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The Neurobiology of Alcohol Consumption and Alcoholism: An Integrative History¹

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Abstract

Studies of the neurobiological predisposition to consume alcohol (ethanol) and to transition to uncontrolled drinking behavior (alcoholism), as well as studies of the effects of alcohol on brain function, started a logarithmic growth phase after the repeal of the 18th Amendment to the United States Constitution. Although the early studies were primitive by current technological standards, they clearly demonstrated the effects of alcohol on brain structure and function, and by the end of the 20th century left little doubt that alcoholism is a “disease” of the brain. This review traces the history of developments in the understanding of ethanol’s effects on the most prominent inhibitory and excitatory systems of brain (GABA and glutamate neurotransmission). This neurobiological information is integrated with knowledge of ethanol’s actions on other neurotransmitter systems to produce an anatomical and functional map of ethanol’s properties. Our intent is limited in scope, but is meant to provide context and integration of the actions of ethanol on the major neurobiologic systems which produce reinforcement for alcohol consumption and changes in brain chemistry that lead to addiction. The developmental history of neurobehavioral theories of the transition from alcohol drinking to alcohol addiction is presented and juxtaposed to the neurobiological findings. Depending on one’s point of view, we may, at this point in history, know more, or less, than we think we know about the neurobiology of alcoholism.

Keywords

Alcohol research history; alcoholism; glutamate; GABA; mesolimbic dopamine system; reward; addiction

I. Introduction

A. Is the Brain the Instigator or Victim of Alcoholism?

Thomas Trotter and Benjamin Rush (in Great Britain and the U.S.A. respectively), were among the pioneers in conceptualizing excessive consumption of alcohol as a “disorder” rather than sinful behavior. Trotter described “the habit of drunkenness” as “a disease of the

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will” (Trotter, 1988). Rush described drunkenness as a disease in which alcohol was the causal agent, loss of control over drinking behavior the characteristic symptom, and total abstinence the only effective cure (Rush, 1808). Without much mention of Rush’s earlier conceptualization of “alcoholism” (a term actually introduced by Magnus Huss in Sweden (Huss, 1849)), temperance leaders revived the notion that alcohol itself was the root of alcoholism and pushed for prohibition of production, sale and distribution of alcohol. Their efforts resulted in the ratification of the Eighteenth Amendment to the U.S. Constitution in 1920 and the beginning of the period of prohibition. This period lasted until 1932 and much reduced scientific inquiry into the nature of alcoholism (if the causative agent is eliminated why study its effects?).

Jellinek et al. (1940) summarized the state of knowledge of the effects of alcohol on the individual as of 1939. In that review, the effects of alcohol (ethanol) on the brain were presented in terms of pathologic consequences as initially described by Wernicke (1881) and Korsakoff (1890a, 1890b). However, during the subsequent twenty years the concept of alcoholism as a disease related to loss of control over alcohol consumption became codified (Jellinek, 1960). In reading Jellinek’s conceptualization of the “disease” of alcoholism and his subtyping of alcoholics, one can assume that the behavioral anomalies that are being described are a product of a malfunctioning brain. In the first edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM) published in 1952, as well as in the second edition published in 1968, the American Psychiatric Association (APA) classified alcoholism among personality disorders and other non-psychotic disorders, implying that aberrant levels of alcohol ingestion were secondary to a personality disorder or response to negative life events. This view was supported by the work of Lawrence Kolb which started in 1923 and is summarized in his book published in 1962 (Kolb, 1962). Kolb’s work with humans and monkeys indicated that addiction was a product of psychopathy and preexisting personality disorders. Thus, excessive alcohol use was viewed as a symptom of some other psychiatric disorder. In other words, diseases of the brain, other than those specifically driving alcohol consumption, were the culprits producing alcoholism.

Alan Leshner (a past Director of NIDA) authored an article in 1997 entitled “Addiction is a Brain Disease: and it Matters” (Leshner, 1997). Leshner focused attention on addiction as a result of drug action on the brain and drug-induced changes in brain function (neuroadaptations) as the underpinning of the behavioral state labeled as addiction. This view has remained popular with neurobiologists and the brain has been considered as the victim of drug consumptive behaviors. But, why take drugs in the first place? It is of some importance that Western culture, including the writings in the Bible (Wallace, 2004), has long distinguished between drinking alcohol in “celebration” (which is condoned if not encouraged), and allowing alcohol to control a person, enslaving him/her to drinking to the point of drunkenness. But drinking in “celebration” is not necessarily an innocuous event. Alcohol drinking engages neurobiological systems that generate pleasure (or in some people aversion), and the extent of pleasure, the desire to continue to receive such pleasure and the untoward results, i.e., addiction, that are evident in a subset of drinking individuals are a result of ethanol’s ability to change the neurochemical processes of brain. Thus, the brain is both an instigator and victim.

B. Alcohol Consumption versus Addiction

Both genetic and environmental factors play important roles in initiation of alcohol drinking and progression to alcoholism. Additionally, the motivational mechanisms leading to an animal’s initial consumption of alcohol are different from the motivation (or lack thereof) in an animal already well experienced with effects of having consumed ethanol, and again quite different from the motivation for drinking in an animal addicted to ethanol and/or having experienced the signs of withdrawal. Thus, the neuroscience of alcohol drinking has

many separate facets and is intimately intertwined with the neuroscience of motivation. If one considers that complex normal motivational components of voluntary behavior, as well as pathological behavior, are a product of brain activity, then books such as Gene Heyman's "Addiction: a Disorder of Choice" (Heyman, 2009) do not represent an attack on the concept of alcoholism as a brain disease. Heyman describes addiction as a "voluntary operant behavior that results from a temporally myopic view of available alternatives in a choice situation". This description sounds like the criterion dealing with the "narrowing of repertoire" component of the diagnosis of alcoholism in the DSM IV.

II. Alcohol Metabolism: Does it Occur in Brain?

The consideration of alcohol as an addictive drug is more complicated than the consideration of other addictive substances because alcohol (ethanol) is also a source of calories through its metabolism, and can be considered a food (Yeomans, 2010, Yeomans *et al.*, 2003). The issue of ethanol contributing a caloric motivation to its consumption has a significant impact on research using "simple" animal models to investigate mechanisms driving excessive drinking (Pohl *et al.*, 2012).

The subject of alcohol metabolism by brain remains controversial to this day. The initial studies to measure brain metabolism of ethanol were performed in systems using brain tissue homogenates and measures of oxygen consumption in a Warburg apparatus, and also by trying to spectrophotometrically measure the conversion of NAD to NADH (see references in Tabakoff and Gelpke, 1975). These studies produced equivocal or negative results. More recent work has used in vivo imaging of brain ethanol to measure metabolism in brain tissue of humans (Xiang and Shen, 2011), and these studies also produced negative results.

A. Acetaldehyde and Condensation Products: Role in Addiction?

Although significant evidence exists that production of the intermediate of alcohol metabolism, acetaldehyde, within the brain is low, or nonexistent, the entry of acetaldehyde into brain from periphery has been another well investigated option for theories reliant on having significant levels of acetaldehyde in brain (Duritz and Truitt, 1966). That acetaldehyde produced in peripheral organs can be a source of brain acetaldehyde has been hotly debated for 30 years (Eriksson, 1983, 2001, 2007, Eriksson and Sippel, 1977), but the opinion on this issue keeps shifting (Deng and Deitrich, 2008). The continued interest in the metabolism of ethanol in brain or the entry of acetaldehyde into brain through circulation is in great part due to the proposals that acetaldehyde, could, in itself, generate reinforcing effects in animals, or could condense with dopamine (and other biogenic amines) in brain to form compounds (e.g., salsolinol) that mediate the transition from controlled drinking to alcoholism. This idea was first introduced by Gerald Cohen and Michael Collins (Cohen and Collins, 1970) and by Virginia Davis (Yamanaka *et al.*, 1970). Collins & Bigdeli (1975) later introduced evidence for salsolinol formation in brain of ethanol-treated animals. Davis also proposed another route for formation of condensation products. Dopamine is metabolized to an aldehyde by monoamine oxidase, and Davis (Davis *et al.*, 1970) demonstrated, in *in vitro* experiments, that acetaldehyde blocked the conversion of the aldehyde derived from dopamine to an acid, by inhibiting aldehyde dehydrogenase. She proposed that the resultant increase in the dopamine derived-aldehyde could instigate the formation of a condensation product between the dopamine-derived aldehyde and dopamine itself to form tetrahydropapaveroline (THP) (Davis *et al.*, 1970). The fact that THP was a precursor of the opioid alkaloids, such as morphine in the opium poppy, drew speculation on possible relations between alcohol and opiate addiction (Myers, 1978). These condensation products (particularly THP) were found by Myers and Melchior (Myers and Melchior, 1977) to promote drinking if administered into brain, but a careful search for endogenous formation of THP and salsolinol or other such compounds in the brain of alcohol intoxicated

animals found no evidence for these compounds being produced at levels amenable to detection by mass spectroscopy (Lee *et al.*, 2010, Shier *et al.*, 1983, Smythe and Duncan, 1985). The conclusion in the 1990's was that such condensation products do not appear in brain during intoxication at levels compatible with concentrations found to be necessary for demonstrated receptor interactions (Britton *et al.*, 1982). The idea that salsolinol is formed, *in vivo*, in an alcohol-consuming animal, is still being pursued (e.g., Deehan *et al.*, 2013), but any link to its being an endogenously formed mediator of alcohol consumption is tenuous.

III. The Molecular Site of Ethanol's Action in the CNS

A. Lipids

At approximately the beginning of the 20th century, a novel conceptualization of the actions of sedative/hypnotics, including ethanol, was introduced. This conceptualization was based on the amphiphilic properties of ethanol (i.e., having both polar and non-polar characteristics). Meyer (1899) and Overton (1901) proposed that the intoxicating effects of ethanol (as well as other anesthetics) derived from their physical chemical properties and their ability to partition into the lipid components of a mixture of oil and water. The ability of ethanol to integrate itself into lipids was extended to the lipid milieu (bilayers) of biological membranes and particularly the plasma membranes of neurons. This conceptualization of ethanol's actions equated ethanol with other anesthetic agents, i.e., ethanol and other anesthetics produce their effects via disruption of the order of brain membrane lipids. The work of Meyer and Overton remained influential for almost 100 years and sophisticated experiments using ethanol and other aliphatic alcohols have been performed. These have been reviewed by Goldstein (1984) and Harris and Hitzemann (1981). Although ethanol was clearly demonstrated to perturb the order of membrane lipid structure, and the effect of ethanol was in some studies correlated with perturbation of function of the proteins residing in the membrane (e.g., voltage sensitive sodium channels (Goldstein, 1984)), the concentrations of ethanol which were necessary to demonstrate effects on membrane lipid order were only compatible with the anesthetic properties of ethanol (i.e., >0.1M ethanol, equivalent to approximately 500 mg%). It was difficult, if not impossible, to ascribe the lower dose effects of ethanol (anxiolytic, disinhibiting, incoordinating, sedative) to membrane lipid disordering effects of ethanol.

