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Protein Kinase C Isozymes and Addiction

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Abstract

Protein kinase C (PKC) has long been recognized an important family of enzymes that regulate numerous aspects of neuronal signal transduction, neurotransmitter synthesis, release and reuptake, receptor and ion channel function, neuronal excitability, development, and gene expression. Much evidence has implicated PKCs in the effects of several drugs of abuse, and in behavioral responses to these drugs. The present review summarizes the effects of both acute and chronic exposure to various drugs of abuse on individual PKC isozymes in the brain. In addition, we summarize recent studies utilizing mice with targeted deletions of the genes for PKCγ and PKCε. These studies suggest that individual PKC isozymes play a role in the development of drug dependence and addiction.

Index Entries: Protein kinase C; isozymes; ethanol; cocaine; amphetamine; opiates; knockout mouse.

Introduction

PKC is a family of 10 structurally related phospholipid-dependent kinases that transduce signals involving lipid second messengers *(1,2).* The first to be isolated were the "conventional" cPKCs (α, βI, βII, and γ), which are activated by calcium and diacylglycerol (DAG). Additional "novel" nPKCs (δ, ε, η, and θ) were subsequently cloned and characterized as being activated by DAG but not by calcium. Finally, two additional "atypical" aPKC isozymes (ζ and λ/ι) were discovered that are insensitive to calcium and DAG. PKC λ and PKCι are the respective human and mouse homologs of the same enzyme. Additional kinases, related to the PKC family are the PKC-related kinases PRK1 and PRK2, which are insensitive to calcium and DAG but show increased activity when bound to activated RhoA GTPase *(3).* Two other enzymes, PKCμ and PKCν, like novel PKCs, are activated by DAG but not by calcium. However, they contain additional functional domains and display a different pattern of substrate specificity, and have now been classified separately as members of the PKD family *(4).*

One of the major signal transduction cascades that leads to PKC activation is the

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phosphoinositide (PI) signaling pathway *(5)*. Stimulation of certain G protein-coupled cell surface receptors activates phospholipase C, which hydrolyzes phosphatidylinositol-4,5-bisphosphate to form inositol triphosphate (IP_3) and DAG. IP_3 binds to intracellular receptors causing release of calcium from stores in the endoplasmic reticulum, whereas DAG binds to and activates most PKC isozymes. DAG may also be formed as a downstream consequence of receptor-mediated activation of phospholipase D *(6)*. In addition, *cis*-unsaturated fatty acids, arachidonic acid, and lysophosphatidycholine produced by phopholipase A_2 can activate or enhance activation of several PKC isozymes *(6–8)*. Likewise, phospatidylinositol-3,4,5-triphhosphate, produced by receptor-mediated activation of PI-3 kinases can activate several PKC isozymes *(9)*.

Before PKCs can respond to second messengers, they must be phosphorylated at three sites in the kinase domain. The first and rate-limiting reaction occurs in the activation loop and is catalyzed by phosphoinositide-dependent kinase 1 (PDK-1), which is in turn regulated by 3′ phosphoinositide products of PI-3 kinase *(10)* This phosphorylation correctly aligns residues at the active site, promoting autophosphoylation of two additional residues at the C-terminus to produce a mature enzyme capable of responding to lipid second messengers. For the cPKCs, PDK-1 phosphorylation is constitutive, whereas for the nPKCs and aPKCs, phosphorylation by PDK1 is under partial regulation by 3′ phosphoinositides providing an additional mode of PKC regulation *(10,11)*.

PKC function is also regulated by subcellular localization, which depends on both kinase:lipid and kinase:protein interactions. PKC activation is generally associated with translocation of PKC from one cellular compartment to another containing lipid activators and proteins that bind the activated form of the enzyme in proximity to substrates. Thus, subcellular fractionation of cells or brain tissue and analysis of PKC content in lipid-containing crude membrane fractions is often used as an indirect measure of PKC activation. Once at the site of a particular substrate, PKCs induce phorphorylation at serine/threonine residues, which in turn alters the function of the substrate protein.

