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Protein Kinase C Isozymes as Regulators of Sensitivity to and Self-Administration of Drugs of Abuse - Studies with Genetically Modified Mice

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Abstract

Studies utilizing targeted gene deletion in mice have revealed distinct roles for individual isozymes of the protein kinase C (PKC) family of enzymes in regulating sensitivity to various drugs of abuse. These changes in drug sensitivity are associated with altered patterns of drug self-administration. The purpose of this review is to summarize behavioural studies conducted on mice carrying targeted deletions of genes encoding specific PKC isozymes (namely the β , γ , δ , and ϵ isozymes), and to critically evaluate the possibility of using pharmacological inhibitors of specific PKC isozymes as modulators of the sensitivity to various drugs of abuse, as well as potential aids in the treatment of substance use disorders.

Keywords

Protein kinase C; isozymes; ethanol; morphine; cannabinoids; targeted gene deletion; mouse

Introduction

The protein kinase C (PKC) family of phospholipid-dependent kinases is comprised of nine genes encoding at least 10 distinct members that are structurally related and transduce signals involving lipid second messengers (Nishizuka, 1988). The ten PKC isozymes are typically divided into subcategories according to their structure, calcium dependence, and lipid activators. The conventional PKCs (cPKCs, α , β I, β II, and γ) are activated by calcium and the lipid signaling intermediate diacylglycerol (DAG). Novel PKCs (nPKCs, δ , ϵ , η , and θ) are activated by DAG but not by calcium. Atypical PKCs (aPKCs, ζ and λ /i) require neither DAG nor calcium but are instead activated by lipids such as phosphatidic acid and arachidonic acid (Hirai and Chida, 2003). PKC λ and PKC ι are the respective human and mouse homologues 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 (Mellor and Parker, 1998). Two other enzymes, PKC μ and PKC ν , are activated by DAG but not by calcium, similar to

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Conflicts of Interest

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nPKCs. However, these enzymes contain additional functional domains and display a different pattern of substrate specificity, and have now been classified separately as members of the protein kinase D family (Ryck *et al.*, 2003).

With the exception of PKC θ , all PKC isozymes are expressed in the brain to varying degrees, and often the degree of expression can vary with developmental stage. Many PKC isozymes are expressed in both neurons and glia. For the precise neuroanatomical localization of individual PKC isozymes, the reader is directed to other published studies for more detailed information (Huang *et al.*, 1988; Young, 1988; Tanaka and Saito, 1992; Naik *et al.*, 2000; Minami *et al.*, 2001). In this review, we will summarize studies that have used targeted deletions of genes encoding specific PKC isozymes to examine their role in regulating the behavioural and biochemical sensitivity to drugs of abuse as well as drug self-administration.

Role of Individual PKC Isozymes in Sensitivity to Drugs of Abuse

Exposure of experimental animals to drugs of abuse such as ethanol, opiates, and psychostimulants can alter the expression or subcellular localization of various PKC isozymes (reviewed in (Olive and Messing, 2004; Newton and Messing, 2006; Newton and Ron, 2007; Lee and Messing, 2008). Behavioural experiments using non-specific PKC inhibitors have suggested roles for this family of enzymes in regulating the behavioural responses to drugs of abuse. For example, nonspecific PKC inhibitors can reduce the locomotor stimulant effects of psychostimulants (Browman *et al.*, 1998; Narita *et al.*, 2004). However, because of the high degree of structural homology between individual PKC isozymes, particularly in the kinase domain, small molecule activators or inhibitors of specific PKC isozymes have been extremely difficult to develop. As a result, researchers have frequently utilized targeted gene disruption and other genetic techniques to determine the precise role of PKC isoforms in addiction-related behaviours (Choi and Messing, 2003; Olive and Ron, 2003).

