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

Roland J. Bainton,1,* Linus T.-Y. Tsai,2,3 Tina Schwabe,4 Michael DeSalvo,1 Ulrike Gaul,4 and Ulrike Heberlein2-3,4*
1Department of Anesthesia
2Department of Anatomy
3Program in Neuroscience
University of California, San Francisco
San Francisco, California 94143
4Laboratory of Developmental Neurogenetics
Rockefeller University
New York, New York 10021

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 ΔFosB, 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; Schen 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 (Andrec 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-
to a control EP

EP1529/H9262 toxicating effects of ethanol, manifested as an in-

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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 μg of volatilized cocaine or 5 μg 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 μg 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.

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tinuously 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 beha-

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 μg), 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 (Rorth et al., 1998), and identified five mutants with a reduced cocaine sensitivity (corresponding to three genes) and seven mu-

tants 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 in-

drugs, manifested as an increased 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 beha-

viors, such as climbing and locomotion (see Experimental Procedures). The EP1529 insertion is responsi-

ble for the aberrant cocaine sensitivity, as precise excision of the EP element restores normal drug sen-

sitivity (data not shown). Sequence analysis by the Berkeley Drosophila Genome Project (http://flybase.bio.indiana.edu/) showed that the EP1529 element is inserted in the first exon of the RB transcript (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 dis-

tinguished 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 (Δ17 and Δ18) 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 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 Δ17 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 that 96 kDa, was recognized by the anti-β antibody; this band is absent in the Δ17 deletion and restored upon expression of a prespliced UAS-moody-β transgene (UAS-β) driven by repo-GAL4 (repo-G4) in the Δ17 background. Similarly, the anti-α antibody recognized a protein of slightly larger than 96 kDa that was absent in Δ17 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 (Δ17 and Δ18, 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 moodyΔ17 and moodyΔ18 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 moodyΔ17 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 5 independent transformant lines); the latter is, however, significantly higher than the 1% viability seen for moodyΔ17 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 moodyΔ17 flies. Analysis of flies carrying transgenes that express only Moody-α or -β (see legend to Figure 2 for details) in the moodyΔ17 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 (Schnabe 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 Bell 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 moodyΔ17 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.
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 lacking 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 moodyΔ17 flies is restored by expression...
of either mooey-α or mooey-β prespliced genomic transgenes to generate flies for behavioral testing. As shown in Figure 4A, flies carrying either of these transgenes in the mooey-117 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 mooey-α and mooey-β. Two independent transformant lines for each of the prespliced constructs (α1, α2, β1, β2) were tested, all showing similarly increased cocaine sensitivity; three independent lines carrying the fourth intron (αβ1, αβ2, αβ3) showed normal cocaine sensitivity. The cocaine sensitivity of mooey-117 flies carrying two transgenes—one encoding the α and the other the β Mooey 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 Mooey does not simply cause resistance to cocaine. Thus, while either Mooey-α or Mooey-β are sufficient for viability, both Mooey forms are required for normal cocaine sensitivity.

Although Mooey appears to be expressed specifically in surface glia and the genomic constructs used for the rescue experiments described above used the endogenous mooey regulatory sequences, some expression and function outside of glia could not be completely excluded. We therefore asked if expression of Mooey alone in glia, driven by the glial-specific repo-GAL4 driver, restores normal cocaine sensitivity to mooey-117 flies. As shown in Figure 4B, expression of either Mooey-α or Mooey-β, or both, resulted in flies with normal cocaine sensitivity (Figure 4B, hatched bars). Again, expression of Mooey 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 mooey is expressed in the context of its normal regulatory sequences, flies require both Mooey-α and Mooey-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-mooey 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 Mooey form can compensate for the lack of the other. Regardless, our data show unequivocally that mooey is required in glial cells to regulate cocaine sensitivity.

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

We identified mooey for its role in regulating cocaine sensitivity. mooey 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 mooey 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).
Drug Sensitivity and Blood-Brain Barrier Function

Figure 5. Requirements for moody Function in Nervous System Insulation

(A) The moody Δ17 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 Δ17 deletion (right 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 Δ17 flies have no eye pigment as they carry the w1118 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 Δ17 flies and all other injected genotypes. No significant differences (p > 0.05) were observed between Ctl, EP1529, and "rescued" Δ17 flies carrying either the moodyα, β, or αβ transgenes. n = 9–10 experiments.

(C) moody is required in the adult fly to maintain proper nervous system insulation. Heat-shocked control flies (hs-GAL4/+; "+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 moodyΔ17 flies showed strong dye penetrance, 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 fluorometry (Figure 5B; see Experimental Procedures). Compared to un.injected flies and injected wild-type controls, moodyΔ17 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 moodyΔ17 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 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 neurexin 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 ensheath 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<sup>-173</sup>; 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 co-injected with Rhodamine B (data not shown). Second, FITC exclusion from the CNS in our behavioral mutants (moody<sup>177</sup> flies carrying either the gen<sup>-α</sup> or gen<sup>-β</sup> transgenes) is indistinguishable from wild-type, when ascertained by either dye-injection or dye-feeding assays. Third, EP1529 flies (Figure 1) and moody<sup>177</sup> flies carrying either the gen<sup>-α</sup> or gen<sup>-β</sup> 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 Rerth EP collection was obtained from G.M. Rubin (Rerth et al., 1998). Approximately 400 X-linked EP lines were screened as hemizygous using moody-α 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” w1118 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 w1118 to be used as controls. Here, we use line EP699 as our wild-type control; these flies show cocaine sensitivity similar to that of the w1118 strain. All strains, including the transformant lines described below, were outcrossed to w1118 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, j17 and j18, 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 (2–3 days posteclosion) or 2–3 days later. Flies were equilibrated at room temperature (−20°C) for 1 hr before behavioral testing. Exposure 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-α 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 SacI-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 j17 and j18 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 manufacturer (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 μl 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 (1:1000) overnight at 4°C and with secondary antibody (1:10000) for 1 hr at room temperature. Visualization of Moody protein was done using Lumi-gen PS-3 chemiluminescence as specified by the manufacturer (Amersham).

Immunohistochemistry
Third-instar CNSs (dissected in PBS) or 10 μm 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-raft, 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 μm confocal sections were taken and stacks of 10–30 sections generated; image analysis was performed using Imars 4.0 (Bilplane) and LSM 510 software (Zeiss).
Dye Injection Assays
CO₂-anesthetized adult flies were injected with thin borosilicate needles containing fluorescent dyes under a dissecting microscrope. The flies were immobilized by suction through a small hole and approximately 0.3 μl 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 μg/ml tetramethylrhodamine dextran (MW 10,000, Molecular Probes D1818) 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 μl 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.

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