Table 1
Brain Regions Showing Highest Expression of PKC Isoforms in the Adult Rat Brain

Isoform	Region
α	Olfactory bulb, hippocampus, cerebral cortex
βI	Cerebral cortex, hippocampus, cerebellum
βII	Cerebral cortex, striatum, hippocampus, cerebellum
γ	Olfactory bulb, hippocampus, cerebellum
δ	Thalamus, septum, cerebellum
ε	Olfactory bulb, cerebral cortex, hippocampus, striatum, cerebellum
η	Hippocampus
ζ	Cerebral cortex, hippocampus, cerebellum
λ/ι	Olfactory bulb, cerebral cortex, hippocampus, cerebellum
μ	Cerebellum

With the exception of the PKCθ, all PKC isozymes are expressed in the brain, but with varying levels across different brain regions and at different times during development. Many are expressed in both neurons and glia. Table 1 shows the regions of highest expression of each individual isozyme in the adult rat brain. The reader is directed to other published studies for more detailed information *(12–16)*.

Effects of Drugs of Abuse on Brain PKC

Drug addiction is most commonly defined as compulsive and excessive drug intake accompanied by narrowing of the behavioral repertoire towards obtaining the drug *(17)*. All drugs of abuse alter neurotransmission in the brain, and current evidence indicates that chronic drug use alters biochemical pathways that subserve normal neuronal signal trans-

duction *(18–20).* Such drug-induced alterations in brain function are thought to alter the rewarding and aversive qualities of drug responses in such a way as to promote compulsive drug use. Since PKC plays a major role in transducing signals carried by lipid second messengers, it is not surprising that drugs of abuse can alter PKC signaling and that PKC can modulate responses to drugs of abuse.

Ethanol

At anesthetic concentrations, ethanol and other aliphatic *n*-alkanols inhibit purified rat brain cPKCs when assayed in a lipid-independent assay using protamine sulfate as activator and substrate *(21).* Ethanol and short-chain alcohols also inhibit PKCα phosphorylation of protamine sulfate when activated in lipid vesicles containing PS and DAG, whereas long-chain alcohols enhance this activity through differential interaction with high- and low-affinity binding sites within the regulatory portion of the kinase *(22).* However, studies using mixed micelles or sonicated lipids and histone as the substrate have failed to demonstrate an effect of ethanol on PKC activity in vitro *(23,24).*

Ethanol has been found to alter PKC localization in some cell systems. For example, brief (30 s) exposure of cultured astroglial cells to 25–200 m*M* ethanol induces translocation of PKC (isoform unknown) from the cytosol to the crude membrane fraction *(25).* In addition, incubation of NG108–15 neuroblastoma-glioma cells with 200 m*M* ethanol for 2 d induces a translocation of PKCδ from the Golgi apparatus to the perinuclear region and a translocation of PKCε from perinuclear region to the cytoplasm *(26).* It is not known whether these changes in subcellular localization are associated with increased phosphotransferase activity and whether they facilitate normal PKC signaling or sequester PKC isozymes away from physiological substrates.

Early in vitro studies by our group revealed that certain cellular responses to chronic ethanol exposure are mediated by PKCδ and PKCε. Exposure of PC12 cells to 50–200 m*M* ethanol for 2–6 d selectively upregulates the expression of the PKCδ and ε in PC12 cells without altering levels of the α, β, or ζ isozymes *(24).* Subsequent studies showed that PKCε mediates up-regulation of N-type calcium channels *(27)* and enhancement of nerve growth factor-stimulated neurite outgrowth *(28,29)* induced by chronic ethanol exposure. In contrast, PKCδ is required for up-regulation of L-type calcium channels by chronic exposure to ethanol in these cells *(30,31).*

Chronic ethanol exposure can also alter PKC abundance in the nervous system. Chronic administration of ethanol to rats (via liquid diet) reduces radiolabelled phorbol ester binding to cortical and hippocampal homogenates *(32,33),* synaptosomes *(34)* and brain slices *(34),* as well as general PKC activity *(35).* However, none of these studies determined which PKC isozymes are involved in these changes. More recently, it was shown that chronic ethanol consumption (via liquid diet) increases membrane bound PKCα and PKCγ in limbic forebrain regions while decreasing total levels of these isozymes in the frontal cortex *(36).* Interestingly, these investigators found no change in levels of PKCε in either region after chronic ethanol, which contrasts with findings in cell culture (*see* above). These studies are limited by heterogeneity of the tissues examined. Also the effects of ethanol on PKC isozyme levels and activity in adult animals may be highly dependent on factors such as cell type, duration of ethanol exposure, and ethanol concentration.

