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Signaling Pathways Mediating Alcohol Effects

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Abstract

Ethanol's effects on intracellular signaling pathways contribute to acute effects of ethanol as well as to neuroadaptive responses to repeated ethanol exposure. In this chapter we review recent discoveries that demonstrate how ethanol alters signaling pathways involving several receptor tyrosine kinases and intracellular tyrosine and serine-threonine kinases, with consequences for regulation of cell surface receptor function, gene expression, protein translation, neuronal excitability and animal behavior. We also describe recent work that demonstrates a key role for ethanol in regulating the function of scaffolding proteins that organize signaling complexes into functional units. Finally, we review recent exciting studies demonstrating ethanol modulation of DNA and histone modification and the expression of microRNAs, indicating epigenetic mechanisms by which ethanol regulates neuronal gene expression and addictive behaviors.

Keywords

Phosphorylation; Signal transduction; Growth factor; Epigenetic; Kinase

1 Introduction

Ethanol is a psychoactive substance with rewarding and sedative-hypnotic properties that stem largely from its acute effects on specific signaling proteins that lead to changes in localization and post-translational modifications, gene expression and neuronal excitability. Neurons adapt to repeated ethanol exposure through homeostatic changes in cellular signaling pathways that serve to maintain nervous system function in the presence of ethanol. Such neuroadaptations are thought to contribute to addiction partly because the absence of ethanol produces an aversive withdrawal state that negatively reinforces continued ethanol consumption. Such neuroadaptive changes include long-term changes in gene expression. This chapter reviews recent advances in the field of signal transduction in *Drosophila melanogaster* and rodents as it relates to alcohol use disorders, with emphasis on mechanisms that hold promise for discovery of new drug targets for treatment. Earlier findings are described in (Ron and Jurd 2005; Newton and Ron 2007; Nagy 2008; Lee and Messing 2008).

2 Serine–Threonine Kinases

Serine/threonine kinases are a large and heterogeneous group of enzymes that phosphorylate protein substrates on serine or threonine residues. Some are receptors (e.g. TGFβ receptors)

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but the majority are intracellular such as protein kinases A, B (also known as AKT), C, calcium/calmodulin-dependent protein kinases and mitogen-activated protein kinases.

2.1 cAMP-Dependent Protein Kinase A (PKA)

PKA plays a key role in learning and memory (Abel and Nguyen 2008) and in behavioral responses to drugs of abuse (Lee and Messing 2008). It exists as an inactive tetramer of two regulatory subunits and two catalytic subunits. Adenylyl cyclase (AC) activation catalyzes the hydrolysis of ATP to cyclic adenosine 3′, 5′-monophosphate (cAMP). cAMP activates PKA by binding to the regulatory subunits, causing their dissociation from catalytic subunits, which then become active (Brandon et al. 1997). All PKA subunits (RIα, RIβ, RIIα, RIIβ, Cα and Cβ) are expressed in distinct but overlapping patterns in the brain (Cadd and McKnight 1989). There are nine AC isoforms and all are regulated by subunits of heterotrimeric G-proteins (Cooper 2003). $G_s\alpha$ activates all except possibly AC8 (Wang and Storm 2003), while $G_{olf}\alpha$ activates AC5, and $G\beta\gamma$ activates AC2, AC4 and AC7. Conversely, $G_{i/o}\alpha$ inhibits AC1, AC5, AC6 and AC8, while $G\beta\gamma$ inhibits AC1. Production of cAMP can also be regulated by protein kinase C (PKC) which inhibits AC6 and activates AC2, AC4 and AC7, and by calcium which inhibits AC5 and AC6, activates AC8 and together with $G_s\alpha$ synergistically activates AC1 (Wang and Storm 2003; Cooper 2003).

2.1.1 Ethanol Regulation of AC/PKA Signaling—Like other addictive drugs, ethanol acutely increases levels of extracellular dopamine in the nucleus accumbens (NAc) (Di Chiara and Imperato 1988), which activates D1 dopamine receptors coupled to G_s and G_{olf}, and leads to activation of AC and PKA. Dopamine also activates D2 receptors coupled to $G_{i/o}$, which inhibits several AC isoforms. Dopamine activation of D2 receptors also releases $G\beta\gamma$ subunits, which stimulate G-protein-regulated inwardly rectifying K^+ (GIRK) channels, and inhibit L–, N–, and P/Q-type calcium channels. The net effect of these actions on ion channel function is to depress neuronal excitability. However, in NAc neurons, $G\beta\gamma$ activation of AC is required for dopamine-stimulated firing, which requires co-activation of D1 and D2 receptors (Hopf et al. 2003).

An important downstream regulator of dopaminergic signaling in striatal neurons is the dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kDa (DARPP-32), which acts as a bidirectional switch that is regulated by phosphorylation (Svenningsson et al. 2005). PKA phosphorylation of Thr-34 makes DARPP-32 a potent inhibitor of the protein phosphatase PP1, which in turn amplifies PKA-mediated phosphorylation of substrates. Cyclin-dependent protein kinase 5 (Cdk5) phosphorylates DARPP-32 at Thr-75, which turns DARPP-32 into an inhibitor of PKA and antagonizes several acute effects of dopamine in the striatum. DARPP-32 appears critical for ethanol reinforcement and reward since mice lacking DARRP-32 show reduced ethanol self-administration and conditioned place preference (Maldve et al. 2002; Risinger et al. 2001).

Ethanol activates AC/PKA/DARPP-32 signaling through several mechanisms. Ethanol increases levels of extracellular dopamine in the NAc (Di Chiara and Imperato 1988; Weiss et al. 1993) by increasing firing of ventral tegmental area (VTA) dopamine neurons (Gessa et al. 1985; Brodie et al. 1990). Ethanol also enhances dopamine D1 receptor-mediated activation of AC (Rex et al. 2008). In addition, ethanol indirectly activates G_{olf}-coupled adenosine A2a receptors by inhibiting adenosine reuptake through type I equilibrative nucleoside transporters, thereby increasing extracellular concentrations of adenosine (Nagy et al. 1990; Choi et al. 2004). Low doses of ethanol and other addictive drugs such as opiates, cannabinoids and nicotine can act synergistically to stimulate ACs through combined effects at A2a receptors, which activate G_{olf}, and dopamine D2 receptors, which cause release of $G\beta\gamma$ subunits (Yao et al. 2003; Yao et al. 2002). These events result in

cAMP response element (CRE)-mediated gene expression not only in the NAc but also in several other limbic brain regions (Asyyed et al. 2006).

An important substrate of PKA is the cyclic AMP response element binding protein (CREB), a transcription factor activated by phosphorylation at Ser-133 by PKA and also by calcium/calmodulin-dependent protein kinase IV, or mitogen-and stress-activated protein kinases (MSK1 and 2) (Lonze and Ginty 2002; Hauge and Frodin 2006). In rats, chronic consumption of ethanol for several weeks decreases Ser-133 phosphorylated CREB (p-CREB) in the striatum (Li et al. 2003) and diminishes the ability of an acute ethanol challenge to increase p-CREB and CREB function in the striatum and cerebellum (Yang et al. 1998a, b). During acute ethanol withdrawal, p-CREB is also decreased in several regions of the cerebral cortex (Pandey et al. 2001). These decreases in p-CREB may relate to increased expression of protein kinase inhibitor α (PKIα), as demonstrated in the prefrontal cortex (PFC), NAc and amygdala by transcriptional profiling of brain tissue from Wistar rats subjected to chronic intermittent exposure for 2 weeks (Repunte-Canonigo et al. 2007). PKIα is a protein that acts as a pseudosubstrate inhibitor of PKA catalytic subunits. Since acute exposure to ethanol activates PKA signaling, up-regulation of PKIα can be considered a homeostatic compensatory response that normalizes PKA signaling during chronic ethanol exposure.

2.1.2 AC/PKA Signaling in Behavioral Responses to Ethanol—Several studies indicate a role for PKA in the intoxicating effects of ethanol. Inhibition of PKA through intracerebroventricular (ICV) administration of the selective PKA inhibitor KT5720 reduces the acute ataxic and hypnotic effects of ethanol in rats (Lai et al. 2007). Likewise, RIIβ knockout mice, which have reduced cAMP-stimulated PKA activity, show decreased sensitivity to hypnotic effects of ethanol (Thiele et al. 2000). In addition, pituitary adenylate cyclase-activating polypeptide (PACAP) knockout mice, which are predicted to have a deficit in PKA signaling (Tanaka et al. 2004), show reduced hypothermic and hypnotic responses to ethanol. These studies suggest that ethanol-induced activation of PKA contributes to acute ataxic, hypothermic and sedative-hypnotic effects of ethanol.

In addition to regulating acute behavioral responses to ethanol, PKA signaling also regulates ethanol consumption. Thus, RIIβ knockout mice show reduced cAMP-stimulated PKA activity and increased ethanol intake (Thiele et al. 2000). Mice haplodeficient for the α and Δ isoforms of CREB show increased ethanol consumption (Pandey et al. 2004), while administration of the PKA inhibitor Rp-cAMPS into the central amygdala (CeA) (Pandey et al. 2003) or NAc shell (Misra and Pandey 2006) of Sprague–Dawley rats increases ethanol consumption and preference. These studies suggest that inhibiting PKA signaling, especially in the CeA and the NAc shell, leads to increased ethanol drinking through a CREB-dependent mechanism. In addition, in rats, long-term alcohol consumption with repeated episodes of deprivation produces a strong down-regulation of PACAP gene expression in the striatum that is reversed by treatment with glycine transport inhibitors, which also reduce relapse drinking in this model (Vengeliene et al. 2010), indicating that PACAP, which lies upstream of PKA, is part of a glycine-regulated gene network that undergoes neuroadaptation during long-term ethanol self-administration to promote relapse.

