

moody Encodes Two GPCRs that Regulate Cocaine Behaviors and Blood-Brain Barrier Permeability in *Drosophila*

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Summary

We identified *moody* in a genetic screen for *Drosophila* mutants with altered cocaine sensitivity. Hypomorphic mutations in *moody* cause an increased sensitivity to cocaine and nicotine exposure. In contrast, sensitivity to the acute intoxicating effects of ethanol is reduced. The *moody* locus encodes two novel GPCRs, *Moody- α* and *Moody- β* . While identical in their membrane-spanning domains, the two *Moody* proteins differ in their long carboxy-terminal domains, which are generated by use of alternative reading frames. Both *Moody* forms are required for normal cocaine sensitivity, suggesting that they carry out distinct but complementary functions. *Moody- α* and *Moody- β* are coexpressed in surface glia that surround the nervous system, where they are actively required to maintain the integrity of the blood-brain barrier in the adult fly. We propose that a *Moody*-mediated signaling pathway functions in glia to regulate nervous system insulation and drug-related behaviors.

Introduction

Cocaine is a highly addictive psychomotor stimulant that elicits a state of enhanced arousal and disinhibition, elevating mood with a rewarding euphoria, while at higher doses it usually induces compulsive stereotypies and psychosis (Gawin, 1991). Prolonged cocaine use commonly results in tolerance to its subjective effects, physical dependence, and eventually to drug abuse and addiction. Cocaine's primary mechanism of action is to block the uptake of monoamines (dopamine, serotonin, and norepinephrine) by inhibiting neuronal plasma membrane transporters, thereby increasing monoamine concentration at the synapse. In mammalian animal models, the acute response to cocaine is predominantly observed as enhanced locomotor activity and stereotypic behaviors. This locomotor stimulant effect of cocaine is mediated primarily by an inhibition of the dopamine transporter (DAT), as mice lacking DAT show enhanced levels of baseline activity

that are insensitive to cocaine administration (Giros et al., 1996). The rewarding effects of cocaine are, however, more complex, involving, in addition to DAT, the serotonin transporter (SERT; Sora et al., 1998, 2001). Further, how the acute stimulant effects of cocaine relate to the long-term changes that underlie addiction is poorly understood. However, emerging evidence suggests that the mechanisms that regulate the acute stimulant effects of psychostimulants are also involved in determining their rewarding properties (reviewed in Laakso et al. [2002]). For example, the locomotor activity of mice lacking both DAT and SERT is not stimulated by cocaine administration; these mice also fail to develop conditioned preference for cocaine, an assay that measures the rewarding effects of the drug (Sora et al., 2001). Conversely, mice lacking FosB or overexpressing Δ fosB, which are supersensitive to the psychomotor stimulant effect of cocaine, also show enhanced place preference for cocaine (Hiroi et al., 1997; Kelz et al., 1999). It is therefore possible that a mechanistic understanding of the relatively simple process of acute drug-induced locomotor stimulation may provide valuable clues about the molecular mechanisms underlying drug reward, reinforcement, and addiction.

Drosophila melanogaster, with its accessibility to genetic, molecular, and behavioral analyses, has been developed as a useful model system for identifying genes that regulate behavioral responses to drugs of abuse, including cocaine (reviewed in Rothenfluh and Heberlein [2002]; Wolf and Heberlein, 2003). Cocaine not only induces motor behaviors in flies that are remarkably similar to those observed in mammals (Bainton et al., 2000; McClung and Hirsh, 1998), but repeated cocaine administration induces behavioral sensitization (McClung and Hirsh, 1998), a form of behavioral plasticity believed to model certain aspects of addiction (Robinson and Berridge, 1993; Schenk and Partridge, 1997). In addition, as in mammals, a role for dopaminergic systems in regulating the behavioral manifestations of cocaine exposure has been demonstrated in flies (Bainton et al., 2000; Li et al., 2000). More importantly, studies in *Drosophila* have led to the identification of genes and pathways whose role in cocaine-related behaviors was unexpected (Hirsh, 2001; Tsai et al., 2004). For example, the circadian gene *period* (*per*) was found to regulate cocaine sensitization in *Drosophila* (Andreic et al., 1999). Subsequently, mice carrying mutations in the homologous *per* genes were also found to have altered cocaine sensitization and conditioned place preference (Abarca et al., 2002).

In order to identify novel molecules and pathways involved in behavioral responses to psychostimulants, we carried out a genetic screen for *Drosophila* mutants with altered acute responses to cocaine. Here, we report the phenotypic and molecular characterization of a gene encoding two G protein-coupled receptors (GPCRs), which we have named *moody*, that functions in glia to regulate the acute sensitivity of flies to cocaine. *moody* is expressed, during development and in adulthood, in glia that surround and insulate the ner-

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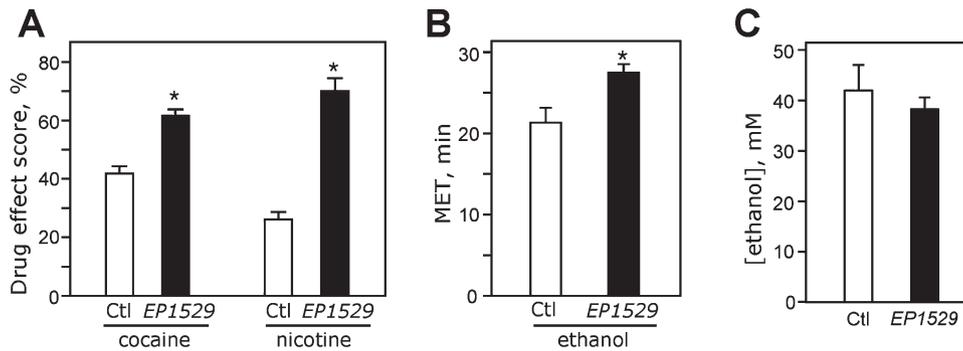


Figure 1. Drug Sensitivity Phenotypes of the *EP1529* Mutation

(A) *EP1529* flies show increased sensitivity to cocaine and nicotine. Male flies carrying the X-linked *EP1529* insertion were exposed to 200 μ g of volatilized cocaine or 5 μ g of volatilized nicotine and tested in the “crackometer” as described in [Experimental Procedures](#). Compared to a control *EP* line (Ctl = *EP369*), *EP1529* shows increased cocaine and nicotine sensitivity. *EP1529* also shows increased cocaine sensitivity when tested at 100 and 400 μ g of cocaine (data not shown). N = 12–20 experiments.

(B) *EP1529* flies show an increased MET, reflecting reduced ethanol sensitivity, from the inebriometer compared to wild-type controls (Ctl = *EP369*). n = 4–8 experiments.

(C) *EP1529* flies show normal ethanol absorption. Ethanol content, quantified after a 30 min exposure to ethanol vapor, was comparable in *EP1529* and Ctl flies. n = 3 experiments.

Asterisks denote significant differences from Ctl ($p < 0.01$) by Student's paired t test assuming equal variance. In all figures, error bars correspond to the standard error of the mean.

vous system. Its function in these glia is required continuously to maintain proper blood-brain barrier (BBB) function. We provide several lines of evidence showing that the behavioral defects observed in *moody* flies are not caused by altered accessibility of the drug to the CNS. We therefore postulate that impaired BBB function leads to changes in nervous system function, which, in turn, cause alterations in drug-related behaviors.