B. Proteins

In 1978 Franks and Lieb (Franks and Lieb, 1978) published a paper in the journal, *Nature*, with the provocative title of "Where do general anesthetics act?" The proposal put forth was that general anesthetics (including ethanol), because of their amphiphilic properties, "probably" act on proteins as well as in the lipid domain of neuronal membranes. Over the next several years, Franks and Lieb continued evaluating the site of anesthetics' actions (Franks and Lieb, 1981, 1984, 1987), and the most striking feature of these studies was the demonstration that certain anesthetics, including ethanol, could "competitively" inhibit the binding of a ligand to a protein in a water solution (e.g., luciferin binding to firefly luciferase (Franks and Lieb, 1984)). This experiment indicated that a lipid environment was not necessary for ethanol's actions and it was concluded that "general anesthetics act by competing with endogenous ligands for binding to specific receptors". Another most important point demonstrated by Franks and Lieb (2004) was that only certain proteins are sensitive to the presence of ethanol or other anesthetics. However, it needs to be remembered that the experiments and conclusions of Franks and Lieb (2004) were fully directed at explaining the anesthetic effect of various molecules, and not the lower dose, behavior-modifying effects of such molecules. Franks and Lieb should, however, receive

full credit for bringing attention to the fact that ethanol can be selective in its actions on particular proteins in the nervous system of animals.

Although the work and conclusions of Franks and Lieb caught the attention of several groups investigating the acute effects of ethanol on the nervous system, the progress on identifying the “ethanol sensitive” proteins was slow, because of lack of needed technology, because of existing theories on ethanol’s abilities to perturb water clathrates which surround proteins, and because of the possibility that ethanol still acts “non-specifically” at lipid-protein interfaces (see references in Tabakoff *et al.*, 1988). In the early 1990’s both technology and conceptualization of the issue made progress possible in trying to identify “specific receptor sites” for ethanol.

Definitive studies were finally performed by John Mihic, and others, in the laboratory of Adron Harris (Mihic *et al.*, 1997), who utilized molecular biology techniques of point mutation and creation of chimeric proteins, and demonstrated that certain amino acids and regions of the α subunit of the GABA-A receptor complex were required for ethanol to potentiate chloride flux through this receptor-gated ion channel. A concern that was expressed about these studies was, again, that the concentrations of ethanol (200 mM, 920 mg%) used to show the importance of the protein sequence of the GABA-A receptor for ethanol’s actions were significantly higher than concentrations of ethanol shown by others to be effective at potentiating GABA-A receptor action in electrophysiological and radioactive chloride flux experiments (i.e., 10–20 mM, 46–92 mg%) (Morrow *et al.*, 1988, Reynolds *et al.*, 1992, Suzdak *et al.*, 1986a, Wakita *et al.*, 2012) (but see below). The further development of studies of the direct effects of ethanol on proteins were aided by developments in NMR and protein X-ray crystallography methods (Howard *et al.*, 2011, Vemparala *et al.*, 2010) which allowed examination of the three dimensional structure of proteins and identification of possible ethanol binding sites. One of the initial studies which charted an ethanol binding site on a protein was that of Kruse *et al.* (2003) using the *Drosophila* odorant-sensing protein called LUSH. This study detailed ethanol (30 mM, 138 mg%) binding to 1.5 Å resolution and brought to light the importance of hydrogen bonding between ethanol and a group of hydroxyl containing amino acids within a receptive “pocket” of LUSH. The studies of ethanol intercalating into the three dimensional structure of LUSH (Kruse *et al.*, 2003), and earlier studies of ethanol interactions with alcohol dehydrogenase (Li *et al.*, 1994), have to be considered in the context that these particular proteins have evolved to interact with ethanol (i.e., alcohol dehydrogenase is an oxidoreductase that metabolizes ethanol in prokaryotes and eukaryotes, and LUSH is a protein that is part of a complex for sensing ethanol as a food source by the fruit fly). A more recent effort at determining direct interactions of ethanol with proteins which are seemingly divorced from an evolved role in the metabolism or sensing of ethanol, led back to studies of pentameric (5 subunit-containing) ion channels. Sauguet *et al.* (2013) used an ion channel protein complex derived from *Gloeobacter violaceus*, which resembles in structure the mammalian GABA-A and strychnine-sensitive glycine channels, to generate crystals containing ethanol or 2-bromoethanol. By mutating specific amino acids and solving the structure of these crystals using x-ray diffraction, these authors noted that a cavity, generated by interaction of two subunits of the model protein, accommodated the interaction of ethanol and the protein, and produced a stabilization of a particular conformation of the pentameric protein structure. They speculated that the stabilized structure was the “open” state of the channel protein, and this stabilization of the “open” state was the mechanism by which ethanol potentiated the function of this mutant channel (Sauguet *et al.*, 2013). Speculation aside, these studies point to an important fact: that being, that ethanol may bind at sites of protein-protein interaction, and ethanol’s ability to bind at such protein interaction sites is highly dependent on the amino acid sequence at the protein-protein interface. Below we will discuss the importance of subunit composition of the GABA-A receptor in determining the sensitivity of this

pentameric ligand-gated ion channel to ethanol, and the studies of Sauguet et al. (2013) may be indicating a mechanism by which specific residues on the interface between specific protein subunits of the GABA-A receptor may dictate the sensitivity of this channel to ethanol.

C. Modification of Protein Function by Phosphorylation

1. Protein Kinase A—Protein phosphorylation, as a mechanism for modifying the function of proteins, was first described by Fischer and Krebs (1955), and the discovery of PKC by Nishizuka (Inoue *et al.*, 1977) and protein kinase A (PKA) by D.A. Walsh et al. (1968), set the stage for considering how ethanol could modulate the activity of signaling cascades through protein phosphorylation. The activity of PKA is under the control of a regulatory protein subunit of this enzyme. When cyclic AMP binds to the regulatory subunit, the catalytic subunit is released from inhibition and allows phosphorylation of cognate substrate proteins to alter their function. The generation of cyclic AMP is catalyzed by the enzyme, adenylyl cyclase, which was discovered by Sutherland and Rall (1960, 1962). Ethanol's actions on adenylyl cyclase activity were first reported in Tabakoff and Hoffman (1979). In that report, acute addition of ethanol was shown to potentiate adenylyl cyclase action when the enzyme was stimulated by dopamine acting through the dopamine D₁ receptor. Adenylyl cyclase isoforms, and PKA, have since been implicated in several effects of ethanol (Cruz *et al.*, 2011, Desrivieres *et al.*, 2011, Ferraro *et al.*, 2006, Lai *et al.*, 2007, Maas *et al.*, 2005, Yang *et al.*, 2003). Interestingly, the Tabakoff group also showed that ethanol's action in enhancing the activity of a particular subtype of adenylyl cyclase was mediated by phosphorylation of adenylyl cyclase by PKC δ (Nelson *et al.*, 2003). This observation illustrated cross-talk between cyclic AMP/PKA and PKC-mediated phosphorylation cascades.

2. Protein Kinase C—The investigations of ethanol's neurobiologic actions through modulation of PKC activity have been significantly influenced by the early work of Gordon et al. (1997) showing that ethanol could alter the cellular localization of PKC ϵ and PKC δ , and the work of Slater et al. on PKC α (Slater *et al.*, 1993, 1997, 2004). The translocation of PKCs between various cellular compartments would either remove these enzymes from proximity to their substrate or put them in the proximity of another substrate whose phosphorylation state and activity could be altered. In 1990 Robert Messing (Messing *et al.*, 1990) followed with his first report on ethanol's action on PKC, and through the next 20 years Messing and his collaborators established PKC (and particularly PKC ϵ) as a substantive contributor to ethanol's acute effects on brain function (e.g., Chou *et al.*, 2010, McMahon *et al.*, 2000). Many of these studies benefited from the construction of mutant mice in which PKC ϵ was inactivated by homologous recombination (knocked out). The lack of PKC ϵ was shown to affect the anesthetic effects of ethanol, the consumption of ethanol, the effects of ethanol on body temperature, and several electrophysiological parameters, as well as ethanol's effects on neuropeptide (CRF)-initiated release of GABA at synapses in the amygdala (see references in Bajo *et al.*, 2008, Shirai *et al.*, 2008). These studies also linked PKC ϵ to a mechanism mediating anxiolytic actions of ethanol (Hodge *et al.*, 1999). Certainly the merging of studies of ethanol's binding sites on PKC isoforms (Das *et al.*, 2009, 2007, 2006) and the biochemical, electrophysiological and behavioral results using PKC ϵ knockout mice, provide a corpus of work that cannot be ignored in relation to the mechanism of acute effects of ethanol. We will continue the discussion of protein phosphorylation in the context of ethanol's actions in the following sections focused on particular neurotransmitter/receptor systems.

D. GABA and the Actions of Ethanol

In a 1975 review entitled “Direct effects of ethanol on the nervous system”, Harold Kalant (Kalant, 1975) wrote “Neurophysiological, neurochemical and behavioral studies of the effects of ethanol on the nervous system have so far failed to identify specific, direct, primary mechanisms of action that may account for the typical pattern of alcohol intoxication *in vivo*.” Since that time, research on two of the major neurotransmitter systems in brain, the γ -aminobutyric acid (GABA) and glutamate neurotransmitter systems, has brought us much closer to identifying the primary pathways for ethanol-induced intoxication as well as the addicting characteristics of ethanol.

The interest in GABA as a mediator of ethanol’s action was in part predicated on the discovery of benzodiazepines by Leo Sternbach in 1954 (Lopez-Munoz *et al.*, 2011) and the demonstration of the spectrum of their behavioral effects. Similarities between the behavioral effects of benzodiazepines, which are known to act through sites on GABA-A receptors, and ethanol’s actions were quite obvious (reduce anxiety, produce sedation, anticonvulsant actions, produce tolerance/addiction), and made the GABA-A receptor a prime candidate for mediating ethanol’s actions.

1. GABA Metabolism and Release—The study of ethanol’s effects on brain GABA function began even before the full acceptance of GABA as an inhibitory neurotransmitter in mammalian brain. The earlier studies which simply measured GABA levels (Hakkinen and Kulonen, 1959) were, however, plagued by problems of rapid post-mortem changes in GABA levels, the effects of stress prior to obtaining brain tissue, and possible biphasic effects of ethanol. These early studies were superseded by studies of ethanol’s effects on GABA release using slices of brain maintained in perfusion chambers. These studies (Carmichael and Israel, 1975) demonstrated that ethanol’s effect on electrically stimulated release of GABA was extremely modest, with inhibition occurring only at high, anesthetic concentrations of ethanol. It is of interest that current work indicates that ethanol can modulate GABA release in certain areas of brain, but ethanol’s actions are concentrated on synaptic events which require effects on pre-synaptic signaling, involving modulators of GABA release (e.g., CRF, Kelm *et al.*, 2011, Nie *et al.*, 2004, Roberto *et al.*, 2003, and see below).

