

A Selective Role for *Lmo4* in Cue–Reward Learning

Rajani Maiya,^{1,2} Regina A. Mangieri,¹ Richard A. Morrisett,¹ Ulrike Heberlein,² and Robert O. Messing^{1,2}

¹Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712, and ²Ernest Gallo Clinic and Research Center, Emeryville, California 94608

The ability to use environmental cues to predict rewarding events is essential to survival. The basolateral amygdala (BLA) plays a central role in such forms of associative learning. Aberrant cue–reward learning is thought to underlie many psychopathologies, including addiction, so understanding the underlying molecular mechanisms can inform strategies for intervention. The transcriptional regulator LIM-only 4 (LMO4) is highly expressed in pyramidal neurons of the BLA, where it plays an important role in fear learning. Because the BLA also contributes to cue–reward learning, we investigated the role of BLA LMO4 in this process using *Lmo4*-deficient mice and RNA interference. *Lmo4*-deficient mice showed a selective deficit in conditioned reinforcement. Knockdown of LMO4 in the BLA, but not in the nucleus accumbens, recapitulated this deficit in wild-type mice. Molecular and electrophysiological studies identified a deficit in dopamine D2 receptor signaling in the BLA of *Lmo4*-deficient mice. These results reveal a novel, LMO4-dependent transcriptional program within the BLA that is essential to cue–reward learning.

Key words: basolateral amygdala; conditioned reinforcement; motivation; reward; transcription

Introduction

The formation of conditioned associations between environmental cues and appetitive and aversive events is important for survival. The BLA plays a central role in such forms of associative learning and is important for the processing of emotions of both positive and negative valence (Everitt et al., 1999; Holland and Gallagher, 1999; Baxter and Murray, 2002). Studies of fear conditioning, in which animals learn to associate a previously neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US), have provided strong evidence for the role of the BLA in emotional learning (LeDoux, 2000). However, the BLA is also implicated in the processing of positive emotions. When a CS is paired with an appetitive US, it acquires motivational value. This acquired value of the CS is displayed in several ways. Cues paired with a reward can elicit approach when presented alone, increase responding to the primary reinforcer, and also serve as conditioned reinforcers supporting the learning of novel instrumental behaviors (Everitt et al., 1999; Holland and Gallagher, 1999; Baxter and Murray, 2002). These properties of reward-paired cues arise from Pavlovian conditioning and can be dissociated in terms of their neuroanatomical correlates. Rats with BLA lesions display deficits in conditioned reinforcement and responding

under second-order schedules of reinforcement, suggesting a critical role for the BLA in attributing motivational properties to reward-paired cues (Cador et al., 1989; Hatfield et al., 1996; Everitt et al., 1999; Baxter and Murray, 2002; Di Ciano and Everitt, 2004). Although much is known about neural structures responsible for imbuing reward-paired cues with incentive properties, the underlying molecular mechanisms remain largely unexplored.

Recent evidence implicates LIM-only (LMO) 4 as an important mediator of BLA-regulated behaviors. Mammalian genomes encode 4 LMO proteins, LMO1–4. LMO proteins interact via their LIM domains with transcription cofactors and regulatory DNA-binding proteins and regulate gene expression either by nucleating the formation of new transcriptional complexes or by disrupting existing complexes through competition with transcriptional coactivators (Heberlein et al., 2009; Matthews et al., 2013). Therefore, depending on the complement of transcriptional coregulators and transcription factors with which they interact, LMO proteins can either activate or repress gene expression. LMO proteins can also affect neuronal function independently of effects on transcription. For example, reducing LMO4 in the hypothalamus and the amygdala increases protein tyrosine phosphatase 1B activity (PTP1B) via increased oxidation of this phosphatase. This regulation of PTP1B is important for hypothalamic leptin and amygdalar endocannabinoid signaling (Qin et al., 2012; Pandey et al., 2013).

Among the 4 mammalian *Lmo* genes, *Lmo4* is highly expressed in brain structures such as the BLA, orbitofrontal cortex (OFC), and NAc that are involved in regulating motivated behaviors (Heberlein et al., 2009; Lasek et al., 2010). Previously, we found that *Lmo4* is expressed predominantly in pyramidal neurons of the BLA (Maiya et al., 2012). Mice with a genetrap (gt) insertion at the *Lmo4* locus (*Lmo4gt/+* mice) express 50% less *Lmo4* than their wild-type (WT) counterparts and display enhanced fear

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Correspondence should be addressed to Rajani Maiya, PhD, College of Pharmacy, C0875, 107 W. Dean Keeton, BME6.116, University of Texas, Austin, TX 78712. E-mail: Rajani.Maiya@austin.utexas.edu.