Results

moody, a Mutant with Increased Sensitivity to Cocaine and Nicotine

To identify novel molecules that may regulate the nervous system's sensitivity to drugs of abuse, we carried out a genetic screen for *Drosophila* mutants with altered responses to volatilized freebase cocaine. Behavior was quantified using a simple assay that measures drug-induced loss of negative geotaxis ([Bainton et al., 2000](#)). Upon exposure to moderate doses of cocaine (~150 μ g), flies show a series of unusual motor behaviors, including reduced locomotion and vigorous circling, which interfere with negative geotaxis, a robust innate behavior of *Drosophila*. We screened a collection of 400 fly strains, each carrying an insertion of the EP element on the X chromosome ([Rørth et al., 1998](#)), and identified five mutants with a reduced cocaine sensitivity (corresponding to three genes) and seven mutants with an increased drug sensitivity (corresponding to six genes; [Tsai et al., 2004](#)). Here, we describe the phenotypic and molecular characterization of *EP1529*, identified by its increased sensitivity to cocaine ([Figure 1A](#)). *EP1529* flies also exhibit increased sensitivity to the effects of volatilized nicotine exposure ([Figure 1A](#)). In contrast, *EP1529* flies are resistant to the acute intoxicating effects of ethanol, manifested as an in-

creased mean elution time (MET) in the inebriometer, an assay that measures ethanol-induced loss of postural control ([Figure 1B](#); [Weber and Diggins, 1990](#); [Moore et al., 1998](#)). *EP1529* flies absorb ethanol normally ([Figure 1C](#)), are fully viable, and show normal baseline behaviors, such as climbing and locomotion (see [Experimental Procedures](#)). The *EP1529* insertion is responsible for the aberrant cocaine sensitivity, as precise excision of the EP element restores normal drug sensitivity (data not shown). Sequence analysis by the Berkeley *Drosophila* Genome Project (<http://flybase.bio.indiana.edu/>) showed that the *EP1529* element is inserted between two predicted genes, CG4322 and CG4313 ([Figure 2A](#)). Further analysis (see below) revealed that the gene disrupted by *EP1529* is CG4322, which we have named *moody*. *moody* encodes a member of a group of three highly related orphan GPCRs, which also include CG4313 and Tre1. Tre1 has been shown to be involved in transepithelial migration of germ cells ([Kunwar et al., 2003](#)), while the function of CG4313 is unknown.

moody Encodes Two Novel GPCRs Generated by Alternative Splicing

Sequence analysis of *moody* cDNAs, isolated from an adult head cDNA library ([DiAntonio et al., 1993](#)), revealed the presence of two alternative 5' exons, RA1 and RB1 (only RA1 was predicted by genome analysis, <http://flybase.net/cgi-bin/>), which are spliced to a common second exon containing the predicted translation start site ([Figure 2B](#)). These two transcripts can be distinguished on Northern blots ([Figure 2C](#)); the longer 3.8 kb *moody*-RB transcript is present in both heads and bodies of adult flies, while the shorter 3.0 kb *moody*-RA transcript is detected only in heads. The *EP1529* element is inserted in the first exon of the RB transcript and leads to a loss of this transcript, while the RA tran-

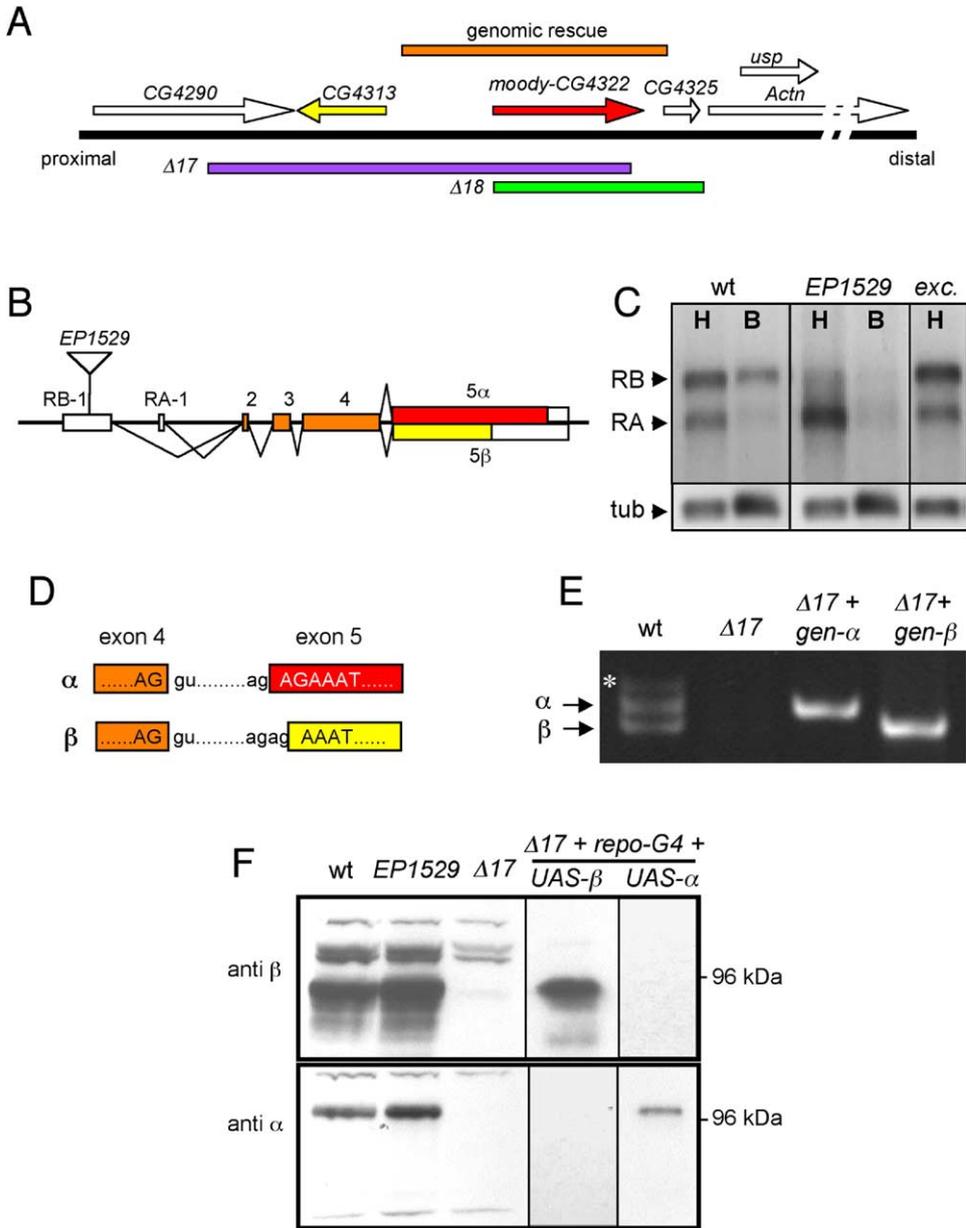


Figure 2. Molecular Characterization of the *moody* Locus

(A) Schematic representation of the genomic region surrounding the *moody* (CG4322) gene (<http://flybase.bio.indiana.edu>). Genes are indicated as horizontal arrows, with the arrow pointing toward the 3' end of each gene. The extent of two chromosomal deletions ($\Delta 17$ and $\Delta 18$) and the sequences contained in the genomic rescue construct are indicated.

(B) Exon-intron structure of the *moody* gene. Two alternative transcription start sites give rise to the RA and RB transcripts; the *EP1529* transposon is inserted in the first noncoding exon of the RB transcript. Alternative splicing of the fourth intron generates the *moody- α* and *moody- β* transcripts. Translation begins in the second exon.

(C) Northern blot analysis of *moody* transcripts. Wild-type (wt) heads (H) contain both the 3.8 kb RB and 3.0 kb RA transcripts, while bodies (B) contain only the RB transcript. The RB transcript is absent in *EP1529* flies; precise excision of the *EP1529* element (*exc.*) restores the RB transcript. A probe from the *tubulin α* gene (*tub*) was used as an mRNA loading control.

(D) Sequence of the exon-intron boundaries of the alternatively spliced intron 4. Exonic sequences are capitalized. The *moody- α* transcript (red box) is two nucleotides longer than the *moody- β* transcript (yellow box).

(E) Analysis of the *moody- α* and *- β* transcripts by RT-PCR. Bands corresponding to *moody- α* and *- β* (and the heteroduplex*) are observed in wild-type (wt). These bands are absent in the $\Delta 17$ deletion and restored upon expression of genomic constructs with a prespliced fourth intron in either the α (*gen- α*) or β (*gen- β*) configuration.