2. GABA-A Receptors: Ligand Binding and Function—Studies of ethanol’s actions on GABA-mediated neurotransmission switched abruptly to investigations of ligand binding to the GABA-A receptor complex when radioactive ligands for the various sites on the GABA-A receptor complex became available. A high volume contributor to such studies was Maharaj Ticku, whose laboratory investigated both the *in vitro* effects of ethanol on GABA-A receptor ligand binding and the effects of ethanol administered *in vivo*, on ligand binding measured *in vitro* (Ticku, 1989, Ticku and Burch, 1980, Ticku *et al.*, 1983). The studies from the laboratory of Ticku and a number of other laboratories (e.g., Greenberg *et al.*, 1984, Liljequist *et al.*, 1986, Ramanjaneyulu and Ticku, 1984, Squires *et al.*, 1983) using muscimol, benzodiazepines, picrotoxinin, THIP, etc. to monitor the state of the GABA-A receptor pre-exposed to, or in the presence of ethanol, were rather disappointing. There seemed to be few effects of ethanol on GABA-A receptor ligand binding properties at concentrations of ethanol below 50 mM (230 mg%). Another technique for monitoring the ion channel function of the GABA-A receptor was introduced by the laboratory of Steven Paul. Suzdak *et al.* (1986b) utilized a preparation that was called “synaptoneurosomes” (pinched off microsacs which were composed of pre- and post-synaptic membrane components). These studies monitored the post-synaptic accumulation of radioactive chloride ($^{36}\text{Cl}^-$) by synaptoneurosomes in response to addition of GABA or other GABA-A receptor agonists. Using this system, Suzdak *et al.* (1986b) demonstrated that 10 mM (46 mg

%) ethanol was able to significantly potentiate the effects of the GABA agonist muscimol in increasing chloride flux into synaptoneurosomes, and that at higher concentrations (25–50 mM) ethanol could, without the addition of a GABA-A receptor agonist, increase chloride entry into synaptoneurosomes. The substantial amount of work that followed, by the laboratory of Adron Harris using the synaptoneurosomes preparation (Allan and Harris, 1987, Harris, 1990), left little doubt that ethanol, at non-anesthetic levels, could potentiate GABA actions at the post-synaptic GABA-A receptor in this preparation and hypothetically increase neuronal membrane hyperpolarization produced by GABA (inhibit neuron activation). The evolving technique which could more definitively assess whether ethanol was actually potentiating the neuronal membrane hyperpolarization produced by GABA was patch-clamp electrophysiological recording. This technique became generally available in the early 1980's. The initial work using patch-clamp electrophysiology produced contentious results. An influential negative finding was published by the group led by Forrest Weight (White *et al.*, 1990). They found no effect of ethanol on GABA_A receptors in slices of hippocampus. On the other hand, Reynolds *et al.* (1992) found clear evidence of ethanol potentiation of GABA action when studying slices of brain tissue and dissociated cortical neurons from several species. Some light was shed on the reasons for these differences by Aguayo *et al.* (1990), who noted that sensitivity to ethanol's action may be related to the distribution of particular subtypes of GABA-A receptors in different cell types.

3. GABA-A Receptor Protein Subunits and Ethanol's Action—The two decades between 1980 and 2000 produced a major shift in the scientific perception of the variety and complexity of enzyme and receptor systems. This shift was predicated on the realization that “families” of proteins which ostensibly perform a similar function, can and do contain an assortment of subunits which impart, to the multi-subunit complex, a variety of regulatory and kinetic characteristics. This variety within a protein family is particularly true of receptor-gated ion channels (Luddens and Korpi, 1995, Traynelis *et al.*, 2010). The realization of this complexity and the emergent knowledge of functional ramifications of modest structural differences between protein subunits (isoforms) and between protein complexes of variant subunit composition, gave a new thrust to the search for “specific receptors” and/or novel mechanisms for ethanol's actions.

Significant information has been gathered on the physical characteristics, protein (subunit) composition, and pharmacologic properties of GABA-A receptors. The GABA-A receptor is a pentameric (five subunit) protein which is composed of particular combinations of α (1–6), β (1–3), γ (1–3), δ , ϵ , and θ subunits (Hevers and Luddens, 1998, Mohler *et al.*, 1998). It is currently believed that the Type 1 GABA-A receptors originally classified by Lippa *et al.* (1981) as receptors mediating sedation, correspond to receptors containing α_1 subunits. The α_1 subunit is often part of a receptor complex that also contains β_2 and γ_1 subunits. One of the first attempts to disentangle the contribution of various subunits of the GABA-A receptor to the overall receptor sensitivity to ethanol was the work by Wafford *et al.* (1993, 1991). This work was focused on alternatively spliced variants of the γ_2 subunit. They demonstrated that the transfection of frog oocytes with a combination of α , β and γ_2L (long) subunits produced a GABA-A receptor which showed potentiation of function by ethanol, while transfection with $\alpha\beta\gamma_2S$ (short, which is missing one of the exons contained in the γ_2L subunit) produced a receptor insensitive to ethanol's actions. However, the phenomenon demonstrated in transfected frog oocytes was not demonstrable in mammalian neurons (Harris *et al.*, 1995).

An important observation was reported in 2002 (Sundstrom-Poromaa *et al.*, 2002) which focused attention on GABA-A receptors located outside the immediate area of the synaptic junction concerned with neurotransmission (the extrasynaptic GABA-A receptors (Brickley

and Mody, 2012)) as targets of ethanol's actions. The extrasynaptic GABA-A receptors are considered to be mediators of "tonic" inhibition of neurons on which they reside (this produces a persistent hyperpolarization of membrane potential when these receptors are activated by GABA which spills over from the synaptic cleft (Semyanov *et al.*, 2004)). The "persistent" inhibition by the extrasynaptic GABA-A receptors is quite distinct from the "phasic" (short open time) inhibition generated by the synaptically located form of the GABA-A receptor. The extrasynaptic GABA-A receptors are differentiated by the subunits which compose these receptors. The γ subunits are not found in the extrasynaptic receptor and the δ subunit is a common component of the extrasynaptic receptor along with α_1 , α_4 , or α_6 subunits (Brickley and Mody, 2012).

The presence of the δ subunit within the array of subunits in the GABA-A receptor generates a receptor that is not responsive to benzodiazepines. The δ subunit-containing GABA-A receptor is, however, significantly more sensitive to ethanol (30–50% potentiation by 10 mM (equivalent to 46 mg%) ethanol (Wallner *et al.*, 2003)) than GABA-A receptors containing the γ subunit (the subunit which is necessary for the actions of benzodiazepines). This finding presents a surprising differentiation of the actions of ethanol and benzodiazepines on the GABA-A receptor complex. At the time of writing this manuscript there were sufficient publications on ethanol's actions on δ subunit containing GABA receptors to warrant several reviews (Belelli *et al.*, 2009, Brickley and Mody, 2012, Olsen, 2011, Wallner and Olsen, 2008), and the consensus seems to well support the contention that the δ subunit is an important feature in determining GABA-A receptor sensitivity to ethanol (but dissenting opinion is also available and this issue was discussed in detail in an issue of the journal Alcohol in 2007 (Volume 41).

Prior work had also indicated the importance of the α_6 subunit of the GABA-A receptor in determining GABA-A receptor sensitivity to ethanol. The association of the α_6 subunit characteristics with sensitivity to the effects (incoordinating) of ethanol was extrapolated from studies of cerebellar tissue from AT and ANT rats. These rats have been selectively bred for many generations to demonstrate genetically determined differences in response to the incoordinating effects of ethanol (AT rats show little effect, ANT rats are highly sensitive (Eriksson and Rossi, 1981)). Esa Korpi and his colleagues (Malminen and Korpi, 1988, Uusi-Oukari and Korpi, 1989) discovered an interesting mutation (Korpi and Seeburg, 1993) in the α_6 subunit that distinguished the AT from the ANT rats. This mutation in the α_6 subunit in the ANT rats resulted in GABA-A receptors which were significantly more sensitive to benzodiazepines (Korpi and Seeburg, 1993), and significantly different in the characteristics for the binding of the purported (Suzdak *et al.*, 1986a) "alcohol antagonist", Ro-15-4513 (Uusi-Oukari and Korpi, 1990). These data led Korpi to suggest that the characteristics of the α_6 subunit of the GABA-A receptor were a significant determinant of ethanol's actions in the AT and ANT rats. Hancher *et al.* (Hancher *et al.*, 2005) demonstrated that the mutation found in the α_6 subunit of the ANT rats increased the ethanol sensitivity (measured electrophysiologically) of GABA-A receptors composed of $\alpha_6\beta_3\delta$ subunits, which are already more sensitive to ethanol than receptors containing α , β and γ subunits (for example). In their studies, only the ethanol-sensitive $\alpha_6\beta_3\delta$ receptors showed increased ethanol sensitivity when the mutation was introduced into the α_6 subunit. This study confirmed the sensitivity of extrasynaptic δ -subunit-containing GABA-A receptors (in cerebellum) to ethanol, and also confirmed a role of the mutation in the α_6 subunit in these receptors in enhancing electrophysiological, as well as behavioral (locomotor activity), sensitivity to alcohol. However, the expression of the α_6 subunit is restricted to the cerebellum in humans as well as other animals, and in other brain regions, the role of the δ receptor subunit may play the more pronounced role in the actions of ethanol.

While extrasynaptic $\alpha_6\beta_3\delta$ -containing GABA-A receptors may mediate tonic inhibitory effects of ethanol on neuron function, some recent studies, in which other mutated GABA-A receptor subunit sequences are inserted into specific loci in the mouse genome ("knock-in" mouse mutant), have shown that mutant α_1 subunits reduce ethanol potentiation of receptor function (electrophysiologic), but mice with these receptors are more sensitive to the anxiolytic effect of alcohol (Lobo and Harris, 2008). This finding should be viewed in the context of the classification of α_1 subunit-containing GABA-A receptors as Type 1 sedative-hypnotic receptors (Rudolph *et al.*, 1999), while other α subunit-containing GABA-A receptors have been associated with anxiolytic actions (Atack, 2011, Auta *et al.*, 2010, Heldt and Ressler, 2010, Rudolph *et al.*, 1999). An interpretation of the findings described by Lobo and Harris (Lobo and Harris, 2008) could be that, as the animal becomes resistant to ethanol's effects at the sedative α_1 subunit-containing receptors, the actions at the anxiolytic receptors become more evident. Additional knock-in experiments show that mutated α_2 subunits affect alcohol taste aversion and alcohol stimulation of motor activity (Blednov *et al.*, 2011), and mutated β_3 subunits affect alcohol tolerance and withdrawal signs in mice (Sanchis-Segura *et al.*, 2007). These mutants were generated because null mutant mice in which a gene is "knocked out", not only may have altered responses to ethanol (Crabbe *et al.*, 1996), but these animals also have been shown to have many compensatory changes in gene expression in addition to the change in expression of the targeted gene. However, even the "knock-in" mice display changes in transcription of other genes (Harris *et al.*, 2011), which impacts the interpretation of the knock-in mouse studies. Furthermore, the overall subunit composition of the GABA-A receptors containing the mutated subunit needs to be considered. One crucial aspect is the fact that receptors containing the δ subunit are extrasynaptic, while those that do not contain the δ subunit are synaptically localized. Results obtained with mutated α_1 and β_3 subunits, for example, can involve synaptic and/or extrasynaptic effects that cannot be distinguished. At this point, it seems clear that the presence or absence of the δ subunit within the GABA-A receptor complex dictates the *in vitro* sensitivity to potentiation of extrasynaptic GABA actions by ethanol. The translation of this phenomenon to the spectrum of *in vivo* actions of ethanol is less clear. The exact contribution of the various subunit combinations in particular brain regions to anxiolytic, sedative, anticonvulsant and other actions of ethanol is not yet defined and will require further work.