Not all studies, however, agree with these findings, but instead report that inhibition of PKA signaling increases acute effects of ethanol and decreases ethanol consumption. For example, mice that are haplodeficient for $G\alpha_s$, that express a dominant negative form of PKA (Wand et al. 2001), or lack the calcium-sensitive adenylyl cyclases AC1 and AC8 (Maas et al. 2005), show a more prolonged ethanol-induced loss of righting and less ethanol consumption than wild type mice. Systemic injection of the A2a receptor antagonist 3, 7-dimethylpropargylxanthine, which is expected to reduce PKA signaling throughout the

striatum, reduces ethanol consumption in Long-Evans (Arolfo et al. 2004) and Wistar rats (Thorsell et al. 2007). In addition, intra-striatal administration of a peptide that inhibits Gβγ and prevents both ethanol-stimulated nuclear translocation of PKA and PKA-stimulated gene expression, decreases ethanol intake in Long-Evans rats (Yao et al. 2002).

The differences between these studies and those that find reduced sensitivity to ethanol and increased ethanol drinking upon inhibition of AC/PKA signaling may relate to differential duration or level of exposure to ethanol, to effects of global versus local pharmacological and genetic manipulations, compensatory effects of gene targeting that alter non-PKA pathways, or, perhaps, effects of genetic background in the different mouse and rat strains used in these studies. However, experiments in selected and inbred lines of rats and mice do support a role for decreased PKA signaling in the amygdala and NAc shell in promoting ethanol drinking. For example, levels of CREB and p-CREB are lower in the NAc shell of high ethanol preferring C57BL/6 mice compared with DBA/2 mice, which are a low ethanol preferring strain. In addition, alcohol-preferring (P) rats show lower levels of pCREB and CREB DNA binding activity in the CeA and medial amygdala (MeA) than alcohol non-preferring (NP) rats (Pandey et al. 1999a).

2.1.3 PKA and CREB Regulation of Anxiety and Ethanol Consumption—Studies by Pandey and colleagues have identified key roles for amygdala PKA, CREB and the CREB-regulated gene neuropeptide Y (NPY) in the co-regulation of anxiety and ethanol drinking. NPY is abundantly expressed in the brain and is anxiolytic when administered into the central nervous system (CNS) (Heilig 2004). Knockout of the NPY gene in mice increases ethanol consumption, while transgenic overexpression of NPY reduces it (Thiele et al. 1998). P rats drink excessively and have lower levels of p-CREB and NPY in the amygdala compared with NP rats; P rats also show greater anxiety-like behavior than NP rats (Pandey et al. 2005). Self-administration of increasing concentrations of ethanol (7–12% over 10 days) in a two-bottle choice paradigm, or injection of 1 g/kg ethanol, normalizes anxiety-like behavior in P rats. These findings are associated with increased p-CREB and expression of NPY in the CeA and MeA of ethanol-treated P rats (Pandey et al. 2005). Infusion of the PKA activator Sp-cAMP into the CeA of P rats increases local p-CREB and NPY levels, decreases ethanol self-administration and normalizes their heightened anxiety-like behavior (Pandey et al. 2005). Conversely, in NP rats, infusion of the PKA inhibitor Rp-cAMP into the CeA decreases local p-CREB and NPY, increases anxiety-like behavior and increases ethanol intake (Pandey et al. 2005). The importance of NPY in these behavioral changes has been demonstrated by infusing NPY into the amygdala of P rats, which mimics the effect of Sp-cAMPs by decreasing anxiety-like behavior and ethanol intake.

Increased anxiety that accompanies alcohol withdrawal is argued to be one of the negatively reinforcing factors that promotes ethanol consumption (Koob 2009). Support for this concept stems from studies of diminished amygdala PKA signaling and NPY expression that accompany ethanol withdrawal in rats. One day after withdrawal from chronic daily intake of ethanol, Sprague–Dawley rats show increased anxiety-like behavior (Pandey et al. 2003), which is associated with decreased p-CREB (Pandey et al. 2003) and NPY (Roy and Pandey 2002) in the CeA and MeA. Sp-cAMPS infused into the CeA normalizes CREB phosphorylation and NPY expression, and prevents withdrawal-induced anxiety in these rats (Pandey et al. 2003; Zhang and Pandey 2003). In ethanol na rats, infusion of the PKA inhibitor Rp-cAMPS into the CeA decreases local p-CREB and NPY and increases both anxiety and ethanol consumption (Pandey et al. 2003; Zhang and Pandey 2003). Furthermore, infusion of NPY into the CeA prevents Rp-cAMPS-induced decreases in ethanol preference (Pandey et al. 2003). Intra-amygdalar infusion of NPY also reduces ethanol intake in P rats after multiple episodes of alcohol deprivation (Gilpin et al. 2003) and reduces withdrawal-induced increases in ethanol consumption in Wistar rats (Gilpin et

al. 2008). These results indicate that deficient PKA and NPY signaling in the amygdala are critical for increased anxiety and drinking that accompany alcohol withdrawal.

2.2 Protein Kinase C (PKC)

The PKC family of serine–threonine kinases mediates signals derived from lipid second messengers. The members of this family share similar catalytic domains but can be subdivided into four classes based on differences in their regulatory domains that alter structure and function (Rosse et al. 2010; Newton 2010). The classical or conventional cPKCs (α, β, γ) are activated by diacylcglycerol (DAG) and calcium. Novel nPKCs (δ, ε, η, θ) are activated by diacylglycerol but not by calcium. Atypical PKCs (ι or λ in mice, and ζ), do not require calcium or diacylglycerol for activation, but can be activated by phosphatidylinositols, phosphatidic acid, arachidonic acid and ceramide, and by interaction with the partitioning defective 6 (PAR6)-CDC42 complex (Hirai and Chida 2003; Rosse et al. 2010). Recently, a fourth group of PKC-related kinases (PKN1, PKN2, PKN3) has been included as a PKC subfamily; they are activated by the small G-proteins Rac and Rho (Rosse et al. 2010).

DAG, generated by activation of phospholipase C (PLC), is the most studied lipid activator of PKC signaling (Fukami et al. 2010). Among the PLC isoforms, activation of β and γ subtypes has been best described. PLCβ is activated by Gβγ or $G\alpha_q$ subunits of heterotrimeric G-proteins released upon ligand binding to G-protein coupled receptors. Activation of receptor tyrosine kinases leads instead to recruitment, tyrosine phosphorylation, and activation of PLCγ. DAG can also be generated as a result of receptor-mediated activation of phospholipase D (Nishizuka 1995). PKC activation is generally associated with translocation of PKC from one cellular compartment to another containing lipid activators and proteins that bind the activated kinase near substrates. Here we summarize recent work on ethanol and PKC, focusing on three PKC isozymes, PKCε, PKCγ and PKCδ (see also section on RACK1).

2.2.1 Ethanol Regulation of PKC Activity—Ethanol has been reported to activate, inhibit or have no effect on PKC activity in vitro, depending on experimental conditions (reviewed in (Stubbs and Slater 1999). Recently, ethanol was reported to bind to PKCε and inhibit PKCε activity when assayed in vitro in the presence of DAG plus phosphatidylcholine and phosphatidylserine (Das et al. 2009). However, using DAG and phosphatidylserine with Triton-X-100 micelles, we previously found that ethanol does not alter the activity of PKC in vitro (Messing et al. 1991), while other literature indicates that ethanol exposure activates PKCε in intact cell systems (Miyame et al. 1997; Jiang and Ye 2003; Qi et al. 2007). Ethanol regulation of PKCε and other PKC isozymes is most likely to be indirect, due to modulation of upstream signaling pathways that generate DAG or that lead to phosphorylation of sites necessary for full kinase activity, such as the C-terminal hydrophobic motifs of PKCε (Wallace et al. 2007) and the cPKCs (Wilkie et al. 2007).

2.2.2 Ethanol Regulation of PKC Localization—In NG108-15 neuroblastoma × glioma cells, ethanol causes translocation of PKCδ from the Golgi to the perinucleus and PKCε from the perinucleus to the cytoplasm (Gordon et al. 1997). Cytosolic translocation of PKCε has also been observed in rat cerebral cortex following acute exposure to ethanol (Kumar et al. 2006). Dopamine D2 receptor agonists stimulate translocation of PKCδ and PKCε to these same sites in NG108-15 and CHO cells stably transfected to express D2 receptors (Gordon et al. 2001), and in cultured rat VTA dopamine neurons (Yao et al. 2010). The effects of ethanol and D2 agonists are synergistic since concentrations of ethanol and agonist that do not cause translocation alone produce robust translocation when administered together (Gordon et al. 2001). Ethanol-translocated PKCε is active (Yao et al. 2008), as

shown by its binding to monoclonal antibody 14E6, which specifically detects the active conformation of PKCε (Souroujon et al. 2004). Translocation of PKCε occurs together with translocation of β′COP (Gordon et al. 2001; Yao et al. 2008), a receptor for activated PKCε (εRACK; RACK2) (Csukai et al. 1997). Ethanol stimulates translocation of this complex through activation of adenosine A2a receptors, and both ethanol and D2 agonist stimulated translocation require activation of PLC, PKCε and PKA; PKA may act by promoting the activation of PLC and by phosphorylating and facilitating the translocation of εRACK (Yao et al. 2008). These results indicate considerable cross talk between PKA and PKCε in synergistic responses to ethanol and dopamine.