(F) Western blots probed with antibodies specific to Moody- β (anti- β , top panel) or Moody- α (anti- α , bottom panel). A prominent band, slightly smaller than 96 kDa, was recognized by the anti- β antibody; this band is absent in the $\Delta 17$ deletion and restored upon expression of a prespliced *UAS-moody- β* transgene (*UAS- β*) driven by *repo-GAL4* (*repo-G4*) in the $\Delta 17$ background. Similarly, the anti- α antibody recognized a protein of slightly larger than 96 kDa that was absent in $\Delta 17$ flies but restored by expression of a prespliced *UAS-moody- α* (*UAS- α*) construct. The total protein levels loaded in the two right lanes, containing extracts from flies overexpressing either Moody- α or Moody- β , are ~20-fold lower than those shown in the three left lanes, which show the endogenous levels of Moody proteins; the nonspecific bands are therefore not seen in the two right lanes.

script is still present and possibly more abundant in the mutant. Precise excision of the *EP1529* transposon restores normal transcript levels (Figure 2C, right lane) and, as mentioned above, normal cocaine sensitivity.

Sequencing of several *moody* cDNAs revealed an additional source of transcript diversity: alternative splicing of the fourth intron generates mRNAs that differ at the exon 4/5 boundary by two bases (Figure 2D), causing exon 5 to be translated in two alternative reading frames. We confirmed the existence of these alternatively spliced transcripts by carrying out RT-PCR reactions with mRNA isolated from adult heads (Figure 2E); we will refer to the longer transcript as *moody- α* and to the shorter one as *moody- β* . These two *moody* transcripts are present in equal amounts in adult heads, as evidenced by RT-PCR (Figure 2E, left lane) and cDNA sequencing. In addition, both the RA and RB transcript undergo alternative splicing of the fourth intron, based on cDNA sequence analysis and the observation that *moody- α* and *- β* are present in the *EP1529* mutant (which lacks RB). The *moody- α* and *- β* transcripts are predicted to encode two proteins that differ substantially in their intracellular C-terminal domains (Figure 2B); *Moody- α* and *Moody- β* are expected to be 671 and 634 amino acids in length, respectively. The seven predicted transmembrane domains, encoded by exons 3 and 4, are identical in both *Moody* proteins.

In summary, *moody* expression is regulated in complex ways; through alternative use of two 5' UTRs and alternative splicing, it generates four transcripts, which encode two proteins that differ substantially in their predicted cytoplasmic C-terminal domains. Furthermore, we have shown that *EP1529* mutants lack the transcripts carrying the RB 5' UTR.

The Function of Either *Moody- α* or *Moody- β* Is Sufficient for Viability

To establish the consequences of complete loss of *moody* function, we generated imprecise excisions of the *EP1529* element and recovered two deletions that remove the entire *moody* gene ($\Delta 17$ and $\Delta 18$, Figure 2A; see Experimental Procedures). These mutations are lethal; only ~1% of homozygous females and hemizygous males survive to adulthood. The few flies that survive are sickly, showing severe motor defects and reduced life span. The lethality associated with *moody* ^{$\Delta 17$} and *moody* ^{$\Delta 18$} is caused by deletion of the *moody* gene, as viability is completely restored by a transgene containing the *moody* coding region and the complete intergenic sequences that flank it, approximately 3 kb and 1 kb of 5' and 3' sequence, respectively (Figure 2A).

To study the potentially distinct functions of *Moody- α* and *- β* , we generated constructs (gen- α and gen- β) that contain the *moody* genomic DNA with prespliced fourth introns (see Experimental Procedures). These constructs, while encoding both the RA and RB transcripts (data not shown), encode only one of the two *Moody* proteins (Figure 2E and below). Transgenes encoding *Moody- β* fully restored viability to *moody* ^{$\Delta 17$} flies (100% of expected progeny obtained with 5 independent transformant lines), while transgenes encoding *Moody- α* showed only partial rescue (20%–25% of expected progeny obtained with five independent transformant lines); the latter is, however, significantly higher than the 1% viability seen for *moody* ^{$\Delta 17$} flies.

Moody Is Expressed in Glia that Form the Blood-Brain Barrier

To determine where *Moody* is expressed and to differentiate between *Moody- α* and *Moody- β* , we generated polyclonal antibodies to their distinct C termini (see Experimental Procedures and Figure 2F). On Western blots, these antibodies recognize proteins of approximately 92 and 96 kDa, which is larger than the predicted molecular weights of 70 and 74 kDa for *Moody- β* and *- α* , respectively. This difference is due to glycosylation of both forms of *Moody*, as it can be eliminated by pretreatment of the extract with N-glycosidase (data not shown). As expected, Western blot analysis of whole-head extracts revealed that both *Moody* forms are absent in *moody* ^{$\Delta 17$} flies. Analysis of flies carrying transgenes that express only *Moody- α* or *- β* (see legend to Figure 2 for details) in the *moody* ^{$\Delta 17$} background confirmed the specificity of both antibodies (Figure 2F, right lanes). Normal levels of both *Moody* proteins were observed in the heads of flies carrying the hypomorphic *EP1529* allele, suggesting that changes are either too subtle to detect and/or localized to subsets of *Moody*-expressing cells.

moody has been shown to be expressed in a subset of embryonic glial cells (Kunwar et al., 2003), those involved in ensheathment and insulation of the nervous system (Schwabe et al., 2005 [this issue of Cell]). We find that in both larvae and adult flies, *Moody* is similarly expressed in glia that insulate the nervous system. One group of *Moody*-expressing glia, the surface glia that surround the CNS, are large and very flat cells held together by pleated septate junctions (reviewed in Bellen et al. [1998]; Bhat, 2003; Hortsch and Margolis, 2003). Figure 3A shows a three-dimensional reconstruction of *Moody*-expressing cells surrounding the larval ventral nerve cord (VNC), which highlights *Moody* localization to regions of cell contact among surface glia. However, *Moody* proteins are also expressed more broadly throughout the surface glia ensheathing the larval VNC and peripheral nerves, as can be observed in thin optical sections (Figures 3B–3D). *Moody- α* and *- β* expression completely overlap in the larval nervous system. The two *Moody* proteins also colocalize to glia ensheathing the adult brain (Figures 3E–3K): expression is observed in cells surrounding the brain and cells lining the cavity that harbors the esophagus. They are also expressed in a layer of cells located between the retina and the brain. These cells express the glial-specific Repo protein (Figures 3L–3O); we therefore believe that these cells correspond to the pseudocartridge glia, which have been shown to be part of the BBB of diptera (Carlson et al., 2000; Shaw and Varney, 1999).

In summary, we have shown that *moody* encodes two stable proteins that are expressed in glial cells that surround the developing and adult nervous system. *Moody* proteins localize to the plasma membrane and are highly enriched in areas of cell-cell contact among surface glia. Consistent with *moody* playing a role in glia is our finding that the lethality of *moody* ^{$\Delta 17$} flies can be completely rescued by expression of a *UAS-moody- α* or *UAS-moody- β* transgene driven by *repo-GAL4*, which drives expression specifically in glia. In contrast, similar experiments using the panneuronal driver *elav-GAL4* failed to restore viability.