PKC β

PKC β is a conventional PKC isoform whose gene is subject to alternative splicing to form two variants, PKC β I and PKC β II. This isozyme has well-established roles in immune system function and insulin signaling (Kawakami *et al.*, 2002). However, there is some evidence that it is also involved in behavioural and neurochemical responses to abused drugs. For example, amphetamine-stimulated dopamine efflux in the ventral forebrain is blocked by the PKC β inhibitor LY379196 (Johnson *et al.*, 2005), a compound that has a high degree of specificity for this isozyme (Jirousek *et al.*, 1996). The behavioural significance of this observation was revealed by experiments using PKC β -null mice, which showed significantly reduced locomotor stimulation following acute amphetamine treatment (3 mg/kg) (Chen *et al.*, 2009). Molecular experiments by the same authors revealed that PKC β is essential for normal and amphetamine-induced alterations in trafficking of the dopamine transporter (DAT) (Chen *et al.*, 2009), providing a possible mechanistic explanation for their behavioural observations. We are not aware of experiments examining the sensitivity of PKC β -null mice to other drugs of abuse.

PKC γ

PKC γ is expressed exclusively in the central nervous system, and mice carrying a targeted deletion of gene encoding PKC γ were generated in the early 1990's to examine its role in long-term potentiation and learning and memory processes (Abeliovich *et al.*, 1993a; Abeliovich *et al.*, 1993b). Subsequently, the behavioural and biochemical effects of ethanol were examined in these mice (Harris *et al.*, 1995). Electrophysiological measurements

showed that cortical microsacs from these mice were insensitive to the ability of low concentrations (15 mM) of ethanol to potentiate the function of the GABA_A receptor as measured by ³⁶Cl⁻ influx. In slice preparations of the hippocampal CA1 region, the ability of 80 mM ethanol to potentiate GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) was absent in PKC γ -deficient mice (Proctor *et al.*, 2003). This reduced sensitivity to ethanol's effects on GABA_A receptor function was demonstrated behaviourally; PKC γ -deficient mice had reduced hypnotic and hypothermic responses to high doses (3.5 g/kg) of peripherally administered ethanol (Harris *et al.*, 1995). Subsequently, it was demonstrated that repeated exposure of PKC γ -deficient mice to ethanol (either by liquid diet consumption or repeated intraperitoneal injections), failed to result in tolerance to the hypnotic and hypothermic effects of high doses of ethanol, although this effect was dependent on the genetic background of the mice used (Bowers *et al.*, 1999, 2000). In the aforementioned studies, behavioural and electrophysiological responses to benzodiazepines were unchanged in PKC γ -null mice. Thus, these studies suggest that PKC γ plays a critical role in regulating behavioural sensitivity to ethanol as well as the ability of ethanol to potentiate GABA_A receptor function, but this may not be a general function of GABA_A receptor modulation by PKC γ . The α_1 and α_4 subunits of GABA_A receptors have been found to co-immunoprecipitate with PKC γ from the rat cerebral cortex (Kumar *et al.*, 2002); however, direct phosphorylation of these subunits by PKC γ has, to our knowledge, not been demonstrated.

With regards to other drugs of abuse, studies conducted on PKC γ -deficient mice have demonstrated a role for PKC γ in regulating sensitivity to opiates. For example, deletion of PKC γ results in increased μ opioid receptor binding in the spinal cord (Narita *et al.*, 2001b) as well as increased μ opioid stimulated G-protein signaling in the lower brainstem (Narita *et al.*, 2001c). These findings suggest a molecular basis for the enhanced μ opioid receptor-mediated analgesia that is found in PKC γ -deficient mice (Narita *et al.*, 2001c; Celerier *et al.*, 2004). Despite the increased sensitivity of PKC γ -deficient mice to the analgesic effects of μ opioid receptor agonists, these mice failed to demonstrate a conditioned place preference to a 5 mg/kg s.c. dose of morphine (Narita *et al.*, 2001a). One possible interpretation of these seemingly counterintuitive findings is that the function of μ opioid receptors expressed in brain regions that mediate the rewarding effects of morphine is not regulated by PKC γ in the same manner as in brain regions that mediate μ opioid-induced analgesia. Alternatively, since PKC γ -deficient mice show deficits in contextual learning (Abeliovich *et al.*, 1993b), it is possible that these mice have an impaired ability to associate the subjective rewarding effects of morphine with the environmental context in which it was administered, thereby rendering them unable to express a conditioned place preference. Further studies on the precise mechanisms and brain regions where PKC γ regulates μ opioid receptor function are needed to clarify this, as are experiments to determine if PKC γ -deficient mice are capable of establishing a conditioned place preference to other conditioned appetitive stimuli.