Prenatal ethanol exposure also been shown to modulate PKC isozyme levels in the brain. Injection of 1 g/kg ethanol into chicken eggs caused time-dependent decreases in overall levels of PKC α, γ, and ε in the postnatal chick brain, with no effects on PKCι *(37).* A recent study examining the effects of pre- and postnatal ethanol exposure on PKC isozymes was also conducted *(38).* In this study, pregnant female rats were fed an ethanol-containing diet for at least 2 mo prior to birth. In addition, the offspring of intoxicated mothers were given an ethanol-containing liquid diet for 8, 30, or 90

postnatal days. Extraction and subcellular fractionation of the cerebral cortex showed increased overall levels of PKC activity, which could be attributed to increases in total and membrane-associated PKCβI, βII and ζ. No changes in the levels of the PKCα, γ, ε and η were found, whereas decreases in the level of PKCδ were observed. The results of these two studies are not easily compared, however, as they not only utilized different species but also used different timing, doses, and durations of ethanol exposure. Nonetheless, they demonstrate the ability of ethanol to alter PKC isozymes in the brain when given during embryonic development.

The predominant inhibitory ligand-gated ion channel in the nervous system is the $GABA_A$ receptor/ionophore complex which, when activated, hyperpolarizes neuronal membranes via increasing chloride influx. Intoxicating concentrations of ethanol can potentiate $GABA_A$ receptor function in several neuronal preparations *(39–41).* Several $GABA_A$ receptor subunits contain consensus sequences for phosphorylation by protein kinases including Src family tyrosine kinases, PKA, PKC, and Ca^{2+}-calmodulin-dependent kinase II *(42),* and evidence suggests that PKC plays an important role in regulating $GABA_A$ receptor sensitivity to ethanol. PKC inhibitors suppress enhancement of $GABA_A$ receptor function by ethanol in hippocampal neurons, suggesting that PKC-mediated phosphorylation is required for ethanol sensitivity in that brain region *(43,44).* Recent studies in knockout mice have implicated PKCγ and ε as key modulators of ethanol's actions at $GABA_A$ receptors (*see* below). PKCγ, but not PKCδ or PKCε, can be co-immunoprecipitated with the α1 and α4 subunits of the $GABA_A$ receptor from rat cerebral cortex and chronic ethanol exposure (in liquid diet) increases PKCγ association with α4 while decreasing its association with α1 *(45).* In addition, the ability of ethanol to potentiate chloride currents generated by another inhibitory receptor, the strychnine-sensitive glycine receptor, in rat ventral tegmental neurons can be blocked by a specific peptide inhibitor of PKCε *(46).* These data provide evidence that PKCγ and PKCε regulate the response of inhibitory ligand-gated ion channels to ethanol.

Psychostimulants

Psychostimulants such as cocaine and amphetamines act in the brain by either inhibiting the reuptake of monoamines via blockade of presynaptic transporters (as is the case for cocaine) or by reversing vesicular and plasma membrane transporters to induce a release of monoamine neurotransmitters (as is the case for amphetamine-related drugs). Psychostimulants are highly addictive, and produce robust behavioral changes in rodents and humans, including arousal, hyperactivity, stereotyped behaviors, and motor sensitization (i.e., increasing motor responses to the drug with each repeated exposure).

Psychostimulants produce hyperactivity primarily by increasing dopamine neurotransmission in the mesolimbic system (i.e., ventral tegmental area—nucleus accumbens pathway) *(47–49).* This process appears to require the activation of PKC, as the locomotor stimulant effects of intra-accumbens amphetamine in rats is attenuated by local infusion of the nonselective PKC inhibitor Ro31-8220 but not the inactive analog bisindoylmaleimide V *(50).* This is presumably due to an inhibition of amphetamine-induced dopamine release, as has been demonstrated by PKC inhibitors in vitro *(50,51).* Similarly, injection of the protein kinase inhibitor H7 intracerebroventricularly *(52)* or into the ventral tegmental area *(53)* reduces acute cocaine-induced hyperlocomotion, as well as cocaine-induced increases in nucleus accumbens dopamine release *(53).* Intrategmental H7 also delays the onset of behavioral sensitization associated with repeated cocaine exposure *(54,55).* However, H7 inhibits the activity of numerous protein kinases aside from PKC, and thus the specificity of these effects on PKC is not certain. Nonetheless, given the established role of PKC in regulation of dopamine transporter function *(56),* investi-

gations into the role of specific PKC isozymes in the locomotor and dopamine-releasing effects of psychostimulants are warranted.