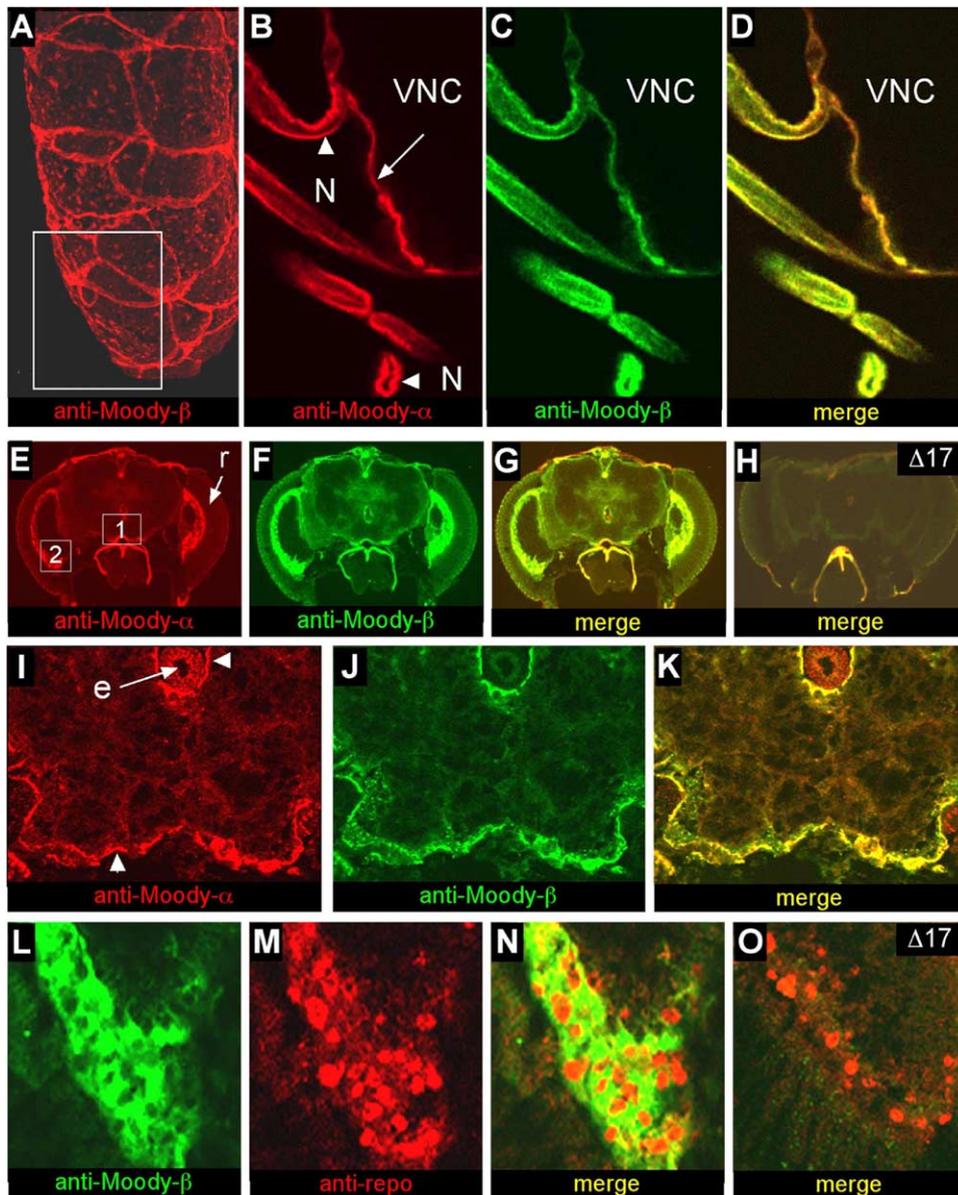


Figure 3. Expression of Moody- α and Moody- β in the Developing and Adult Nervous System

(A–D) Moody is expressed in surface glia. Ventral nerve cords (VNCs) of third-instar larvae were stained with antibodies recognizing Moody- β (red, [A]) and analyzed by confocal microscopy. Three-dimensional reconstructions show that Moody- β is highly expressed in regions of cell contact between surface glia, which form a mesh of large, flat cells that surrounds the entire nerve cord. (B)–(D) show an optical section (0.5 μ m thick) through a region of the VNC (approximate location is indicated in the box in [A]) stained with antibodies recognizing Moody- α (red) and - β (green). Both Moody proteins are expressed in a thin layer of cells surrounding the VNC (arrow) and peripheral nerves ([N], arrowheads). (E–K) Moody- α and - β are coexpressed in adult surface glia. Frontal cryosections of adult heads were stained with antibodies against Moody- α (red, [E and I]) and Moody- β (green, [F and J]). Expression of the two Moody proteins completely overlaps in wild-type flies (E–G) and is absent in $\Delta 17$ flies (H). (I–K) Higher-magnification views of the region shown in box 1 (E) highlights expression in the surface glia that ensheath the brain (vertical arrowhead) and the cavity containing the esophagus (e; horizontal arrowhead). Staining of the esophagus with anti-Moody- α is nonspecific, as it is also observed in the $\Delta 17$ mutant (not shown); parts of the head cuticle show strong autofluorescence (H). (L–O) Moody is expressed strongly in pseudocartridge glia located between the retina (r) and the central brain. Frontal cryosections of adult heads were stained with anti-Moody- β (green, [L]) and anti-Repo (red, [M]); a high-magnification view of the region highlighted in box 2 (E) is shown. Expression of Moody- β surrounds large Repo-positive nuclei, confirming the glial specificity of Moody expression. These glia are still present in the $\Delta 17$ mutant (O).

Both Moody- α and Moody- β Are Required for Normal Cocaine Sensitivity

We next addressed the role of the two Moody proteins in cocaine-induced behaviors. *EP1529* flies, while lack-

ing the RB transcript, produce both Moody- α and - β (Figure 2F) and are for that reason not useful for this purpose. We therefore took advantage of the fact that the viability of *moody* ^{$\Delta 17$} flies is restored by expression

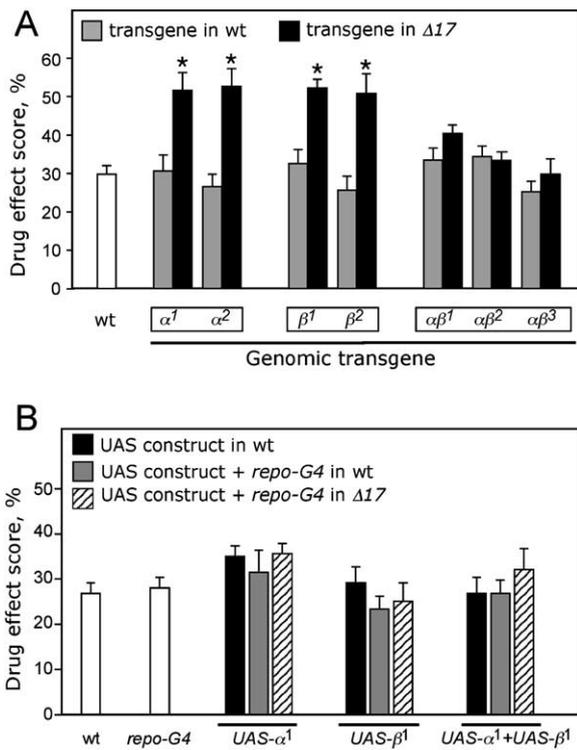


Figure 4. Requirements for *moody* Function in Cocaine Sensitivity
(A) Both *Moody- α* and *Moody- β* are required for normal cocaine sensitivity. Flies carrying genomic transgenes with prespliced introns 4 in either the α or β configuration were exposed to 150 μ g of cocaine and tested for cocaine sensitivity in the crackometer. Two independent transgenes each for *Moody- α* (α^1 and α^2) and *Moody- β* (β^1 and β^2) failed to restore normal cocaine sensitivity to $\Delta 17$ flies (black bars). In contrast, expression of three independent transgenes carrying the “native” genomic construct, containing the fourth intron and thus encoding both *Moody- α* and β ($\alpha\beta^1$ - $\alpha\beta^3$), restored normal cocaine sensitivity to $\Delta 17$ flies (black bars). Neither transgene affected the cocaine sensitivity of wild-type flies (gray bars). One-way ANOVA with Newman-Keuls post hoc tests revealed significant differences between flies carrying any of the α or β genomic transgenes in the $\Delta 17$ background (nonrescued flies) and their appropriate controls, which carry the same transgenes in a wild-type background ($p < 0.001$). No significant differences were observed between flies carrying any of the three genomic $\alpha\beta$ transgenes in the $\Delta 17$ background (rescued flies) and their controls carrying the same transgenes in a wild-type background ($p > 0.05$). $n = 12$ experiments.

