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Protein Kinase C Deficiency-induced Alcohol Insensitivity and Underlying Cellular Targets in *Drosophila*

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Summary

Multiple subtypes of protein kinase C (PKC) isozymes are implicated in various neurological disorders including alcohol insensitivity, a trait strongly associated with alcoholism in humans, but molecular and cellular mechanisms underlying the PKC activities remain poorly understood. Here we show that functional knockdown of conventional, novel or atypical PKC in the fly nervous system each resulted in alcohol insensitivity. Neuroanatomical mapping of conventional Ca^{2+} -sensitive PKC53E activity uncovers a previously uncharacterized role of *Drosophila* serotonin neurons in alcohol sensitivity. The deficiency of PKC53E but not novel Ca^{2+} -independent PKC98E appears to reduce synaptic serotonin levels, since acute inhibition of serotonin reuptake by citalopram and Prozac reversed alcohol insensitivity in flies expressing PKC53E double-stranded RNA in serotonin neurons. Together, findings from this and our previous studies indicate that PKC53E and PKC98E differentially regulate fly alcohol sensitivity through independent modulation of conserved serotonin and neuropeptide Y-like systems.

Introduction

Insensitivity to alcohol intoxication in adolescents is a strong predictor of human alcoholism. However, our understanding of underlying genetic and neural mechanisms remains rudimentary. *Drosophila*, like mammals, display stereotyped behavioral responses to alcohol intoxication including hyperexcitation, uncoordination and sedation. In addition, a growing number of conserved genes and molecular pathways have been identified that mediate alcohol-related behaviors in flies and rodents. For example, it has been shown that neuropeptide Y (NPY) family peptides are critical for alcohol sensitivity. Overexpression of NPY or its fly counterpart, neuropeptide F (NPF), causes hypersensitive responses to ethanol sedation in both flies and mice (Thiele et al., 1998, Wen et al., 2005). Conversely, deficiencies in NPY/NPF signaling leads to decreased ethanol sensitivity. More recently, genetic analysis of the epidermal growth factor signaling pathway in *Drosophila* has lead to the discovery of its novel role in regulation of alcohol sensitivity in both insects and mammals (Corl et al., 2009). These findings provide compelling evidence that *Drosophila* is a powerful model for investigating mechanisms underlying alcohol use disorders.

The conserved PKC family comprises three subtypes of isozymes: conventional Ca^{2+} /DAG-responsive PKC, novel Ca^{2+} -independent/DAG-responsive PKC and atypical PKC. These

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kinases differentially mediate diverse intracellular signaling processes across evolution, and have been implicated in multiple neurological disorders including acute alcohol sensitivity (Harris et al., 1995, Hodge et al., 1999, Newton and Ron, 2007, Choi et al., 2008). Ethanol has been shown to rapidly induce PKC activity in the specific regions of the rodent brain (Deitrich et al., 1989, Mahadev and Vemuri, 1998). The demonstration of ethanol-binding sites in the regulatory domains of several conventional and novel PKCs has raised the possibility that PKC may serve as the molecular link between ethanol sensing and cellular responses (Slater et al., 1997, Das et al., 2006).

In the mouse model, genetic knockout of three conventional or novel isozymes has resulted in altered behavioral responses to alcohol intoxication (Harris et al., 1995, Hodge et al., 1999, Choi et al., 2008), but the underlying mechanisms remain unknown. The pleiotropic effects of PKC on diverse cellular processes in various tissues have also made it difficult to interpret the complex and, in some cases, opposing phenotypes of such knockout mice. Therefore, identification of physiologically relevant cellular targets of PKC responsible for observed alcohol phenotypes remains both challenging and critically important for advancing our understanding of alcohol-related behaviors.