Psychostimulants also produce a phenomenon known as conditioned place preference (CPP), where subjects that are repeatedly exposed to cocaine in a particular environment will demonstrate increased preference for that environment (over a neutral or saline-paired environment) in a drug-free state. This presumably results from the subject's ability to pair or associate the pleasurable effects of the drug with the environment in which the drug is received. There is some evidence that PKC is involved in cocaine CPP, as intracerebroventricular injection of H7 or the more selective PKC inhibitor chelerythrine in rats inhibits the development of a cocaine CPP *(52).* However, these investigators found that PKC was only involved in the *consolidation* of cocaine CPP (i.e., establishing the cocaine-environment associations), as these PKC inhibitors were effective in blocking cocaine CPP only when administered immediately *after* the cocaine-environment pairings, and not when administered *before* the drug-environment pairings or prior to the post-training CPP test session. Thus, PKC appears to be involved in drug-associated memory formation, which is not surprising given the role of PKC in long-term potentiation and learning and memory *(57–60).*

To our knowledge, only one study has been conducted on the role of individual PKC isozymes in the establishment of psychostimulant-environment associations. Thomas and Everitt *(61)* trained rats to press a lever in order to receive an intravenous infusion of cocaine that was accompanied by a distinct environmental stimuli (i.e., illumination of a light). A control group of rats were exposed to cocaine and the environmental stimuli, but in a noncontingent manner (i.e., presentation of the light or tone did not depend on whether the rat executed the lever press for the cocaine infusion). After a 3-d period when no cocaine or environmental cues were given, the rats were placed back in the training environment to determine if they would perform the operant task to obtain cocaine-associated cues. Examination of PKCγ mRNA expression by *in situ* hybridization revealed that animals that had learned the light stimulus was predictive of cocaine infusion demonstrated increased PKCγ mRNA abundance in the amygdala and decreased abundance in the prefrontal cortex. Interestingly, no changes in PKCγ mRNA levels were observed in the hippocampus, despite evidence that PKCγ in this structure is important for various forms of learning and memory *(62–67).* However, rats that were presented associations between cocaine and the light stimulus in a noncontingent manner showed changes in PKCγ mRNA levels in the nucleus accumbens and anterior cingulate cortex. Results of this study indicate not only that PKCγ in multiple brain regions may contribute to the formation of drug-environment associations, but also that distinct neural circuits may underlie such associations based on whether drug-associated environmental cues are predictive of obtaining the drug itself.

Repeated cocaine administration has been reported to alter the levels of individual PKC isoforms in a heterogeneous manner. Steketee and colleagues found that repeated administration of cocaine (15 or 30 mg/kg/d for at least 4 d) produced an overall increase in PKC activity in the ventral tegmental area *(68),* but did not produce any changes in absolute levels of PKCα, βI, βII, or γ in this region. However, repeated cocaine exposure did increase levels of membrane associated PKCβI in the frontal cortex, and decreased levels of PKCα and PKCβII in the striatum when assayed 24 h after the last injection *(69).* In a more recent study utilizing oligonucleotide microarrays with posthoc immunoblot confirmation, Vrana and co-workers found that a more prolonged administration of cocaine to rats (45 mg/kg/d for 14 d) caused increases in PKCα and PKCε mRNA in the hippocampus of rats *(70).*

The recreational drug 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") is a substituted amphetamine that acts by inducing massive release and impaired reuptake of synaptic serotonin and dopamine. Recent evidence

suggests that MDMA may also alter PKC signaling in the brain. Acute administration of MDMA to rats increases membrane bound PKC in cortical synaptosomes, as measured by phorbol ester binding *(71,72);* however, the PKC isozymes involved in this are currently unknown. Nonetheless, these effects appear to be dose- and time-dependent *(72)* and require intact nerve terminals that release serotonin *(71),* which in turn activates receptors that are coupled to phosphoinositide/PKC signaling, such as 5-$HT_{2A/2C}$ receptors *(72,73).*

Opiates

Opiate drugs such as morphine and heroin act on one or more of three subtypes of receptors, namely μ, δ, and κ opioid receptors. While these opioid receptors are primarily linked to cyclic AMP and protein kinase A signaling pathways, recent evidence suggests that the μ receptor, which is crucial for the actions of morphine and heroin, may also be linked to phospholipase D and possibly PKCε *(74,75).*