(B) *Moody* expression in glia is sufficient for normal cocaine sensitivity. Flies carrying the glial-specific *repo-GAL4* (*repo-G4*) driver and UAS transgenes encoding either *Moody- α* (*UAS- α^1*), *Moody- β* (*UAS- β^1*), or both (*UAS- α^1 + UAS- β^1*) were tested for cocaine sensitivity as described above. Expression of these *moody* transgenes in the $\Delta 17$ background restored not only viability, but also normal cocaine sensitivity (hatched bars). Overexpression of either or both *Moody* forms in the glia of wild-type flies had no effect on cocaine sensitivity (gray bars). One-way ANOVA with Newman-Keuls post hoc tests revealed no significant differences ($p > 0.05$) between flies in each group. $n = 12$ experiments. It should be noted that expression of *Moody* under the control of the *repo-GAL4* driver is approximately 20 times higher than that achieved by its own regulatory sequences contained in the genomic constructs used in the experiments shown in (A) (data not shown).

of either *moody- α* or *moody- β* prespliced genomic transgenes to generate flies for behavioral testing. As shown in Figure 4A, flies carrying either of these trans-

genes in the *moody* ^{$\Delta 17$} null background (Figure 4A, black bars) showed increased cocaine sensitivity similar to that observed with *EP1529* flies. In contrast, completely normal cocaine behaviors were displayed by flies carrying the genomic construct containing the fourth intron, thus able to encode both *Moody- α* and *Moody- β* . Two independent transformant lines for each of the prespliced constructs (α^1 , α^2 , β^1 , β^2) were tested, all showing similarly increased cocaine sensitivity; three independent lines carrying the fourth intron ($\alpha\beta^1$, $\alpha\beta^2$, $\alpha\beta^3$) showed normal cocaine sensitivity. The cocaine sensitivity of *moody* ^{$\Delta 17$} flies carrying two transgenes—one encoding the α and the other the β *Moody* form—was also normal (data not shown), eliminating the possibility that our prespliced transgenes were defective. Expression of none of these transgenes affected the behavior of otherwise wild-type flies, showing that overexpressing *Moody* does not simply cause resistance to cocaine. Thus, while either *Moody- α* or *Moody- β* are sufficient for viability, both *Moody* forms are required for normal cocaine sensitivity.

Although *Moody* appears to be expressed specifically in surface glia and the genomic constructs used for the rescue experiments described above used the endogenous *moody* regulatory sequences, some expression and function outside of glia could not be completely excluded. We therefore asked if expression of *Moody* solely in glia, driven by the glial-specific *repo-GAL4* driver, restores normal cocaine sensitivity to *moody* ^{$\Delta 17$} flies. As shown in Figure 4B, expression of either *Moody- α* , β , or both, resulted in flies with normal cocaine sensitivity (Figure 4B, hatched bars). Again, expression of *Moody* in glia of wild-type flies (Figure 4B, gray bars) did not simply reduce the flies’ sensitivity to the effects of cocaine. It should be noted that the *repo-GAL4* driver is expressed in all developing and mature glia (Xiong et al., 1994), not only those involved in nervous system insulation.

In summary, we show that when *moody* is expressed in the context of its normal regulatory sequences, flies require both *Moody- α* and *Moody- β* for normal cocaine sensitivity. This suggests that these related proteins play distinct roles in regulating cocaine sensitivity and/or that a functional receptor may involve both protein forms. In the *repo-GAL4/UAS-moody* flies, the protein is overexpressed greater than 20-fold based on Western blots (data not shown). It is therefore possible that under these nonphysiological conditions, one *Moody* form can compensate for the lack of the other. Regardless, our data show unequivocally that *moody* is required in glial cells to regulate cocaine sensitivity.

The Function of Either *Moody- α* or *Moody- β* Is Sufficient for Normal Blood-Brain Barrier Function

We identified *moody* for its role in regulating cocaine sensitivity. *moody* was identified independently as a gene expressed in embryonic glia, where it functions in the establishment of the BBB (Schwabe et al., 2005). In the complete absence of *moody* function, the surface glia form abnormally and fail to insulate the nervous system properly; this interferes with larval motor behaviors required for hatching, causing lethality (Schwabe et al., 2005).

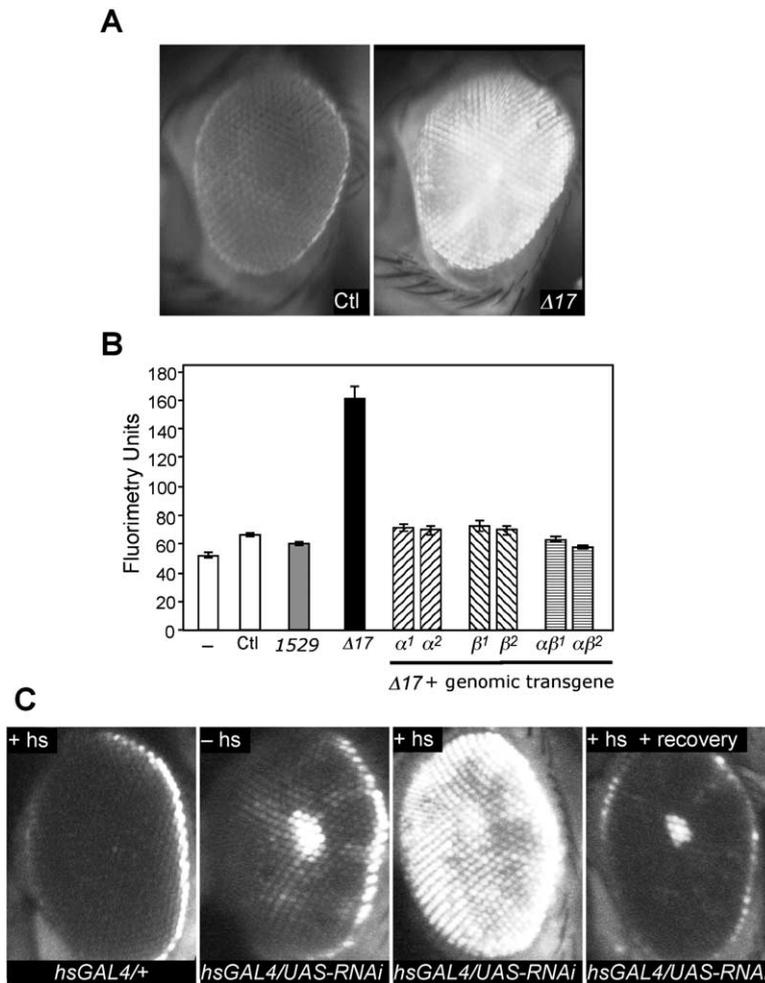


Figure 5. Requirements for *moody* Function in Nervous System Insulation

(A) The *moody* $\Delta 17$ mutation disrupts the blood-brain barrier. A fluorescent dye was injected into the abdomens of adult flies and its accumulation in the retina observed by fluorescence microscopy. The dye is excluded from the retina of wild-type flies (right panel) but penetrates the eye when injected into flies carrying the $\Delta 17$ deletion (left panel). Dye accumulation in the head was seen within seconds of dye injection and the distributions shown were stable for several days after injection. Both Ctl and $\Delta 17$ flies have no eye pigment as they carry the w^{1118} mutation.

(B) Dye penetration into the central nervous system was quantified by fluorimetry of dissected brains 24 hr after abdominal dye injection. Compared to uninjected flies (-, white left bar), injected control flies (Ctl = *EP369*) showed a small increase in fluorescence, which is likely due to accumulation of dye in the hemolymph that surrounds the esophagus (data not shown). One-way ANOVA with Newman-Keuls post hoc tests revealed a significant difference ($p < 0.001$) between $\Delta 17$ flies and all other injected genotypes. No significant differences ($p > 0.05$) were observed between Ctl, *EP1529*, and "rescued" $\Delta 17$ flies carrying either the *moody* α , β , or $\alpha\beta$ transgenes. $n = 9-10$ experiments.