4. GABA-A Receptor Phosphorylation and Ethanol's Action—Both PKA and various isoforms of PKC can phosphorylate subunits of the GABA-A receptor and modify receptor function (Kumar *et al.*, 2009). As described earlier, protein phosphorylation by PKA and PKC has been implicated in a number of acute alcohol actions, and PKA and PKC have also been implicated in ethanol effects on GABA-A receptor function. Treatment of cultured rat brain neurons with ethanol (50 mM) for 4 hours decreased the surface expression of GABA-A receptor α_1 subunits, and this effect was mediated by the PKC γ isoform (Besheer *et al.*, 2006, Kumar *et al.*, 2012, Kumar *et al.*, 2010, Weiner *et al.*, 1997). In contrast, this group found that PKC γ also mediated ethanol-induced increases in GABA-A receptor α_4 subunit surface expression in cultured neurons (Werner *et al.*, 2011). PKA also affects GABA-A receptor trafficking (Arancibia-Carcamo and Kittler, 2009), and PKA was reported to mediate ethanol (50 mM)-induced increases in GABA-A receptor α_1 subunit surface expression (Carlson *et al.*, 2013, Kumar *et al.*, 2012). These results suggest that ethanol can alter GABA-A receptor function by modulating the subunit composition of GABA-A receptors, through a protein phosphorylation mechanism that affects GABA-A receptor trafficking. Such results could explain previous reports of sensitization of GABA-A receptors to ethanol via PKA activation (Freund and Palmer, 1997) or PKC activation (Weiner *et al.*, 1997). As noted earlier, there have also been numerous studies of ethanol's effects on GABA-A receptors and behavior using PKC null mutant mice. For instance, the

effects of ethanol on GABA-A receptor phosphorylation and function were reduced in brains of mice lacking PKC ϵ (Qi *et al.*, 2007). Besheer *et al.* (Besheer *et al.*, 2006) found that ethanol consumption was not affected by GABAergic compounds in PKC ϵ null mutant mice. It must be kept in mind that compensatory transcriptional effects occur in null mutant mice that can confound interpretation of results. Nevertheless, there is ample evidence (Kumar *et al.*, 2009) that phosphorylation of GABA-A receptor subunit proteins can affect the *in vitro* and *in vivo* effects of ethanol on GABA-A receptor function and cell surface expression, and on behavioral effects of ethanol mediated by GABA-A receptors. Interestingly, an unbiased transcriptional analysis also suggested that GABA-A receptor trafficking is involved in the predisposition to alcohol consumption by rodents and humans (Tabakoff *et al.*, 2009).

5. What Does Ethanol's Action at the GABA-A Receptor Have to Do With Drinking?

—If one accepts the premise that particular subunits of the GABA-A receptor are determinant in the ethanol sensitivity of the GABA-A receptor (potentiation of its inhibitory actions), then one can hypothesize that brain areas containing the δ subunits as part of the GABA-A receptor may be more sensitive to ethanol. The δ subunits are expressed in striatal areas of brain and particularly the nucleus accumbens (NAc), as well as thalamus, cortex, hippocampus and cerebellum (Pirker *et al.*, 2000, Schwarzer *et al.*, 2001). As will be discussed later, the NAc is an important relay station for integrating signals predicting reward, and its output determines behavioral action in response to such reward signals. Nie *et al.* (2011) recently reported that reducing the number of δ subunit containing GABA-A receptors, specifically, in the dorsomedial shell region of the NAc, by injection of RNAi (inhibitory RNA), could reduce alcohol preference in mice. It may seem a bit incongruous that reducing the number of ethanol sensitive GABA-A receptors results in a decrease in ethanol drinking. But, this observation underscores some of the anatomical specificity of ethanol's effects, and ethanol potentiation of the GABA effect in a very circumscribed area of brain may be critical for maintaining ethanol consumption. The shell region of the NAc is heterogeneous in its input and output relationships (Groenewegen *et al.*, 1999, Pennartz *et al.*, 1994). The injection of muscimol (a GABA-A receptor agonist) into rostral sites of the medial shell region of the NAc robustly stimulates food consumption and caloric intake (Richard *et al.*, 2013). Muscimol is considered to act on medium spiny neurons of the medial shell region to inhibit their activity. The medium spiny neurons are GABAergic neurons which innervate the ventral pallidum, the VTA and the lateral hypothalamus (see below for effect of stimulation of the lateral hypothalamus). The mechanism by which the injection of muscimol into the medial shell region of the NAc can produce activation of neurons in the areas targeted by the medium spiny neurons, is referred to as disinhibition, i.e., activation of GABA-A receptors on the medium spiny neurons of the medial shell will inhibit these projection neurons and result in decreased GABA input and disinhibition of neurons in target structures. It is known that the medial shell region of the NAc contains GABA-A receptors which contain the δ subunit of the GABA receptor complex. Thus, if ethanol is present, it can potentiate the effects of GABA specifically in this region, exaggerate the inhibition of the medium spiny neurons and produce a more intense disinhibition of neurons in areas such as the lateral hypothalamus, VTA and ventral pallidum. The effects of such disinhibition can well be related to increased consumptive behavior as reported by Richard *et al.* (2013), and, conversely, to the decrease in consumption of ethanol in mice in which the δ subunit of the GABA-A receptor has been significantly diminished in the medial shell of the NAc (Nie *et al.*, 2011). The lack of the δ subunit would reduce the disinhibition of appetitive behavior by GABA in combination with ethanol.

In this regard, it is particularly interesting that γ -hydroxybutyric acid (GHB), which has been characterized as an agonist at GABA-B receptors (Andresen *et al.*, 2011, Carai *et al.*, 2008,

Schep *et al.*, 2012, Vienne *et al.*, 2010), reduces alcohol consumption in animal models (Colombo *et al.*, 2012), and is used to treat alcohol dependence and craving in Europe (Addolorato *et al.*, 2011, Maremmani *et al.*, 2011). Recently, GHB has been found to be a partial agonist at GABA-A receptors that contain α , β and δ (but not α , β and γ) subunits (Absalom *et al.*, 2012). As a partial agonist, GHB could reduce the maximal response to GABA at δ subunit-containing receptors, leading to less intense disinhibition of neurons in target structures of the medium spiny neurons and reduced alcohol consumption. It remains to be seen whether baclofen, known as a GABA-B receptor agonist, which reduces alcohol consumption in animal models (Kemppainen *et al.*, 2012, Maccioni *et al.*, 2012, Moore and Boehm, 2009, Peters *et al.*, 2013, Quintanilla *et al.*, 2008, Villas Boas *et al.*, 2012, Walker and Koob, 2007), but shows very modest effects in humans (Addolorato *et al.*, 2011, Brennan *et al.*, 2013), can also interact with δ subunit-containing GABA-A receptors.

E. Dopaminergic Neurons as Signalers of Reward

To this point, all addictive drugs have been shown to activate dopaminergic neurons whose cell bodies reside in the ventral tegmental area (VTA) of brain (the A-10 nucleus) (Arias-Carrion *et al.*, 2010). The projections of the dopaminergic cells travel to the nucleus accumbens (NAc), pre-frontal cortex (PFC), and the amygdala. The history of mapping the dopaminergic neurons of brain began with the development of histochemical methods by Falck and Hillarp (1962) and the extensive application of these methods by Dahlstrom and Fuxe (1964). The dopaminergic pathway from the VTA to the NAc, the mesolimbic pathway, has been well established as a central component of brain systems that alert the individual to the possibility of reward in response to an action by the individual (reinforcement) (Berridge, 2007). The mechanisms by which most of the addictive drugs, except for ethanol, activate mesolimbic dopaminergic systems have been described to involve specific “receptors” for these addictive drugs on the dopaminergic neurons of the VTA which project to the NAc, or on the terminals of the dopaminergic neurons in the NAc. The current work with ethanol, however, indicates an indirect mechanism which involves the inhibition of GABA function in a particular area of brain.

1. What Happens When the Dopamine Neurons are Released from Inhibition?

—Initially, ethanol effects on dopamine turnover and release were measured using neurochemical techniques in brain slices *in vitro* or in areas of brain after ethanol administration to the animal. One of the earlier studies which demonstrated the dopamine releasing properties of ethanol was published by Seeman and Lee (1974). This phenomenon was, however, brought into question by other studies which measured dopamine synthesis and turnover (which was expected to increase, concomitantly with dopamine release) in brain areas containing dopaminergic terminals or cell bodies, or whole brain (Bacopoulos *et al.*, 1978, Corrodi *et al.*, 1966, Gysling *et al.*, 1976, Hunt, 1981). These studies found either no effect or even decreases in dopamine synthesis or turnover after ethanol administration. Attempts were then made to examine the direct effects of ethanol both on the terminals of dopaminergic neurons in the NAc of anesthetized rats and on dopaminergic cell bodies in slices of the VTA *in vitro*. The measures in the NAc were carried out using dialysis techniques to measure dopamine release when ethanol was added to the dialysate and administered directly into the NAc (Wozniak *et al.*, 1991). There was a statistically significant increase in dopamine release, but the lowest concentration of ethanol at which this effect was noted was 1% (~1000 mg %).

Studies using electrophysiological measures of depolarization and discharge (firing) of dopaminergic neurons which were isolated from afferent input by preparation of VTA slices, have also been performed (Brodie and Appel, 1998, Koyama *et al.*, 2007), but examination of ethanol concentrations which consistently produce a significant increase in firing

frequency in these preparations indicates that concentrations of 40 mM or more (184 mg %) are necessary. Contrary to the studies where ethanol was applied directly into the NAc or added to slices of the VTA *in vitro*, systemic administration of ethanol produced an increase in firing of dopaminergic neurons in the VTA and increases in dopamine release in NAc and other areas of the striatum (Di Chiara and Imperato, 1988, Gessa *et al.*, 1985). Doses as low as 0.5 gm/kg administered to rats produced increases in dopamine release in the NAc (Di Chiara and Imperato, 1988) (the expected blood or brain ethanol concentrations after injection of 0.5 gm ethanol/kg in the rat would be ~4–9 mM (~20–40 mg%), at 15–30 minutes after the injection).