U. Heberlein's present address: HHMI, Janelia Research Campus, 9700 Helix Drive, Ashburn, VA 20147.

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learning. BLA-specific knockdown of *Lmo4* recapitulates this phenotype. These findings suggest that LMO4 functions in the BLA to negatively regulate fear learning.

Because the BLA is also involved in the processing of positive emotions, we investigated the role of LMO4 in cue–reward learning and the ability of reward-paired cues to influence goal-directed behavior. Our results reveal that global or BLA-specific reduction in LMO4 leads to a selective deficit in conditioned reinforcement, enhanced neuronal excitability, and reduced dopamine D2 receptor (D2R) expression and D2R-mediated signaling in the BLA.

Materials and Methods

Animals. The generation and characterization of mice with a *gt* insertion at the *Lmo4* locus have been described previously (Heberlein et al., 2009; Lasek et al., 2010). We used 8- to 12-week-old, male, heterozygous *Lmo4gt* (*Lmo4gt/+*) mice and their WT littermates on a C57B6/J background for behavioral and biochemical experiments, 5- to 8-week-old male and female *Lmo4gt/+* mice and their WT littermates for electrophysiological studies, and 8- to 12-week-old C57BL/6J mice for shRNA experiments. Mice were group housed until surgery, after which they were housed individually. For behavioral experiments and tissue collection for molecular and biochemical studies, mice were housed under a 12 h light/dark cycle with lights on at 7:00 AM and were euthanized for experiments between 9:00 AM and 2:00 PM. For electrophysiological studies, mice were housed under a 12 h light dark cycle with lights on at 12:00 AM. Mice were habituated to this cycle for a minimum of 14 d. Mice were euthanized for electrophysiological experiments between 10:00 AM and 4:00 PM; recordings were performed between 12:00 PM and 1:00 AM.

Lentiviral shRNA constructs and stereotaxic surgery. The design and cloning of small hairpin RNAs against *Lmo4* (designated as sh*Lmo4*) and control shRNA (designated as shCon) that does not target any gene in the mouse genome have been described previously (Lasek and Azouaou, 2010; Lasek et al., 2010; Maiya et al., 2012). Male C57BL/6J mice were infused bilaterally with 1 μ l of lentivirus (10^7 – 10^8 pg/ μ l) using a Hamilton syringe, as described previously (Lasek and Azouaou, 2010; Lasek et al., 2010; Maiya et al., 2012). The coordinates for BLA were A/P = -1.6 , M/L = ± 3.1 , D/V = -4.8 . The coordinates for the NAc were A/P = $+1.43$, M/L = ± 0.9 , and D/V = -4.55 . After injection, mice were allowed to recover for 2 weeks before behavioral testing. For investigating the role of the BLA in cue–reward learning, a total of 40 animals were injected (20 mice/shRNA). Ten animals were not included in the study due to off target or unilateral placements. For NAc knockdown experiments, a total of 28 animals were injected (14/group), of which six were excluded due to off-target or unilateral placements.

Quantitative PCR. WT and *Lmo4gt/+* mice were killed and brains were rapidly frozen in isopentane and sectioned at 300 μ m thickness using a cryostat. Sections were frozen on dry ice and the BLA was punched from frozen sections using a 0.75-mm-diameter stainless steel micropunch (Stoelting). RNA was extracted using the RNeasy kit from Qiagen according to the manufacturer's instructions. Complementary DNA (cDNA) was generated from 1 μ g of mRNA using the High Capacity cDNA Reverse Transcription Kit from Life Technologies. Gene expression was analyzed by quantitative RT-PCR using an Applied Biosystems Viia7 thermal cycler with TaqMan probes (Life Technologies) that detects *Drd1* (Mm0260146_s1), *Drd2* (Mm00438545_m1), and *Drd3* (Mm00432887_m1). Expression levels were normalized to mouse *Gapdh* (Mm99999915_g1). Amplification reactions contained 5 μ l of cDNA template, 1 \times Universal PCR Master Mix, 100 nM each of forward and reverse primers, and 200 nM FAM- or VIC-labeled probe in a final volume of 10 μ l. Relative expression levels were quantified by normalizing target amplicon Ct values to *Gapdh*.