PKC δ

Exposure of PC12 cells to ethanol increases the expression of PKC δ (Messing *et al.*, 1991). To our knowledge, only one study has been conducted examining the effect of targeted deletion of the gene encoding PKC δ on sensitivity to ethanol. Choi and colleagues (Choi *et al.*, 2008) showed that mice carrying a null mutation for the PKC δ gene displayed a marked resistance to the ataxic effects of ethanol (1.5-2 g/kg i.p.) as assessed on a rotarod. Sensitivity to the hypothermic and hypnotic effects of ethanol was also reduced. The ataxic effects of the general GABA_A receptor potentiators pentobarbital and pregnanolone were also reduced, but ataxic responses to the benzodiazepine flunitrazepam were normal. Since flunitrazepam acts only at GABA_A receptors containing the γ_2 subunit, these data suggested that PKC δ may regulate ethanol potentiation of GABA_A receptors that do not contain this

subunit, the most abundant of which are extrasynaptic, contain the δ subunit, and regulate tonic inhibition of neurons. In this same study, electrophysiological experiments in brain slices revealed that ethanol potentiated these tonic currents in neurons located in the thalamus and hippocampus, both of which are brain regions implicated in ataxia. However, this potentiation of tonic GABA-mediated currents was not found in PKC δ -null mice, suggesting that PKC δ regulation of GABA $_A$ receptors containing the γ_2 subunit may contribute to ethanol-induced ataxia. In the same study, the authors found that the ataxic effects of ketamine were normal in PKC δ null mice, but to our knowledge, the sensitivity of PKC δ null mice to psychostimulants, opiates, nicotine, and other abused substances has not yet been examined.

PKC ϵ

Numerous studies have implicated PKC ϵ in regulating sensitivity to various drugs of abuse. PKC ϵ -deficient mice were first generated in the late 1990's and demonstrated a role for this isozyme in nociception (Khasar *et al.*, 1999). Not long after the initial characterization of these mice, Hodge and colleagues demonstrated that PKC ϵ -deficient mice displayed supersensitivity to the behavioural and biochemical effects of ethanol and other positive allosteric modulators of the GABA $_A$ receptor (Hodge *et al.*, 1999; Hodge *et al.*, 2002). PKC ϵ -deficient mice lacked any evidence for compensatory up-regulation of other PKC isozymes as measured by Western blot analysis.

Crude synaptosomes (microsacs) isolated from the frontal cortex of wild-type and PKC ϵ -deficient mice showed similar GABA $_A$ receptor responsiveness (as measured by $^{36}\text{Cl}^-$ uptake) to orthosteric agonists such as muscimol. However, the addition of allosteric potentiators of GABA $_A$ receptor function, such as ethanol, benzodiazepines, barbiturates, and GABAergic neurosteroids dramatically increased $^{36}\text{Cl}^-$ uptake in tissue from PKC ϵ -deficient mice (Hodge *et al.*, 1999; Hodge *et al.*, 2002). The enhanced GABA $_A$ receptor sensitivity was reproduced in microsacs from wild type mice following preincubation with the PKC ϵ translocation inhibitor peptide $\epsilon\text{V1-2}$, which had no effect on $^{36}\text{Cl}^-$ uptake in microsacs from PKC ϵ -deficient mice, verifying that the enhanced GABA $_A$ receptor sensitivity was indeed as result of loss of PKC ϵ . Using slice electrophysiology, it was later demonstrated that ethanol (80 mM) enhanced GABA $_A$ receptor-mediated IPSCs in the CA1 region of the hippocampus of PKC ϵ null mice but not wildtype littermates (Proctor *et al.*, 2003). This enhanced sensitivity of GABA $_A$ receptors to positive allosteric modulators such as ethanol, benzodiazepines, barbiturates, and GABAergic neurosteroids in PKC ϵ -deficient mice was paralleled by behavioural supersensitivity to these drugs. For example, PKC ϵ null mice showed prolonged hypnotic effects of high doses ethanol and benzodiazepines as measured by the loss-of-righting reflex (LORR), and increased locomotor stimulant effects of lower doses of ethanol (Hodge *et al.*, 1999; Hodge *et al.*, 2002). Locomotor responses to the NMDA receptor antagonist MK-801 did not differ between PKC ϵ -deficient and wildtype mice, suggesting normal functioning of NMDA receptors in these mice. Other behavioural indices of increased GABA $_A$ receptor sensitivity and/or functioning in PKC ϵ -deficient mice have been manifested by reduced anxiety-like behaviours (Hodge *et al.*, 2002), reduced ethanol withdrawal severity (Olive *et al.*, 2001), and reduced acute functional tolerance to the ataxic effects of ethanol (Wallace *et al.*, 2007).