(C) *moody* is required in the adult fly to maintain proper nervous system insulation. Heat-shocked control flies (*hsGAL4/+*, "+hs" panel) did not reveal fluorescent dye penetration into the retina, showing that the heat treatment does not disrupt nervous system insulation. Some dye penetration was observed in experimental flies (*hsGAL4/UAS-RNAi*), which express a *moody*-specific RNA

interference transgene (*UAS-RNAi*), even in the absence of heat shock ("-hs" panel). When exposed to heat shocks during adulthood (*hsGAL4/UAS-RNAi*, "+hs" panel), these flies showed strong dye penetration into the retina, implying breakdown of the blood-brain barrier. The dye was excluded from retinas if injected into experimental flies that had recovered from heat shock for 4 days (*hsGAL4/UAS-RNAi*, "+hs + recovery" panel). Similar data were obtained when using the fluorimetric assay ([B]; data not shown).

To determine if the role of *moody* in regulating cocaine sensitivity is related to its function in establishing the BBB, we assayed surface glia integrity in flies with cocaine sensitivity defect. For this purpose, we injected adult fly abdomens with a fluorescent dye (see [Experimental Procedures](#)) and observed diffusion of the dye into the retina, which signals an impaired BBB (Figure 5A). In control flies, the dye failed to penetrate the retina (only a narrow rim of fluorescence was observed surrounding the retina), while the retinas of *moody* $\Delta 17$ flies showed strong dye penetration, revealing a breakdown of barrier function. For a quantitative assessment of dye penetration into the brain, we dissected the brains of dye-injected adult flies and measured dye penetration by fluorimetry (Figure 5B; see [Experimental Procedures](#)). Compared to uninjected flies and injected wild-type controls, *moody* $\Delta 17$ flies showed a large increase in brain fluorescence, reflecting a disruption of the blood-brain barrier. This defect was completely rescued by expression of genomic transgenes expressing either *Moody*- α , *Moody*- β , or both (Figure

5B). Flies carrying the *EP1529* mutation showed dye penetration indistinguishable from wild-type controls.

In summary, we show that flies with an apparently normal BBB show abnormal cocaine sensitivity. Specifically, while both *Moody*- α and *Moody*- β are required for normal cocaine sensitivity, either form alone is sufficient to confer normal nervous system insulation. The observation that *EP1529* flies display normal brain insulation reinforces the dissociation between cocaine sensitivity and blood-brain barrier formation (see [Discussion](#)).

Moody Function Is Actively Required in the Adult Fly to Maintain Normal Nervous System Insulation

Analysis of surface glia development in *moody* $\Delta 17$ flies revealed that, while present in the mutant, the shape and size of the cells is abnormal (Schwabe et al., 2005). To determine if *moody* function is required to maintain BBB integrity in the adult fly, we used transgenic RNA interference (RNAi; Carthew, 2001) to disrupt *moody* expression in adults. Specifically, we generated a *UAS-*

moody-RNAi construct that targets all *moody* transcripts and induced its expression in the adult fly with the heat-inducible *hs-GAL4* transgene; nervous system insulation was measured with the dye-injection assay described above (Figure 5A). Flies carrying the *UAS-moody-RNAi* transgene and *repo-GAL4* did not survive, showing that RNA interference causes a strong reduction in *moody* expression. Heat-shock treatment did not affect BBB function of control flies (*hs-GAL4/+*; “+hs” panel). A small amount of dye accumulation was observed in experimental flies in the absence of heat shock (*hs-GAL4/UAS-RNAi*; “-hs” panel), likely due to leaky expression of GAL4. Heat treatment of experimental flies, however, led to a complete breakdown of the barrier as visualized by strong dye penetration into the retina (*hs-GAL4/UAS-RNAi*; “+hs” panel). Interestingly, this breakdown in nervous system insulation was reversible; experimental flies subjected to heat shock and allowed to recover for 4 days prior to dye injection showed normal barrier function (*hs-GAL4/UAS-RNAi*; “+hs + recovery” panel). We conclude that Moody expression in surface glia is required not only for their proper development (Schwabe et al., 2005), but also in the adult fly to actively maintain the integrity of the BBB.

Discussion

In a screen for mutants with altered sensitivity to acute cocaine exposure, we identified a mutation that disrupts a GPCR that we have called Moody. Molecular analysis of the *moody* locus revealed that it encodes two proteins, Moody- α and - β , that differ in their long C-terminal domains. Both forms of Moody are coexpressed in glia that surround and insulate the nervous system and are both found at regions of cell-cell contact. Partial loss of *moody* function causes increased sensitivity to cocaine and nicotine, and reduced sensitivity to ethanol-induced loss of postural control. Complete loss of function results in lethality due to defective insulation of the nervous system (Schwabe et al., 2005). Transient inhibition of *moody* expression in the adult fly causes a reversible disruption of the BBB, indicating that *moody* function is continuously required to insulate the nervous system. The role of *moody* in drug sensitivity can, however, be dissociated from its role in nervous system insulation: while either Moody- α or - β are sufficient for normal blood-brain barrier formation, both protein forms are needed for flies to show normal cocaine sensitivity. We postulate that a Moody-mediated signaling pathway functions in surface glia to regulate both nervous system insulation and drug-related behaviors.

We show that the *moody* locus encodes two proteins that differ in their C-terminal domains; these predicted cytoplasmic regions are 271 and 230 amino acids long for Moody- α and - β , respectively. The two Moody proteins are found in nearly equal amounts and are coexpressed during development and adulthood in glia that ensheath the nervous system. Interestingly, coexpression is accomplished by an unusual alternative splicing event that leads to translation from overlapping reading frames. This mechanism likely insures stoichiometric

amounts of the two protein products, which in turn may be important for normal function. Although either Moody protein is sufficient for viability and normal blood-brain barrier formation, both proteins are needed for flies to respond normally to acute cocaine exposure. This likely reflects the high degree of sensitivity of behavioral outputs to changes in organismal physiology. Why would normal drug sensitivity require both Moody forms? It is possible that the two proteins interact with distinct downstream signaling pathways or that their optimal function, maturation, and/or stability requires the formation of heterodimers; these possibilities are currently under investigation. Regardless of the exact functional significance of the two Moody forms, their existence is likely to be important, as their presence is conserved in *Drosophila pseudoobscura*, a species that diverged from *Drosophila melanogaster* some 30 million years ago. The Moody- α C-terminal domain shows 58% identity and 63% similarity, while the Moody- β -specific domain shows 48% identity and 61% similarity between the two *Drosophila* species.

In insects, including *Drosophila*, the nervous system is insulated from the humoral environment through a glial-dependent blood-brain and blood-nerve barrier, which plays a crucial role in its electrical and chemical insulation. Septate junctions between the surface glial cells are believed to form the structural basis for these barriers and to be functionally equivalent to vertebrate paranodal junctions found at nodes of Ranvier. Indeed, many molecular components of septate junctions—including the cell adhesion molecules gliotactin (a member of the neuroligin family), Neurexin IV, and Contactin—are also found at paranodal junctions of myelinated nerves (reviewed in Bellen et al. [1998]; Bhat, 2003; Hortsch and Margolis, 2003). The GPCR Moody was identified by two completely independent means: as a gene expressed in embryonic glia that ensheathes the nervous system (Kunwar et al., 2003; Schwabe et al., 2005) and as a mutation that alters the sensitivity of adult flies to acute cocaine administration (this study). Schwabe et al. (2005) postulate that the Moody GPCR signals through trimeric G proteins, which, in turn, regulate the cortical actin cytoskeleton, the proper development of septate junctions, and the formation of the blood-brain barrier.

Interestingly, we find that Moody continues to be expressed in the surface glia of the adult fly, where it functions to maintain the integrity of the BBB; transient reduction of *moody* expression causes a reversible disruption in nervous system insulation. We therefore believe that, in addition to its role in establishment of the BBB, *moody* functions continuously to regulate its degree of permeability. What signals would Moody normally respond to in order to carry out its functions? One possibility is that the ligand is delivered via the hemolymph that bathes the nervous system. The hemolymph is not only rich in certain ions (such as K⁺), but also contains nutrients, amino acids, hormones, neuropeptides, and various proteins involved in clotting and the immune response (Karlsson et al., 2004). Alternatively, the ligand may be produced by the underlying neurons, or by neighboring glia. In mammals, the permeability of the blood-brain barrier can be altered by hypoxia-ischemia and by various substances released

under pathological conditions, including the amino acids glutamate and aspartate, ATP, histamine, serotonin, and various peptides (reviewed in Ballabh et al. [2004]). Several of these substances signal via receptors of the GPCR superfamily, although the mechanisms by which this signaling leads to altered BBB function remain poorly understood. The identification of the Moody ligand and downstream signaling pathway, and the physiological conditions that modulate their function, should provide interesting new insights into the mechanisms that regulate nervous system insulation.