Cue–reward learning. Cue–reward learning, conditioned reinforcement, and instrumental conditioning were tested as described previously (Mead and Stephens, 2003). Mice were food restricted to 85–90% of their body weight and were then trained to associate the presentation of discrete cues with a sucrose reinforcer in operant chambers (Med Associ-

ates). Cues consisted of a 10 s presentation of lights with white noise and were delivered on a variable interval schedule with a mean interval of 120 s. Reinforcer (0.01 ml of a 15% sucrose solution) was delivered 5 s after cue presentation. Head entry rates into the reward port during the presentation of the cue and during the intertrial interval (ITI) were recorded. Training consisted of 11 sessions (1 session/d) lasting 2 h each. Mice were then tested for goal tracking (1 h session) by presenting them with cues previously paired with the reinforcer once every 2 min. The reinforcer was not delivered during this session. Head entries into the reward port were monitored during the presentation of the cue and during the ITI. Immediately after the goal tracking session, mice were subjected to another conditioning session, as described above. Conditioned reinforcement was tested the day after goal tracking. Mice were placed in operant chambers and presented two nosepoke ports. Responses on the active port resulted in presentation of the cue previously paired with the reward. Responses on the inactive port had no consequence. The assignment of active and inactive ports was counterbalanced across genotypes and shRNAs. The number of active and inactive nosepokes was monitored in a 1 h session.

Instrumental conditioning. The day after testing for conditioned reinforcement, mice underwent instrumental conditioning. On day 1 of training, mice were placed in operant chambers with one active lever. Responses on the active lever resulted in the delivery of 0.01 ml of a 15% sucrose reinforcer on a fixed ratio 1 (FR1) schedule, by which one active lever response results in one delivery of the reinforcer. The number of lever presses made in a 3 h session was recorded. On days 2–6, a second, inactive lever was introduced into the chamber. The assignment of active and inactive levers was counterbalanced across genotypes and shRNAs. As before, responses on the active lever resulted in reinforcer delivery on an FR1 schedule, whereas responses on the inactive lever had no consequence. The percentage of active lever presses in a 2 h session was monitored every day from days 2–6.

Experimentally naive mice were trained to nosepoke on an FR1 schedule for a sucrose reinforcer. Mice were subjected to 1 h magazine training sessions in operant chambers (Med Associates) for 2 d, during which sucrose reinforcer (10 μ l) was delivered on a random interval schedule with a mean interval of 120 s. Mice were subsequently trained to nosepoke on an FR1 schedule to obtain a sucrose reinforcer. Each active nosepoke resulted in the 10 s presentation of a tone and light cue that coterminated with the delivery of sucrose. Inactive nosepokes had no programmed consequence. The assignment of active and inactive nosepokes was counterbalanced within and across genotypes. Mice were subjected to an overnight conditioning session on day 1 and 2 h conditioning sessions on days 2–8. Active and inactive nosepokes were recorded through all sessions.

Two-bottle choice sucrose consumption. Naive mice were individually housed for 1 week and given 24 h access to 2 bottles of water. Mice were then presented with one bottle containing sucrose solution and another containing water. The concentration of sucrose was escalated every 2–3 d in the following order: 2%, 6%, and 15%. The amount of sucrose and water consumed was measured every day and the positions of the bottles were alternated every day to control for side preferences. Drinking volumes were corrected for spillage by subtracting the amount of liquid lost from water and sucrose bottle placed on empty cages. The amount of sucrose consumed was normalized to body weight. Preference was expressed as the ratio of the amount of sucrose solution consumed to the total amount of fluid consumed.

Immunohistochemistry. To identify the extent of lentiviral infection, mice were anesthetized with Euthasol and perfused transcardially with 0.9% saline to remove blood and then with 4.0% paraformaldehyde (PFA) in PBS for 5 min. Brains were removed, fixed in 4% PFA overnight at 4°C, and transferred to 30% sucrose solution at 4°C, where they remained for 1–2 d until they were sectioned. Brains were mounted using Tissue-Tek OCT (Ted Pella) and 50 μ m free-floating sections were cut using a cryostat. The sections were then pretreated with 3% H₂O₂ for 10 min, followed by 50% ethanol twice for 10 min each. Sections were blocked with 10% normal donkey serum (NDS; Jackson ImmunoResearch) for 30 min and incubated with mouse anti-GFP monoclonal antibody (3E10; Life Technologies) diluted 1:1500 in PBS with 0.1%

Triton X-100 for 48 h. Sections were washed for 5 min 3 times with PBS and then incubated with 2% NDS for 10 min. Biotin-conjugated donkey anti-mouse secondary antibody (diluted 1:250; Jackson ImmunoResearch) was incubated with sections for 2–3 h, followed by ExtrAvidin-Peroxidase (1:2500; Sigma-Aldrich) for 1–2 h. Diaminobenzidine was used for brown color detection of the GFP immunoreactivity. Sections were washed with PBS, mounted on gelatin-coated slides, and dried. Slides were counterstained with cresyl violet.

