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Lmo Genes Regulate Behavioral Responses to Ethanol in *Drosophila melanogaster* and the Mouse

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Abstract

Background—Previous work from our laboratory demonstrated a role for the *Drosophila* Limonly (*dLmo*) gene in regulating behavioral responses to cocaine. Herein, we examined whether *dLmo* influences the flies' sensitivity to ethanol's sedating effects. We also investigated whether 1 of the mammalian homologs of *dLmo, Lmo3*, is involved in behavioral responses to ethanol in mice.

Methods—To examine *dLmo* function in ethanol-induced sedation, mutant flies with reduced or increased *dLmo* expression were tested using the loss of righting (LOR) assay. To determine whether mouse *Lmo3* regulates behavioral responses to ethanol, we generated transgenic mice expressing a short-hairpin RNA targeting *Lmo3* for RNA interference-mediated knockdown by lentiviral infection of single cell embryos. Adult founder mice, expressing varying amounts of *Lmo3* in the brain, were tested using ethanol loss-of-righting-reflex (LORR) and 2-bottle choice ethanol consumption assays.

Results—We found that in flies, reduced *dLmo* activity increased sensitivity to ethanol-induced sedation, whereas increased expression of *dLmo* led to increased resistance to ethanol-induced sedation. In mice, reduced levels of *Lmo3* were correlated with increased sedation time in the LORR test and decreased ethanol consumption in the 2-bottle choice protocol.

Conclusions—These data describe a novel and conserved role for *Lmo* genes in flies and mice in behavioral responses to ethanol. These studies also demonstrate the feasibility of rapidly translating findings from invertebrate systems to mammalian models of alcohol abuse by combining RNA interference in transgenic mice and behavioral testing.

Keywords

Ethanol Sedation; Alcohol Consumption; *Drosophila* Lim-Only; *Lmo3*; Transgenic RNA Interference

COMPLEX GENETIC AND environmental components contribute to the risk of development of alcoholuse disorders (AUDs). A variety of genes have been identified that contribute to a higher risk for AUDs (Gelernter and Kranzler, 2009). These include genes involved in ethanol metabolism, such as *ALDH2* and *ADH1B*, as well as several receptors and signaling molecules regulating neurotransmission, such as the $GABA_A$ receptor (Ducci and Goldman, 2008). However, a complete understanding of the genetic risk factors contributing to alcoholism remains elusive. We use the fruit fly *Drosophila melanogaster* as

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a model organism to identify novel genes that regulate behavioral responses to ethanol. There are a number of similarities between behavioral responses to ethanol in flies and mammals. For instance, flies exhibit increased locomotion in response to a low dose of ethanol, and exhibit motor incoordination and sedation in response to higher doses (Guarnieri and Heberlein, 2003). Recent studies from our laboratory have demonstrated the utility of using *Drosophila* to identify novel genes that modify behavioral responses to ethanol and to translate these findings to mammalian models of AUDs (Corl et al., 2009).

In an unbiased genetic screen for mutants with altered sensitivity to cocaine, we identified mutations in the *Drosophila* LIM-only (*dLmo*, also known as *Beadex*, *Bx*) gene, which encodes a transcriptional regulator consisting of 2 tandem LIM domains (Kadrmas and Beckerle, 2004). Reduced *dLmo* function increases behavioral responsiveness to cocaine, whereas increasing *dLmo* levels has the opposite effect (Tsai et al., 2004). Herein, we report that *dLmo* also modulates sedation in response to ethanol in a bidirectional manner. To translate these findings to a mammalian system, we used RNA interference (RNAi) to reduce expression of 1 of the mammalian homologs of *dLmo*, *Lmo3*. We employed a technique to rapidly generate transgenic mice globally expressing short-hairpin RNAs (shRNAs) targeting *Lmo3* by infecting single-cell embryos with lentivirus (Lois et al., 2002; Rubinson et al., 2003; Tiscornia et al., 2003). This method produced founder mice with varying degrees of *Lmo3* knockdown. When tested for behavioral responses to ethanol, transgenic mice exhibited altered ethanol sedation and consumption that correlated with *Lmo3* expression levels. These results suggest a role for *Lmo3* in ethanol-related behaviors in mice, and demonstrate the feasibility of rapidly validating novel genes, identified in invertebrate systems, in behavioral responses to ethanol in mammals.