A study by Reuben Gonzales and his colleagues (Yim *et al.*, 1998) did much to put the various *in vitro* and *in vivo* results into perspective. The study used a design similar to that of Wozniak *et al.* (1991) to administer ethanol directly into the NAc and simultaneously measure dopamine release in this location, but then, in animals (rats) with similarly placed cannulae, ethanol was administered systemically and dopamine release in the NAc was again measured. With direct, local perfusion, ethanol concentrations above 500 mM were needed to see increases in dopamine release, while with systemic administration, doses of 1 gm/kg (resulting blood concentrations ~10–15 mM; 46–70 mg%) were sufficient to see a significant release of dopamine in the NAc. One can interpret the sum of the *in vivo* and *in vitro* studies on the effects of ethanol on mesolimbic dopaminergic systems to indicate that ethanol at low or modest doses can activate these dopaminergic systems, but that the effects of ethanol are indirect and originate in systems that impinge on the dopamine neuron cell bodies in the VTA.

A source of inhibitory input from the hypothalamus to the interneurons of the VTA was proposed by Roger Nicoll and colleagues in 1980 to explain the activation of dopaminergic neurons in the VTA by opiates. The data generated (Nicoll *et al.*, 1980) supported a model in which opiates inhibited GABA interneuron activity in the VTA, thus releasing the dopamine neurons from the tonic inhibition exerted by the GABA interneurons (Figure 1A). A significant literature accumulated by the end of the 20th century (reviewed by Cowen and Lawrence (Cowen and Lawrence, 1999)) indicating that ethanol could enhance the release of opioid peptides in brain. This information was used by Xiao *et al.* (2007) and Xiao and Ye (2008) to demonstrate that ethanol-induced potentiation of firing of VTA dopamine neurons could be blocked by naloxone (μ opioid receptor antagonist) and that silencing the GABA interneurons by administration of a μ opioid receptor agonist (DAMGO) resulted in excitation of dopamine neuron firing (Figure 1B). This provided both a mechanism by which ethanol could induce activity in a system (mesolimbic dopamine) that would predict reward, and explained why opiate antagonists such as naltrexone could be used as a treatment for alcoholism and could interfere with the rewarding properties of ethanol (Weinrieb and O'Brien, 1997). Interestingly, the GABA-A receptors in the VTA do not contain δ subunits (Ciccarelli *et al.*, 2012, Okada *et al.*, 2004); therefore, a direct potentiating effect of ethanol on the response to GABA released from the GABA interneurons may not play a prominent role in this brain region.

F. Ethanol and Glutamate

The consideration of glutamate as a neurotransmitter began in a way similar to that of GABA. Early studies in the 1950's using chemical analysis of brain established that glutamate existed at high concentrations in brain. The first indication that glutamate may have properties that directly impact brain electrophysiology were published by Hayashi (1954). His work indicated that glutamate may be an excitatory transmitter substance, since its introduction directly into brain produced convulsions. These studies were independently substantiated using cat spinal neurons, in the laboratories of J.C. Eccles, by Curtis and

colleagues (Curtis *et al.*, 1960). The transmitter function for glutamate still remained obscure, until synthetic analogues such as N-methyl-D-aspartate (NMDA) were synthesized such that stereoselectivity of glutamatergic receptors could be demonstrated (Curtis and Watkins, 1965), and the mechanisms of terminating glutamate's action in the synapse (uptake into glia) were elucidated (Curtis and Johnston, 1974). Even during the early stages of ascertainment of the neurotransmitter role of glutamate, there were indications that multiple types of glutamate receptors were present in the CNS (McCulloch *et al.*, 1974), and it became obvious over the next decade that glutamate receptors were of several varieties. The first definitive distinction of glutamate receptors could be made on whether they were receptor gated ion channels (ionotropic receptors) or G-protein coupled (metabotropic) receptors. The ionotropic subtypes of glutamate receptors were subdivided into NMDA, kainate and quisqualate (AMPA) subtypes, based on the agonists (by the same names) that were selective for each subtype (Evans *et al.*, 1979, Traynelis *et al.*, 2010). Of this group, the NMDA subtype of glutamate receptor gained the most prominence, because it was found to be necessary for transformation of high frequency signals into what can be considered neuronal short term memory (i.e., long term potentiation, LTP) (Collingridge *et al.*, 1983). The NMDA receptor also has the interesting property in that it requires both glutamate and a second ligand, glycine, for activation.

1. Ethanol's Inhibition of NMDA Receptor Function—A group led by Paula Hoffman at NIAAA, NIH was, in 1988, studying ethanol's actions on guanylate cyclase. They noticed that ethanol could inhibit the initial obligatory step in the activation of cyclic GMP production, i.e., the entry of calcium into the neuron. Calcium entry was being initiated in their studies by addition of NMDA to cerebellar granule neuron culture (Hoffman *et al.*, 1989). The NMDA receptor had recently been described as a calcium gating entity on neuronal membranes (Mayer *et al.*, 1984, Nowak *et al.*, 1984) and was shown to be present on cerebellar granule cells (Novelli *et al.*, 1987). With the use of more selective agonists and antagonists, it became obvious that ethanol was inhibiting the function of the NMDA receptor, in a selective manner, to reduce calcium entry into neurons. The group headed by Forrest Weight, also at NIAAA, NIH, was asked to perform an electrophysiological assessment of the effects of ethanol. Lovinger, White and Weight were quickly able to substantiate ethanol's effects on NMDA receptor-mediated currents in hippocampal neurons, and a paper describing their experiments was published in March of 1989 (Lovinger *et al.*, 1989), with the final version of the work of the Hoffman group appearing in June, 1989 (Hoffman *et al.*, 1989). Soon thereafter, reports by Dildy and Leslie (1989) and Woodward and Gonzales (1990) also demonstrated ethanol-induced inhibition of NMDA receptor function in brain cells from newborn rats and in rat striatal slices, respectively. All of these reports demonstrated that concentrations of ethanol as low as 10–25 mM (46–115 mg %), were able to significantly inhibit NMDA receptor function.

2. NMDA Receptor Subunit Proteins and Ethanol's Actions—The NMDA receptor gated ion channel resembles the GABA-A receptor in the fact that it is a multi-subunit complex currently known to be composed of GluN1 (which is obligatory for activity), GluN2A, GluN2B, GluN2C, and GluN2D subunits (Traynelis *et al.*, 2010). There are also GluN3 subunits, but these form receptors that can be activated by glycine in the absence of glutamate. By comparison to the effect of ethanol on the GABA-A receptor, which is influenced significantly by receptor subunit composition, the effect of ethanol on NMDA receptor function is more modestly influenced by subunit composition. In the adult human and rodent forebrain, NMDA receptors are composed primarily of GluN1 and GluN2A and/or GluN2B subunits. There are eight possible splice variants of GluN1, and the C0 cassette, which is conserved in all splice variants, does affect ethanol sensitivity of the NMDA receptor (Wills and Winder, 2013). In addition, several mutations within the transmembrane

regions of GluN1 and GluN2A have been found to affect ethanol sensitivity to a degree (Honse *et al.*, 2004, Ren *et al.*, 2003, Ren *et al.*, 2007, Ronald *et al.*, 2001, Smothers and Woodward, 2006). Interestingly, the regulation of ethanol sensitivity by GluN1 is only observed in the presence of the GluN2A subunit (Anders *et al.*, 2000, Mirshahi *et al.*, 1998, Xu *et al.*, 2011). There has also been a substantial interest in the role of GluN2B in ethanol sensitivity. As reviewed in Wills and Winder (Wills and Winder, 2013), although initial studies suggested that GluN2B-containing receptors were most sensitive to ethanol, differential sensitivity between GluN2A and GluN2B-containing receptors appears to depend on the expression system used, as well as the splice variant of the obligatory GluN1 subunit.

3. NMDA Receptor Phosphorylation and Ethanol's Actions—NMDA receptor function is highly regulated by phosphorylation and de-phosphorylation catalyzed by serine/threonine kinases and phosphatases and protein tyrosine kinases and phosphatases (see review by Lee and Messing, 2008). These post-translational modifications affect receptor activity and trafficking (Chen and Roche, 2007). Therefore, the action of ethanol on protein kinases and protein phosphorylation, as detailed earlier, became of particular interest with regard to the mechanism by which ethanol could affect NMDA receptor function. PKA can directly phosphorylate a serine residue on the GluN1 subunit and increase NMDA receptor function (Ron, 2004). PKA can also phosphorylate GluN2A and GluN2B, although the consequences of these modifications are less clear (Chen and Roche, 2007, Sanz-Clemente *et al.*, 2013). Lin *et al.* (Lin *et al.*, 2006) published data that activation of PKA reduces the ability of ethanol to inhibit NMDA receptor function in a time-dependent manner. The maintenance of PKA-mediated phosphorylation of the NMDA receptor in the NAc (via the DARPP-32 protein) is a crucial component of ethanol's "rewarding" effect, and is discussed later.

Woodward and Gonzales (1990) noted that glycine, a co-agonist at the NMDA receptor, could competitively reverse the inhibitory effects of ethanol on the NMDA receptor in the striatal slice preparations. A further examination of ethanol's interactions with glycine at the NMDA receptor in cerebellar granule cells, by Rabe and Tabakoff (1990), indicated that ethanol was acting as a "competitive inhibitor" of glycine and changing glycine's affinity at the NMDA receptor.