Electrophysiology. Mice were lightly anesthetized with isoflurane and their brains were rapidly removed and placed in ice-cold (4°C) oxygenated high-sucrose cutting solution containing the following (in mM): 210 sucrose, 26.2 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 6 MgCl₂, 2.5 CaCl₂, and 11 dextrose, bubbled with 95% O₂/5% CO₂. Coronal slices (250 μm) containing the BLA were sectioned in ice-cold, high-sucrose cutting solution using a Leica VT1000S vibrating microtome and then placed into artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 26 NaHCO₃, 1 NaH₂PO₄, 4.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 dextrose, and 0.075 Na-metabisulfite bubbled with 95% O₂/5% CO₂, pH 7.4. Slices were maintained in ACSF at room temperature for at least 1 h before recording. Recordings were conducted at 30–31°C in ACSF bubbled with 95% O₂/5% CO₂ and pumped into the recording chamber at ~2.0 ml/min. Dopamine-containing ACSF (10 μM) was made fresh daily by dissolving 9.5 mg of dopamine in 250 ml of ACSF. Recording electrodes (4-inch thin-wall glass, 1.5 outer diameter/1.12 inner diameter; World Precision Instruments) were made using a Brown-Flaming model P-88 electrode puller (Sutter Instruments) to yield resistances between 3 and 6 MΩ and contained the following (in mM): 120 KMeSO₄ (ACROS Organics) 10 NaCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 14 phosphocreatine, pH 7.3 with KOH. Unless otherwise noted, chemicals were obtained from Sigma-Aldrich or Fisher Scientific.

Cells were visually identified based on their morphology using the MRK200 Modular Imaging system from Siskiyou mounted on a vibration isolation table. Recordings were acquired using a CV203BU headstage and Axopatch 200B amplifier (Molecular Devices), filtered at 1 kHz, and digitized at 5 kHz via a Digidata 1440A interface board using Clampex 10.3 (Molecular Devices). Cells were included in analyses based on parameters measured immediately after obtaining whole-cell configuration (series resistance <25 MΩ and a resting membrane potential less than -65 mV) and firing patterns in response to depolarizing current injections that were consistent with features reported previously for BLA principal neurons (Kröner et al., 2005).

Data were analyzed using Clampfit 10.3 software (Molecular Devices). Input resistance was determined from the average of the voltage responses to small hyperpolarizing current pulses (-20 pA; 150 ms duration) delivered 150 ms before depolarizing current pulses. Excitability was measured by applying depolarizing intracellular current pulses (500 ms duration) of increasing amplitude (from 40 to 360 pA in 40 pA steps) once every 5 s to determine rheobase (minimum injected current that elicited an action potential) and the number of action potentials fired at each current amplitude. The action potential threshold was defined as the membrane potential at the initial point of rapid voltage deflection and action potential amplitude was measured from this voltage. Action potential half-width was the duration of the action potential at half-amplitude. To evaluate the effect of dopamine on excitability, we delivered the previously described series of depolarizing current injections once every 4 min for at least 12 min to establish a stable baseline. Dopamine was then washed on for ~4 min and recording continued for at least 25 min thereafter.

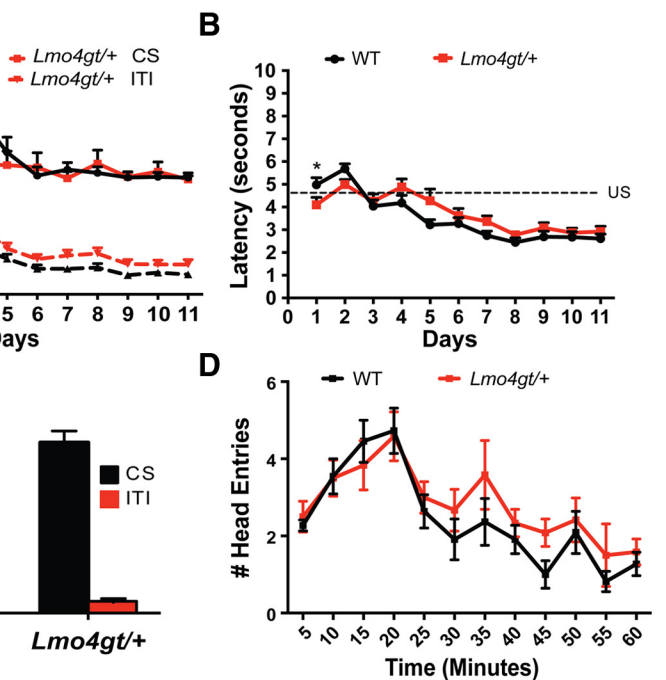


Figure 1. *Lmo4gt/+* and WT mice show similar cue–reward learning. **A**, WT and *Lmo4gt/+* mice increased head entry rates during presentation of the cue and decreased head entry rates during the intertrial interval ITI (* $p = 0.006$; # $p = 0.02$; ## $p = 0.01$ by Bonferroni test; $n = 15$ WT, $n = 18$ *Lmo4gt/+* mice). **B**, Latency for head entries into the reward port after cue onset decreased over time in both groups. * $p = 0.048$, Bonferroni test. **C**, During goal tracking, both genotypes increased head entries during presentation of the cue. **D**, Within-session extinction of goal tracking was similar in both genotypes.

mine was then washed on for ~4 min and recording continued for at least 25 min thereafter.