MATERIALS AND METHODS

Drosophila Culture and Strains

All flies were maintained on standard cornmeal and molasses agar food at 25°C and 70% humidity. *EP1306* was originally isolated from a collection of X-linked EP lines (Rorth, 1996), and showed reduced *dLmo* expression and increased cocaine sensitivity (Tsai et al., 2004). Bx^J is a well-characterized overexpression allele of *dLmo*/*Bx* (Shoresh et al., 1998), and showed reduced behavioral responses to cocaine (Tsai et al., 2004). Both lines were out-crossed for at least 5 generations to a w^{1118} stock isogenic for Chromosomes II and III; these "*w*; iso" flies served as the genetic background control for experiments.

Drosophila Behavioral Assays and Ethanol Absorption

Ethanol sedation (loss of righting, LOR) assays were performed essentially as previously described (Corl et al., 2009; Rothenfluh et al., 2006). Samples of 25 to 30 male flies were allowed to equilibrate for approximately 10 minutes to humidified air in the booz-o-mat apparatus (Wolf et al., 2002) before starting exposure to a 100:50 mixture of ethanol vapor:humidified air (100:50 E:A). Ethanol exposure commenced at 0 minutes of the assay and was continuous thereafter. At 2-minute intervals, each tube of flies (8 tubes per assay) was twirled and the number of flies that appeared unable to right themselves was scored by an experimenter blinded to sample identity. Typical assay duration was 30 minutes. To measure ethanol absorption, samples of 25 flies were exposed to 100:50 E:A for 15 minutes and were immediately snap-frozen on dry ice and processed to determine internal ethanol concentration using a kit (Genzyme Diagnostics, Framingham, MA).

Animals

C57BL/6J mice were used for single cell embryo injections and subsequent behavioral testing. CD1 females were used for embryo implantation. Animals were group housed. Food

and water were provided at all times, and animals were on a 12-hour light-dark cycle. All animal protocols were approved by the Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee.

Design and Production of Lentivirus Expressing shRNAs

Three different 19-nucleotide small interfering RNAs were designed to target mouse *Lmo3* (NM_207222) using the Wistar siRNA design tool (Levenkova et al., 2004). The three 19-nucleotide targeting sequences are listed in the Supplemental Table S1. Target sequences, including the shScr control sequence, were incorporated into hairpins and cloned into the lentiviral vector pLL3.7 as described previously (Lasek et al., 2007). The shScr sequence encodes a nonspecific hairpin, predicted to not target any gene in the mouse genome. Lentivirus was produced from shScr and shLmo3.8 plasmids in 293FT cells using the ViraPower™ packaging mix (Invitrogen, Carlsbad, CA) as described previously (Lasek et al., 2007).

Testing shRNA Efficacy in Neuro-2a Cells

Neuro-2a cells (American Type Culture Collection, Manassas, VA) were grown in DMEM plus 10% fetal bovine serum and 5% CO_2. Cells were seeded into 12-well dishes, and transfected with pLL3.7 plasmids containing shLmo3 and shScr sequences using Lipofectamine™ 2000 and Opti-MEM media (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, RNA was isolated using the RNeasy® Mini Kit according to manufacturer's instructions (Qiagen, Valencia, CA). Total RNA was treated with RNase-free DNase (Promega, Madison, WI) to remove genomic DNA contamination.

Quantitative PCR

cDNA synthesis and TaqMan® quantitative PCR (qPCR) were performed as described in Lasek and colleagues (2007). The sequences of the mouse *Lmo1, Lmo2,* and *Lmo3* primers are listed in the Supplemental Table S1. Predesigned primers to *Lmo4* (Assay ID Mm00495373) and mouse *Gapdh* (product number 4352932E) were purchased from Applied Biosystems (Foster City, CA).