In addition to *moody*, our behavioral screens for drug sensitivity mutants identified a hypomorphic allele of *loco* (7-88; R.J.B., J. Niclas, and U.H., unpublished data). 7-88 flies show reduced sensitivity to acute cocaine administration. *loco* encodes an RGS (regulator of G protein signaling) protein whose normal function is to terminate signaling by GPCRs (Neer, 1995; Ross and Wilkie, 2000). Interestingly, *loco* has been implicated in nervous system insulation (Granderath et al., 1999) and, more recently, shown to function together with *moody* in the development of the BBB (Schwabe et al., 2005). Our observation that mutations in *moody* and *loco* cause opposite cocaine sensitivity defects is therefore consistent with their molecular functions. Interestingly, the drug resistance seen with the *loco* 7-88 mutation (and flies heterozygous for the null allele *loco*^{Δ13}; R.J.B., unpublished data) suggests that overactivation of the Moody-GPCR pathway can cause the opposite effect as its inhibition, implying that the pathway is under both positive and negative control, providing a broad range of physiological and behavioral regulation. Our ability to discern such subtle physiological changes likely reflects the exquisite sensitivity of behavioral phenotypes to changes in organismal physiology, such as alterations in BBB function, and further demonstrates the utility of psychoactive compounds as probes into CNS function.

Could the increased cocaine (and nicotine) sensitivity seen with the *EP1529 moody* mutation be caused by an altered drug accessibility to the nervous system? As in vertebrate systems the *Drosophila* respiratory system is the most accessible route for drug entry in acute exposure paradigms; drug volatilization ensures quick and relatively homogeneous delivery to a population of flies. Drugs enter the tracheal system that is connected to the environment through spiracles at the cuticular surface. The tracheal network then divides into smaller tubes, or tracheoles, which make links throughout the organism to the hemolymph and to end organs, such as the brain (reviewed in Manning and Krasnow [1993]). We have no reason to believe that *moody* mutations affect the delivery of drugs via the tracheal system, as *moody* does not appear to be expressed in these cells, and the tracheal system is therefore expected to function normally in *moody* mutants. *moody* does, however, play a role in the development and function of surface glia that insulate the nervous system from the hemolymph that bathes it. Drugs delivered via the hemolymph would need to cross this barrier to access the nervous system. We do not believe, however, that increased drug accessibility—caused by a dysfunctional BBB—is the cause of drug phenotypes observed with

moody mutations. First, molecules such as cocaine and nicotine freebase—neutral compounds with molecular weights (MW) of 303 and 163 Da, respectively—readily cross the blood-brain barrier in mammals, and we suspect the same to be true in flies. Indeed, we find that Rhodamine B (a neutral compound with a MW of 600 Da) readily crosses the BBB of wild-type flies as assayed by the dye-injection assay, while FITC (a negatively charged compound of MW 450 Da) is excluded. Rhodamine B's ability to penetrate into the CNS is not due to a toxic effect of the compound on the BBB, as FITC is still excluded when coinjected with Rhodamine B (data not shown). Second, FITC exclusion from the CNS in our behavioral mutants (*moody*^{Δ17} flies carrying either the *gen-α* or *gen-β* transgenes) is indistinguishable from wild-type, when ascertained by either dye-injection or dye-feeding assays. Third, *EP1529* flies (Figure 1) and *moody*^{Δ17} flies carrying either the *gen-α* or *gen-β* transgenes (data not shown), are resistant to the acute intoxicating effects of ethanol. Since ethanol readily crosses cell membranes, a defect in the BBB should have no effect on ethanol's ability to access its targets in the nervous system. Indeed, we find that ethanol absorption is completely normal in *EP1529* flies (Figure 1). Finally, downregulation of *moody* expression in flies carrying the *UAS-moody-RNAi* and *hsGAL4* transgenes, a manipulation that clearly disrupts BBB integrity (Figure 5), causes resistance, not sensitivity, to acute cocaine exposure (data not shown); we currently do not understand the bases for this resistance. Regardless, the data listed above argue strongly that the behavioral defects observed in *moody* flies are not caused by altered drug accessibility to its sites of action in the nervous system.

Rather, we postulate that in our behavioral mutants the “state” or responsiveness of the nervous system has changed due to a chronic yet subtle alteration in blood-brain barrier function. This could be caused, for example, by a chronic alteration in the ionic composition of the CNS or changes in the concentrations of various small molecules (such as neurotransmitters) that may leak into or out of the CNS. Interestingly, these proposed adaptations have opposite effects on the flies' response to cocaine and nicotine (increased sensitivity) and ethanol (reduced sensitivity). This divergence is not too surprising, as our unbiased genetic screens have identified several mutants that differentially affect the response to psychostimulants and ethanol. Thus, a particular set of changes in the nervous system—caused either by its altered insulation or by single-gene mutation—can have distinct effects on the organism's response to drugs. Further studies of the mechanisms of Moody signaling and its downstream effects in glia should begin to reveal how the BBB and the molecules that regulate its permeability interact with the nervous system to regulate behavior. Interestingly, a recent study in *Drosophila* identified a signaling pathway—involving the neuropeptide Amnesiac and the neurotransmitter transporter Inebriated—that functions to regulate the growth of peripheral perineurial glia in response to signals from motorneurons (Yager et al., 2001). It is possible that reciprocal interactions occur between surface glia and the mature nervous system to regulate behavioral responses to drugs of

abuse. In mammals, claudin-5, a cell-adhesion molecule found in tight junctions of epithelial cells that form the BBB, has been implicated in normal BBB function. Specifically, claudin-5-deficient mice show an increased permeability to small molecules (Nitta et al., 2003). Interestingly, the human claudin-5 locus (CLDN5) has been associated with vulnerability to schizophrenia (Sun et al., 2004). Taken together with our study, these observations warrant a closer examination of the role of the BBB in nervous system function and the etiology of mental illness.

Experimental Procedures

Drosophila Culture and Genetics

All flies were raised and maintained on standard cornmeal molasses agar at 25°C and 70% humidity. Repo-GAL4 (V. Auld) and UAS-MoesinGFP (D. Kiehart) were obtained from published sources. The Rørth EP collection was obtained from G.M. Rubin (Rørth et al., 1998). Approximately 400 X-linked EP lines were screened as hemizygous males for cocaine sensitivity in the crackometer (see below). Lines that showed phenotypes that deviated from the collection mean by greater than 1.5 standard deviations were outcrossed into our "control" w^{1118} genetic background (which is isogenic for the second and third chromosomes) and then retested for cocaine sensitivity. In addition, a group of lines that showed normal cocaine sensitivity (near the mean of the distribution) were also outcrossed to w^{1118} to be used as controls. Here, we use line EP369 as our wild-type control; these flies show cocaine sensitivity similar to that of the w^{1118} strain. All strains, including the transformant lines described below, were outcrossed to w^{1118} for five generations prior to behavioral testing. Excisions of EP1529 were generated using standard genetic crosses. Several lines carrying precise excisions of the EP1529 element, characterized by PCR analysis and DNA sequencing, showed normal cocaine sensitivity. Two lethal excision lines, $\Delta 17$ and $\Delta 18$, were shown to be allelic and to carry imprecise excisions of the EP1529 element and adjacent genomic DNA (Figure 2).