PKC can phosphorylate the GluN1, GluN2A and GluN2B subunits (Chen and Roche, 2007, Qiu *et al.*, 2011, Ron, 2004, Sanz-Clemente *et al.*, 2013). Snell *et al.* (Snell *et al.*, 1994a) demonstrated that activation of PKC in cerebellar granule cells inhibited NMDA receptor-induced increases in intracellular calcium by reducing the potency of NMDA and, to a greater extent, the potency of glycine to activate the NMDA receptor. Furthermore, it was shown that the extent of ethanol's inhibition of NMDA receptor activity in these cells was dependent on the activity of PKC (PKC inhibitors reversed the effect of ethanol). These experiments showed that, in cerebellar granule cells, both ethanol and PKC reduced glycine affinity at the NMDA receptor. This result suggested that the inhibitory effect of ethanol on NMDA receptor function may be mediated through PKC, and involves reduced affinity of glycine at the GluN1 subunit, potentially as a result of PKC phosphorylation of GluN1. However, in other cell types (those from trigeminal nuclei), as well as in neurons from other brain regions (prefrontal cortex, hippocampus), and in *Xenopus* oocytes expressing whole brain mRNA or mRNA for the GluN1 and GluN2B subunits, activation of PKC increased NMDA receptor function (Chen and Huang, 1991, Grosshans and Browning, 2001, Urushihara *et al.*, 1992), and in cultures of cerebral cortical cells, activation of PKC did not inhibit the function of the NMDA receptor, and treatment with ethanol did not affect the potency of glycine to activate the NMDA receptor (Bhave *et al.*, 1996). These results suggest that the effect of PKC, or ethanol's effect through PKC, on the NMDA receptor may

depend on the splice variant of GluN1 and/or on the other NMDA receptor subunits that are expressed in a particular cell type or brain region, as well as on the PKC isoform(s) present. It is important to note, for example, that while GluN2A and GluN2B are the NMDA receptor subunits mainly present in adult forebrain, GluN2C is abundant in cerebellar granule cells (Monyer *et al.*, 1992), but has also been shown to be expressed in other brain areas (e.g., striatum) (Raeder *et al.*, 2008). In heterologous expression systems, receptors containing GluN2C subunits can be inhibited by activation of PKC (Grant *et al.*, 2001), and this could be a determinant of the mechanism of ethanol's effect on NMDA receptor function in the cerebellar granule cells or in the striatal slice (Snell *et al.*, 1994a, Woodward and Gonzales, 1990).

The tyrosine kinase, Fyn, targets the GluN2B subunit of the NMDA receptor, and Miyakawa *et al.* (1997) demonstrated that Fyn-kinase activity was a critical component of determining whether ethanol could inhibit the function of NMDA receptors. The activation of Fyn reduces sensitivity of the NMDA receptor to ethanol, and mice in which Fyn has been knocked out are more sensitive to the sedative-hypnotic effects of ethanol and develop less acute functional tolerance to ethanol (Boehm *et al.*, 2003, Miyakawa *et al.*, 1997). A recent review of this research has been provided by Trepanier *et al.* (2012). Yaka *et al.* (2003a) examined the issue of Fyn mediated phosphorylation of NMDA receptors in more depth and provided data that Fyn is complexed, at the neuronal plasma membrane, with an anchoring protein called RACK1 in the proximity of the NMDA receptor complex. Ethanol inhibits the NMDA receptor, but simultaneously releases RACK1, from its tethering, allowing for phosphorylation of the GluN2B subunits by Fyn, which subsequently reduces ethanol's actions (acute tolerance). The importance of these phosphorylation events for modulating the reinforcing actions of ethanol is discussed in the following section.

IV. Integration of Ethanol's Actions on the Pathways Signaling Reinforcement

Material presented above indicates that ethanol can activate the mesolimbic dopaminergic pathway by disinhibiting the dopamine neurons in the VTA by releasing them from inhibition by GABA neurons. The disinhibiting effect of ethanol on dopaminergic neuron activity may well arise from ethanol's actions in the hypothalamus and involve opioid neuron projections from the hypothalamus to the VTA. The hypothalamus is a primitive area of brain and is intimately involved in processing interoceptive and exteroceptive sensory input to generate survival promoting actions. To generate such actions, animals, including humans, have evolved neural systems that generate positive and/or negative reinforcement. The concept of positive and negative reinforcement was introduced by Thorndike (1901) under the rubric of the Law of Effect. This concept indicated that "rewarded" behavior becomes "imprinted" through learning whereas behavior which is punished is eliminated from an organism's repertoire of behavior. Skinner operationalized this concept by introducing the means to quantitate positive and negative reinforcement (the "Skinner box", in which an animal learns to activate, by lever pressing, a presentation of a "reward" or generate an escape from noxious stimuli). Skinner also introduced the now famous statement "behavior is controlled by its consequences" (Skinner, 1938). The Skinnerian approach to the generators of behavior was modified to introduce the idea of intervening brain systems in generating reinforcement. Hebb (1949) postulated that stimuli can act as positive reinforcers, if they elicit brain physiological events that give rise to subjective sensations, which animals experience as rewarding (i.e., pleasurable). Another component (and an important one), in Hebb's explanation of the drivers of behavior was the inclusion of the concept that the interpretation of any particular stimulus at any point in time is defined, not by features of the external environment, but by the prevailing condition of the organism (the

interoceptive state, Hebb, 1949). The incentive value or motivational strength of a stimulus may thus, be modulated by the level of thirst, hunger, etc. An important demonstration in this regard is the coupling of electrical brain stimulation of the lateral hypothalamus (a technique introduced by Olds and Milner, 1954) with modulation by hunger. The animal's intensity of responding for electrical stimulation in this area of brain is modified as the level of hunger is modified. Manipulations that increase hunger (demonstrated in other experiments by increases in food intake, e.g., by ventromedial hypothalamic lesions or insulin injections), increase responding for brain electrical stimulation and manipulations which produce satiety reduce responding for brain electrical stimulation of the lateral hypothalamus (Margules and Olds, 1962). Such results indicate that electrical brain stimulation acts through pathways related to natural drives, and which are activated by natural rewards. Thus, one would think that by mapping sites in brain that maintain operant responding for electrical brain stimulation one could map the origins, targets and trajectories of neural pathways mediating responses to naturally rewarding and aversive stimuli.

Such electrical brain self-stimulation studies have identified several brain areas within which electrical stimulation is reinforcing (in other words an animal's behavior, in this case bar pressing, will be maintained if the bar pressing produces an electric stimulus to the particular area of brain). The first such area identified by Olds and Milner (1954) and Olds (1962) was the lateral hypothalamus, but the placement of electrodes provided concerns that this stimulation actually activated dopaminergic neuron projections travelling in the medial forebrain bundle (MFB). The projections of the MFB innervate both limbic and cortical areas (prefrontal cortex) and a plethora of work demonstrated that the areas receiving projections through the MFB (nucleus accumbens, septum, amygdala, prefrontal cortex and others) were areas in which the contingent delivery of electrical stimulation was reinforcing. However, additional studies did demonstrate that the original importance ascribed to the lateral hypothalamus for the reinforcing effects of electrical self-stimulation was not erroneous. In this brain area, even the stimulation of a single cell, under some conditions, will result in reinforcement (see review by Rolls, 1981).

Morphine, cocaine, nicotine, etc. have all been shown to increase responding for brain stimulation reward by increasing the rewarding potency of stimulation (Wise, 1996). Although each drug has its own initial site of action, these actions converge on the same sites and pathways which mediate brain self-stimulation reward (i.e., dopaminergic pathways). The ability of ethanol to stimulate dopamine release in the nucleus accumbens and lateral striatum has already been mentioned and data on ethanol's potentiation of brain self-stimulation reward are also available (Bain and Kornetsky, 1989, Moolten and Kornetsky, 1990). However, the consistency of results with ethanol in this area of investigation is much less than with other addictive drugs. The laboratory of Conan Kornetsky, using rats as experimental subjects, demonstrated that intragastric or intraperitoneal administration of ethanol did not produce changes in brain self-stimulation reward parameters that would indicate that ethanol administration was rewarding. On the other hand, oral self-administration of ethanol produced both a decrease in threshold for self-stimulation, and changes in rate of stimulation, indicative of the rewarding properties of ethanol (Moolten and Kornetsky, 1990). This type of result distinguishes ethanol from other addictive drugs and points to a distinct mechanism (taste? smell?) by which ethanol initiates the reinforcing cascade in animals.

A. The Medium Spiny Neuron as an Integrator of Dopaminergic and Glutamatergic Signals (the Signals for Reward)

The NAc is positioned as an integrator of a number of influences related to "reward" (something desired because it produces a conscious experience of pleasure, also termed "liking"), and contains the systems that control an individual's response to a signal that

“reward” may be attainable (Graybiel, 1990) (the NAc is the site of integration of inputs from prefrontal cortex, VTA, amygdala, hippocampus, etc.). Ninety percent or more of the neurons receiving this information in the NAc and the dorsal striatum are called medium spiny neurons (MSN; the name is derived from the morphology of the cell bodies) and MSN are GABAergic cells.

The MSN are constantly summing excitatory and inhibitory information on the “spines” of their dendritic arborization and hundreds to thousands of inputs are processed to provide a binary (discharge/no discharge) output. Additional modification of signal strength can arise from synapses on the cell body of a MSN and through axo-axonal connections (Hausser *et al.*, 2000). Of major interest in signaling related to reward and the process of addiction is the integration of glutamatergic, dopaminergic and GABAergic signals by medium spiny neurons. As an example, one can consider the input to these neurons from dopaminergic neurons of the VTA, the glutamatergic input from the prefrontal cortex or amygdala and GABAergic input from interneurons located within the NAc. In many cases, the glutamate (NMDA) and dopamine D-1 receptors are located on the same dendritic spine, while GABA neurons can terminate on the dendritic shafts or the soma of the MSN. The GABA interneurons of the NAc also receive excitatory glutamatergic input from the cortex and can provide temporally delayed inhibition of the MSN. This architecture provides ample opportunity for temporal and spatial integration of incoming signals to the MSN. As will be described later, the integration of signals in the NAc may well mediate an individual’s actions in response to reward or possible punishment.

As already stated, ingestion of ethanol can increase dopamine release in the NAc by a mechanism localized in the VTA, but additional actions of ethanol on glutamate-dopamine signal integration at the level of the NAc dictate much of the individual’s response to ethanol. In this regard, the output from the NAc depends not only on the immediate (millisecond) temporal and spatial summation of signals, but on more protracted events which can occur within seconds or minutes and determine the characteristics of ethanol’s electrophysiological and behavioral effects during a single session of intoxication. These events are dictated by intracellular (MSN) elements which alter receptor sensitivity to transmitters (e.g., desensitization, receptor dimerization), and also regulate the sensitivity of a receptor to ethanol’s actions.

Phosphorylation of glutamatergic (NMDA, AMPA) receptors is a major mechanism controlling the strength of excitatory input to the MSN (Qi *et al.*, 2010). These neurons contain a protein referred to as DARPP-32 (dopamine- and cAMP-regulated phosphoprotein with 32 kDa molecular weight). This protein was originally discovered by Paul Greengard (Greengard *et al.*, 1999) and is a mediator of a variety of biochemical actions within the neuron in response to stimulation of dopamine (D-1) receptors (Svenningsson *et al.*, 2004). The D-1 subtype of dopamine receptor is coupled through a Gs protein to activation of adenylyl cyclase, generating an increase in intracellular cAMP. The cAMP can, in turn, activate protein kinase A (PKA), which phosphorylates DARPP-32 on threonine-34 (Thr³⁴). This dopamine-initiated cascade converts DARPP-32 into a form which inhibits protein phosphatase-1 and thereby maintains phosphorylation of other proteins which control ion channel function and the regulation of gene expression (Qi *et al.*, 2010) (Figure 2).