Generation of Transgenic Mice Expressing shRNAs

C57BL/6J mice were superovulated at 3 to 4 weeks of age, and embryos were collected. High-titer lentivirus ($>10^7$ pg p24 antigen/ml) in a volume of 10 to 100 pl was injected into the perivitelline space of single-cell embryos using a pulled glass pipette, as described in Lois and colleagues (2002) and implanted into pseudopregnant CD1 females. Of 141 injected embryos, we obtained 63 pups, 39 derived from shLmo3.8 injections and 24 derived from shScr injections. To confirm genomic insertion of the lentivirus, we isolated DNA from tail clips using standard methods. PCR amplification was performed with primers against the pLL3.7 vector and green fluorescent protein (GFP) encoded by the lentiviral vector. Primer sequences are listed in the Supplemental Table S1. Of the 63 pups analyzed, 13 (6 females, 7 males) of 39 (33%) shLmo3.8-injected animals and 9 (5 females, 4 males) of 24 (37.5%) shScr-injected animals were positive for virus by genotyping. Twelve shLmo3.8 and 8 shScr animals were used for behavioral testing.

Processing of Transgenic Mouse Brains and GFP Fluorescence Imaging

After the completion of behavioral tests on transgenic mice, animals were euthanized with CO_2 and decapitated. Brains were rapidly removed and cut in half through the midline. Half of the brain was placed in 4% paraformaldehyde for 24 hours to post-fix for GFP imaging and then stored in phosphate buffer. The other half of the brain was dissected into forebrain,

midbrain, and cerebellum sections, and snap-frozen in liquid nitrogen for subsequent RNA isolation using Trizol® (Invitrogen) and qPCR. To image GFP fluorescence, 40-μm sagittal brain sections were cut using a vibrating blade microtome (Leica Microsystems, Bannockburn, IL) and mounted on slides. Images were acquired using a Zeiss confocal microscope, and visualized using Zeiss LSM software (Carl Zeiss MicroImaging, LLC, Thornwood, NY).

Ethanol Loss-of-Righting-Reflex and 2-Bottle Choice Consumption Assays

Mice aged 9 to 16 weeks were tested first for ethanol-induced loss-of-righting-reflex (LORR) at 3 doses of ethanol (3.2, 3.6, and 4.0 g/kg) in a random-order balanced design with a 1 week interval between each dose. The same mice were used for the 2-bottle-choice ethanol consumption experiment, and were allowed to rest for 2 weeks after the final LORR test. LORR and 2-bottle choice ethanol consumption experiments were performed as described in Kapfhamer and colleagues (2008). Both males and females were tested; we did not detect a significant difference in *Lmo3* expression between males and females, so data from males and females were combined in the analysis.

RESULTS

Mutations in Drosophila dLmo *Affect Ethanol Sedation*

To investigate whether *dLmo* might play a role in ethanolinduced sedation, the LOR assay was used to test control flies and 2 *dLmo* mutants: Bx^J, with increased levels of *dLmo*, and *EP1306*, with reduced *dLmo* (Tsai et al., 2004). When exposed to ethanol, *EP1306* mutant flies sedated more quickly, showing a significantly reduced ST50 (time to 50% sedation) of 12.1 minutes compared with 17.1 minutes for the *w;iso* control flies. Bx^J flies, by contrast, exhibited increased resistance to sedation, with an ST50 of 19.8 minutes (Fig. 1*A*,*B*). Ethanol absorption was not altered in the mutants (Fig. 1*C*). Thus, *dLmo* negatively regulates the flies' sensitivity to ethanol-induced sedation, and this regulation is bidirectional.

