for connecting the NMDAR to the cytoskeleton (for reviews, see O'Brien and others 1998; Husi and others 2000; Walikonis and others 2000). More than 80 proteins have been identified within the PSD complex (Husi and others 2000; Walikonis and others 2000), and the orchestration of their interactions is beginning to unravel. In this review, I concentrate on proteins that directly interact with the cytoplasmic tail of the NMDAR subunits (Fig. 3). The major scaffolding protein in the PSD is PSD-95 (for review, see Gomperts 1996). PSD95, as well as its homologous proteins PSD93, SAP97, and SAP 102, directly interacts with NR2A and NR2B via its first two PDZ domains (Fig. 3). The PSD95/NR2A/B association has been extensively

studied and is thought to be important for the compartmentalization of the NMDAR complex within the PSD, for channel clustering, and for the recruitment of signaling proteins in close proximity to the NMDAR (Kornau and others 1995; Niethammer and others 1996; Bassand and others 1999). However, a recent study by Lim and others (2003) used peptides that disrupt the PSD95/NMDAR interaction, and the authors reported a moderate inhibition of clustering of the receptor but no changes in NMDAR-mediated currents, suggesting that the interaction may not be important for the short-term activity of the channel (Lim and others 2003).

Another example of an anchoring protein important for the regulation of the phosphorylation state and function of the NMDAR is Yotiao. Yotiao is a 230-kDa protein that was identified by a two-hybrid screen for NR1-interacting proteins (Lin and others 1998). Yotiao was also identified as a PKA-anchoring protein (A kinase anchoring protein) that associates with PP1 (Westphal and others 1999). Yotiao interacts with active PP1, and thus under resting conditions, the phosphatase keeps NR1 in a dephosphorylated state. However, when the cAMP/PKA signaling pathway is activated, the catalytic subunit of PKA phosphorylates NR1 to overcome the activity of the phosphatase, leading to PKA-mediated enhancement of channel activity (Westphal and others 1999).

As mentioned in section 3, the scaffolding protein RACK1 also interacts with the NMDAR (Yaka and others 2002), and the association between RACK1 and the NMDAR is subunit specific. Only NR2B binds RACK1, unlike NR2A (Fig. 4A) and NR1 (Yaka and others 2002). NR2B is a substrate of Fyn kinase, and RACK1 simultaneously binds both substrate and kinase. RACK1 prevents the ability of the kinase to phosphorylate the channel, leading to the inhibition of NMDAR-mediated excitatory postsynaptic potentials (Yaka and others 2002). The release of RACK1 from the NMDAR complex via activation of the cAMP/PKA pathway allows Fyn to specifically phosphorylate NR2B, and this phosphorylation in turn enhances the function of the channel (Yaka, He, and others 2003).

Other proteins that directly interact with the NMDAR are kinases such as CaM kinase II and cytoskeleton-associated proteins (Fig. 3). CaM kinase II directly interacts with NR2B, and the interaction results in the facilitation of the translocation and targeting of the kinase and in the maintenance of calcium and calmodulin-independent kinase activity, leading to NR2B phosphorylation (Bayer and others 2001). The NMDAR is linked to the cytoskeleton at least in part via the cytoskeleton-associated proteins α -actinin 2 and spectrin, which interact with both NR2B and NR1 subunits (Wyszynski and others 1997; Wechsler and Teichberg 1998). In the presence of calcium, calmodulin competes with and prevents α -actinin 2 from binding to NR1, presumably leading to NMDAR rundown and to redistribution of the channel (Wyszynski and others 1997).