Since the discovery of the opposing altered biochemical and behavioural supersensitivity to ethanol in PKC γ - and PKC ϵ -deficient mice, it has been hypothesized that the resulting phenotypes of these mice are a result of an altered phosphorylated state of the GABA $_A$ receptor, as there is substantial evidence that PKC regulates GABA $_A$ receptor function (Song and Messing, 2005). This hypothesis was tested by Qi and colleagues (Qi *et al.*, 2007), who demonstrated that PKC ϵ directly phosphorylates the serine 327 residue of the γ_2 subunit of the GABA $_A$ receptor, and that limiting this phosphorylation produced an

enhanced electrophysiological sensitivity of these receptors to ethanol and benzodiazepines. Other possible mechanisms by which responsiveness to ethanol and other allosteric modulators of GABA_A receptor function is altered by PKC isozymes include regulation of GABA_A receptor cell surface expression, intracellular trafficking, and degradation (Song and Messing, 2005). In addition, PKC ϵ null mice have increased extracellular levels of endogenous taurine (Olive *et al.*, 2000), a sulfonated amino acid that potentiates GABA_A receptor function.

PKC ϵ also appears to mediate the ability of ethanol to potentiate glycine receptor function. Jiang and Ye demonstrated that the PKC ϵ peptide translocation inhibitor ϵ V1-2 actually decreased the ability of low concentrations of ethanol (10 mM) to potentiate glycine receptor function in slices of the ventral tegmental area (Jiang and Ye, 2003). These findings are of interest, since GABA_A and glycine are both ligand-gated chloride channels, but their function appears to be regulated by PKC ϵ in opposite manners. In addition, PKC ϵ regulation of glycine receptor responses to ethanol may represent a non-GABAergic mechanism by which this isozyme regulates ethanol sensitivity.

In addition to regulating sensitivity to abused substances that target the GABA_A receptor (i.e., ethanol, benzodiazepines, and barbiturates) or glycine receptors (i.e., ethanol), recent studies have shown that PKC ϵ also regulates behavioural and biochemical sensitivity to opiates and cannabinoids. For instance, PKC ϵ -deficient mice are supersensitive to the rewarding, reinforcing, and supraspinal analgesic effects of morphine (Newton *et al.*, 2007). However, the mechanisms underlying these observations are unclear, as this study demonstrated that brain μ opioid receptor densities and G-protein coupling are unaltered in PKC ϵ -deficient mice. PKC ϵ -deficient mice also display supersensitivity to the analgesic and hypothermic effects of the cannabinoid receptor 1 (CB₁) receptor agonist WIN55,212-2 but not the structurally unrelated CB₁ agonist CP55,940 (Wallace *et al.*, 2009). Tolerance to the behavioural effects of WIN55,212-2 was also enhanced in PKC ϵ -deficient mice. These latter behavioural findings were paralleled by evidence of increased CB₁ receptor binding affinity. Thus, in addition to the GABA_A receptor, PKC ϵ also appears to regulate sensitivity to μ opioid and cannabinoid receptor agonists; however, further studies are needed to determine if these observations are a result of direct interaction of PKC ϵ with μ opioid or CB₁ receptors.

Role of Individual PKC Isozymes in Self-Administration of Drugs of Abuse

PKC α

Post-mortem analysis of brains from heroin addicts has shown a reduction in the level of PKC α expression (Busquets *et al.*, 1995; Garcia-Sevilla *et al.*, 1997), indicating that the expression level of at least one PKC isozyme is altered in human addicts. Also, a microarray study demonstrated reduced PKC α expression in the nucleus accumbens of rats which had been trained to self-administer ethanol (Rodd *et al.*, 2008). However despite these intriguing genetic findings we are not aware of any behavioural studies examining the effects of PKC α knockdown or knockout on the self-administration of, or sensitivity to, drugs of abuse.