Integration of glutamate and dopamine neurotransmission is a major role of the striatal MSNs (Fernandez *et al.*, 2006, Qi *et al.*, 2010, Surmeier *et al.*, 2007). Glutamate input to MSNs is transduced by NMDA and AMPA receptors which are, as discussed earlier, regulated by an array of kinases and phosphatases, and the phosphorylation state of the glutamate receptors is maintained by DARPP-32. The impact of this phenomenon on the actions of ethanol has been well demonstrated within the MSNs of the NAc. Similar to

ethanol's actions on other cell types and other brain areas, ethanol can, initially, inhibit glutamate-induced activation of the NMDA receptors in the NAc (Zhang *et al.*, 2005). But, this action would be the antithesis of what would be expected if one accepts the tenet that addictive drugs activate the MSNs in the NAc (Gass and Olive, 2008), and would certainly be counterintuitive if one considers that a major component to transitioning to addiction is the development of memory (LTP) within the NAc and related areas, which depends on glutamatergic activity (Kauer and Malenka, 2007). A seminal finding to resolve this conundrum was published by Risinger *et al.* (2001) and this finding was extended and amplified by Richard Morrisett. DARPP-32 knockout mice demonstrated a significant reduction in the positive reinforcing effects of ethanol, compared to wild type mice, while showing no decrement in response to aversive properties of ethanol (Risinger *et al.*, 2001). Such findings indicated that DARPP-32 was a participant in expression of the "rewarding" effects of ethanol. Maldve *et al.* (2002), through electrophysiologic measures of ethanol actions, showed acute inhibition of NMDA receptor mediated currents, but this inhibition was quickly allayed or completely negated if dopaminergic input to the MSN was activated. Maldve *et al.* (2002) went on to show that the dopamine effects are mediated by DARPP-32, and that in DARPP-32 knock-out mice, activation of D-1 dopamine receptors did not change (did not override) the inhibition of NMDA receptors by ethanol. This phenomenon can be explained by the findings of Snyder *et al.* (1998) which demonstrate that phosphorylation of DARPP-32 and its block of protein phosphatase 1 protects the GluN1 subunit phosphorylation state, and this would result in resistance of the NMDA receptor to ethanol actions. It is an interesting revelation that ethanol's activating effect in the VTA, which produces a stimulation of dopamine release in the NAc, is necessary to, in turn, reduce ethanol's inhibitory actions on NMDA receptors in the NAc (Figure 2). This balancing act allows for excitatory glutamatergic signals to MSNs in the presence of ethanol. The effects of ethanol to increase phosphorylation of DARPP-32 on Thr³⁴ are rapid, and when ethanol is administered *in vivo* to mice, maximal increases in Thr³⁴ phosphorylation of DARPP-32 are evident within a few minutes (Donohue *et al.*, 2005). Another event involving what can be referred to as acute tolerance to ethanol's actions on the NMDA receptor within the MSNs has a slower onset, and involves phosphorylation of the GluN2B subunit of the NMDA receptor by Fyn kinase in the dorsal striatum (Wang *et al.*, 2007). This phosphorylation event takes approximately 40 minutes to develop after addition of ethanol to tissue containing MSNs of the dorsal striatum (Wang *et al.*, 2007), and has a two-pronged effect. The first is the development of resistance to ethanol-induced inhibition of the NMDA receptor (Yaka *et al.*, 2003a), and the second is the production of sustained, enhanced responses to glutamate (Long Term Facilitation) after ethanol is removed from the assay system. Both of these events would lead to enhancement of glutamatergic transmission, during the presence of ethanol and after its washout from dorsal striatum. This chronology and pattern of effect in the dorsal striatum differs from what Morrisett and his group (Maldve *et al.*, 2002) observed in the ventral striatum (i.e., NAc). After a single ethanol administration, the MSN in the NAc demonstrate a phenomenon called long-term depression (LTD, see below) which can be interpreted as a diminution of glutamate responsiveness. It is only after chronic administration of ethanol, *in vivo*, to animals that one can demonstrate LTP (or LTF) in the MSNs of the ventral striatum, i.e., NAc (Jeanes *et al.*, 2011). Ethanol's acute actions are distinct between the different areas of the striatum and the final common path to responding to the possibility of reward, which involves the activation of striatal input into the putamen, thalamus and other brain regions is regulated in a complex fashion after acute and chronic ethanol intoxication.

V. The Transition to Dependence

The dominant theories which try to explain the loss of control over alcohol ingestion concentrate on loss of pre-frontal cortical executive control over the intake of ethanol

(Everitt and Robbins, 2005), a shift in salience of alcohol-related cues (Robinson and Berridge, 2008), or the conversion of positive reinforcement to negative reinforcement (Koob and Le Moal, 2008) during chronic periods of alcohol drinking. In any case, on a neurochemical level, one is confronted with changes in glutamatergic signaling in either its strength or location. It is also important to note that there is good evidence that the reinstatement of drug seeking behavior (relapse) is mediated by different anatomical locations in brain, depending on which drug is responsible for the addiction. Cocaine reinstatement depends critically on glutamatergic function in the NAc core (Kalivas *et al.*, 2009), while reinstatement of opiate use or ethanol use is dependent on NAc shell function (Bossert *et al.*, 2012, Liu *et al.*, 2012). The shell region of the NAc also seems to be important for alcohol preference, as discussed earlier (Nie *et al.*, 2011).

All of the “loss of control” theories have a “learning” component as a basic premise. The importance of neuronal learning and memory (reviewed by McGaugh and Izquierdo, 2000) is particularly evident in the work of Everitt and his colleagues, who focus on emotional and procedural forms of memory in addiction (Everitt *et al.*, 2007). The concept of addiction as a form of learned behavior (memory) can be traced back to the use of Skinnerian (operant behavior) techniques with human addicts (Wikler, 1961, 1968) and Pavlovian conditioning (Lynch *et al.*, 1976) with other animals, to determine the reinforcing properties and “relapse” with addictive drugs. Abraham Wikler started to advocate in the late 1940’s that opiate addiction (self-administration) and relapse are a form of conditioned response to negative feelings of withdrawal and that such “learned responses” perpetuate the cycle of drug dependency. Wikler’s early work is well summarized in his review (Wikler, 1961). A similar proposal was championed by Griffith Edwards and Milton Gross (1976) for alcohol addiction, in which they contended that continued heavy drinking was a conditioned response to relieve and avoid withdrawal symptoms. Their paper in 1976 was also the first to use the word “salience” in regard to importance of drinking to the alcohol addicted individual. The expansion of the concept that learning and memory were an integral part of alcohol addiction was promoted by studies of alcohol tolerance which considered alcohol tolerance as a form “biologic memory”. The work of the groups led by Harold Kalant (Kalant, 1998) and by Hoffman and Tabakoff (Tabakoff and Hoffman, 1983, 1989) used pharmacologic approaches to manipulate alcohol tolerance in animals, and through these experiments established that the neuroadaptations that result in alcohol tolerance had pharmacologic characteristics very similar to learning and memory (e.g., learning and memory of a conditioned avoidance task, etc. (de Wied, 1976)). Learning can be most easily considered as a change in behavior in response to prior experience (thus can be conceptualized either on a single neuron level or the level of the whole organism). One of the main components of synaptic plasticity (a form of neuronal “learning”) is the phenomenon referred to as long term potentiation (LTP) (see above). LTP is a long-lasting (hours/days) enhancement in efficacy of signaling between neurons and the phenomenon was first described by Terje Lømo and co-workers in 1966 (a retrospective on this discovery is contained in (Lomo, 2003)). The original work was done in an experimental system using the rabbit hippocampus and this brain area remained the favorite of electrophysiologists and neurochemists studying this rudimentary form of “memory”. More recent work has, however, revealed that nearly all neurons in brain can exhibit LTP and thus “learn” from past experience. Another form of neuronal “learning” was described by Lynch, *et al.* (1977) and was labeled as long-term depression (LTD). In this form of “learning”, prior experience leads to a diminished efficacy of synaptic transmission in a synapse which had previously undergone activation. One of the common features of LTP and LTD is their reliance on synaptic glutamatergic transmission and the presence of ionotropic and certain metabotropic glutamate receptors at such synapses. The determining features of whether the result of synaptic activation is LTP or LTD is the strength of glutamatergic input (frequency and quantitative release), the quantity of calcium that enters the neuron, and activation of certain

intracellular kinases or phosphatases (Bear and Malenka, 1994, MacDonald *et al.*, 2006, Soderling and Derkach, 2000). Lower levels of glutamatergic stimulation and lower levels of calcium entry result in LTD while higher frequency of stimulation of glutamate receptors results in LTP (Abarbanel *et al.*, 2002). The conversion of “short term” (hours or even days) memory, as exemplified by LTP or LTD, into long term memory (months, years, lifetime) requires protein synthesis (transcription/translation) as initially proposed by Sam Barondes and Harry Cohen (Barondes and Cohen, 1968). Barondes and Cohen (1968) also introduced the idea that “arousal” of an animal may be an important component of conversion of short-term to long-term memory. A tremendous volume of pioneering and clarifying work was done over the last 45 years, on the biochemical mechanisms of conversion of short-term memory to long-term memory by Eric Kandel and his many colleagues and an excellent review of this work and the history of contribution of others to this area is contained in (Kandel, 2012). This review ends with the conclusion that cAMP, PKA, CRE, CREB-1, CREB-2 and CPEB are critical elements in control of the protein synthesis necessary for long-term memory. Given the early assertions that addiction and memory may be mechanistically similar, it is not surprising that some of the more recent work on molecular mechanisms of addiction (e.g., Hyman, 2005, Hyman *et al.*, 2006, Madsen *et al.*, 2012) follows in close step with the pioneering work of those describing the phenomena and mechanisms of short-term and long-term memory. Notably, the key role of protein kinases in modulating the acute effects of ethanol on some of its “target” proteins (GABA-A and NMDA receptors) supports the concept that post-translational protein modification is an initiating step in the process that leads to ethanol addiction.

The early contentions that withdrawal signs are an unconditioned stimulus that generates the conditioned response (and memory) that drives drug (alcohol) consumption (Edwards and Gross, 1976, Schulteis *et al.*, 1995), brings to light the possible involvement of stress and stress related hormones in addiction and learning (Howland and Wang, 2008). The physiologic events occurring during ethanol withdrawal certainly produce a significant elevation in circulating glucocorticoids (Tabakoff *et al.*, 1978) and it has been well documented that adrenal hormones influence memory consolidation that relates to emotionally significant experiences while impairing “working memory” which is necessary for frontal cortical executive (behavioral control) function (Shansky and Lipps, 2013). The effects of the glucocorticoids on memory consolidation have been considered to involve noradrenergic and dopaminergic activation of, and integration of, information in the basolateral amygdala.