Ethanol and NMDAR

The NMDAR system has been implicated in disease states associated with alcohol abuse including withdrawal, craving, and relapse, and the NMDARs are major mediators of the development of tolerance and dependence to ethanol (for review, see Kumari and Ticku 2000). For example, studies in rodents show that various NMDAR antagonists block the development of rapid tolerance (Khanna and others 1993), decrease ethanol intake (Holter and others 2000), and reduce the intensity of ethanol withdrawal symptoms (for review, see Bisaga and others 2000). (For a detailed review of ethanol-mediated behavior paradigms, see Olive and Ron 2003). Evidence that NMDARs are also involved in craving and/or relapse includes the finding that the NMDAR is a site of action of acamprosate, one of the few drugs with anticraving properties used for human alcoholics (Spanagel and Zieglansberger 1997). In addition, long-term administration of an NMDAR antagonist decreases ethanol intake in a relapse model (Holter and others 2000). Because the NMDAR is one of the major sites of action

of ethanol, understanding the molecular mechanisms that mediate the actions of ethanol on the NMDAR are of great interest.

The expression level of NMDAR subunits upon prolonged exposure of cells of rodents to ethanol has been extensively investigated. Numerous studies report an upregulation of NMDAR subunits on chronic administration of ethanol, and this upregulation has been linked to the hyperactivation of NMDAR channel observed upon ethanol withdrawal (for review, see Kumari and Ticku 2000). However, thus far, the results have not been entirely conclusive. For example, chronic ethanol was found to increase the levels of NR2B in the forebrain but not cerebral cortex (Narita and others 2000), in contrast to other studies that found that long-term voluntary ethanol intake increases the NR2B subunit in the cortex (Henniger and others 2003) and in cortical neurons (Kumari and Ticku 1998). Five days' incubation of cortical neurons with ethanol also resulted in increased level of NR1 spliced variants containing the C2' cassette (Kumari 2001). A more recent study suggests that the expression level of NR1, but not NR2, is altered in the rat amygdala after chronic exposure to ethanol (Floyd and others 2003). Taken together, these results suggest that ethanol alters NMDAR function differently in various brain regions by changing the subunit composition of the channel. This is likely to be due to alteration in the signaling pathway upon exposure to ethanol, as described in section 8.

The actions of ethanol on channel activity were also determined by electrophysiology measurements. Acutely, ethanol has been shown to inhibit NMDAR responses in a slice preparation (Lovinger and others 1989) and in vivo (Simson and others 1991). In addition, ethanol also inhibits hippocampal LTP (Morrisett and Swartzwelder 1993), and these actions could explain the episodes of amnesia after alcohol binge drinking (White and others 2000). Several studies conducted in transfected fibroblasts, HEK293 cells, and *Xenopus* oocytes (frog eggs) have alluded to the possibility that different combinations of NMDAR subunits show differing degrees of ethanol sensitivity. For example, combinations of NR1 with either NR2A or NR2B are more sensitive to ethanol compared to combinations that include either NR2C or NR2D. In addition, transfection of Fyn, but not Src, rescued cells expressing NR1/NR2A, but not NR1/NR2B, from inhibition by ethanol (for review, see Allgaier 2002). Although taken together, these findings and others suggest that the effect of ethanol on the NMDAR depends on subunit composition, these types of studies should be interpreted with caution because fibroblast cells and frog eggs are missing essential neuronal-specific proteins and cofactors that influence the activity of the NMDAR in the absence and presence of ethanol.

Ethanol's inhibitory effects on NMDAR channel activity suggest a direct interaction between ethanol and the NMDAR. Indeed, early studies implied that ethanol attenuates the activity of the NMDAR channel by reducing the potency of the coagonist glycine (Hoffman and others 1989; Rabe and Tabakoff 1990). Other studies in heterologous systems, such as transfected HEK293 cells and *Xenopus* oocytes, suggest other sites of direct interactions of ethanol with NMDAR subunits (Peoples and Weight 1995; Peoples and Stewart 2000; Ronald and others 2001; Ren and others 2003). In addition, indirect mechanisms strongly influence the short- and long-term effects of ethanol on the NMDAR. For example, in a series of studies in which the inhibitory effects of ethanol were determined in *Xenopus* oocytes expressing different deletion mutants of NR1, the C0 domain was found to be important for conferring ethanol inhibition of the NMDAR channel (Mirshahi and others 1998). Interestingly, the C0 domain is the site that mediates the interaction between NMDAR and both the cytoskeleton protein α -actinin-2 and calmodulin (Fig. 3) (Zhang and others 1998; Krupp and others 1999; Leonard and others 2002), suggesting that ethanol's actions may result in changes in the interaction between the NMDAR and its binding partners, as described in section 9.