shRNAs Reduce Lmo3 Expression in Cell Culture

To begin the investigation of whether the mammalian *Lmo* genes are involved in behavioral responses to ethanol, we targeted *Lmo3* in the mouse using RNAi. *Lmo3* is 1 of 4 mammalian homologs of *dLmo*, and is expressed at high levels in brain regions important for behavioral responses to ethanol, particularly in the forebrain (Bulchand et al., 2003; Hinks et al., 1997). We designed 3 independent shRNAs specific for *Lmo3* (Fig. 2*A*). To test their ability to reduce *Lmo3* expression, lentiviral plasmids encoding each *Lmo3* shRNA (shLmo3) were transfected into mouse Neuro-2a cells in parallel to the control shScr plasmid, which encodes a scrambled shRNA sequence that does not target any mouse gene (Lasek et al., 2007). Two days after transfection, RNA was isolated and subjected to first-strand cDNA synthesis and real-time qPCR. We found that *Lmo3* levels were significantly reduced by 30 to 43% in cells transfected with each of the shLmo3 plasmids compared with the shScr control (Fig. 2*B*). Transfection of shLmo3 plasmids had no detectable effect on the expression of the closely related *Lmo* family members *Lmo4* (Fig. 2*C*), *Lmo1* (Fig. 2*D*), and *Lmo2* (data not shown). These results indicate that these shRNAs specifically attenuated expression of *Lmo3* without affecting expression of the other *Lmo* genes.

Transgenic Mice Display a Range of Lmo3 Expression in the Brain

To knock down *Lmo3* expression in mice, we injected single- cell embryos with lentivirus expressing either shLmo3.8 or shScr. We reasoned that infection of individual mice at the embryo stage with shLmo3 lentivirus would yield animals with a range of *Lmo3* expression

due to variation in the number of viruses integrated into the genome of each founder (Lois et al., 2002) as well as potential effects of the integration site, generating an allelic series for behavioral testing. After behavioral testing (see below), the brains of founder animals were analyzed for expression of GFP, which is encoded by the integrated virus. We found that GFP was widely expressed in the brains of animals containing either integrated shScr or shLmo3.8 lentivirus. GFP fluorescence was visible as bright spots in cell bodies and processes of neurons in many brain regions, including cells in the cortex, nucleus accumbens, and cerebellum (Fig. 3*A*–*F*). No gross differences were observed in the levels and patterns of GFP fluorescence between groups of animals that had been injected with shScr or shLmo3.8 lentivirus. However, the levels of GFP fluorescence varied among individual animals within each group. To quantify the amount of GFP and *Lmo3* transcript in individual animals, we performed qPCR using RNA isolated from the forebrain of each founder animal (Fig. 3*G*). Within the shScr group, GFP expression (normalized to *Gapdh* expression as a control for input RNA) ranged from 0.08 to 1.92; comparable levels of GFP expression were observed in the shLmo3.8 group (range: 0.03 to 1.64). Infection with shScr did not affect expression of *Lmo3*, as *Lmo3* expression varied from 0.74 to 0.85 in these animals, similar to the levels of *Lmo3* observed in animals that did not contain integrated virus (data not shown). In contrast, expression of *Lmo3* ranged from 0.41 to 0.86 in shLmo3.8-infected animals, indicating that shLmo3.8 was, at least in some animals, effective in reducing levels of *Lmo3* in vivo. Importantly, GFP expression was inversely correlated with *Lmo3* expression in shLmo3.8- infected mice (Fig. 3*G*, $R = -0.628$, $p = 0.029$), indicating that higher levels of integrated shLmo3.8 virus correlated with decreased *Lmo3* expression in the brains of these mice.