Western blotting. *Lmo4gt/+* mice and their WT littermates were administered 1 mg/kg quinpirole hydrochloride (Sigma-Aldrich) or saline intraperitoneally. Thirty minutes later, mice were killed by cervical decapitation, their brains were rapidly removed, and the BLA was dissected using a 1 mm biopsy punch. Tissue was flash frozen in liquid nitrogen and stored at -80°C until further processing. Tissue was homogenized in 1% SDS containing 2 μM okadaic acid using a glass pestle. The homogenate was boiled for 10 min and centrifuged at 10,621 × g for 5 min. Supernatant was collected and protein concentrations were measured using the bicinchonnic acid assay method (Life Technologies). Protein (40 μg) was resolved on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After transfer, the membrane was blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween (TBST) for 1 h, followed by overnight incubation with 1:1000 dilution of anti P-Thr³⁰⁸ AKT antibody (Cell Signaling Technology) in 5% BSA in TBST. Immune complexes were detected by appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch), followed by chemiluminiscent detection (Supersignal-West Pico; Life Technologies). Membranes were stripped and probed using an antibody against AKT (1:1000 in 5% milk in TBST; Cell Signaling Technology). Densitometric analyses was performed using ImageJ (Schneider et al., 2012). Phosphoprotein levels were normalized to total protein levels.

Statistical analyses. Electrophysiological data were analyzed using IBM SPSS Statistics 21. Other data were analyzed using GraphPad Prism 6.0. Statistical significance was defined as $p < 0.05$ for group comparisons using an unpaired, two-tailed t test or two-way ANOVA with a Bonferroni *post hoc* test, as appropriate. Summary data are presented as mean values ± SEM.

Results

To determine whether LMO4 is important for cue–reward learning, we first investigated the ability of WT and *Lmo4gt/+* mice to associate cues with the delivery of 15% sucrose as an appetitive reinforcer (Fig. 1A). Both groups increased the rate of head en-