Such crosstalk is important in the VTA where drugs of abuse increase extracellular levels of dopamine and thereby activate D2 autoreceptors on dopaminergic neurons. This event leads to the activation of PKC (most likely PKCε) and PKA, which phosphorylate and up-regulate tyrosine hydrolase (TH) and increase production of dopamine (Yao et al. 2010). Up-regulation of TH activity by cocaine is essential for the ability of ALDH2 inhibitors to reduce cocaine self-administration in rodents (Yao et al. 2010). ALDH2 inhibitors impair metabolism of dopamine, resulting in the generation of tetrahydropapaveroline (THP), a potent inhibitor of TH, especially phosphorylated TH. It is likely that the ability of ALDH2 inhibitors to reduce ethanol self-administration (Arolfo et al. 2009) also requires PKCε and PKA-mediated up-regulation of dopamine production to generate THP, although this possibility remains to be tested.

2.2.3 PKCε Regulation of $GABA_A$ Receptors and Intoxication—The intoxicating effects of ethanol last much longer in PKCε knockout mice than in wild type mice due to impaired development of acute functional tolerance to ethanol in the knockout (Wallace et al. 2007). Phenotypic and biochemical studies using PKCε knockout mice and selective peptide inhibitors and activators of PKCε have demonstrated that PKCε reduces the response of $GABA_A$ receptors to several positive allosteric modulators, including neurosteroids, benzodiazepines and ethanol (Hodge et al. 1999; Hodge et al. 2002). Two mechanisms appear to account for this modulation. First, PKCε phosphorylates the γ2 subunit of $GABA_A$ receptors at Ser-327, and when this site is phosphorylated, synaptic $GABA_A$ receptors show reduced activation by benzodiazepines and by ethanol (Qi et al. 2007). This phosphorylation event is important for behavior since development of acute functional tolerance is associated with increased Ser-327 phosphorylation and reduced effects of ethanol on cerebellar $GABA_A$ receptors (Qi et al. 2007; Wallace et al. 2007). Second, PKCε phosphorylates the N-ethylmaleimide-sensitive factor (NSF) at Ser-460 and Thr-461 (Chou et al. 2010). Phosphorylation at these sites increases NSF activity and binding to PKCε and alters $GABA_A$ receptor trafficking, resulting in fewer receptors at the synapse (Chou et al. 2010). Thus, inhibiting PKCε facilitates inhibitory synaptic transmission in general by increasing the density of synaptic $GABA_A$ receptors through a reduction in NSF activity and specifically enhances the positive allosteric effects of ethanol and benzodiazepines by decreasing the phosphorylation of GABA γ2 subunits.

2.2.4 PKCε and Ethanol-Induced GABA Release—Ethanol stimulates GABA release in the CeA through a mechanism that requires activation of type 1 corticotrophin releasing factor (CRF) receptors (CRF_1Rs) (Nie et al. 2004). CRF is an anxiogenic neuropeptide that is upregulated in the amygdala of ethanol-dependent rodents where it promotes excessive ethanol consumption through actions at CRF_1Rs (Chu et al. 2007; Sommer et al. 2008). Furthermore, a polymorphism in the *Crhr1* promoter that is accompanied by increased abundance of *Crhr1* transcripts in several limbic areas has been identified in Marchigian–Sardinian Preferring (msP) rats genetically selected for high alcohol preference (Hansson et al. 2007). PKCε knockout mice, which show reduced anxiety-like behavior (Hodge et al. 2002) and low levels of ethanol self-administration (Hodge et al. 1999; Olive et al. 2000),

also have an ~50% reduction in levels of CRF in the CeA (Lesscher et al. 2008). Furthermore, absence or inhibition of PKCε prevents CRF or ethanol-stimulated GABA release in the CeA (Bajo et al. 2008). Therefore, PKCε is important not only for the production of CRF but also for CRF_1R signaling that controls ethanol-induced GABA release in the CeA and regulates both anxiety and ethanol consumption (Lesscher et al. 2008; Lesscher et al. 2009).

2.2.5 PKCγ and Ethanol-Mediated $GABA_A$ Receptor Trafficking—Like PKCε, PKCγ is widely expressed in the CNS (Naik et al. 2000), and also regulates $GABA_A$ receptors and behavioral responses to ethanol. In contrast to PKCε knockout mice, PKCγ knockout mice are less sensitive to acute effects of ethanol, consume more ethanol and show impaired development of chronic tolerance to ethanol compared with wild type mice (Bowers et al. 1999; Bowers et al. 2000; Bowers and Wehner 2001). Exposure to ethanol for several hours causes internalization of α1 subunits of $GABA_A$ receptors in cerebral cortex (Kumar et al. 2003) and hippocampus (Liang et al. 2007), which may play a role in the hyperexcitablity that appears during ethanol withdrawal. In a recent study it was found that PKCγ co-immunoprecipitates with α1 subunits and this association is increased after 4 h of exposure to ethanol (Kumar et al. 2010). Treatment with short-interfering RNAs targeted against PKCγ prevented ethanol-induced decreases in the abundance of α1 subunits in cultured cortical neurons (Kumar et al. 2010), suggesting that PKCγ mediates ethanol-induced decreases in α1. However, while suggestive, these results should be viewed as preliminary since although three siRNAs were used against PKCγ in this study, it appears that they were administered together, not separately. Also, an inhibitory peptide derived from the pseudosubstrate sequence of PKCβ had no effect on α1 subunit trafficking which is puzzling since this peptide appears to also inhibit PKCγ activity (Correia et al. 2003). Finally, ethanol treatment results in the inhibition of PKCβII translocation and thus prevents proper substrate phosphorylation (Ron et al. 2000).

2.2.6 PKCδ and Sensitivity to Ethanol Intoxication—PKCδ is expressed in several brain regions that regulate ethanol intake (Merchenthaler et al. 1993; Choi et al. 2008) including the CeA (Koob et al. 1998; Finn et al. 2007; Funk et al. 2006; Primeaux et al. 2006; Moller et al. 1997; Hyytiä and Koob 1995), the hippocampus (Adell and Myers 1994; Huttunen and Myers 1987; Martin-García et al. 2007), the bed nucleus of the stria terminalis (BNST) (Walker et al. 2003; Hyytia et al. 1999) and the lateral septum (Ryabinin et al. 2008). Acute ethanol exposure alters the distribution, whereas chronic exposure increases the abundance and translocation of PKCδ in neural cell lines (Messing et al. 1991; Gordon et al. 1997), suggesting that PKCδ participates in responses to ethanol. This hypothesis has been confirmed in PKCδ knockout mice(Chou et al. 2004), which are less sensitive to the acute motor-impairing effects of ethanol (Choi et al. 2008). This resistance is most obvious at a dose of ethanol (1.5 g/kg) that produces blood ethanol concentrations of 150–240 mg/dl (32–51 mM) in mice (Gentry et al. 1983); similar blood levels impair coordination in humans (Messing 2007). These findings suggest that PKCδ is involved in neuronal signaling pathways that increase acute sensitivity to ethanol at ethanol doses that produce moderate intoxication.

2.2.7 PKCδ and Tonic GABA Currents—Although $GABA_A$ receptors are considered primary targets for ethanol, demonstration of direct effects at concentrations lower than those that produce anesthesia has been historically difficult (Harris et al. 1997; Harris et al. 1995; Criswell and Breese 2005). However, recent electrophysiological studies have provided evidence of low dose ethanol effects at $GABA_A$ receptors in the hippocampus, cerebral cortex, NAc and CeA (Weiner and Valenzuela 2006). Evidence from electrophysiological recordings of recombinant receptors expressed in *Xenopus oocytes*

(Sundstrom-Poromaa et al. 2002; Wallner et al. 2003) and of native receptors in hippocampal dentate gyrus granule cells (Wei et al. 2004; Fleming et al. 2007) suggest that $GABA_A$ receptors formed by the subunit combination of α4βxδ are sensitive to low (1–30 mM) concentrations of ethanol. Concentrations of 3–20 mM produce mild intoxication in humans and 17 mM is equivalent to a blood alcohol level of 80 mg/dl (Messing 2007). It must be noted, however, that some investigators have been unable to replicate these findings (Yamashita et al. 2006; Borghese et al. 2006). The basis for this discrepancy could be related partly to differences in phosphorylation state of the receptor, as discussed below.

$GABA_A$ receptors that contain δ subunits are extrasynaptic and modulate the inhibitory tone of neurons by responding to ambient GABA levels, as opposed to synaptic receptors, which contain γ2 subunits instead of δ subunits, and provide rapid, phasic inhibition by responding to stimulated release of GABA at synapses (Farrant and Nusser 2005; Wei et al. 2003; Glykys and Mody 2007; Glykys et al. 2007). $GABA_A$ receptors that contain δ subunits have a high affinity for GABA and a slow rate of desensitization, properties that are useful for tonic regulation of inhibition. Our recent work indicates that PKCδ regulates the ethanol sensitivity of tonic inhibitory GABA currents (Choi et al. 2008). Thus, tonic currents in thalamic and hippocampal neurons of PKCδ knockout mice show no response to 30 mM ethanol. Ethanol regulation of tonic GABA current is mediated by a direct effect of ethanol on extrasynaptic $GABA_A$ receptors rather than on mechanisms that regulate extracellular concentrations of GABA (e.g. GABA transporters) since, in mouse L(tk-) fibroblasts that express α4β3δ $GABA_A$ receptors, ethanol enhancement of GABA currents is also PKCδ-dependent (Choi et al. 2008). These findings suggest that PKCδ facilitates ethanol intoxication by enhancing ethanol's action at extrasynaptic $GABA_A$ receptors, possibly through phosphorylation of receptor subunits.