Lmo3 Expression Correlates with Levels of Behavioral Responses to Ethanol

To determine if *Lmo3* is involved in behavioral responses to ethanol, we tested transgenic founder mice expressing shScr or shLmo3.8 for their response to the acute sedative / hypnotic effect of ethanol in the LORR test. We observed a significant negative correlation between expression levels of *Lmo3* and sedation time at a dose of 3.2 g/kg ethanol (Fig. 4*A*, $R = -0.663$, $p = 0.019$), suggesting that reduced expression of *Lmo3* leads to increased sensitivity to the sedating effect of this dose of ethanol. Sedation time at higher doses of ethanol (3.6 and 4 g/kg) was not significantly correlated with *Lmo3* expression (data not shown). The same animals were tested 2 weeks after the completion of the LORR test for voluntary ethanol consumption using a 2-bottle choice protocol. Significant positive correlations were observed between *Lmo3* expression and ethanol consumption at 6% (Fig. 4*B*, $R = 0.719$, $p = 0.008$), 10% (Fig. 4*C*, $R = 0.691$, $p = 0.013$), and 14% ethanol ($R = 0.591$, $p = 0.043$, data not shown). Although not significant, we observed a trend correlating *Lmo3* expression with consumption at 2 additional ethanol concentrations, 3% ($R = 0.540$, $p = 0.07$) and 20% ($R = 0.527$, $p = 0.078$). To determine if *Lmo3* levels were specifically correlated with ethanol consumption or were more generally associated with drinking behavior, we examined the correlation between *Lmo3* expression and water consumption, and observed no significant relationship (Fig. 4*D*, $R = 0.353$, $p = 0.261$). Together, these results indicate that lower levels of *Lmo3* are associated with decreased voluntary ethanol consumption in mice.

DISCUSSION

The work presented here demonstrates that the *Drosophila Lmo* gene, *dLmo*, and its mouse homolog *Lmo3* regulate behavioral responses to alcohol. We found that responses in the fly broadly paralleled to those in the mouse. Reduced *dLmo* expression (observed in the *EP1306* mutant) caused increased sensitivity to the sedating effect of ethanol. Similarly, in the mouse, reduced *Lmo3* expression correlated with increased sedation time in the LORR

assay. In addition to its effect on ethanol sedation, reducing *Lmo3* levels correlated with decreased ethanol consumption, suggesting that *Lmo3* may play a role in alcohol preference in mammals.

Lmo3 is 1 of 4 *Lmo* genes in the mouse. We have studied another member of this family, *Lmo4*, in behavioral responses to cocaine and ethanol. We found that mice with a 50% reduction in *Lmo4* levels exhibit enhanced locomotor sensitization to cocaine (Lasek et al., 2010). However, these mice did not show any differences in several behavioral assays related to alcohol, including locomotor stimulation, LORR, and 2-bottle choice ethanol consumption (David Kapfhamer, personal communication). Interestingly, however, *Drosophila dLmo*, which is the only *Lmo* gene in flies, regulates behavioral responses to both cocaine and ethanol. These findings suggest that inmammals, the role of the *Lmo* genes in regulating responses to abused drugs has diverged such that different homologs function in pathways that are drug-specific.

How might the *Lmo* genes regulate behavioral responses to ethanol? *dLmo* and *Lmo3* are expressed in several brain regions in flies and in mice. Gross abnormalities in the brain have not been observed in either *dLmo* mutant flies, the shLmo3 transgenic mice described here, or *Lmo3* knockout mice, which are viable, fertile, and exhibit normal organ development (Tsai et al., 2004; Tse et al., 2004). dLMO and LMO3 are members of the LIM-only family of proteins, whose function is to modulate transcription by binding to DNA-binding proteins through their 2 tandem LIM domains (Kadrmas and Beckerle, 2004). In vertebrates, LMO proteins are thought to play important roles in specifying cell fate, particularly in the developing nervous system (Bulchand et al., 2003; Joshi et al., 2009; Lee et al., 2008; Remedios et al., 2004). One possibility is that LMO3 affects the patterning of specific brain structures during development, such as the cortex or amygdala (Bulchand et al., 2003; Remedios et al., 2004), through its ability to regulate transcription. Subtle changes in patterning during development might later affect behavioral responses to ethanol in adults. Little is known regarding the molecular mechanism of LMO3 function or its transcriptional targets, although binding partners of LMO3 include the neuronal transcription factor HEN2 (Aoyama et al., 2005), p53 (Larsen et al., 2010), and the calcium and integrin binding protein, CIB (Hui et al., 2009). In the adult animal, *Lmo3* expression is increased by seizure activity in the hippocampus (Hinks et al., 1997). *Lmo3* expression is also increased by dopamine treatment of glial cell cultures (Shi et al., 2001). The mechanism of LMO3 function in behavioral responses to ethanol has not been studied. It is tempting to speculate that *Lmo3* expression/and or function is regulated by ethanol in the adult brain, and that *Lmo3* in turn modulates behavioral responses to ethanol in the adult animal. As an attempt to address this possibility, we examined *Lmo3* expression in response to ethanol in the cortex, striatum, hippocampus, cerebellum, and olfactory bulb by qPCR, and observed no significant changes following single or repeated ethanol injections (Amy W. Lasek, Ulrike Heberlein, and David Kapfhamer, unpublished results), suggesting that *Lmo3* expression is not regulated by ethanol, at least under these conditions. Future studies will examine the function of LMO3 in ethanol-related behaviors in more detail.