Ethanol and Posttranslation Modifications

The discovery that posttranslation modifications are important in the regulation of the NMDAR raised the possibility that ethanol's actions are mediated, at least in part, by changing the activities of kinases and phosphatases. Indeed, activation of PKC in cerebellar granule cells was found to decrease the potency of glycine binding to the NMDAR, resulting in an inhibition of NMDAR activity similar to that of ethanol (Snell and others 1994). These results suggest that activation of a PKC isozyme by ethanol contributes to ethanol's effects on the NMDAR channel function. Interestingly, a recent report suggested that the priming of the NMDAR for endocytosis is due to the interaction of the NMDAR with glycine (Nong and others 2003), raising the possibility of ethanol's involvement in NMDAR internalization.

Several groups have been investigating the effects of ethanol on the phosphorylation state of NMDAR subunits. In cortical slices, an increase in phosphorylation of NR1 on serine 897 and a decrease of tyrosine phosphorylation of NR2 subunits were observed (Ferrani-Kile and others 2003). This decrease was reversed in the presence of tyrosine phosphatase inhibitors, suggesting the activation of tyrosine phosphatase(s) in the presence of ethanol (Ferrani-Kile and others 2003). Alvestad and others (2003) reported that exposure of hippocampal slices to ethanol results in decreased basal phosphorylation levels of the NMDAR via activation of a tyrosine kinase phosphatase. However, we, and others, have found that in hippocampal but not cortical neurons, acute ethanol exposure results in a specific increase in tyrosine phosphorylation of NR2B by Fyn (Miyakawa and others 1997; Kalluri and Ticku 1999; Yaka, Phamluong, and others 2003). A possible explanation for these apparently conflicting results is a scenario in which ethanol alters the activity of a kinase or a phosphatase depending on the activation state of the neurons and the basal phosphorylation state of the NMDAR subunit. Hence, in the presence of ethanol, nonphosphorylated NR2B may be phosphorylated by Fyn; however, when phospho-NR2B levels are already high under basal conditions, ethanol activation of a phosphatase may overcome the activity of Fyn, leading to dephosphorylation of the subunit.

Ethanol and Compartmentalization

From the studies described in sections 7 and 8, it is clear that ethanol acts differently in diverse brain regions, on different subunit compositions, and via various signaling pathways. The question arising from these observations is how specificity in ethanol's actions can be achieved. The following two examples describe how the defined compartmentalization of proteins in different brain regions determines the specific actions of ethanol.

RACK1

As described in section 6, RACK1 is a scaffolding protein that localizes Fyn in close proximity to NR2B but inhibits the ability of Fyn to phosphorylate the channel until the appropriate signal occurs (Yaka and others 2002; Yaka, He, and others 2003). Interestingly, formation of this trimolecular complex is not ubiquitously observed in the brain. In the hippocampus, RACK1 is localized at the plasma membrane, where it scaffolds Fyn to the NMDAR (Fig. 4B). However, in the cortex, RACK1 is found mainly in the cell soma (Fig. 4B), and therefore it is not capable of compartmentalizing Fyn with the NMDAR (Yaka, Phamluong, and others 2003). This specific compartmentalization of Fyn to the NMDAR complex via RACK1 has broad implications for the actions of ethanol on the channel. In the hippocampus, ethanol treatment results in the release of RACK1 from the NMDAR complex in a mechanism that depends on the activation of the cAMP/PKA signaling cascade (Yaka, Phamluong, and others 2003) (Fig. 5A). The dissociation of the trimolecular complex enables Fyn to phosphorylate NR2B (Yaka, Phamluong, and others 2003). These changes lead to phosphorylation-dependent enhancement of NMDAR channel activity during exposure to ethanol, to a rebound potentiation of the