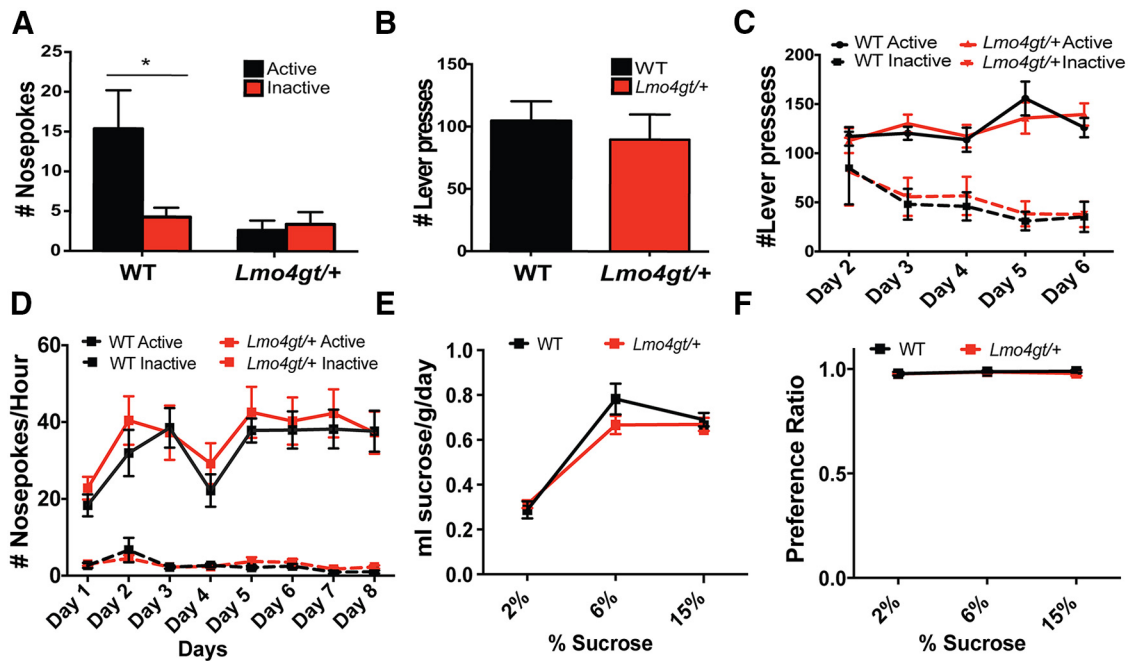


Figure 2. *A*, *Lmo4gt/+* mice show a selective deficit in conditioned reinforcement. WT mice made significantly more active than inactive nosepokes, whereas *Lmo4gt/+* mice did not discriminate between active and inactive ports. (* $p = 0.01$, Bonferroni test; $n = 14$ WT and $n = 13$ *Lmo4gt/+* mice). *B*, The number of reinforcers obtained on day 1 of instrumental conditioning was similar in both genotypes. *C*, With training, both WT and *Lmo4gt/+* mice increased the number of active and decreased the amount of inactive lever presses. ($n = 7$ /genotype). *D*, Both WT and *Lmo4gt/+* mice were equally proficient in performing a nosepoke response on an FR1 schedule to obtain a sucrose reinforcer ($n = 8$ WT and $n = 13$ *Lmo4gt/+*). *E*, Significant differences were not observed in the amount of sucrose consumed or in the preference ratio (*F*) between WT and *Lmo4gt/+* mice ($n = 7$, *Lmo4gt/+*; $n = 8$ WT).

tries into the reward port during cue presentation ($F_{\text{Day}(10,310)} = 17.67, p < 0.0001$). Although the increase in head entries was greater in WT mice early in training ($F_{\text{Genotype} \times \text{Day}(10,310)} = 2.03, p = 0.0301$), WT and *Lmo4gt/+* mice differed significantly only on conditioning day 4. Importantly, head entry rates during cue presentation were identical for both genotypes from day 5 through the end of the training period. Conversely, during the ITI (Fig. 1*A*), mice of both genotypes decreased head entries into the reward port with training ($F_{\text{Day}(10,310)} = 63.98, p < 0.0001$). Although this decrease was slightly greater in WT mice on days 3 and 4 of training ($F_{\text{Genotype} \times \text{Day}(10,310)} = 1.992, p = 0.03$), the number of head entries did not differ between genotypes after day 4.

We also investigated the latency to make head entries into the reward port after cue onset (Fig. 1*B*). With training, both WT and *Lmo4gt/+* mice reduced their latencies ($F_{\text{Day}(10,310)} = 41.18, p < 0.0001$). Although there was a slight delay in latency reduction in *Lmo4gt/+* mice ($F_{\text{Genotype} \times \text{Time}(10,310)} = 4.064, p < 0.0001$), differences between the genotypes were small and apparent only on days 2 and 5; latencies were identical thereafter. Finally, we examined the ability of reward-paired cues to elicit approach to the reward port (“goal tracking”; Fig. 1*C*). Both WT and *Lmo4gt/+* mice showed more head entries into the reward port during cue presentation than during the ITI ($F_{\text{Cue}(1,64)} = 356.46, p < 0.0001$; $F_{\text{Genotype}(1,64)} = 1.93, p = 0.17$; $F_{\text{Genotype} \times \text{Cue}(1,64)} = 2.75, p = 0.1$). We also compared within-session extinction of goal tracking responses in WT and *Lmo4gt/+* mice (Fig. 1*D*). Head entries into the reward port during CS presentation decreased similarly in both genotypes over time ($F_{\text{Time}(11,231)} = 10.23, p < 0.0001$; $F_{\text{Genotype}(1,21)} = 1.247, p = 0.28$; $F_{\text{Genotype} \times \text{Time}(11,231)} = 0.59, p = 0.84$). These results indicate that both acquisition and extinction of cue–reward learning are intact in *Lmo4gt/+* mice.

We next investigated the ability of reward-paired stimuli to act as conditioned reinforcers and support the acquisition of novel instrumental tasks. Mice were placed in operant chambers with two nosepoke ports. Nosepokes into the active port resulted in the presentation of the cue that was previously paired with the reward, whereas nosepokes into the inactive port had no consequence. WT, but not *Lmo4gt/+* mice, made significantly more active than inactive nosepokes ($F_{\text{Genotype} \times \text{Nosepoke}(1,50)} = 4.61, p = 0.03$; Fig. 2*A*). This result was not due to impaired locomotion because we found previously that *Lmo4gt/+* mice are not impaired in exploration of a novel open field (Lasek et al., 2010). This behavioral deficit also cannot be explained by reduced motivation to consume sucrose because, in the home cage, WT and *Lmo4gt/+* mice consumed similar amounts of sucrose (Figs. 2*E,F*). Such a deficit could arise from an inability to learn the association between the stimulus and the reward, an inability to assign motivational properties to reward-paired cues, or impairments in instrumental conditioning because conditioned reinforcement requires the learning of a novel instrumental task. Our results shown in Figure 1, *A–C*, clearly indicate that *Lmo4gt/+* mice do not have deficits in learning of cue–reward associations. To distinguish between the other two possibilities, we investigated instrumental conditioning. On the first day of conditioning, WT and *Lmo4gt/+* mice showed a similar ability to lever press for sucrose on an FR1 schedule (Fig. 2*B*). On subsequent days (day 2–6), a second, inactive lever that did not result in reinforcer delivery was introduced and the mice had to learn to discriminate between active and inactive levers (Fig. 2*C*). With training, both genotypes learned to press the active lever equally well ($F_{\text{Day}(4,48)} = 3.67, p = 0.01$; $F_{\text{Genotype}(1,12)} = 0.001, p = 0.96$; $F_{\text{Genotype} \times \text{Day}(4,48)} = 0.96, p = 0.44$) and to decrease their responses on the inactive lever ($F_{\text{Day}(4,48)} = 5.05, p = 0.002$; $F_{\text{Genotype}(1,12)} = 0.04, p = 0.85, F_{\text{Genotype} \times \text{Day}(4,48)} = 0.1, p = 0.98$).