In this study, we use the genetically tractable *Drosophila* model to investigate the potentially conserved roles of neural PKC isozymes and their cellular targets in acute alcohol response. We show that the neural activities of all three PKC subtypes are required for fly alcohol sensitivity. Functional mapping of conventional PKC53E activity has led to the discovery of a previously uncharacterized role of the *Drosophila* serotonin system in alcohol sensitivity. We also provide evidence that PKC53E deficiency-induced alcohol insensitivity is likely due to the reduction of synaptic serotonin levels, which can be reversed pharmacologically using selective serotonin reuptake inhibitors (SSRIs). Finally, findings from this and our previous work demonstrate that acute alcohol sensitivity in *Drosophila* requires a second evolutionarily conserved neurotransmitter system in addition to the NPY-like system, each respectively modulated by conventional PKC53E and novel PKC98E.

Experimental Procedures

Flies

Synchronized fly eggs were collected onto apple juice agar plates with yeast paste. Larvae and adults were reared on the same food at room temperature with exposure to natural lighting. Adult females, synchronized by collecting flies enclosed within a 12-h period, were aged for 7 days. All fly lines are in the *w¹¹¹⁸* genetic background. The *Ddc*-GAL4 line drives expression in both serotonergic and dopaminergic neurons (Li et al., 2000), while the *TH*-GAL4 line drives expression only in dopaminergic neurons (Friggi-Grelin et al., 2003). The UAS-*nsyb-GFP* encodes GFP fused with neuronal synaptobrevin (Vosshall et al., 2000). The UAS-*PKCi* encodes a pseudo-substrate fragment inhibitory to PKC isozymes (Broughton et al., 1996), 1996). The UAS-*PKC53E^{dsRNA}*, UAS-*PKC98E^{dsRNA}* and UAS-*DaPKC^{dsRNA}* encode double strand RNA sequences of respective PKC isozymes (Dietzl et al., 2007). The UAS-*shi^{ts1}* encodes a semi-dominant-negative form of dynamin (Kitamoto, 2002).

Behavioral assay

The procedures for behavioral assay were described previously (Chen et al., 2008). Unless indicated otherwise, 1ml of 45% ethanol solution was applied to each behavioral test. After exposing 20 female flies to ethanol vapor, the number of uncoordinated or anesthetized flies was recorded at specified time intervals. At least 3 independent trials were performed per experiment. Data were analyzed by using PROC PHREG in statistical software SAS 9.1 (SAS Institute, Cary, NC).

Pharmacological treatment of flies

Prozac (fluoxetine hydrochloride), citalopram (citalopram hydrobromide), 5-HTP (5-hydroxy-L-tryptophan), and α -MTP (α -methyl-DL-tryptophan) were purchased from Sigma (St. Louis, MO). Drugs were mixed with autoclave-killed yeast paste food. The concentration of Prozac or citalopram is 0.1% (w/w) in food. The concentration of 5-HTP or α -MTP was ca. 50 mM (1%, w/w) in food. Flies were fed with drugs for 72 hours prior to behavioral assays.

Quantitative RT-PCR (qRT-PCR)

The procedures of RNA extraction, first-strand cDNA synthesis, and qRT-PCR were described previously (Chen et al., 2008). The quantification of *PKC53E*, *PKC98E*, *DaPKC* and *gapdh2* transcripts in fly heads was done by using TaqMan primer Dm01816273_m1, Dm02151736_g1, Dm01843305_g1 and Dm0184377_s1, respectively (Applied Biosystems Inc, Foster City, CA). The relative RNA quantification was normalized against *gapdh2*. Significance was analyzed by using PROC GLM with *post hoc* Tukey's test.

Ethanol content assay

The assays were performed as previously described (Moore et al., 1998, Wen et al., 2005). The ethanol assay kit was from Genzyme Diagnostics P.E.I. Inc (Charlottetown, Canada). The protein assay kit was from Thermo Scientific (Rockford, IL).