Invertebrate model systems, such as *Drosophila* and *Caenorhabditis elegans*, provide an opportunity to identify novel genes that modulate acute responses to alcohol (Wolf and Heberlein, 2003). However, validation of these genes in mammalian systems, in which more complex aspects of alcoholrelated behaviors can be modeled, is necessary to provide relevance to human AUDs. Herein, we have used a technique to rapidly generate transgenic gene knockdown animals for behavioral testing. In theory, founder animals can be bred to maintain gene knockdown (Lois et al., 2002), although we found by breeding 1 of our founders that GFP fluorescence was maintained only through the first generation and appeared to be silenced in the second and third generations (data not shown). This may be

due to silencing at the lentiviral integration site, perhaps due to toxicity from high shRNA expression (Cao et al., 2005; Grimm et al., 2006).

Traditional methods for testing gene function in mice have relied on the use of constitutive and conditional gene knockouts and classical transgenic animals. Producing these mice is expensive and time-consuming, and may not yield an easily interpretable behavioral phenotype due to potential developmental compensations from the complete loss of gene function. In addition to the relative ease and speed of their generation, mice with gene knockdown by shRNA may be advantageous, as gene function is not completely eliminated, thus circumventing potential lethality associated with null alleles as well as the above-mentioned compensatory mechanisms. By utilizing transgenic mice expressing lentiviral-delivered shRNAs, animals with different degrees of gene knockdown can be produced rapidly as a screening tool to complement the generation of traditional gene knockout animals. As we have done here, founder mice may be examined for correlations in behavioral responses to ethanol as a first determinant of whether a gene may play a role in alcoholrelated behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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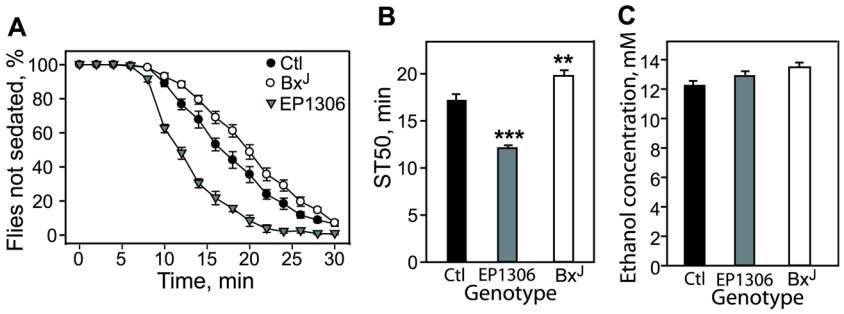

Fig. 1.

dLmo/Bx mutants display altered resistance to ethanol sedation and no change in ethanol absorption. (**A**) Ethanol sedation curves indicating that EP1306 flies show increased sensitivity and Bx^J flies show decreased sensitivity to ethanol sedation in the loss of righting (LOR) assay when compared with control (*Ctl*) flies. *Ctl* flies were in the w;iso genetic background. (**B**) The median sedation time (ST50)—the time required for half of the ethanol-exposed flies to show LOR—was calculated by linear interpolation. Error bars represent SEM, and asterisks denote statistical significance by one-way ANOVA followed by post hoc Holm-Sidak testing (**$p < 0.01$; ***$p < 0.001$; $n = 9$ to 12, where n is the number of samples, not the number of flies). (**C**) Samples of mutant and control flies were exposed to ethanol vapor under identical conditions as for behavioral assays, and were snap-frozen after 15 minutes and processed for internal ethanol absorption. No significant difference was seen between mutant and control flies ($n = 10$ or 11).