channel activity when ethanol is washed out, and to the development of acute desensitization (Yaka, Phamluong, and others 2003) (Fig. 5A). However, in the cortex, because Fyn is not compartmentalized near NR2B (Fig. 4B), ethanol exposure does not result in changes in the phosphorylation state of the subunit (Kalluri and Ticku 1999; Yaka, Phamluong, and others 2003) and ethanol treatment leads only to the inhibition of channel activity but not to rebound potentiation following ethanol washout or to the development of acute desensitization (Yaka, Phamluong, and others 2003) (Fig. 5B). Together, these results suggest that in brain regions where the phosphorylation state of NR2B is regulated by the compartmentalization of Fyn via RACK1, ethanol's actions on the NMDAR channel are the sum of two opposing activities: 1) an increase in activity due to the release of RACK1 and the phosphorylation of NR2B by Fyn and 2) a decrease in activity due to the direct inhibitory activity of ethanol on the NMDAR as previously documented (Lovinger and others 1989). In addition, these results suggest a mechanism by which ethanol alters the phosphorylation state of a specific NMDAR subunit. Finally, this specificity of ethanol activity on the NR2B subunit in some brain regions but not in others is likely to contribute to the behavioral effects of ethanol, such as acute sensitivity to the hypnotic effects of ethanol, that are likely to be mediated via hippocampal Fyn and NR2B (Miyakawa and others 1997; Yaka, Tang, and others 2003).

DARPP-32

DARPP-32 is a 32-kDa protein expressed predominantly in the medium spiny dopaminergic neurons of the neostriatum, and it is an important regulator of numerous functions including the regulation of the NMDAR. The activity of DARPP-32 depends on its phosphorylation state. Activation of the cAMP/PKA pathway leads to DARPP-32 phosphorylation on serine 34, and as a phosphorylated protein, DARPP-32 is a potent inhibitor of the phosphatase PP1 (for a review, see Greengard and others 1999). As described in section 5, activation of the cAMP/PKA pathway also leads to the phosphorylation of NR1 by PKA (Fig. 2), and the phosphorylation contributes to the enhancement of channel activity, whereas PP1 dephosphorylates the subunit. This NR1 phosphorylation, together with the inhibition of PP1 by phospho-DARPP-32, keeps the NR1 subunit in a phosphorylated state (Fig. 6). Activation of the NMDAR and the increase in calcium results in the activation of calcineurin, which dephosphorylates both the NR1 subunit and DARPP-32 and leads to a reduction in channel activity (for a review, see Greengard and others 1999).

Maldve and others (2002) reported that this DARPP-32/PP1 cascade is an important regulatory mechanism for neostriatal NMDARs in the presence of ethanol (Fig. 6). Ethanol, like other drugs of abuse, increases dopamine release in the neostriatum (Koob and others 1998). Maldve and colleagues (2002) found that the activation of the cAMP/PKA pathway via the dopamine D1R leads to both phosphorylation of NR1 and DARPP-32. The combination of NMDAR phosphorylation and the inhibition of PP1 results in the reduction of the inhibitory actions of ethanol on the NMDAR (Fig. 6). Interestingly, ethanol itself (via a mechanism that has not yet been identified) mediates the phosphorylation of DARPP-32, contributing to the enhancement of NMDAR channel activities. Importantly, this disinhibition of NMDAR channel activity via ethanol and the release of dopamine is likely to be an important factor in NMDAR-dependent LTP, a process considered important for long-term neuroadaptations to ethanol (Maldve and others 2002). In addition, Edwards and others (2002) reported that although in vivo administration of a dopamine D1R agonist resulted in an increase in the phosphorylation state of both NR1 and DARPP-32, treatment with dopamine D2 receptor (D2R) agonist (which results in an inhibition of the cAMP/PKA pathway) significantly reduced the activities of the D1R agonist when both drugs were coadministered. Ethanol, however, reversed the inhibitory effect of D2R activation, suggesting that ethanol in the neostriatum is synergizing with dopamine D1R to increase NR1 and DARPP-32 phosphorylation, and by doing so, ethanol is masking the effects of activation of the dopamine D2Rs (Edwards and others 2002). Last, the