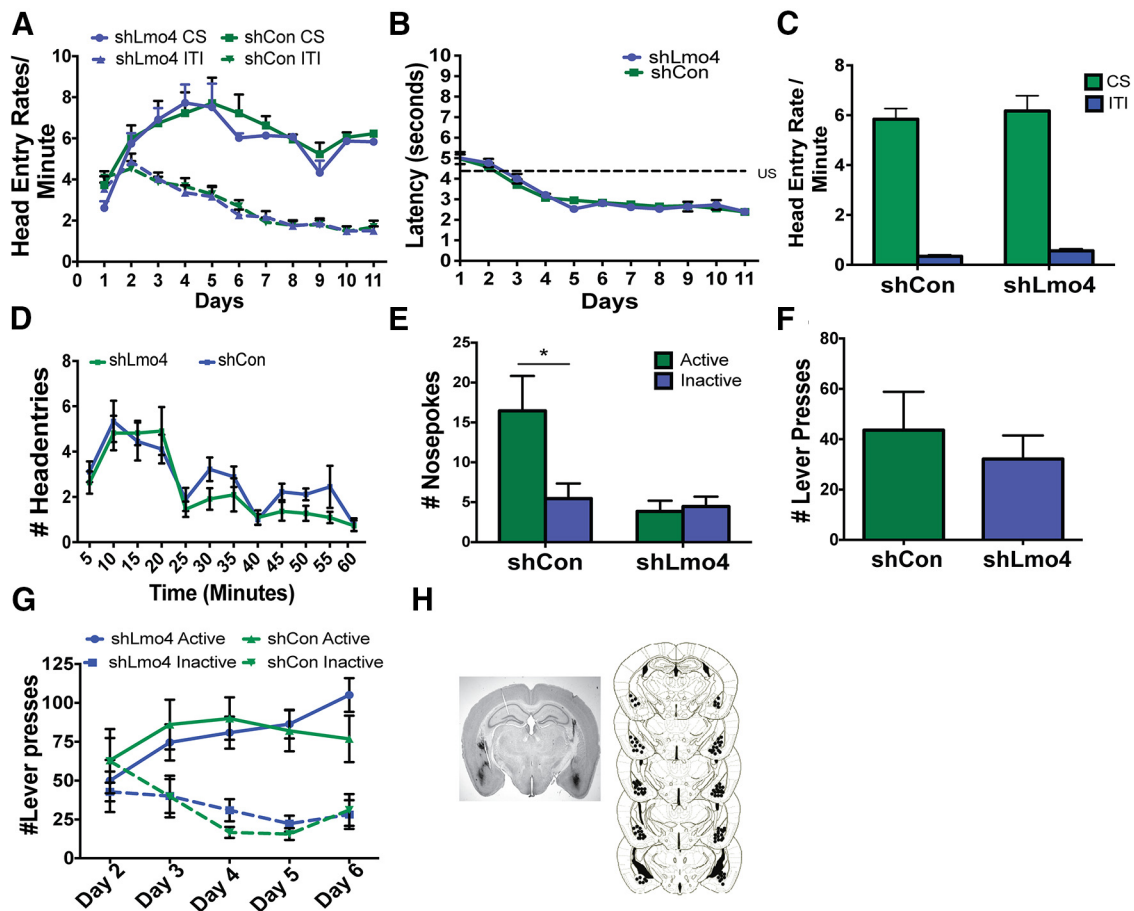


Figure 3. Knockdown of *Lmo4* in the BLA does not affect cue–reward associations. **A**, During conditioning, mice injected with shCon ($n = 13$) or shLmo4 ($n = 15$) made more head entries during presentation of the cue than during the ITI. **B**, No differences were observed in latencies to enter the reward port after cue onset between shCon- and shLmo4-injected mice across training sessions. **C**, During goal tracking sessions, both groups made more head entries during the presentation of the cue than during the ITI. **D**, Differences were not observed between the groups in extinction of goal tracking. **E**, During the conditioned reinforcement session, shCon-injected mice made significantly more active than inactive nosepokes, whereas shLmo4-injected mice failed to discriminate between active and inactive nosepokes ($*p = 0.002$, Bonferroni test, $n = 13$). **F**, No significant difference was observed in the number of reinforcers obtained between the groups on day 1 of the instrumental conditioning session. **G**, Both shCon- and shLmo4-injected mice increased the number of active lever presses and decreased the number of inactive lever presses with training. **H**, Left, Coronal section showing GFP immunoreactivity indicating viral infection in the BLA. Right, Serial reconstruction of microinjection sites.