2.3 Extracellular Signal-Regulated Kinases (ERKs)

ERKs are serine–threonine protein kinases that are members of the mitogen-activated protein kinase (MAPK) family. There are two isoforms, p44 ERK1 and p42 ERK2, with functions that partly overlap. Both are widely expressed in limbic brain regions including in the mesolimbic dopaminergic system, amygdala and prefrontal cortex (Lein et al. 2007). ERKs are activated by a Ras–Raf–MEK signaling cascade that is activated by receptor tyrosine kinases (see section below on Receptor Tyrosine Kinases) or by calcium influx through NMDA and voltage-gated calcium channels. The function of these ion channels can be enhanced by PKA-mediated phosphorylation resulting from activation of dopamine D1 receptors (Lu et al. 2006; Pascoli et al. 2011). Since ERK activity is increased by dopamine and glutamate receptor stimulation, it may function as a coincidence detector that combines information about rewards and contextual information during the development of addiction (Girault et al. 2007).

Since MEK phosphorylation activates ERK1 and ERK2 (ERKs), ERK activity can be indirectly assayed by measuring MEK phosphorylation of ERKs using phospho-specific antibodies. Using this approach, previous studies have reported that acute ethanol exposure (3.5 g/kg) in adult rats inhibits ERKs in the cerebral cortex, hippocampus and cerebellum (Chandler and Sutton 2005) and that continuous or intermittent exposure to ethanol vapor for 12 days also inhibits ERKs in the amygdala, cerebellum, dorsal striatum, hippocampus and PFC (Sanna et al. 2002). We recently found that acute systemic administration of 2 g/kg of ethanol to C57BL/6 mice did not alter ERK phosphorylation in the NAc (Neasta et al. 2011a). However, Ibba et al. (Ibba et al. 2009) detected ERK activation in the BNST, CeA and the NAc 15 min after intragastric gavage of 1 but not 2 g/kg ethanol; both basal and ethanol-stimulated ERK activity could be blocked by a dopamine D1 receptor antagonist. In addition, Neznanova and colleagues (Neznanova et al. 2009) observed that alcohol-preferring AA rats showed rapid and transient dephosphorylation of ERK1/2 upon acute

ethanol challenge in the medial prefrontal cortex, and to a lesser degree in the nucleus accumbens, whereas alcohol non-preferring ANA rats did not. Therefore, it is possible that, under certain conditions, ethanol stimulation of dopaminergic signaling activates ERKs. In addition, ethanol can indirectly activate ERKs by up-regulating BDNF-mediated signaling in the dorsal striatum (see section below on BDNF). The mechanisms responsible for inhibition of ERKs by ethanol elsewhere are not known.

Few studies have examined ERK activity in ethanol-dependent rodents. An older report found that ERK activity is increased during ethanol withdrawal in the dorsal striatum, cerebellum and especially in the amygdala (Sanna et al. 2002). ERK activation can induce transcription of the immediate-early gene *c-fos*. Acute administration of ethanol stimulates *c-fos* in several brain regions, but only in the MeA is this ERK-dependent (Hansson et al. 2008). However, in ethanol-dependent rats, induction of *c-fos* by an ethanol challenge is inhibited in orbital frontal cortex and NAc shell through an ERK-dependent mechanism (Hansson et al. 2008), suggesting that in these brain regions ERKs are part of a homeostatic response that suppresses ethanol-induced *c-fos* expression mediated by other signaling pathways. Overall, ethanol's effects on ERK signaling are heterogeneous and depend not only on the brain region studied but also on whether animals are in an ethanol-naïve or -dependent state. The net effect of ERK signaling may be to suppress ethanol intake, since recent evidence indicates that systemic administration of the MEK inhibitor SL327 increases operant self-administration of ethanol in C57BL/6J mice (Faccidomo et al. 2009), and this inhibitory mechanism of ERKs on ethanol consumption may be mediated via BDNF (see section on BDNF).

2.4 P13K, AKT and GSK3beta

Phosphatidylinositol-3-kinase (P13K) is a lipid kinase that phosphorylates phosphatidylinositides (PtdIns) at the plasma membrane leading to the recruitment of the downstream serine and threonine kinases, 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT, to the membrane, where PDK1 phosphorylates and activates AKT. The PI3/AKT pathway contributes to diverse biological functions such as cell survival and growth (Brazil and Hemmings 2001; Engelman 2009), and in the CNS, AKT phosphorylates the β2 subunit of the $GABA_A$ receptor leading to increased membranal localization of β2 containing $GABA_A$, thereby increasing $GABA_A$ receptor-mediated synaptic transmission (Wang et al. 2003). Interestingly, several independent investigations in flies and rodents recently indicated an important role for the P13K/AKT pathway in ethanol's actions. Specifically, inhibition of P13K in the NAc reduced binge drinking in C57BL6 mice (Cozzoli et al. 2009) and in rats (Neasta et al. 2011a). These results suggest that ethanol treatment results in the activation of P13K in the NAc, but the mechanism underlying P13K activation is not yet clear. One possibility is that ethanol activates a small Ras family G-protein upstream of P13K. Ras proteins (H-Ras, K-Ras and N-Ras) cycle between active GTP-bound and inactive GDP-bound forms. Active GTP-bound Ras interacts with several effector proteins, and among them is P13K. Interestingly, we previously observed that acute ex vivo treatment of hippocampal slices with ethanol leads to a robust activation of H-Ras (Suvarna et al. 2005).

As menioned above, AKT is activated in response to the activation of P13K. AKT activation can be measured by phosphorylation of AKT on threonine 308 and serine 473. Systemic administration of 0.75 g/kg ethanol to young adult (3-week) mice increases phosphorylation of AKT at Thr-308 in the striatum (Bjork et al. 2010). Administration of a higher dose (1.5 g/kg) increases AKT Thr-308 phosphorylation measured 45 min later in the medial prefrontal cortex, but not in the NAc of AA rats selectively bred to drink high levels of ethanol (Neznanova et al. 2009). On the other hand, we found that systemic administration of ethanol (2 g/kg) leads to the phosphorylation of AKT at both threonine 308 and serine

473 in the NAc of adult (9-week old) mice (Neasta et al. 2011a). AKT is also phosphorylated (and thus activated) in the NAc of high ethanol drinking Long-Evans rats (Neasta et al. 2011a). The activation of AKT by ethanol is likely to be an important contributor to mechanisms that lead to ethanol-drinking behaviors as the blockade of the AKT pathway within the NAc decreases excessive voluntary consumption and self-administration of ethanol in heavy drinking rats (Neasta et al. 2011a). Finally, using *Drosophila* as a model system, the Heberlein group conducted an elegant set of experiments suggesting that the P13K/AKT pathway contributes to the sensitivity of flies to the sedative actions of ethanol. Specifically, neuronal overexpression of PDK1, the catalytic subunit P13K or AKT increased the duration of ethanol sedation, whereas over expression of the dominant negative form of P13K or RNAi-mediated knockdown of AKT decreased the sensitivity of flies to the acute hypnotic actions of ethanol (Eddison et al. 2011). Together, the studies described above strongly suggest that the P13K/AKT signaling pathway is a key contributor to mechanisms that underlie phenotypes such as excessive ethanol drinking.

The serine/threonine kinases glycogen synthase kinase-3 α and β (GSK-3α and GSK-3β) (Jope and Johnson 2004) are important and well-characterized substrates of AKT, and phosphorylation of GSK-3α on serine 21 and GSK-3β on serine 9 by AKT results in the inhibition of GSK-3 kinase activity (Jope and Johnson 2004). Neznanova et al. did not observe changes in the level of GSK-3β phosphorylation in the NAc of AA rats in response to systemic administration of ethanol, although an increase in GSK3β phosphorylation was detected in the prefrontal cortex (Neznanova et al. 2009). We recently observed that systemic administration of ethanol (2 g/kg) as well as recurring cycles of voluntary consumption of high amounts of ethanol followed by periods of withdrawal in rats lead to an increase in level of phosphorylated GSK-3α and GSK-3β in the NAc (Neasta et al. 2011a). The contribution of GSK-3α or GSK-3β inhibiton to ethanol's actions in the CNS is yet to be determined.