PKC β

Despite the reported roles for PKC β in regulation of DAT trafficking and amphetamine sensitivity mentioned above, drug self-administration and place preference have not, to our knowledge, been examined in PKC β null mice or in conjunction with the PKC β -specific inhibitor LY379196. This is perhaps unfortunate since the inhibition of PKC β would be hypothesized to block the behavioural effects of amphetamines and thus perhaps be a therapeutic strategy for the treatment of psychostimulant addiction.

PKC γ

The reduced sensitivity to ethanol exhibited by PKC γ -deficient mice is paralleled by increased ethanol consumption in a two bottle choice paradigm (Bowers and Wehner, 2001). No differences in saccharin or nicotine consumption were noted, indicating that the effects were specific to ethanol. We are unaware of any studies to date that have examined differences patterns of self-administration of, or place preference for, opiates, nicotine, or psychostimulants in PKC γ -null mice. This is unfortunate, as there is evidence that PKC γ null mice exhibit increased impulsivity in an appetitive nosepoke task (Bowers and Wehner, 2001), and impulsivity is a behavioural trait associated with risk for developing addiction (Everitt *et al.*, 2008; Crews and Boettiger, 2009).

PKC δ

Similarly, we are unaware of any studies that have examined differences voluntary self-administration of psychostimulants, opiates, nicotine, ethanol, or other abused substances in PKC δ -deficient mice and wildtype mice. However, such studies are certainly warranted, since inhibition of PKC δ function by siRNA knockdown or dominant-negative protein expression in cultured midbrain cells increases the phosphorylation and activity of tyrosine hydroxylase (Zhang *et al.*, 2007), the rate-limiting enzyme involved in the synthesis of dopamine. The effects of PKC δ inhibition are indirect, resulting from a decrease in phosphorylation of PP2A, a phosphatase that dephosphorylates tyrosine hydroxylase thereby reducing its activity. The net result of PKC δ inhibition might therefore be expected to be an increase in dopamine levels and this was indeed found in the striatum of PKC δ null mice (Zhang *et al.* 2007). These findings are of interest in light of the well-established involvement of mesocorticolimbic dopaminergic transmission in regulating drug reinforcement and incentive learning (Di Chiara *et al.*, 2004; Wise, 2004; Koob and Volkow, 2010).

PKC ϵ

In a two-bottle choice paradigm, PKC ϵ -deficient mice consumed approximately 75% less alcohol and showed reduced ethanol preference as compared with wildtype mice (Hodge *et al.*, 1999). PKC ϵ -deficient mice did not differ in their total fluid consumption or intake of saccharin or quinine containing fluids. Thus, reduced intake was specific for ethanol. Restoration of PKC ϵ expression by means of tetracycline-regulated transgenic “rescue” increased ethanol intake in PKC ϵ -deficient mice to levels similar to those observed in wildtype mice (Choi *et al.*, 2002), suggesting that reduced alcohol consumption in PKC ϵ -deficient mice is due to loss of PKC ϵ in adult neuron rather than developmental alteration of other neural substrates underlying the motivation to consume ethanol. In an operant self-administration paradigm, PKC ϵ -deficient mice also showed an approximate 50% reduction in ethanol self-administration as compared with wild type mice (Olive *et al.*, 2000). In this study PKC ϵ null mice also demonstrated a blunted alcohol deprivation effect (i.e., increased ethanol consumption immediately following a period of imposed abstinence). Place preference studies have revealed that PKC ϵ null mice exhibit an increased sensitivity to the aversive effects of ethanol but reduced sensitivity to the rewarding effects of ethanol (Newton and Messing, 2007). Recent studies using site-specific inhibition of PKC ϵ function (via siRNA knockdown of PKC ϵ expression or infusion of a cell-penetrant form of ϵ V1-2) have shown the activity of this enzyme in the amygdala, but not nucleus accumbens, mediates ethanol consumption or reinforcement (Gass and Olive, 2009; Lesscher *et al.*, 2009). A genetic screening study using inbred mouse strains identified higher levels of PKC ϵ expression in strains that show high levels of ethanol expression. This study was conducted on mice which had not been exposed to ethanol, suggesting that PKC ϵ levels are correlated with predisposition to high/low ethanol intake (Mulligan *et al.*, 2006).