The work of Garcia-Sevilla and colleagues has provided robust evidence that chronic exposure to opiate drugs such as heroin and morphine alter brain levels of various PKC isozymes. While an acute exposure of rats to morphine (30 mg/kg) up-regulates cortical levels of PKCα and β *(76),* chronic exposure to morphine (10–100 mg/kg/d for 5+ d) results in reductions in cortical levels of these isozymes *(76–78).* These effects are reversed by spontaneous or naloxone-induced withdrawal *(76–78).* Similar effects of acute and chronic treatment with other opiate drugs (i.e., heroin, methadone) have also been observed *(78).* In addition, the frontal cortex of brains from humans addicted to heroin or other opiates has shown similar decreases in PKCα *(77,79),* PKCβ *(77),* but not in the atypical isoform PKCζ *(79).* Thus, levels of PKCα and PKCβ appear to be consistently reduced following chronic opiate exposure in both rodents and humans. The functional consequence of this loss of PKCα and β is currently unknown and needs to be determined.

The data from studies by Garcia-Sevilla et al. are in contrast to those of Narita et al. *(80),* who found that intermittent morphine exposure (1–5 mg/kg, every other day for 3 d) produced no changes in PKCα, βI, βII and ε in the limbic forebrain of mice, but did significantly increase PKCγ levels. No changes in lower midbrain levels of any PKC isozymes were detected. Interesting, this same study also found that intracerebroventricular administration of the PKC inhibitor calphostin C reduced the conditioned place preference produced by repeated morphine exposure (*see* below).

Prenatal exposure to opiates such as heroin has also been shown to alter PKC isozyme expression. When pregnant mice were given heroin 10 mg/kg/d subcutaneously on gestational d 9–18, the hippocampi of offspring mice showed no changes in total levels of PKCα and PKCγ *(81).* However, this study did reveal that prenatal heroin exposure resulted in blunted translocation of PKCγ, but not PKCα, from the cytosol to the membrane in response to cholinergic stimulation of the hippocampus with carbachol. This study emphasizes an important point—that prenatal exposure to opiates (or other drugs of abuse) may not change overall levels of individual PKC isozymes, but may alter their function indirectly via changes in neurotransmitter receptor-mediated stimulation.

Lessons From Mice Lacking Individual PKC Isozymes

Although mice with targeted deletions for several of the known PKC genes have been generated *(82),* the only studies to date examining addiction-related phenomena have been performed in mice with targeted mutations in PKCγ and PKCε.

PKCγ Null Mutant Mice

PKCγ is expressed exclusively in the nervous system and mice lacking this isozyme were generated over a decade ago to examine its role in

long-term potentiation and memory *(64,65).* A few years later the behavioral and biochemical effects of ethanol and other drugs active at $GABA_A$ receptors were examined in these mice *(83)* with some interesting findings. Cortical microsacs from these mice showed no differences in the effects of the direct $GABA_A$ agonist muscimol (2 and 30 μ*M*) on $^{36}Cl^-$ flux, but the addition of ethanol (15 m*M*) to this preparation potentiated $GABA_A$ receptor function in tissue from wild-type but not PKCγ null mutant mice. However, other positive allosteric modulators such as flunitrazepam (0.1–1 μ*M*) and pentobarbital (10–30 μ*M*) were still able potentiate $GABA_A$ receptor function in the presence of 2–3 μ*M* muscimol in both wild-type and PKCγ null mice, indicating a degree of specificity for ethanol. Behaviorally, PKCγ null mice demonstrated reduced hypnotic and hypothermic responses to 3.5 g/kg ethanol when compared with wild-type mice. But in parallel to the in vitro findings on $GABA_A$ receptor function, hypnotic responses to pentobarbital (62 mg/kg) and hypothermic responses to pentobarbital (50 mg/kg) and flunitrazepam (100 mg/kg) were not different from wild-type controls. These data suggest that PKCγ null mice are specifically less sensitive to ethanol but not to other positive allosteric modulators of the $GABA_A$ receptor. These genotypic differences could not be explained by differences in rates of blood ethanol clearance in PKCγ null mutant mice, which has been reported to be similar to that of wild-types *(83,84).* The mechanisms underlying PKCγ regulation of ethanol-induced changes in $GABA_A$ receptor function are unknown, but are thought to involve altered receptor function brought on by reduced PKCγ phosphorylation of an unknown substrate that regulates $GABA_A$ receptor sensitivity to ethanol.