The word allostasis (“maintaining stability through change” (Sterling and Eyer, 1988), was applied to drug addiction by Koob and Le Moal (2001). Allostasis is a departure from the concept of homeostasis within an organism as described by Cannon (1929). Homeostasis is the stability of physiologic systems that are critical for maintaining life. Allostasis is a process that supports homeostasis, by responding to perturbations in homeostasis, and allowing for change that results in a new homeostatic set point. The involvement of stress mediators such as glucocorticoids in allostatic mechanisms has been emphasized by Bruce McEwen (McEwen and Wingfield, 2003), and the involvement of neural mechanisms responding to stress (e.g., CRF) in the etiology of addiction, has been the linchpin of the development of a theory for addiction by Koob and his colleagues (Koob, 2013). This theory and others are still reliant on the involvement of “memory” in the development of addiction but the approach of Koob also relies on the withdrawal-induced development of negative affect as the driving force for the development of addiction (Figure 3).

VI. The Integration of Theory and Fact for the Future

In light of history, we find it safe to say, that the transition from alcohol drinking to alcohol addiction, involves learning and memory and is mediated by glutamatergic and GABAergic signaling with necessary choreography by the dopaminergic systems. GABA-A receptors may be particularly important neurobiological predictors of genetic susceptibility to alcohol dependence, given the findings that the genes coding for certain subunits of these receptors have been found in human genetic association studies to be associated with alcohol dependence (Enoch *et al.*, 2009). To experience the subjective aspects of reward, an individual has to be exposed to sensory stimuli, either interoceptive or exteroceptive. The current neurobiologic concepts of addiction differ in the fact, that one set of theories of addiction emphasizes that the transition between the experience of reward and compulsive drug taking (development of addiction) is an increase in the desire to experience the pleasurable sensations produced by the drug (alcoholism). This can be described as an increased salience of positive drug response related cues. The variant on this theme, is that the transition involves increased salience of cues that are related to the relief of the negative consequences of abstinence. Interestingly, the exteroceptive cues that drive the drug taking behavior in either case may be similar, but the interoceptive cues for initiating craving would have to differ. For instance, alcohol may be excessively consumed to potentiate the expected positive ethanol effects, versus ethanol being consumed to allay the negative interoceptive cues. At the current time, Koob and Volkow (2010) have argued eloquently to support the relief of negative consequences as being the salient feature of loss of control drinking in an addicted individual. The theory proposed by Berridge and colleagues, however argues that the compulsive drug-taking behavior that characterizes addiction is not motivated either by the desire for pleasure or the desire to relieve withdrawal, but by sensitization of brain systems that affect motivated behavior in the absence of subjective pleasure (the incentive salience of the drug) (Robinson and Berridge, 2001)

VII. The Future: Systems Biology

It is exciting to experience the satisfaction of face validity of the hypothesis describing the psychological and anatomical components of addictive drug taking behavior and neuroadaptation leading to addiction. It is also exciting to see the detail in which the response to ethanol of many relevant neurobiologic components has been studied (as described earlier). The challenge currently is to integrate information to provide a “systems biology” approach for proper interpretation. Systems biology involves studying the interactions and interplay of many levels of biological information. All current hypotheses invoke the concerted participation of multiple (if not most) brain areas and a plethora of neurobiological processes in the behavior of drinking, either socially or uncontrollably. Brain imaging technology has progressed from being able to provide only structural information about brain, to technology that provides information on magnitude of neural activity in various parts of brain through measures of energy expenditure (PET), to measures of receptor density (PET, SPECT), to more novel techniques in which particular types of neurons can be visualized in small animals (Schulte *et al.*, 2012). The levels and dynamics of particular chemicals, including some neurotransmitters in brain, can also be imaged by use of high field NMR (Shapiro *et al.*, 2010). And, all of this type of information can be computationally integrated into comprehensive views of brain function in a living animal or human performing cognitive tasks, or reacting to cues, or receiving “reward”. The longitudinal collection of such information will provide fundamental insight into the role of brain as an instigator or victim of addiction. The first neuroimaging technique, i.e., pneumoencephalography, was introduced by Walter Dandy in 1919 (Dandy, 1919a, 1919b). This technique, although highly dangerous to the subject, was utilized to demonstrate cortical and cerebellar damage in alcoholics as well as an enlargement of the third ventricle.

The ventricular enlargement was an early demonstration of “brain shrinkage” in alcoholics. The development of much safer technology which is referred to as computerized tomography (CT) scanning became available in the 1970’s (Ambrose, 1973, Hounsfield, 1973), and was quickly applied to the studies of brain of alcoholic subjects (Bergman *et al.*, 1980, Carlen *et al.*, 1978, Fox *et al.*, 1976). These studies demonstrated that chronic, excessive alcohol consumption does result in a decreased brain volume (shrinkage), but this “shrinkage” was, at least in part, reversible with abstinence (Carlen *et al.*, 1978, Pfefferbaum *et al.*, 1995). Major efforts were also exerted to ascertain whether the changes in brain anatomy correlate with cognitive deficits seen in alcoholics, but such a correlation seems minimal (particularly if using tests of IQ, or similarity tests, or Object Assembly Tests). On the other hand, tests of information processing related to abstract concepts and problem solving revealed a relationship between frontal lobe malfunction in alcoholics and deficits in such tests (see Moselhy *et al.*, 2001 for review). The frontal lobe deficits were best demonstrated by positron emission tomography (PET). This technique’s history and its application to studies with alcoholics is well reviewed by Bühler and Mann (2011). The link between brain imaging studies and the substance of most of this chapter is best illustrated by the use of PET technology by which functional information about brain in either an intoxicated or sober state can be gathered. An excellent example and review of such work is presented by Goldstein and Volkow (2002). This work demonstrates the relationships between damage to the frontal lobes, not only in alcoholics, but also other drug addicted individuals, and impaired response inhibition and salience attribution. It is interesting that most of the neurochemical systems and anatomical areas linked to intoxication and addiction in other sections of this chapter come into play in the brain imaging examinations of addiction. Very substantive utilization of brain imaging technology has even occurred to test various theories of addiction (Haber and Knutson, 2010, Liu *et al.*, 2011) and even to ascertain the brain functional correlates of “moral judgment” using functional magnetic resonance imaging (fMRI) (Greene *et al.*, 2001). The challenge for better anatomical resolution and integration of information across technologies still remains. A final frontier is the application of “omics” technology (genomics, transcriptomics, proteomics, etc.) to brain tissue. Studies with brains of rodents and non-human primates have already demonstrated the power of such technology in determining predisposing factors for various levels of alcohol intake (e.g., Tabakoff *et al.*, 2008, 2009), but studies with humans are hindered by obvious concerns about ethical and technical issues regarding accessibility to the brain. A really futuristic approach is currently in its nascent stages, and that is *in vivo* measures of gene transcriptional patterns using specific promoters attached to fluorescent reporters (Hong *et al.*, 2011).

The rationale for a systems approach is not to attempt to use more complicated technology for technology’s sake, but an acquiescence to the fact that human behavior, be it addiction or morality, is complicated and dynamic. To understand, to prevent harm or to treat a disease of behavior will take thorough integrated knowledge of the organ we call brain.

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Highlights

- Review of the history of developments in understanding the neurobiology of ethanol
- Historical perspective of ethanol's effects on GABA, glutamate and dopamine systems
- Juxtaposition of history of neurobehavioral theories of addiction and neurobiology

A

B

Figure 1. Dopaminergic Neurons as Signalers of Reward

A. The dopaminergic projections from the ventral tegmentum (ventral tegmental area, VTA) to the NAc, i.e., the mesolimbic pathway, represent a central component of the “reward” pathway. The dopaminergic neurons in the VTA project to the NAc, amygdala, prefrontal cortex and other limbic areas. Opiate peptides (β -endorphin, β -END) released from the hypothalamus inhibit the activity of VTA GABAergic interneurons, the cell bodies of which reside in the rostromedial tegmentum, and increase VTA dopaminergic neuron activity. B. Ethanol increases the release of hypothalamic opiate peptides, further inhibiting the GABAergic interneurons, and leading to enhanced dopamine release through this disinhibition mechanism. At the same time, ethanol can inhibit NMDA receptor function, decreasing excitatory input to the VTA GABAergic interneurons, which further disinhibits VTA dopamine neuron activity.

Figure 2. Ethanol's Action on Glutamatergic Activation of Nucleus Accumbens Medium Spiny Neurons (MSN)

Left Panel. Phosphorylation of NMDA receptors controls the strength of excitatory input to the MSN. When dopaminergic activity at the D1 dopamine receptor is low, protein phosphatase-1 (PP1) activity is relatively high, leading to dephosphorylation of the NMDA receptor, and reduced excitatory input to the MSN. Ethanol also inhibits NMDA receptor function in the presence of low dopaminergic input, and these factors both reduce the output of the MSN. Right Panel When dopamine release is high, stimulation of D1 receptors increases cAMP levels in the MSN, which promotes phosphorylation of DARPP-32 on threonine-34 (T34), converting DARPP-32 into a form that inhibits PP-1. This results in maintained phosphorylation of the NMDA receptor, which in turn produces resistance to ethanol inhibition of NMDA receptor function. Overall, ethanol, by activating DA release in the VTA (see Figure 1), leads to an inhibition of its actions on NMDA receptor function in the NAc, resulting in increased excitatory input to the MSN.

Figure 3. Stages of Addiction

The three panels illustrate a composite of the theoretical frameworks discussed in the text on the progression from the initial drinking experience to addiction in susceptible individuals.

A. Initial Experience. During the initial experience with ethanol, interoceptive and exteroceptive cues become associated with a pleasurable result (positive reinforcement). Ethanol directly activates the “pleasure signals” at the level of the hypothalamus and the VTA to initiate the attention to and processing of the ethanol-related events. The salience and valence of the ethanol-related events as processed through the prefrontal cortex, hippocampus and amygdala, determine the motivation for further ethanol ingestion. A stronger motivation can be interpreted as a greater level of “liking”. B. Habituation. During

the stage of habituation, learning and expectation lead to the strengthening of certain sensory cues which drive and respond to ethanol ingestion. Activation of “pleasure signals” from the hypothalamus and VTA become strongly associated with the particular sensory cue (relief of stress, anxiety, or maybe even the taste of a good wine). The intake of the alcoholic beverage at this stage can result in either positive or negative reinforcement to drive further drinking. The drinking becomes more automated with diminished influence of the prefrontal cortex and the strengthening of activity of sub-cortical regions: nucleus accumbens and dorsal striatal connections to the pallidum, and input from the amygdala to the nucleus accumbens strengthening the sub-cortical initiation of motor action to obtain more ethanol.

C. Dependence. In the alcohol-addicted state, the sensory cues become the untoward effects of not having ethanol in the body. The ethanol intake alleviates these negative sensory states (negative reinforcement) and the system learns that pleasure can be derived from alleviation of these negative states. The salience of this experience, modulated through the amygdala, drives motivation and drinking is at this point a repetitive act to prevent exposure to negative states (“wanting”).