The only study that has explored potential alterations in patterns of intravenous drug self-administration in PKC ϵ -deficient mice was conducted by Newton and colleagues (Newton *et al.*, 2007). In this study, wildtype and PKC ϵ -deficient mice were trained to self-administer morphine via the intravenous route, and it was found that PKC ϵ -deficient mice developed morphine self-administration at a low dose (0.1 mg/kg/infusion) that did not generate self-administration behaviour in wild-type mice, consistent with the μ opioid supersensitivity phenotype in PKC ϵ -deficient mice. The abundant animal literature about PKC ϵ and drug self administration/sensitivity led to its inclusion on a panel of markers for haplotype analysis of addiction-related genes (Hodgkinson *et al.*, 2008).

Do PKC isozyme modulators have a future in the treatment of addiction?

The evidence presented above suggests that pharmacological manipulation of PKC isozymes may produce beneficial effects in the treatment of addiction. For example, a PKC ϵ inhibitor may reduce alcohol intake while also increasing sensitivity to the analgesic effects of opiates. A PKC β inhibitor may inhibit the acute effects of amphetamine and possibly other psychostimulants while manipulation of PKC δ activity might result in alterations in the activity of tyrosine hydroxylase and thus altered dopamine levels. Unfortunately there are no pharmacological compounds that are available and approved for use in humans that target individual PKC isozymes, although peptide reagents that alter the function of PKC δ and PKC ϵ are in advanced phases of development for the treatment of cardiac conditions (Yonezawa *et al.*, 2009). The PKC β inhibitor ruboxistaurin (LY333531, from the same chemical class as the aforementioned LY379196) is in advanced clinical trials for the treatment of diabetic retinopathy (Danis and Sheetz, 2009), but no studies to date have examined its effects on addiction-related behaviours.

PKC isozymes have functional roles in many biological systems, in addition to the responses to drugs of abuse described here. Indeed, PKC ϵ alone has proposed roles in a wide spectrum of diseases and disorders, including cancer (Basu and Sivaprasad, 2007; Gorin and Pan, 2009), cardiac disorders (Churchill *et al.*, 2008), inflammation (Aksoy *et al.*, 2004), pain (Khasar *et al.*, 1999) and anxiety (Hodge *et al.*, 2002; Lesscher *et al.*, 2008). PKC δ has been implicated in many of these systems and is also a key promoter of apoptosis (Reyland, 2007). These studies suggest that, even when isozyme-specific reagents become available, they will have to be used with care due to the potential for affecting multiple systems.

An additional path to the development of treatments for addiction, using knowledge gained in the studies described in this review, may be to map the signaling pathways in which specific PKC isozymes operate. Each PKC isozyme appears to phosphorylate multiple substrates (e.g., see (Newton and Messing, 2010). Manipulating the interaction between a kinase and its substrate may produce more focused effects than systemic manipulation of the kinase alone. For example, regulation of GABA $_A$ receptors by PKCs is clearly a mechanism by which these kinases contribute to addictive behaviours. It might be expected that manipulation of PKC-GABA $_A$ receptor interactions would produce mostly neurological effects. In addition, the described specificity of PKC isozymes for receptors containing certain GABA $_A$ receptor subunits (γ_2 -containing receptors for PKC ϵ , δ -containing receptors for PKC δ) may provide an additional level of control for unwanted effects on multiple biological systems.

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Table 1

Summary of phenotypes of mice with genetic alterations in PKC isozyme expression

PKC isozyme	Phenotype
β	reduced amphetamine-stimulated locomotor activity and ventral forebrain dopamine release
γ	reduced behavioral and GABA _A receptor sensitivity to ethanol; increased ethanol consumption; increased μ opioid receptor binding and μ -mediated analgesia; lack of conditioned place preference to morphine
δ	reduced behavioral and GABA _A receptor sensitivity to ethanol
ϵ	increased behavioral and GABA _A receptor sensitivity to ethanol; reduced ethanol withdrawal severity, ethanol-stimulated mesolimbic dopamine release, acute functional tolerance to ethanol, and ethanol consumption; increased behavioral sensitivity to morphine and cannabinoids; self-administration of ultra-low doses of morphine