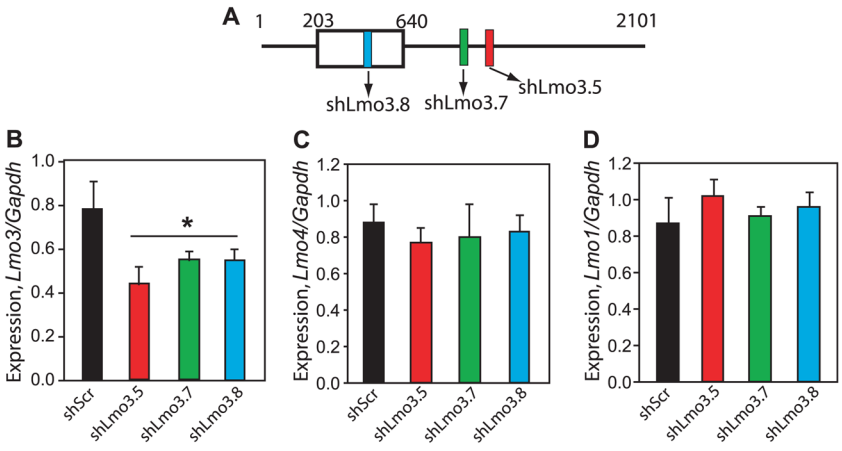

Fig. 2.

Efficacy of short-hairpin RNAs (shRNAs) targeting *Lmo3* for RNA interference in Neuro-2a cells. (**A**) Schematic illustrating the position of shRNAs targeting the *Lmo3* transcript. Colored boxes show shRNA location, open box illustrates the protein coding region, and numbers indicate the nucleotide position along the *Lmo3* mRNA. (**B**) Expression of *Lmo3* in Neuro-2a cells transfected with lentiviral plasmids expressing shRNAs targeting *Lmo3* (shLmo3.5, shLmo3.7, and shLmo3.8) or the control shScr. RNA was isolated 2 days after transfection and subjected to qPCR. *Lmo3* expression is normalized to expression of the housekeeping gene, *Gapdh*. Asterisk indicates a significant difference between the shLmo3 constructs and shScr by one-way ANOVA (**p* = 0.007; *n* = 3). (**C, D**) *Lmo4* (**C**) and *Lmo1* (**D**) expression in transfected Neuro-2a samples described in (**B**).

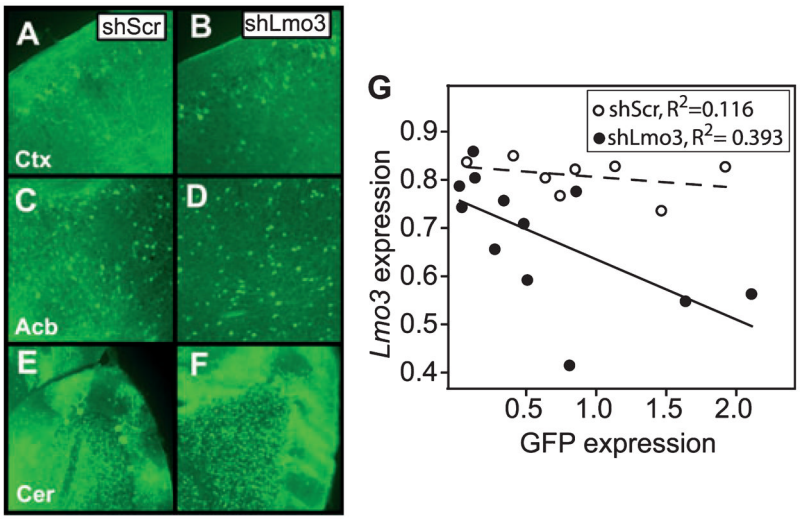

Fig. 3.