Results

Roles of conventional, novel and atypical PKCs in alcohol sensitivity

To investigate the regulatory roles of PKC isozymes in fly ethanol sensitivity, we attempted to knock down the activity of Ca^{2+} -sensitive PKC53E, Ca^{2+} -independent PKC98E and atypical DaPKC in the nervous system. A pan neural *elav-GAL4* driver was used to direct expression of UAS-PKCi, which encodes a pseudo-substrate peptide that competitively inhibits different subtypes of PKC (Broughton et al., 1996), and the double-stranded RNA (dsRNA) of *PKC53E*, *PKC98E* and *DaPKC* (Figure 1). The assay involves exposing 20 adult flies to an increasing concentration of ethanol vapor in a sealed bottle and scoring for ethanol-induced uncoordination and sedation. We found that flies deficient in multiple (*elav-GAL4/UAS-PKCi*) or a single PKC activity (e.g., *elav-GAL4/UAS-PKC53E^{dsRNA}*) were significantly more resistant to acute ethanol intoxication than the paired controls (e.g. UAS-PKCi alone). We also found that each dsRNA construct reduced the transcripts level of the corresponding PKC isozyme ((Chen et al., 2008); Figure S1). These results suggest that the neural activity of each of the three isozymes is required for ethanol sensitivity.

Mapping of PKC53E activity to serotonin neurons

To determine how PKC53E regulates fly ethanol response, we expressed *PKCi* or *PKC53E^{dsRNA}* in different subsets of neurons using various GAL4 drivers. We found that the fly lines expressing PKCi or *PKC53E^{dsRNA}*, driven by *Ddc-GAL4*, displayed significantly reduced alcohol sensitivity (Figure. 2A, C). However, expression of *PKC98E^{dsRNA}* directed by the same driver showed no alteration in ethanol sensitivity. Importantly, Ca^{2+} -independent PKC98E, but not PKC53E, has been shown to modulate the activity of NPF neurons, the only previously known neurotransmitter system critical for fly ethanol sensitivity (Wen et al., 2005, Chen et al., 2008). Thus, our result suggests that PKC98E and PKC53E differentially regulate alcohol sensitivity through mutually exclusive modulation of NPF and *Ddc-GAL4* neurons.

Since *Ddc-GAL4* is predominantly expressed in both dopamine and serotonin neurons (see Figure S3 of supplemental data), we also tested whether dopamine neuron-specific *TH-GAL4*-

driven expression of *PKCi* or *PKC53E^{dsRNA}* has a similar effect on ethanol behavior. We found that the ethanol sensitivity of *TH-GAL4/UAS-PKC53E^{dsRNA}* flies was similar to those of paired controls (Figure 2B, D). This finding suggests that serotonin neurons have a previously uncharacterized role in fly ethanol insensitivity, and PKC53E regulates serotonin signaling.

Acute regulation of ethanol sensitivity by serotonin neurons

To provide evidence that PKC53E positively regulates serotonin signaling, we performed two additional experiments. First, we expressed, in *Ddc-GAL4* neurons, *UAS-shi^{ts1}*, which encodes a temperature-sensitive, semidominant-negative form of dynamin that blocks neuronal activity at a restrictive temperature (Kitamoto, 2002). When assayed at the restrictive temperature (30°C), *Ddc-GAL4/UAS-shi^{ts1}* but not *TH-GAL4/UAS-shi^{ts1}* flies showed significantly reduced ethanol sensitivity (Figure 3), suggesting that functional knockdown of *PKC53E* attenuates activity of serotonin neurons which are acutely required for fly response to ethanol intoxication.

Reversal of ethanol insensitivity by acute inhibition of serotonin reuptake

In the second experiment, we attempted to treat PKC53E deficiency-induced ethanol insensitivity by feeding *Ddc-GAL4/UAS-PKC53E^{dsRNA}* flies with citalopram or Prozac, two SSRIs used clinically as antidepressants. Strikingly, each pharmacological treatment restored ethanol sensitivity of *Ddc-GAL4/UAS-PKC53E^{dsRNA}* flies to the normal level (Figure 4A, B). These results suggest that PKC53E deficiency leads to a significant reduction in the synaptic serotonin level. On the other hand, the drug treatment of wild type flies did not significantly alter ethanol sensitivity (Figure 4C), suggesting that the level of synaptic serotonin is normally at or near saturation for fly ethanol sensitivity.