A few years later it was demonstrated that after repeated exposure to ethanol (either by liquid diet consumption or repeated intraperitoneal injections), PKCγ null mutant mice do not become tolerant to the ability of ethanol to produce hypothermia or hypnosis, as measured by loss of the righting reflex (LORR) *(85,86).* This is consistent with the earlier findings of Harris et al. *(83)* that PKCγ null mutant mice display reduced sensitivity to ethanol. However, this lack of behavioral tolerance to ethanol was originally performed in PKCγ null mutant mice on a mixed C57BL/6J × 129/SvJ genetic background, and interestingly, this behavioral phenotype was no longer evident when mice were backcrossed to C57BL/6J mice for six generations. One of the behavioral phenotypes (decreased LORR) reappeared when the mice were subsequently outcrossed to a C57BL/6J × 129/SvEvTac mixed genetic background. These studies emphasize the importance of alleles in the genetic background that can modify or mask phenotypes induced by a null mutation (for a further discussion of this issue, *see* refs. *87,88*).

In humans *(89,90)* and rodents *(91,92)* there is often an inverse relationship observed between sensitivity to the acute effects of ethanol and alcohol consumption. The reduced ethanol sensitivity phenotype of PKCγ null mice was associated with increased ethanol consumption in the two bottle choice paradigm *(93).* No differences in saccharin or nicotine consumption were noted, indicating that the effects were specific to ethanol. Also consistent with reduced sensitivity to ethanol, PKCγ null mutant mice showed decreased sensitivity to the anxiolytic effects of ethanol in the elevated plus maze test *(94).* Ironically, however, PKCγ null mutant mice were more sensitive to the anxiolytic effects of flunitrazepam in the light–dark box test, despite earlier reports that their hypothermic responses to flunitrazepam were similar to that of wild-types *(83).* PKCγ null mice also show decreased basal levels of anxiety-like behavior as measured in three separate testing paradigms *(95),* which may account for their altered sensitivity to flunitrazepam observed in the light–dark box test.

Electrophysiological experiments have revealed further alterations in response to ethanol in PKCγ null mutant mice. In hippocampal slices, application of 80 m*M* ethanol to the CA1 region enhances $GABA_A$ receptor-mediated inhibitory postsynaptic current (IPSC)

amplitude in wild-type mice but not PKCγ null mutant mice *(84)*, providing an electrophysiological basis for reduced sensitivity to ethanol in these mice. Interestingly, opposite results were found in PKCε null mutant mice (*see* below).

To our knowledge, only one study on the effects of drugs of abuse other than ethanol has been conducted using PKCγ null mutant mice. Narita and colleagues *(80)* demonstrated that PKCγ null mutant mice fail to display a conditioned place preference for morphine following 3 d of training at 5 mg/kg. These results are interesting, because the authors also showed that intracerebroventricular administration of the PKC inhibitor calphostin C prior to each drug conditioning session attenuated morphine-induced place preference. These data suggest a role for PKCγ in the conditioned rewarding effects of opiates.

PKCε Null Mice

Since previous work in cultured cells suggested that PKCε mediates certain actions of ethanol (*see* above) we generated PKCε null mice to determine the function of this isozyme in responses to ethanol in vivo *(96,97)*. The brains of these mice displayed no differences in protein levels of other PKC isozymes (as detected by Western blot analysis), indicating a lack of developmental compensation in the absence of PKCε. However, changes in expression of other related genes have not yet been examined. Behaviorally, we found that PKCε null mice consumed approx 75% less alcohol and showed reduced ethanol preference compared with wild-type mice in a two-bottle choice paradigm *(96)*. There were no differences in total fluid consumption or intake of saccharin or quinine containing fluids. Thus, reduced intake was specific for ethanol. Restoration of PKCε by means of tetracycline-regulated transgenic expression elevated alcohol intake to levels observed in wild-type mice, indicating that reduced alcohol consumption in PKCε null mice is due to loss of PKCε in adult neurons *(98)*. Compared with wild-type mice, PKCε null mice also self-administered about 50% less ethanol in an operant self-administration paradigm *(99)*.

Rodents show increased consumption when allowed to self-administer ethanol after a period of forced abstinence ("alcohol deprivation effect") *(100)*. When mice were allowed to self administer ethanol only 2 d per wk, PKCε null mice self-administered 60% less ethanol than did wild-type mice following 104 h of alcohol deprivation *(99)*. These findings were associated with less severe alcohol withdrawal seizures *(101)* and markedly reduced dopamine release in the nucleus accumbens following acute systemic administration of 1 or 2 g/kg ethanol *(99)*. These results suggest that the positive rewarding and reinforcing properties of alcohol, as well as the negative effects of alcohol withdrawal, are reduced in PKCε null mice.