Characterization of transgenic mice expressing shLmo3.8 or shScr. (**A–F**) Green fluorescent protein (GFP) fluorescence in representative sagittal adult brain sections of transgenic mice infected at the single-cell embryo stage with lentivirus encoding shLmo3.8 (**B, D, F**) or shScr (**A, C, E**). GFP expression from the viral vector is visible in cell bodies (bright spots) and processes of neurons throughout the brain, including the cortex (**A, B**, Ctx), nucleus accumbens (**C, D**, Acb), and cerebellum (**E, F**, Cer). Hazy green areas represent background autofluorescence from the tissue, rather than infection per se. (**G**) Expression of GFP and *Lmo3* transcript levels were negatively correlated in the forebrains of transgenic shLmo3.8 (closed circles, $n = 12$), but not shScr (open circles, $n = 8$) mice. GFP (*x*-axis) and *Lmo3* (*y*-axis) expression values were normalized relative to expression of the housekeeping gene *Gapdh*.

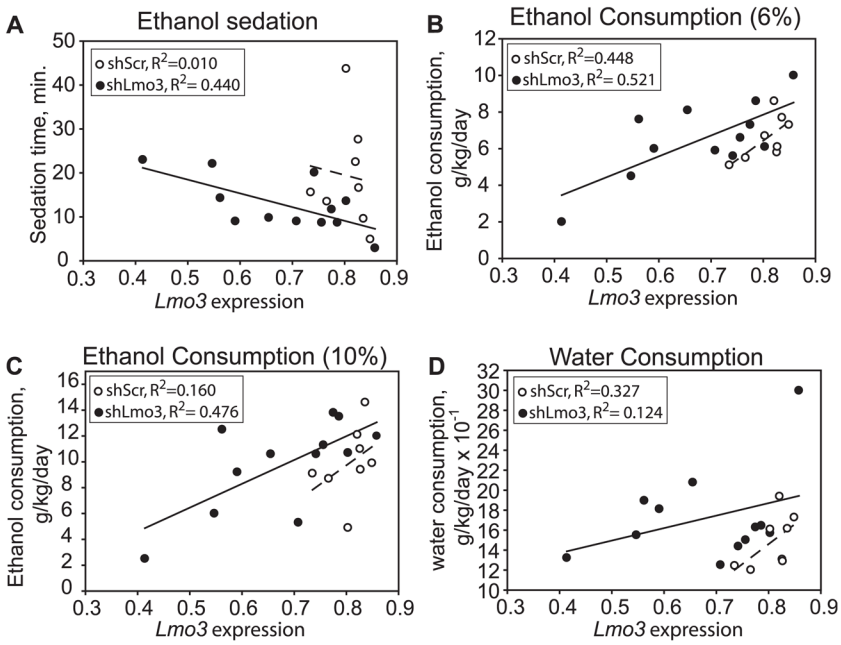

Fig. 4.

Correlations between *Lmo3* expression and behavioral responses to ethanol in transgenic shLmo3.8 and shScr mice. In all graphs, normalized *Lmo3* expression is plotted on the *x*-axis and the behavioral response on the *y*-axis. Mice expressing shLmo3.8 (n = 12) are represented by closed circles and shScr (n = 8) by open circles. (**A**) Negative correlation between *Lmo3* expression and ethanol sedation in the loss-of-righting-reflex test with a 3.2 g/kg dose of ethanol. (**B, C**) Positive correlation between *Lmo3* expression and 2-bottle choice ethanol consumption of 6% (**B**) and 10% (**C**) ethanol. (**D**) Water consumption and *Lmo3* expression were not significantly correlated.