2.5 mTOR

A very important downstream target of AKT is the serine/threonine protein kinase, mammalian target of rapamycin (mTOR) (Hay and Sonenberg 2004). mTOR signals through two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Hoeffer and Klann 2010). The two complexes have unique protein compositions consisting of adaptor proteins, enzymes and substrates (Hoeffer and Klann 2010). mTORC1 plays an essential role in initiating local synaptic protein translation, synaptic plasticity, learning and memory (Costa-Mattioli et al. 2009). We recently found that mTORC1 within the NAc is a novel contributor to molecular mechanisms underlying ethanol drinking (Neasta et al. 2010). Systemic administration of ethanol and high levels of ethanol intake activated mTORC1-mediated signaling in the NAc of mice and rats. In addition, levels of the AMPA receptor subunit GluR1 and the scaffolding protein Homer, two synaptic proteins whose translation is regulated by mTORC1 (Slipczuk et al. 2009), were up-regulated in these rodents (Neasta et al. 2010). Importantly, the FDA-approved inhibitor of mTORC1, rapamycin, decreased ethanol induction of Homer translation, reduced ethanol-induced locomotor sensitization and place preference, and reduced excessive ethanol intake and seeking (Neasta et al. 2010). Interestingly, the translation of postsynaptic density protein 95 (PSD-95), and the activity-regulated cytoskeleton-associated protein (Arc) have been linked to the mTORC1 pathway (Lee et al. 2005; Takei et al. 2004). Both proteins play a role in neuroadaptations underlying ethanol's actions (Carpenter-Hyland and Chandler 2006; Pandey et al. 2008b; Moonat et al. 2011; Camp et al. 2011). Therefore, it would be interesting to determine if ethanol-induced activation of mTORC1 signaling results in translation of these and other synaptic proteins.

3 Tyrosine Kinases

Tyrosine kinases are a large and diverse family of proteins that can be subdivided into two groups: receptor tyrosine kinases (RTK), and the non-receptor tyrosine kinases (NRTK). Both groups share a highly conserved kinase domain and unique protein or lipid binding domains.

3.1 Receptor Tyrsosine Kinases

RTKs are membrane-spanning receptors composed of an extracellular N-terminal region, a membrane-spanning region, and an intracellular C-terminal region, which contains the catalytic domain. Most receptors dimerize upon ligand binding allowing auto- and trans-phosphorylation to occur. Tyrosine phosphorylation at the C-terminus of the receptor serves as a docking site for adaptor proteins, which, in turn, recruit enzymes that initiate the activation of a signaling cascade. The most well-characterized ligands that interact and activate RTKs are growth factors, and in recent years several growth factors have been associated with molecular and behavioral adaptations in response to ethanol.

3.1.1 EGF—Epidermal growth factor (EGF) and its receptor (EGFR) are expressed in adult neurons in brain regions such as the hippocampus, cerebellum and cerebral cortex (Wong and Guillaud 2004). In the hippocampus EGF enhances the activity of the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) and increases long-term potentiation (LTP) in CA1 pyramidal neurons (Wong and Guillaud 2004). EGF also plays a protective role in stimulating the survival of rat cortical and dopaminergic neurons (Wong and Guillaud 2004). Recent evidence suggests a contribution of EGFR-mediated signaling to ethanol's actions in the CNS. A forward genetic screen in *Drosophila* identified mutants in the gene *happyhour*, which display a high level of resistance to ethanol intoxication (Corl et al. 2009). The protein encoded by *happyhour* shows strong homology to mammalian Ste20 family kinases and acts to inhibit EGFR-mediated activation of ERK (Corl et al. 2009). In addition, inhibitors of the EGFR signaling increase the sensitivity of both flies and mice to the intoxicating properties of ethanol (Corl et al. 2009). Interestingly, incubation of cultured cancer cells with ethanol (43 mM) produces a rapid and robust phosphorylation and activation of the EGFR and of ERK1/2, and an inhibitor of the EGFR blocks both phosphorylation events (Forsyth et al. 2010), providing a direct link between ethanol and EGFR-mediated signaling. In rats, systemic administration of EGFR inhibitors reduces voluntary consumption of ethanol but not sucrose (Corl et al. 2009). Together, these results indicate that the EGFR is part of an evolutionary conserved signaling pathway activated by ethanol exposure that contributes to mechanisms underlying ethanol intoxication as well as consumption.

3.1.2 Insulin—Insulin is not produced in the brain, but circulating insulin crosses the blood–brain barrier. Insulin receptors (IRs) are expressed in both astrocytes and neurons in brain regions such as the hypothalamus, hippocampus, cerebellum, amygdala and cerebral cortex. Insulin's main roles in the CNS are the control of food intake and cognitive functions such as memory (Laron 2009). In *Drosophila*, activation of insulin signaling in the CNS plays a regulatory role in ethanol-mediated intoxication, as inhibition of the pathway or reduction in the function of insulin-producing cells increases the severity of fly intoxication (Corl et al. 2005). Although these results are intriguing, the role of insulin in ethanol intoxication needs to be confirmed in mammals.

3.1.3 GDNF—The glial-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor β (TGF-β) superfamily. Although GDNF was originally identified in a glial cell line (Lin et al. 1993), it is mainly expressed in neurons of the adult

brain (Pochon et al. 1997; Barroso-Chinea et al. 2005). Binding of GDNF to its co-receptor, GFRα1 leads to the recruitment of the RTK, Ret, to the GFRα1-GDNF complex (Jing et al. 1996), and Ret is then activated by autophosphorylation (Durbec et al. 1996). The main signaling pathways that are downstream of Ret activation are ERK1/2, P13K and PLCγ (Airaksinen and Saarma 2002). GDNF is highly expressed in the NAc, and its receptors (Ret and GFRα1), are localized in the VTA (Trupp et al. 1997). We recently showed that dopaminergic terminals in the nucleus accumbens retrogradely transport GDNF to the VTA, where the growth factor increases the spontaneous activity of dopaminergic neurons, resulting in an increase in dopamine overflow in the NAc (Wang et al. 2010a). Accumulating evidence suggests that GDNF in the mesolimbic dopaminergic system plays an important inhibitory role in ethanol-drinking behavior. A single administration of GDNF into the VTA of Long-Evans rats results in a very rapid and sustained reduction of ethanol intake in two-bottle choice continuous access and operant self-administration paradigms (Carnicella et al. 2008, 2010; Carnicella and Ron 2009). Interestingly, GDNF's actions are specific for ethanol and are not due to a general reduction of reward or changes in locomotor activity, as the growth factor has no effect on operant self-administration of sucrose (Carnicella et al. 2008). Importantly, intra-VTA infusion of GDNF 10 min before the beginning of an operant session blocks the reacquisition of operant responding for ethanol after a period of extinction (Carnicella et al. 2008). Activation of the GDNF pathway in the VTA results in the phosphorylation of ERKs (Carnicella et al. 2008; Wang et al. 2010a), which is required to reduce ethanol consumption, since inhibition of ERKs in the VTA blocks GDNF inhibition of ethanol self-administration (Carnicella et al. 2008). Finally, mice haploinsufficient for GDNF or its receptor, GFRα1, consume more ethanol after a period of abstinence compared with wild type littermates (Carnicella et al. 2009b). In addition, these mice exhibit increased place preference for ethanol compared with wild type mice (Carnicella et al. 2009b). These results suggest that endogenous GDNF-mediated signaling contributes to mechanisms that protect against addiction by suppressing or delaying the development of ethanol reward and limiting relapse to drinking.

3.1.4 BDNF—The brain derived neurotrophic factor (BDNF) belongs to the nerve growth factor (NGF) family of neurotrophic factors. BDNF and its receptor TrkB are widely distributed throughout the brain, and the BDNF/TrkB pathway plays an important role in neuronal proliferation, differentiation and survival, as well as synaptic plasticity (Lewin and Barde 1996; Yoshii and Constantine-Paton 2010). More recently, BDNF has been implicated in psychiatric disorders such as depression and anxiety (Martinowich et al. 2007). In addition, a growing body of literature suggests a role for BDNF in drug addiction (Ghitza et al. 2010), and we and others generated evidence that suggests a unique role for BDNF in regulating behavioral responses to ethanol. Specifically, a reduction in *BDNF* gene expression in BDNF heterozygous knockout mice (Hensler et al. 2003; McGough et al. 2004), or inhibition of the BDNF receptor TrkB (Jeanblanc et al. 2006), increases ethanol consumption and preference. Moreover, acute systemic administration of ethanol and voluntary intake of moderate amounts of ethanol, through two-bottle choice or operant self-administration paradigms, increases *BDNF* expression in the dorsal striatum of mice and rats (McGough et al. 2004; Jeanblanc et al. 2009; Logrip et al. 2009). Ethanol-mediated increases in *BDNF* mRNA result in increased synthesis of BDNF and activation of ERK (Logrip et al. 2008), and to increased expression of downstream genes, such as the dopamine D3 receptor and dynorphin (Jeanblanc et al. 2006; Logrip et al. 2008). Interestingly, BDNF in the dorsal striatum, in turn, acts as an endogenous inhibitor of ethanol consumption (McGough et al. 2004; Jeanblanc et al. 2009), and this action is localized to the dorsolateral striatum (Jeanblanc et al. 2009), a brain region associated with habit learning (Yin and Knowlton 2006). In contrast, long-term, daily ethanol intake in C57BL6 mice results in a breakdown of this protective homeostatic pathway in the dorsal striatum (Logrip et al.

2009). In addition, chronic, high levels of ethanol intake decrease cortical BDNF mRNA (Logrip et al. 2009). These results are in line with previous data showing that a decrease in cortical BDNF can be detected 24 h after withdrawal from chronic ethanol treatment (Pandey et al. 1999b).