It has been shown that ingestion of food containing 50 mM of serotonin precursor 5-HTP caused 15- to 20-fold increases in serotonin level in the fly head (Dierick and Greenspan, 2007). We found that wild type flies fed with ca. 50 or 100 mM 5-HTP food consistently displayed a small but significant increase in ethanol sensitivity (Figure 4D). In addition, flies treated with ca. 50 or 100 mM serotonin synthesis inhibitor α -MTP, which reduces the serotonin level by about two fold (Dierick and Greenspan, 2007), showed no significant changes in ethanol sensitivity. Taken together, these findings suggest that ethanol sensitivity of normal flies is relatively independent of the fluctuations in tissue serotonin levels.

Discussion

We have shown that the acute alcohol sensitivity of *Drosophila* depends on all three subtypes of PKC isozymes in the nervous system. Functional analysis of a conventional PKC (PKC53E) has led to the discovery of a previously uncharacterized role of *Drosophila* serotonin neurons in acute alcohol response. Our findings suggest that further characterization of the cellular targets of different PKC isozymes related to ethanol sensitivity in the *Drosophila* model may hold great promise for gaining novel insights into the genetic and neural mechanisms underlying alcohol dependence.

In addition to the conserved NPY-like system (Wen et al., 2005), we have identified the serotonin system as another major signaling component in the fly neural circuit for alcohol sensitivity. In this and our previous studies (Chen et al., 2008), we have also shown that serotonin and NPY-like signaling activities are differentially modulated by conventional PKC53E and novel PKC98E in a mutually exclusive manner (Figure 5). It has been reported that conventional PKC γ and novel PKC δ knockout mice display reduced sensitivity to ethanol intoxication (Harris et al., 1995, Choi et al., 2008). However, how the deficiencies of these two PKC isozymes impact mouse alcohol sensitivity are not yet clear. Since both serotonin and NPY signaling systems have been implicated in alcohol sensitivity of rodents (Myers and

Veale, 1968, McBride et al., 1990, Thiele et al., 1998), it will be interesting to examine whether PKC γ and PKC δ isozymes also differentially modulate the serotonin and NPY pathways.

We have shown that acute inhibition of serotonin signaling with SSRIs reversed the alcohol insensitivity of *Ddc-GAL4/UAS-PKC53E^{RNAi}* flies, suggesting that the PKC53E deficiency reduces serotonin levels at presynaptic sites. In mammalian cells, PKC activity has also been shown to downregulate serotonin reuptake by its transporters (Qian et al., 1997, Jayanthi et al., 2005). On the other hand, Ca²⁺-independent PKC98E may be specialized to regulate neuropeptide release. Consistent with this notion, PKC-1, the *C. elegans* homolog of PKC98E, has been shown to promote neuropeptide secretion (Sieburth et al., 2007). Ethanol has been shown to bind and activate mammalian PKC isozymes (Slater et al., 1997, Das et al., 2006, Das et al., 2009). It would be interesting to determine whether PKC53E and PKC98E may function as intracellular ethanol sensors to mediate cellular responses.

Although acutely blocking neurotransmission of serotonin neurons by a dominant-negative form of dynamin (encoded by *shi^{ts1}*) caused significant reduction of fly alcohol sensitivity, feeding wild type flies with up to 100 mM α -MTP failed to show any significant effect. One possible explanation for these observations is that the serotonin receptor(s) responsible for fly ethanol sensitivity may bind to serotonin with a high affinity. Despite the fact that feeding of α -MTP reduces the serotonin level in head tissues by about two fold (Dierick and Greenspan, 2007), it may not reduce sufficiently the serotonin levels at presynaptic sites to trigger ethanol insensitivity. Consistent with this notion, we also found that feeding flies with 50 mM or 100 mM 5-HTP, which increases the serotonin level in head tissues by 15-20 fold, had similarly smaller effects (Figure 4D).