Behavioral Assays

For all behavioral assays, groups of 15 male flies were collected under CO₂ anesthesia 0–2 days posteclosion and tested 2–3 days later. Flies were equilibrated at room temperature (~20°C) for 1 hr before behavioral testing. Exposures to volatilized cocaine and nicotine were carried out as described before (Bainton et al., 2000; McClung and Hirsh, 1998). Flies were then transferred to a glass cylinder to quantify startle-induced negative geotaxis (the crackometer assay) as previously described (Bainton et al., 2000; Tsai et al., 2004). The drug effect score corresponds to the average (measured every min over 5 min) number of flies that remained on the bottom of the cylinder, expressed as percent of the total number of flies. Ethanol sensitivity was measured in the inebriometer as described previously (Moore et al., 1998). Significance was established using either Student's paired t tests assuming equal variance or one-way ANOVAs with Newman-Keuls post hoc tests carried out in GraphPad Prism 4 (GraphPad, San Diego, California). Error bars in all experiments correspond to the standard error of the mean. In all behavioral experiments, the experimenter was blind to the genotype of the flies and all genotypes were tested on several different days. All genotypes were tested for baseline locomotion and startle-induced climbing and found to be normal.

Molecular Biology and Biochemistry

Isolation of moody cDNAs and Generation of UAS Constructs

A *Drosophila* head cDNA library (obtained from T. Schwarz) was screened with a probe unique to the fourth exon of *moody*. Positive clones were isolated and sequenced, leading to the identification of the *moody*- α and *moody*- β transcripts; both transcripts were found at similar frequency. Full-length clones for each of the *moody* transcripts were then cloned into the pUAST vector (Brand and Perrimon, 1993) to generate UAS- α or UAS- β constructs, and

germline transformants were obtained by standard procedures (Rubin and Spradling, 1982).

Construction of Genomic Rescue and RNAi Constructs

A 9.4 kb genomic EcoRV (partial)-StuI (partial) fragment containing the complete *moody* gene and all intragenic sequences (Figure 2) was cloned into the pCW8 vector to generate the *gen*- $\alpha\beta$ construct. The genomic *moody* constructs with prespliced fourth introns in either the α or β configuration (*gen*- α and *gen*- β) were generated by replacing a 3 kb SacII-XbaI fragment of the genomic clone with an equivalent fragment from either the *moody*- α or - β cDNAs. The UAS-RNAi construct was made from two PCR-generated *moody* fragments. One fragment contained 1.3 kb of genomic DNA spanning exon 3, intron 3, exon 4, and intron 4 and the other contained 0.6 kb of exon 4. The fragments were ligated sequentially into pUAST in an orientation that would generate an inverted repeat and thus a double-stranded RNA species.

RT-PCR and Northern Blots

To verify the presence of *moody*- α and - β transcripts in the heads of adult flies, PCR primers were designed to be located adjacent to (within 3 bp) and span the fourth intron. The primer for the reverse transcriptase (RT) reaction was complementary to sequences located 70 bp 3' to the fourth intron. Head mRNA isolation and RT-PCR reactions were carried out as described previously (Sullivan et al., 2000). Northern blots were carried out using mRNA isolated from the heads or bodies of flies of various genotypes as indicated in the text using previously described methods (Sullivan et al., 2000) and probed with a radio-labeled fragment specific to exon 4.

Mapping of the moody Deletions

PCR primers were designed to recognize sequences surrounding the *moody* gene at ~1 kb intervals. Genomic DNA from $\Delta 17$ and $\Delta 18$ flies was isolated and used as a template. Once a PCR product that spanned the deletion was obtained, it was cloned into the TOPO TA vector (Invitrogen) and sequenced, identifying the exact extent of each deletion.

Antibody Production and Western Blots

The predicted C-terminal regions encoding Moody- α and Moody- β were amplified by PCR and cloned into the pGEX4T-1 vector to produce in-frame fusions with GST. GST fusion proteins were produced and purified as specified by the manufacturers (Pharmacia). The purified fusion proteins were injected into rat (α) or rabbit (β) (Covance Pharmaceuticals). Anti-Moody antibodies were purified by adherence to purified fusion protein immobilized on PVDF membranes. For Western blots, fly heads, or bodies were homogenized in 5 μ l of loading buffer (0.125 M Tris base, 2% SDS) per fly and samples run on 8% polyacrylamide gels. Proteins were transferred to PVDF membranes (Amersham) by electroblotting; membranes were incubated with affinity-purified primary antibody (1:100) overnight at 4°C and with secondary antibody (1:1000) for 1 hr at room temperature. Visualization of Moody protein was done using LumiGen PS-3 chemiluminescence as specified by the manufacturer (Amersham).

Immunohistochemistry

Third-instar CNSs (dissected in PBS) or 10 μ m cryosections of adult fly heads were fixed in 3.7% paraformaldehyde in 200 mM sodium phosphate buffer (pH 7.2) containing 1 mM CaCl₂ for 30 min. After several washes in PBS + 0.3% Triton X-100 (PBS-TX), samples were transferred into primary antibody and incubated overnight at 4°C. After additional washes in PBS-TX, samples were incubated with secondary antibodies for 4 hr at room temperature. Samples were stored and mounted in 80% glycerol. Primary antibodies were as follows: affinity-purified rabbit anti-Moody- β (1:15 dilution), affinity-purified rat anti-Moody- α (1:10 dilution), mouse anti-GFP (1:250, Molecular Probes), mouse anti-Repo (1:10 dilution) (Xiong et al., 1994). Secondary antibodies were as follows: FITC goat anti-rabbit, Rhodamine goat anti-rat, and Texas Red goat anti-mouse (Jackson Immuno Research Laboratories) were used at a 1:100 dilution in PBS-TX; alexa-Fluor 488 goat anti-mouse (Molecular Probes) was used at a 1:200 dilution in PBS-TX. For the three-dimensional reconstructions shown in Figure 3A, confocal images were acquired using a Zeiss LSM 510. 0.5 μ m confocal sections were taken and stacks of 10–30 sections generated; image analysis was performed using Imaris 4.0 (Bitplane) and LSM 510 software (Zeiss).

Dye Injection Assays

CO₂-anesthetized adult flies were injected with thin borosilicate needles containing fluorescent dyes under a dissecting microscope. The flies were immobilized by suction through a small hole and approximately 0.3 μ l of dye was injected using a micromanipulator into the soft tissue between two abdominal segments of the exoskeleton. For visualization of eyes of intact animals, flies were injected with 50 mg/ml tetramethylrhodamine dextran (MW 10,000, Molecular Probes D1816) and photographed 2 hr later on a Zeiss M2BIO microscope fitted with an Ikegame SKC-141 high-resolution camera. For quantification of dye absorption into the brain, flies were injected as described above with 50 mg/ml eosin dextran (MW 10,000, Molecular Probes D1807). Brains were dissected in PBS 18 hr after injection and placed in Corning Costar Special Optics 96-well plates (two brains per well) containing 50 μ l 0.1% SDS. Fluorescence was measured using a Tecan SpectraFluor Plus Reader. For the experiments shown in Figure 5C, flies were heat shocked (60 min at 37°C) five times over 2 days. Dye was injected 24 hr after the last heat shock and dye penetration into the retina analyzed 18 hr later.

Acknowledgments

We would like to thank Rajana Van and Viktor Kharzia for help with immunohistochemistry and confocal microscopy, Tom Schwarz for the head cDNA library, and Mark Eddison and Adrian Rothenfluh for insightful comments on the manuscript. We are also indebted to Michelle Starz-Gaiano and Ruth Lehmann for helpful discussions during the early phases of this project. R.J.B. would also like to thank Cynthia Kenyon, Elizabeth Blackburn, Ron Vale, Pat O'Farrell, and Peter Walter for sharing their resources and wisdom, Laura Mitic, Reed Kelso, Nico Sturmann, and Steve Rodgers for helpful hints and encouragement, Nasima Mayer and Robert Threlkeld for tireless efforts with RT-PCR, Northernblots, and Westerns, and Fahima Mayer for help with dye injections and brain dissections. This work was funded by grants from NIH to R.J.B. (DA444906-33821), U.H. (DA14809 and AA10035), and U.G. (EY011560).

Received: March 22, 2005

Revised: May 20, 2005

Accepted: July 18, 2005

Published: October 6, 2005

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