Elegant studies by Pandey and colleagues suggest that BDNF in the CeA and MeA plays a protective role against anxiety and ethanol consumption during ethanol withdrawal. Reducing BDNF in the amygdala increases anxiety and ethanol consumption in rats (Pandey et al. 2006; Pandey et al. 2008b). In contrast, the anxiolytic actions of ethanol are associated with increased expression of BDNF, as well as BDNF-induced expression of Arc in the CeA and MeA, and infusion of BDNF in the CeA reverses ethanol withdrawal-induced anxiety (Pandey et al. 2008b). Interestingly, BDNF mRNA and protein levels are lower in the extended amygdala of P rats compared with NP rats, which is consistent with BDNF's role in suppressing ethanol intake (Prakash et al. 2008).

Consistent with these results in rodents, Heberlein and colleagues (Heberlein et al. 2010) recently reported that the level of BDNF in the serum of alcohol-dependent patients is negatively correlated with the severity of withdrawal symptoms. In addition, a single nucleotide polymorphism (Val66Met) in the *BDNF* gene, which leads to a reduction in BDNF function (Chen et al. 2004), has been linked with an earlier onset of alcoholism (Matsushita et al. 2005), and a recent human study reported a higher risk of relapse in ethanol-dependent patients with this polymorphism (Wojnar et al. 2009).

3.2 The Src Family of Protein Tyrosine Kinases

The Src family of protein tyrosine kinases (Src PTKs) are intracellular, membrane-bound enzymes that play an important role in various cellular functions. Four members of the family are expressed in the brain (Src, Fyn, Lyn and Yes) (Kalia et al. 2004). Src and Fyn have been heavily implicated in modulation of NMDARs and synaptic plasticity (Salter and Kalia 2004). Fyn is also an important mediator of neurite outgrowth and myelination by oligodendrocytes (Beggs et al. 1994; Bodrikov et al. 2005; Brackenbury et al. 2008; Sperber et al. 2001), and has been implicated in Alzheimer's disease (Lee et al. 2004; Chin et al. 2005). Lyn was reported to interact with the Na^+/K^+ ATPase and to phosphorylate its α3 subunit (Wang and Yu 2005). Lyn was also shown to negatively regulate NMDAR activity (Umemori et al. 2003). A link between Lyn and the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) in the cerebellum has also been suggested (Hayashi and Huganir 2004). Very recently, we found that Lyn negatively regulates the release of dopamine in the mesolimbic system (Gibb et al. 2011). Although Yes is highly expressed in the basal ganglia (Walaas et al. 1993), its role in the CNS has yet to be determined.

3.2.1 Src and Fyn in Ethanol Modulation of NMDA Receptors—Interestingly, ethanol exposure results in opposing actions on the activity of Src and Fyn. Acute treatment of hippocampal neurons with ethanol inhibits Src (Suvarna et al. 2005), but activates Fyn (Yaka et al. 2003b). Src inhibition, in turn, results in the internalization of the NR2A subunit of the NMDAR (Suvarna et al. 2005), and this mechanism contributes to the acute inhibitory actions of ethanol on channel function (Suvarna et al. 2005). In the hippocampus, ethanol stimulates Fyn-mediated phosphorylation of NR2B, which contributes to the development of acute tolerance to ethanol both ex vivo and in vivo (Ron 2004; Ron and Jurd 2005). Fyn is also activated in the dorsal striatum in response to ethanol (Wang et al. 2007; Wang et al. 2010b). Interestingly, ethanol-stimulated phosphorylation of NR2B is not observed in a structurally related brain region, the NAc (Wang et al. 2007). Furthermore, Fyn-mediated activation and NR2B phosphorylation results in long-term facilitation (LTF) of NR2B-

containing NMDARs in the dorsal striatum of ethanol exposed rodents (Wang et al. 2007). This LTF of NMDAR activity is centered in the dorsomedial striatum (DMS) (Wang et al. 2010b), a brain region implicated in goal-directed behaviors (Yin et al. 2005). Importantly, repeated daily systemic administration of ethanol leads to prolonged activation of Fyn, increased NR2B phosphorylation and membrane localization of the receptor subunit in the DMS (Wang et al. 2010b). These events are associated with a long-lasting increase in the activity of the NR2B-NMDARs in this brain region (Wang et al. 2010b). Furthermore, inhibition of NR2B–NMDARs or Src family PTKs in the DMS but not the DLS or the NAc significantly decreases operant self-administration of ethanol and reduces ethanol-primed reinstatement of ethanol seeking (Wang et al. 2007; Wang et al. 2010b). Together, these results suggest that Fyn phosphorylation of NR2B and subsequent up-regulation of NMDAR function within the DMS contribute to the maladaptive synaptic changes that promote excessive ethanol intake and relapse.

In contrast to the above results, a recent study by Wu and colleagues (Wu et al. 2010) showed that tyrosine phosphorylation of NR2B in the hippocampus is markedly reduced in rats fed a liquid ethanol diet. The discrepancy between Wu's results and ours could be due to differences in paradigms. It is also important to note that the basal level of NR2B phosphorylation in Wu's study was rather high, while we did not detect a significant basal level of NR2B phosphorylation in the dorsal striatum of control rats (Wang et al. 2007; Wang et al. 2010b).

3.2.2 Lyn and Rewarding Properties of Ethanol—As mentioned above, we recently found that Lyn negatively regulates the release of dopamine in SHSY5Y human neuroblastoma cells and in the mouse NAc (Gibb et al. 2011). Acute exposure of rodents to ethanol causes a rapid increase in extracellular dopamine in the NAc (Gonzales et al. 2004), and we found that overexpression of the active form of Lyn in the VTA blocks ethanol-mediated dopamine overflow. Dopamine transmission is a contributor to ethanol reward-related behaviors (Gonzales et al. 2004), and we observed an inverse relationship between the protein level and the activity of the kinase versus the rewarding properties of ethanol (Gibb et al. 2011). Place preference for ethanol was increased in Lyn knockout mice compared with wild type littermates (Gibb et al. 2011) but was reduced in mice overexpressing an active form of Lyn in VTA neurons compared with control mice (Gibb et al. 2011). Together, these results suggest that Lyn contributes to a mechanism that controls the extracellular levels of DA, and by doing so, the kinase reduces the rewarding properties of ethanol.

4 Scaffolding Proteins

Scaffolding proteins are a diverse group of proteins that allow for the orchestration of multiple signaling events, and provide a focal point of interaction between signaling proteins such as kinases, phosphatases, their substrates, intracellular organelles, the cytoskeletal network and the plasma membrane. Scaffolding proteins also provide platforms that allow spatially and temporally segregated events to occur. Changes in the protein–protein interactions between scaffolding proteins and their associated binding partners are potentially important consequences of neuroadaptation to ethanol. Here we review the contribution of three scaffolding proteins that play important roles in the actions of ethanol on the adult brain, although other proteins such as β-arrestin, and A-kinase anchoring proteins are likely to also be involved in mediating ethanol's effects.

4.1 RACK1

RACK1 is a scaffolding protein that is highly expressed in the CNS (Ashique et al. 2006) and was originally identified as an anchoring protein of PKCβII (Ron et al. 1994). The

RACK1 amino acid sequence is characterized by seven WD40 repeats that form a seven-blade β-propeller structure (Coyle et al. 2009; Smith et al. 1999), enabling the protein to interact with a large number of binding partners including several enzymes such as Fyn kinase (Yaka et al. 2002), focal adhesion kinase (FAK) (Kiely et al. 2009), receptor protein tyrosine phosphatase μ (PTPμ) (Mourton et al. 2001), the cyclic AMP-specific phosphodiesterase isoform PDE4D5 (Yarwood et al. 1999), as well as with the intracellular tails of receptors like the insulin-like growth factor 1 receptor (IGF-1R) (Kiely et al. 2002; Hermanto et al. 2002), the inositol 1, 4, 5-triphosphate receptor (Patterson et al. 2004) and the NR2B subunit of NMDARs (Yaka et al. 2002). Interestingly, the intracellular compartmentalization of RACK1 changes in response to stimuli. For example, cellular stress such as hypoxia and heat shock results in RACK1 association with cytoplasmic stress granules (Arimoto et al. 2008). In contrast, upon activation of PKCβII, RACK1 shuttles active PKCβII to its site of action (Ron et al. 1999), whereas activation of PKA induces translocation of RACK1 to the nucleus (Yaka et al. 2003a; He et al. 2010). Exposure of cells to ethanol changes the intracellular localization of RACK1 via a mechanism that requires activation of PKA (Ron et al. 2000; He et al. 2002; Yaka et al. 2003b; Wang et al. 2007). One of the consequences of RACK1 nuclear localization is the inhibition of PKCβII translocation which was observed both in cultured cells and in vivo (Ron et al. 2000). Furthermore, under basal conditions, RACK1 interacts with and localizes Fyn kinase in close proximity to the NR2B subunit of the NMDAR, but inhibits the ability of Fyn to phosphorylate the channel until the appropriate signal occurs (Yaka et al. 2002; Yaka et al. 2003a; Thornton et al. 2004). Formation of this tri-molecular complex is not ubiquitous in the brain; it is found in hippocampus and dorsal striatum, but not in the cerebral cortex or NAc (Yaka et al. 2003a; Wang et al. 2007) (see also section on Src, Fyn and modulation of NMDAR function in response to ethanol). In the hippocampus and dorsal striatum, acute ex vivo ethanol treatment releases RACK1 from the NMDAR complex, which enables Fyn to phosphorylate NR2B (Yaka et al. 2003b; Wang et al. 2007). In addition, ethanol-stimulated translocation of RACK1 to the nucleus increases expression of *BDNF* in hippocampal and dorsal striatal neurons (McGough et al. 2004). In SHSY5Y cells, nuclear RACK1 localizes at the promoter IV region of the *BDNF* gene, resulting in chromatin modifications that lead to promoter-controlled *BDNF* exon IV transcription (He et al. 2010). It will be of great interest to determine if RACK1-dependent epigenetic modulation of *BDNF* expression contributes to ethanol's actions in the brain (see also section on Epigenetic regulation of gene expression). Finally, in vivo evidence suggests that RACK1-mediated increases in BDNF levels in the dorsal striatum are part of a homeostatic pathway that regulates behaviors such as voluntary ethanol intake and ethanol sensitization (McGough et al. 2004; Jeanblanc et al. 2006) (see also section on Receptor Tyrosine Kinases). In summary, RACK1 provides an example of how changes in the compartmentalization of a scaffolding protein, as well as modifications in protein–protein interactions, can lead to the inhibition or activation of numerous signaling pathways in response to ethanol exposure.