contribution of DARPP-32 to ethanol-mediated behaviors was tested using the DARPP-32 deletion mutant mice (DARPP-32^{-/-}) (Risinger and others 2001). DARPP-32^{-/-} mice consume less ethanol compared to the wild-type control mice and are less responsive to the rewarding actions of ethanol as measured by the conditioned place preference paradigm (Risinger and others 2001), further implicating DARPP-32 as an important specific regulator of ethanol's behavioral effects. In summary, although the NR1 subunits are ubiquitously expressed throughout the brain, this kind of process will occur only in DARPP-32/D1R-containing neurons.

Concluding Remarks

In this review, the effects of ethanol on the NMDAR system were used as an example of how a small diffusible molecule such as ethanol can act differently in various neuronal populations. In both examples (RACK1 and DARPP-32), ethanol alters signaling cascades that regulate the phosphorylation state and activity of the NMDAR, and these have important implications for ethanol-mediated behaviors. In one example (RACK1), the compartmentalization of signaling and scaffolding proteins within neurons determines the actions of ethanol. The second example (DARPP-32) suggests that the restricted expression of a protein within specific populations of neurons has significant implications for the actions of ethanol. These examples are clearly not limited to the NMDAR system but are likely to determine how ethanol regulates its targets in general. It is very plausible, however, that the activities of ethanol within brain regions are much more refined and are likely to be on the level of different populations of neurons within a specific neuroanatomical area depending on the varying composition of genes or pre- or postsynaptic proteins within different neurons. Recent advances in technologies allowing the identification of genes within single neurons, as well as proteomics, which allows the determination of large-scale protein networks, will allow us to explore these possibilities in the future.

Acknowledgements

Work described in the article is supported by funds provided by the state of California for medical research on alcohol and substance abuse through the University of California, San Francisco, and by the National Institute on Alcohol Abuse and Alcoholism (R01AA/MH13438-O1A1). The author thanks Dr. Richard Morrisett of the University of Texas at Austin for the DARPP-32 diagram, Steven Messing and Lisa Daitch for technical assistance, and Drs. Patricia Janak, Rachel Jurd, Claire Thornton, and Rami Yaka for careful reading of the manuscript. Thanks also to Merck Sharp and Dohme, Essex, England, for providing the Ltk-cell lines and Khanhky Phamluong and Dr. Viktor Kharazia for generating Figures 4a and 4b, respectively.

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Fig. 1.

Ethanol alters the function of a defined number of targets. Depicted is the structure of ethanol (*center*) and examples of its direct and indirect protein targets. These include ligand-gated ion channels such as the *N*-methyl-D-aspartate (NMDA) and GABA_A receptors; voltage-gated ion channels such as calcium and potassium ion channels; the adenosine transporter; kinases such as protein kinase A (PKA), protein kinase C (PKC), and Fyn; and transcription factors such as c-fos.

Fig. 2.

The *N*-methyl-D-aspartate (NMDA) receptor subunits are phosphorylated on serine and tyrosine residues. Depicted are the amino acid sequences of the cytoplasmic tails of rat NR1, NR2A, and NR2B subunits. The N-terminus faces the membrane, and the C terminus is facing inward. The kinase is depicted on the top of each rectangle, and the amino acid residue is depicted at the bottom. PKC, protein kinase C; PKA, protein kinase A.

Fig. 3.