Decreased self administration of ethanol in PKCε null mice was associated with enhanced sensitivity to the acute hypnotic effects of ethanol, as measured by the duration of the ethanol induced LORR *(96,102)*. Blood ethanol concentrations following intraperitoneal injection of 4 g/kg ethanol were similar in wild-type and PKCε null mice, indicating that this response was not due altered ethanol clearance. Since many of the acute effects of ethanol, including sedation and ataxia, are postulated to be due to actions at $GABA_A$ receptors, we examined responses to other drugs that act at these receptors. LORR duration was also prolonged in PKCε null mice in response to pentobarbital, diazepam, and the neurosteroid pregnanolone. Since sedatives can elicit locomotor activation at low doses, we also examined the effect of $GABA_A$ receptor agonists on locomotor activity. We found that ethanol (2 g/kg) elicited much greater locomotor activation in PKCε null mice than in wild-type mice. Diazepam (0.5–4.0 mg/kg), which also allosterically enhances the action of GABA at $GABA_A$ receptors, elicited much greater locomotor activation in PKCε null mice. However, muscimol, a direct-acting $GABA_A$ receptor agonist, elicited a similar degree of locomotor activation at 0.4 mg/kg in both genotypes. These findings suggest that $GABA_A$ receptors in PKCε null

mice are supersensitive to positive allosteric modulators but not to direct agonists of $GABA_A$ receptors.

To test whether $GABA_A$ receptor function is actually altered in these mice, we measured $^{36}Cl^-$ uptake in crude synaptosomes (microsacs) isolated from frontal cortex *(96,102).* Direct activation of receptors by muscimol evoked similar responses in wild-type and PKCε null mice, but ethanol, flunitrazepam, and the neurosteroid alphaxalone caused much greater enhancement of muscimol (1 μ*M*) -stimulated $^{36}Cl^-$ uptake in microsacs from PKCε null mice. This enhanced sensitivity could be reproduced in microsacs from wild-type mice following preincubation with the peptide εV1-2, which specifically inhibits PKCε *(103).* This peptide had no effect on $^{36}Cl^-$ uptake in microsacs from PKCε null mice. These results indicate that supersensitivity to allosteric activators results from loss of PKCε in adult tissue, and not from a developmental abnormality. Ethanol (80 m*M*) also enhanced proximal $GABA_A$ receptor-mediated inhibitory postsynaptic currents in CA1 hippocampal neurons from PKCε null mice but not in neurons from wild-type mice *(84)*. Thus, supersensitivity to ethanol is not limited to receptors found in frontal cortex but can also be detected in hippocampal neurons from PKCε null mice using electrophysiological methods. Taken together, these findings demonstrate that PKCε inhibition enhances $GABA_A$ receptor function, and suggest that PKCε normally suppresses regulation of $GABA_A$ receptors by positive allosteric modulators.

Consistent with known effects of $GABA_A$ receptors on anxiety, PKCε null mice showed reduced anxiety-like behavior in an open field and on the elevated plus maze *(102).* In addition, these mice showed reduced levels of plasma ACTH and corticosterone, indicating reduced function of the hypothalamic–pituitary–adrenal (HPA) axis. This was due to reduced stimulation from outside the HPA axis rather than from impaired HPA function since responses to corticotrophin releasing factor and the corticosterone synthesis inhibitor, metyrapone, were normal. Treatment with a low dose of the $GABA_A$ receptor antagonist, bicuculline, increased corticosterone levels and induced behavior resembling wild-type mice on the elevated plus maze. These studies suggest that absence of PKCε leads to increased $GABA_A$ receptor sensitivity to an endogenous positive modulator (e.g., neurosteroids), thereby reducing anxiety-like behavior and suppressing the HPA axis. Given that anxiety promotes relapse drinking in humans, and that stress can induce relapse drinking in alcohol-deprived rodents, reduced anxiety may contribute to decreased alcohol consumption in PKCε null mice.