4.2 PSD-95

The postsynaptic density protein of 95 kDa (PSD-95) is a core scaffolding protein that clusters NMDARs at glutamatergic synapses, connecting the receptors to the cytoskeleton and to signaling proteins that regulate channel function (Kim and Sheng 2004). PSD-95 has been implicated in synaptic plasticity underlying learning and memory (Kim and Sheng 2004). A recent study shows that PSD-95 knockout mice exhibit greater signs of ethanol intoxication and show less voluntary ethanol intake than wild type littermates (Camp et al. 2011). Although both genotypes showed similar levels of ethanol preference, wild type, but not PSD-95 knockout mice, maintained their preference for ethanol when tested 14 days later. Surprisingly, the deficits attributed to deletion of PSD-95 do not seem to involve altered NMDAR function since MK801, an NMDAR antagonist-enhanced ethanol

intoxication to a similar extent in both genotypes (Camp et al. 2011). These results suggest that PSD-95 contributes to the level of ethanol intoxication, which can influence ethanol intake. In addition, these results imply that PSD-95 contributes to reward memory. However, the mechanism by which PSD-95 contributes to ethanol's actions in vivo has yet to be unraveled.

4.3 Homer

Homer proteins are structurally related scaffolding proteins that are the products of three independent genes, Homer1, 2 and 3 (Fagni et al. 2002). These genes can give rise to constitutively expressed long isoforms (Homer1b, c, d, Homer 2a, b and Homer3) and an immediate-early gene (short) isoform (Homer1a) (Soloviev et al. 2000). The long Homer isoforms contain a coil–coil domain and leucine zipper motifs allowing them to assemble as multimers (Hayashi et al. 2006). Homer proteins contain the protein–protein interaction binding motif Enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) that enables the direct interaction of homers with various proteins. Homer proteins connect ion channels and receptors to intracellular calcium storage, the cytoskeleton (Thomas 2002; Sala et al. 2001), and to various signaling cascades including ERK (Mao et al. 2005) and P13K (Rong et al. 2003).

Several studies by Szumlinski and colleagues indicate that the Homer2 isoform plays an important role in ethanol's actions. Consumption of high levels of ethanol increases Homer2 expression in the NAc of mice, and this increase persists even 2 months after the last ethanol-drinking session (Klugmann and Szumlinski 2008; Cozzoli et al. 2009). Our finding that high levels of ethanol intake increase Homer proteins in the NAc via mTORC1 (Neasta et al. 2010) (see section on mTOR) may provide a mechanism for ethanol-mediated induction of Homer2 protein levels, although the antibodies we used did not differentiate between the long isoforms of Homer. Homer2 knockout mice consume less ethanol than wild type mice in a two-bottle choice continuous access paradigm, and do not develop ethanol place preference or locomotor sensitization to ethanol (Szumlinski et al. 2005). Homer2 knockout mice instead show ethanol place aversion and an increased hypnotic response to high doses of ethanol compared with wild type mice. In addition, Homer2 knockout mice do not show certain characteristic neurochemical changes associated with repeated ethanol administration (Szumlinski et al. 2005). In line with these findings, knockdown of Homer2 in the shell of the NAc reduces binge drinking in C57BL/6 mice (Cozzoli et al. 2009) and both the behavioral and neurochemical abnormalities in Homer2 knockout mice can be rescued by an administration of an adeno-associated virus (AAV) expressing Homer2 into the NAc (Klugmann and Szumlinski 2008). Interestingly, the contribution of the Homer gene to ethanol's actions has also been observed in *Drosophila*; flies lacking the Homer gene show increased sensitivity to the sedative actions of ethanol and do not develop acute tolerance to ethanol (Urizar et al. 2007).

5 Epigenetic Regulation of Gene Expression

Transcription factors and other regulatory proteins regulate gene expression by binding to specific sites in the genome. Layered on top of this process are epigenetic mechanisms that control the way genomic DNA is packaged into chromatin and regulate access of transcription factors to target DNA sequences. These mechanisms regulate DNA methylation, covalent modification of histones and positioning of nucleosomes, and have the potential to produce long-lasting changes in gene expression that are self-perpetuating in the absence of the signals that initiate them. Chromatin changes may be transient or long lasting, mitotically transmissible, and in some cases inherited through meiosis to the next generation (Youngson and Whitelaw 2008; Dulac 2010). Epigenetic mechanisms have recently become topics of intense interest in the addiction field since they could produce persistent

neuroadaptations that underlie drug tolerance and addiction (Tsankova et al. 2007). This field of research is still young and ethanol-induced modifications of histone and DNA are just now being identified. Here we describe a few recent examples in the nervous system.

5.1 DNA Methylation

Methylation of cytosine bases in DNA is mainly restricted to CpG dinucleotides and plays an important role in silencing of genes, inactivation of one X chromosome in females and in genomic imprinting of parental alleles. Proteins such as MECP2, which contain a methyl-CpG-binding domain (MBD) can inhibit, or in some cases facilitate, gene expression by binding to methylated CpG islands commonly located in gene promoter regions. DNA methyltransferases (DNMTs) catalyze DNA methylation and are critical for normal development. The enzymes are also expressed in post-mitotic neurons and recent evidence using DNMT inhibitors suggests that DNMTs mediate neuronal plasticity associated with memory (Miller and Sweatt 2007).

Although DNA methylation had been thought to be stable once established, recent evidence indicates that it can be dynamically regulated. For example, in response to maternal care, the glucocorticoid receptor promoter undergoes demethylation leading to increased expression of the receptor and a reduced response of the hypothalamic-pituitary axis to stress in the offspring (Szyf et al. 2005). The mechanisms responsible for DNA demethylation in adult neurons are not yet known.

In humans, chronic alcoholism is associated with increased circulating levels of homocysteine, probably due to impaired homocysteine metabolism (Bleich and Hillemacher 2009). Homocysteine is methylated to yield methionine, which can be metaboilized to S-adenosyl-L-methionine (SAM); SAM is a methyl group donor for DNMTs. This mechanism may explain why chronic exposure to alcohol can lead to hypermethylation and transcriptional silencing of some genes. For example, maternal ingestion of ethanol before or during gestation in mice leads to hypermethylation and reduced gene expression at the epigentically sensitive Agouti viable yellow (A_{vy}) allele of the *Agouti* gene in the offspring (Kaminen-Ahola et al. 2010). Hypermethylation has also been found in cell cycle genes of ethanol exposed neural stem cells (Hicks et al. 2010). These findings suggest that DNA hypermethylation contributes to teratogenic effects of alcohol.

Ethanol exposure can also lead to demethylation and increased expression of certain genes, such as the *NR2B* subunit of NMDA receptors. Specifically, the abundance of the NR2B subunit is persistently up-regulated in C57BL/6 J mouse cortical neurons following chronic intermittent ethanol (CIE) treatment and, a recent analysis suggests that the mechanism involves DNA demethylation of CpG islands within the 5′ regulatory region of the *Grin2b* gene (Qiang et al. 2010). CIE treatment decreased the association of MeCP2 with chomatin and with regulatory regions of the gene. Conversely, methylation of these regions in vitro decreased binding of the CREB transcription factor to the *Grin2b* promoter. Treatment with SAM to promote DNA methylation prevented CIE-induced demethylation of the *Grin2b* promoter and CIE-induced increases in NR2B mRNA. The mechanism for this effect appeared to involve a CIE-mediated decrease in the level of mRNA for the DNA methyltransferase Dnmt1 that persists for at least 5 days after ethanol treatment; however, how this decrease occurs is not yet known.

5.2 Histone Acetylation and Up-Regulation of Neuropeptide Y

Covalent modification of histone is another mechanism for epigenetic regulation of gene expression. Modifications occur at N-terminal tail regions of histones and alter histone-DNA and histone–histone binding. Identified modifications include acetylation, methylation,

phosphorylation, ubiquitination, ADP-ribosylation and SUMOylation (Kouzarides 2007). Most is known about histone acetylation in regulating neuronal gene expression. Histone acetyltransferases (HATs) add acetyl groups to specific lysine residues, which relaxes local chromatin structure and permits transcription factor binding to DNA (Tsankova et al. 2007). For example, the CREB binding protein (CBP) is a HAT and its recruitment by CREB facilitates CREB-mediated gene expression. Conversely, histone deacetylases (HDACs) remove acetyl groups, thereby promoting chromatin condensation and decreasing transcription.