Compartmentalization of proteins with the *N*-methyl-D-aspartate receptor (NMDAR) via a direct association. Depicted are the direct interactions of proteins with the NR1 or NR2 subunits. These include scaffolding proteins of the postsynaptic density (PSD) 95 family, which include PSD95, PSD93, SAP97, and SAP102. Other scaffolding proteins that interact directly with the NMDAR are RACK1 (NR2) and Yotiao (NR1). Included also are kinases such as CaM kinase II (NR1, NR2) and the tyrosine kinase cAbl (NR2D only), the regulatory protein calmodulin (NR1), and cytoskeleton-associated proteins neuronal intermediate filament (NFL) that interact with NR1, spectrin that binds NR2, and α -actinin that associates with both. Not shown is the large group of proteins that indirectly associate with the NMDAR.

Fig. 4.

A, Anti-RACK1 antibodies co-immunoprecipitate NR2B and Fyn but not NR2A from L(-tk) cell homogenates. Immunoprecipitations were carried out in L(-tk) cells, homogenates generated from cells that stably expressed NR1 together with NR2B or NR2A (kindly provided by Merck Sharp and Dohme, Essex, England). Immunoprecipitations were performed using mouse monoclonal IgM anti-RACK1 antibodies (lanes 1 and 4) and antimouse IgM antibodies (lanes 3 and 6). The control mouse IgM and anti-RACK1 antibodies in the absence of the immunoprecipitated protein are also included (lanes 7 and 8). Samples were separated on a 10% SDS-PAGE gel and probed with anti-NR2A (*first panel*), anti-NR2B (*second panel*), anti-Fyn (*third panel*) and anti-RACK1 antibodies (*fourth panel*). The presence of RACK1, Fyn, NR2B, and NR2A in L(-tk) cell homogenates was verified by Western blot analysis (lanes 2 and 5). As shown in the figure, anti-RACK1 antibodies co-immunoprecipitated Fyn but not NR2A from cell homogenates (lane 1); however, the trimolecular complex was apparent in RACK1 immunoprecipitations from L(-tk) cells expressing NR1/NR2B (lane 4).

Representative of three experiments. **B**, RACK1 is compartmentalized to different sections of the hippocampus and cortex. Slices that were 35-µm thick were obtained from 2-month-old male C57Bl/6 mice. Immunoperoxidase staining for RACK1 in the neocortex (*left*) and CA1 region of hippocampus (*right*) were obtained with an upright Nikon E600 microscope, with the SPOT-2 Digital Imaging System (Technical Instrument, San Francisco, CA). Cell bodies in the neocortex are densely stained. However, in most CA1 pyramidal cells, only a thin band of RACK-1 immunoreactivity can be detected near the cell's plasma membrane. Some interneurons and glial cells in both the cortex and hippocampus show dense somatic staining.

Fig. 5.

Specific scaffolding of Fyn to the *N*-methyl-D-aspartate receptor (NMDAR) determines ethanol modulation of channel function. *A*, In the hippocampus, RACK1 scaffolds Fyn to the NR2B subunit but prevents the phosphorylation of the subunit by Fyn (*left*). Ethanol, in addition to the direct inhibition of NMDAR function, causes the release of RACK1 from NR2B and Fyn and promotes an enhancement of channel activity via tyrosine phosphorylation of the NR2B (*right*). *B*, In the cortex, Fyn is not compartmentalized to the NMDAR (*left*). Ethanol directly inhibits NMDAR function, but NR2B phosphorylation is not affected (*right*). As a result, ethanol-induced inhibition of NMDAR function is greater in the cortex than in the hippocampus.

Fig. 6.

Phospho-DARPP-32 reduces ethanol inhibition of *N*-methyl-D-aspartate receptor (NMDAR) channel activity. Activation of G α s-coupled dopamine D1 receptors (D1Rs) leads to the activation of adenylate cyclase and subsequent generation of cAMP leading to the activation of protein kinase A (PKA). PKA in turn phosphorylates both the NR1 subunit and DARPP-32, which inhibits the activity of the phosphatase PP1. The combination of kinase phosphorylation and phosphatase inhibition leads to an enhancement of NMDAR activity that negates the direct inhibition of the channel by ethanol.