PKCε is activated by bradykinin and contributes to bradykinin-mediated sensitization of nociceptors to heat *(104).* In addition, PKCε null mice show decreased nociceptor sensitization by epinephrine *(97).* Moreover, subcutaneous administration of a peptide inhibitor of PKCε (εV1-2 peptide) reduces nociceptor sensitization induced by epinephrine, NGF, or carrageenan in rats. These results indicate a key role for PKCε in nociceptor sensitization. Chronic ethanol exposure causes excitatory changes in specific membrane currents in cultured sensory neurons *(105).* Using a rat model of alcohol-induced hyperalgesia *(106)* we found that this hyperalgesia is acutely attenuated by nonselective protein kinase C (PKC) inhibitors and by the selective peptide inhibitor of PKCε, εV1-2, injected intradermally at the site of nociceptive testing. Chronic ethanol exposure also induced a higher level of PKCε in dorsal root ganglia (DRG) from ethanol-fed rats, supporting a role for enhanced PKCε signaling in nociceptors in ethanol-induced hyperalgesia. When ethanol was withdrawn following 12 wk of chronic administration, a marked enhancement of hyperalgesia developed. Treatment with εV1-2 peptide reversed alcohol-induced hyperalgesia with an efficacy similar to that achieved using bisindoylmaleimide I, a PKC inhibitor that is not isozyme-selective *(107).* This suggests that PKCε is the sole PKC isozyme that mediates pain associated with alcohol-induced hyperalgesia.

Conclusions

It is clear that several drugs of abuse perturb the localization and abundance of PKC. However, there is preciously little data on the effects of abused drugs on specific PKC isozymes. Considering that different isozymes may have totally opposite effects, as exemplified by PKCγ and PKCε regulation of $GABA_A$ receptor sensitivity to ethanol, viewing the PKC family as a unit without regard to isozyme specificity is inadequate. In addition, although several drugs of abuse alter membrane localization or PKC abundance, studies of PKC function are seriously lacking. Whereas PKC translocation to lipid rich subcellular compartments is necessary for PKC activation, relocalization of PKCs does not necessarily equate with their activation. One approach, not utilized yet in the addiction field is to use autophosphorylation as a marker for PKC isozyme activitation. It would also be helpful to identify isozyme-specific substrates to use as markers of PKC activation. Until the effects of abused drugs on PKC isozyme function are known, the meaning of studies that examine changes in PKC localization and abundance in response to drugs of abuse is unclear.

Studies utilizing null mutant mice have, in contrast, shed much light on the roles of PKCγ and PKCε in host responses to ethanol. In particular, findings in PKCε null mice suggest that inhibitors of PKCε might be useful in reducing ethanol consumption, anxiety, and alcohol-induced hyperalgesia. Caveats associated with gene deletion studies may temper such a conclusion, though several phenotypes in PKCε null mice have been confirmed in inbred lines and using pharmacological and gene reconstitution approaches, making it less likely that phenotypes in PKCε null mice are due to developmental abnormalities or genetic background effects. The fact that PKCε and PKCγ null mice demonstrate opposite phenotypes with regards to ethanol sensitivity and consumption underscores the need for individualized study of PKC isozymes in drug addiction. Future studies with other drugs and with additional PKC null mutants *(82)* will undoubtedly provide us with a more complete picture of the role of PKC isozymes in behavioral responses to drugs of abuse.

The genetic component of alcoholism vulnerability appears to be related to allelic variants of alcohol metabolizing enzymes *(108)*, high levels of impulsivity or disinhibition *(109)*, comorbid psychiatric disorders such as schizophrenia and bipolar disease *(110,111)* and a low level of response (LR) to alcohol *(112)*. LR can be determined by observing a reduced effect of ethanol on self-rated subjective responses and on objective measures such as balance and blood cortisol or prolactin levels at a given blood ethanol concentration *(110)*. LR has also been assessed through self-report of a relatively high number of drinks required to generate specific effects of ethanol *(113)*. An inverse association between LR and alcohol preference has also been observed in some inbred strains of mice and rat *(114)* and in several knockout mouse lines including null mutants for PKCγ and PKCε. Studies using C57BL/6 × DBA/2 or long sleep × short sleep recombinant inbred mouse strains have not identified quantitative trait loci for alcohol consumption or ataxic and sedative-hypnotic responses to alcohol that lie near the mouse PKCε gene on chromosome 17E4. However, it is very interesting that the human PKCε gene on chromosome 2p21 lies within a region recently identified in linkage studies of alcoholism susceptibility or low LR to ethanol *(115,116)*. Future studies will determine whether allelic variants of PKCε contribute to alcoholism risk in humans.

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