The serotonin and NPF neurons appear to define two parallel neuronal pathways critical for fly ethanol sensitivity. However, it remains unclear how these two pathways mediate the behavioral effects of ethanol. One possibility is that serotonin and NPF signaling may act upon a common set neurons expressing both serotonin and NPF receptors in defined brain regions that control motor coordination/consciousness (Yuan et al., 2005, Yuan et al., 2006). We are currently investigating this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Downregulation of Neural PKC Isozymes Attenuates Ethanol Sensitivity

A) Inhibition of multiple PKC isozymes by expressing a pseudo-substrate peptide PKCi in the nervous system. B-D) RNA interference mediated knockdown of PKC53E, PKC98E and DaPKC, respectively. The asterisks indicate the data set that is statistically different from controls ($PKCi$: $\chi^2_{1,4} = 36.53$, $P < 0.0001$; $PKC53E^{dsRNA}$: $\chi^2_{1,4} = 21.04$, $P < 0.0001$; $PKC98E^{dsRNA}$: $\chi^2_{1,4} = 11.32$, $P < 0.001$; $DaPKC^{dsRNA}$: $\chi^2_{1,4} = 12.00$, $P < 0.001$). Cox's regression model-based Wald test was used for the analysis of anesthetization data in this and other figures. The selectivity of each RNAi line against the corresponding PKC isozyme was confirmed using qRT-PCR ((Chen et al., 2008), Figure S1 of supplemental data). All PKC knockdown flies also exhibited delayed display of ethanol-induced uncoordination (data not shown). PKC deficient flies also showed normal pharmacokinetics of ethanol catabolism (see Figure S2 of supplemental data).

Figure 2. PKC53E but not PKC98E Deficiency in Serotonin Neurons Results in Ethanol Insensitivity

A, B) Inhibition of PKC activity by a pseudo-substrate peptide in *Ddc*-GAL4 but not *TH*-GAL4 neurons leads to ethanol sensitivity. C) RNA interference-mediated knockdown of *PKC53E* but not *PKC98E* activity in *Ddc*-GAL4 neurons caused ethanol insensitivity. D) Knockdown of *PKC53E* or *PKC98E* activity in *TH*-GAL4 neurons showed no altered ethanol sensitivity. *Ddc*-GAL4/*UAS-PKCi*: $\chi^2_{1,4} = 33.11$, $P < 0.0001$; *Ddc*-GAL4/*UAS-PKC53E^{dsRNA}*: $\chi^2_{1,4} = 31.36$, $P < 0.0001$.

Figure 3. Conditional Inhibition of *Ddc*-GAL4 and *TH*-GAL4 Neurons Reveals an Acute Requirement of Serotonin Signaling in Ethanol Sensitivity

A) Expression of a temperature-sensitive, semidominant negative form of dynamin (UAS-*shi*^{ts1}) in *Ddc*-GAL4 and *TH*-GAL4 neurons did not reduce ethanol sensitivity at a permissive temperature (23 °C). B) *Ddc*-GAL4/UAS-*shi*^{ts1} but not *TH*-GAL4/UAS-*shi*^{ts1} flies displayed decreased ethanol sensitivity. *Ddc*-GAL4/UAS-*shi*^{ts1}: $\chi^2_{1,4} = 49.88$, $P < 0.0001$. 1 ml of 30% ethanol solution was applied in each test.

Figure 4. Identification of the Cellular Targets of PKC53E Activity Underlying the Ethanol Insensitive Phenotype

A, B) Treatment of *Ddc-GAL4/UAS-PKC53E^{dsRNA}* flies with Prozac or citalopram restores ethanol sensitivity. *Ddc-GAL4/UAS-PKC53E^{dsRNA}*: $\chi^2_{1,4} = 10.11$, $P = 0.0015$. C) Treatment of wild type flies with Prozac or citalopram. D) Treatment of wild type flies with the serotonin precursor (5-HTP) and serotonin synthesis inhibitor (α -MTP). 5-HTP treated *w¹¹¹⁸* flies: $\chi^2_{1,4} = 10.84$, $P = 0.001$ (50 mM); $\chi^2_{1,6} = 16.97$, $P < 0.0001$ (100mM). 1 ml of 40% ethanol solution was applied in each test.

Figure 5. A Schematic Presentation of the Differential Role of PKC53E and PKC98E in Ethanol Sensitivity

In this model, it is proposed that conventional PKC53E and novel PKC98E modulate independently conserved serotonin and NPF signaling activities, which may function, in parallel, through their receptor neurons to positively regulate ethanol sensitivity.