Recently, Pandey and colleagues (Pandey et al. 2008a) reported that the anxiolytic effect of ethanol is associated with increased levels of CBP, histone acetylation and NPY in the rat CeA and MeA, whereas ethanol withdrawal is associated with increased anxiety-like behavior and decreased CBP, histone acetylation and NPY in these brain regions. To investigate whether changes in histone acetylation are causally related to anxiety and NPY expression, the authors treated ethanol-withdrawn rats with trichostatin A (TSA), an HDAC inhibitor. TSA restored histone acetylation and NPY expression, and reduced anxiety-like behavior in rats undergoing ethanol withdrawal. These findings suggest a link between these events, but these results should be viewed with caution given the action of HDACs on other genes, as well as the limited specificity of TSA (Dulac 2010).

5.3 MicroRNA

MicroRNAs (miRNAs) are a large family of non-coding RNAs that may control translation from as many as 60% of all protein-coding transcripts (Hicks et al. 2010). Primary miRNA transcripts show internal complementarity and thus adopt a stem-loop structure. These precursors are processed by ribonucleases to form mature 21–23 bp miRNAs that form miRNA-induced silencing complexes (miRISCs) by associating with the proteins Argonaute and GW182 [glycine-tryptophan (GW) repeat-containing protein of 182 kDa]. miRNAs regulate protein synthesis by base-pairing to target mRNAs, most commonly at their 3′-untranslated region, allowing miRISC-mediated repression of translation, or induction of deadenylation and degradation of mRNA.

Recent studies have documented ethanol-induced changes in up to 3% of miRNAs in models of alcohol-induced liver disease and teratogenesis, but less is known about ethanol regulation of miRNA in the adult nervous system (Miranda et al. 2010). A recent, in-depth study by Pietrzykowski and colleagues identified miR-9 as a key regulator of ethanol-sensitive BK channel splice variants that contributes to ethanol tolerance (Pietrzykowski et al. 2008). The BK channel is a high conductance calcium- and voltage-dependent potassium channel that is potentiated by ethanol (Treistman and Martin 2009). In the rat supraoptic nucleus and striatum these channels develop tolerance to ethanol, resulting from decreased ethanol sensitivity and reduced channel density. The decrease in BK channel density is associated with decreased mRNA encoding the main pore-forming subunit of the channel, and involving, in particular, those mRNA splice variants that recognize miR-9 and encode for subunits that are most ethanol-sensitive. Analysis of other predicted miR-9 target transcripts with a known role in ethanol's actions revealed 8 whose expression was decreased and 2 whose expression was increased by exposure to 20 mM ethanol for 15 min. These targets encode several proteins of interest for understanding addiction, such as clock, the dopamine D2 receptor, the β2 subunit of $GABA_A$ receptors and the β1 subunit of voltage-gated calcium channels (Pietrzykowski et al. 2008). How ethanol rapidly increases levels of miR-9 is not known, but may involve increased expression or processing of its precursor.

6 Summary

In this chapter, we covered progress that has been made on elucidating signaling pathways such as those involving PLC/PKC and P13K/AKT/mTORC1 that underlie or maintain behaviors associated with alcohol use disorders, as well as cascades initiated by growth factors such as GDNF that act in the opposite direction. We emphasized the role of signaling molecules such as protein kinases that control post-translational modifications in response to alcohol exposure in rodents, as protein kinases hold great promise as therapeutic targets for CNS diseases (Chico et al. 2009). Most research and development efforts on kinases as drug targets have been focused on oncology. However, signaling cascades are shared across cell types, and information generated in other systems can be of potential use for alcohol use disorders. For example, the EGFR inhibitor, TARCEVA® (Erlotinib), is used to treat non-small-cell lung cancer, yet was reported by Heberlein and colleagues to reduce voluntary consumption of ethanol (Corl et al. 2009) (Fig. 1). Another example is the mTORC1 inhibitor, rapamycin (sirolimus), which is used clinically to prevent rejection in organ transplantation yet was recently found in preclinical rodent models to reduce excessive ethanol intake and seeking (Neasta et al. 2010) (Fig. 1). In addition to mTORC1, its upstream kinase activators AKT and P13K, which show promise as potential drug targets in rodent models (Cozzoli et al. 2009; Neasta et al. 2011b) (Fig. 1), are currently being targeted by pharmaceutical companies for the treatment of various types of cancers (LoPiccolo et al. 2008). In addition, other signaling targets that are potentially of great interest for drug development are PKCε, PKCδ and Fyn (Hodge et al. 1999; Khasar et al. 1999; McMahon et al. 2000; Yaka et al. 2003b; Yaka et al. 2003c; Wang et al. 2007; Wang et al. 2010b), as well as HDAC (Pandey et al. 2008a) (Fig. 1). Of interest are the very recent advances that are being made in the development of small molecules that disrupt protein–protein interactions between signaling and scaffolding proteins (Arkin and Whitty 2009; Blazer and Neubig 2009) that may allow a high degree of desirable specificity in inhibitor action. Another intriguing possibility is the use of FDA-approved drugs such as cabergoline (Dostinex), which are approved for other indications but show promise in preclinical rodent models (Carnicella et al. 2009a) (Fig. 1). In summary, the examples described above put forward the possibility of developing small-molecule inhibitors or activators of specific signaling molecules as novel treatments for alcohol use disorders.

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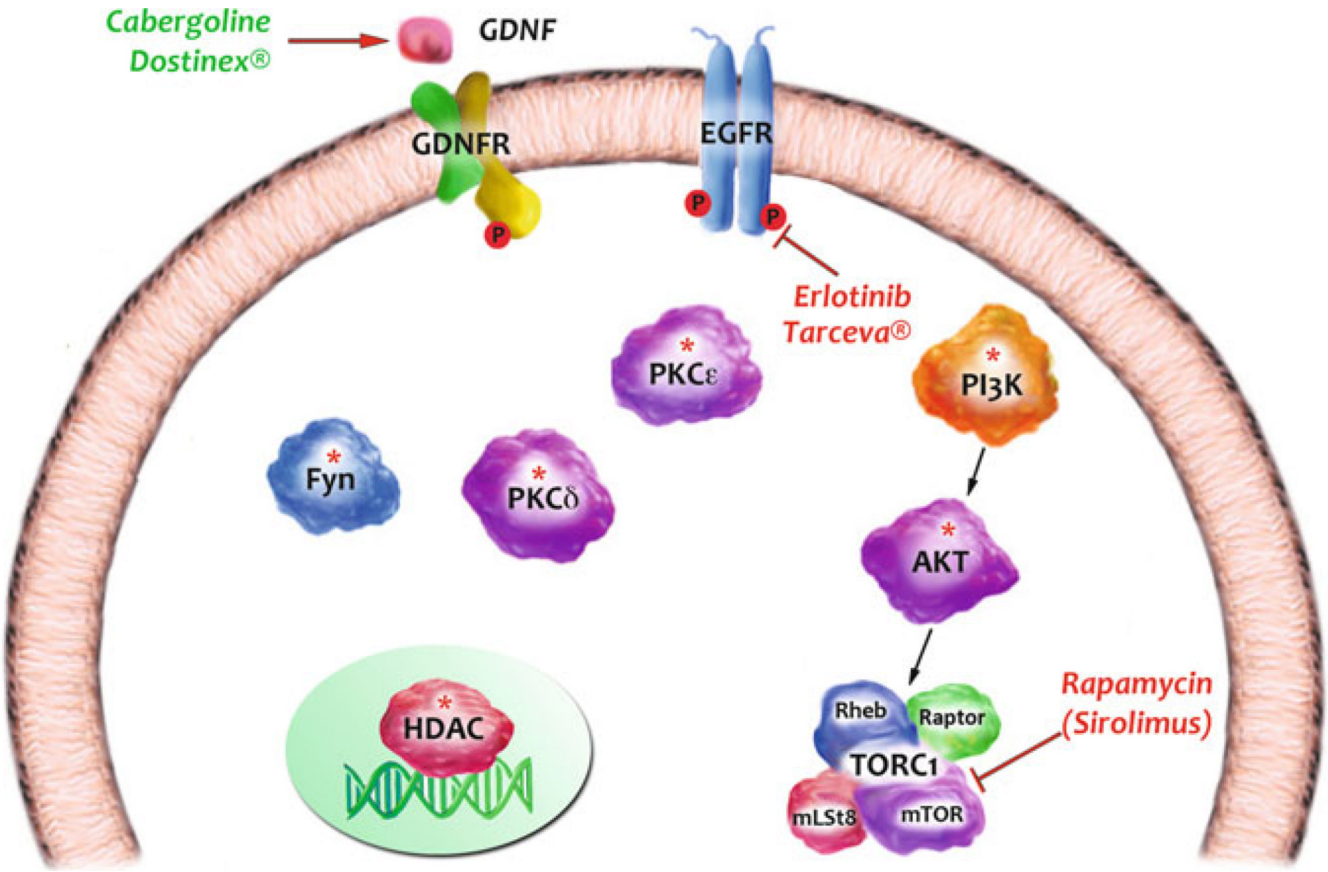

Fig. 1.

Ethanol alters the function of membrane and intracellular enzymes. Inhibitors or up-regulators of these targets can be developed as novel therapeutics for the treatment of alcohol use disorders. Depicted are examples of such targets. *Asterisks* denote targets for which inhibitors are in development. *Red* depicts FDA-approved kinase inhibitors. *Green* depicts an FDA-approved medication that up-regulates the expression of a protective gene