We also compared nosepoke responding for sucrose on an FR1 schedule in WT and *Lmo4gt/+* mice (Fig. 2D). Both genotypes increased active nosepoke responding with training ($F_{\text{Day}(7,152)} = 3.077$, $p = 0.005$; $F_{\text{Genotype}(1,152)} = 1.534$, $p = 0.2174$; $F_{\text{Genotype} \times \text{Day}(7, 152)} = 0.1596$, $p = 0.99$). Inactive nosepoke responding remained low in both genotypes and decreased with training ($F_{\text{Day}(7,133)} = 5.011$, $p < 0.0001$; $F_{\text{Genotype}(1,19)} = 0.2735$, $p = 0.61$; $F_{\text{Genotype} \times \text{Day}(7,133)} = 1.032$, $p = 0.42$). These results indicate that *Lmo4gt/+* mice are not impaired in acquisition of a discriminative instrumental conditioning task. Together, our results indicate that the deficit in conditioned reinforcement in *Lmo4gt/+* mice is due to impaired ability to assign motivational value to reward-paired stimuli rather than general impairments in instrumental learning, sucrose preference, extinction learning, or motor performance.

This selective deficit in conditioned reinforcement that we observed in *Lmo4gt/+* mice is reminiscent of rats with BLA lesions (Baxter and Murray, 2002). Therefore, we investigated whether knockdown of *Lmo4* in the BLA would be sufficient to reproduce this phenotype. We used an shRNA that we found reduces *Lmo4* mRNA in the BLA by 44% and also reduces LMO4 protein (Maiya et al., 2012). An shCon not targeting any gene in the genome was used as a control (Maiya et al., 2012). Knockdown of *Lmo4* in the BLA did not impair the acquisition of cue–

reward learning (CS: $F_{\text{Day}(10,260)} = 10.95$, $p < 0.0001$; $F_{\text{shRNA}(1,26)} = 0.4158$, $p = 0.5247$; $F_{\text{shRNA} \times \text{Day}(10,260)} = 0.5130$, $p = 0.88$; Fig. 3A) or the rate of head entry into the reward port during the ITI across training sessions ($F_{\text{Day}(10,260)} = 40.93$, $p < 0.0001$; $F_{\text{shRNA}(1,26)} = 0.06882$, $p = 0.8$; $F_{\text{shRNA} \times \text{Day}(10,260)} = 0.5827$, $p = 0.82$). There were also no differences between the groups in latency to enter the reward port ($F_{\text{Day}(10,260)} = 50.77$, $p < 0.0001$; $F_{\text{shRNA}(1,26)} = 0.08$, $p = 0.8$; $F_{\text{shRNA} \times \text{Day}(10, 260)} = 0.6166$, $p = 0.8$; Fig. 3B), head entry rates into the reward receptacle during the presentation of the cue alone in a goal tracking session ($F_{\text{Cue}(1,56)} = 255.11$, $p < 0.0001$; $F_{\text{shRNA}(1,56)} = 1.324$, $p = 0.25$; $F_{\text{shRNA} \times \text{Cue}(1,56)} = 0.28$, $p = 0.6$; Fig. 3C), or in the rate of extinction of goal tracking responses ($F_{\text{Time}(11,198)} = 12.97$, $p < 0.0001$; $F_{\text{shRNA}(1,18)} = 3.468$, $p = 0.08$; $F_{\text{shRNA} \times \text{Time}(11,198)} = 0.6662$, $p = 0.77$; Fig. 3D). However, conditioned reinforcement was impaired in mice that received the shRNA against *Lmo4* ($F_{\text{Nosepoke} \times \text{shRNA}(1,48)} = 5.2$, $p = 0.03$; Fig. 3E). Significant differences were not observed between the two shRNAs in the number of active lever presses made on day 1 of conditioning (Fig. 3F). Analyses of active lever presses on days 2–6 indicate a significant genotype \times shRNA interaction ($F_{\text{shRNA} \times \text{Day}(4,112)} = 2.949$, $p = 0.0232$). This interaction is likely driven by small differences in the number of active lever presses made by shLmo4-injected mice on days 2 and 6 compared with shCon mice. However, these differences were not significant by *post hoc*

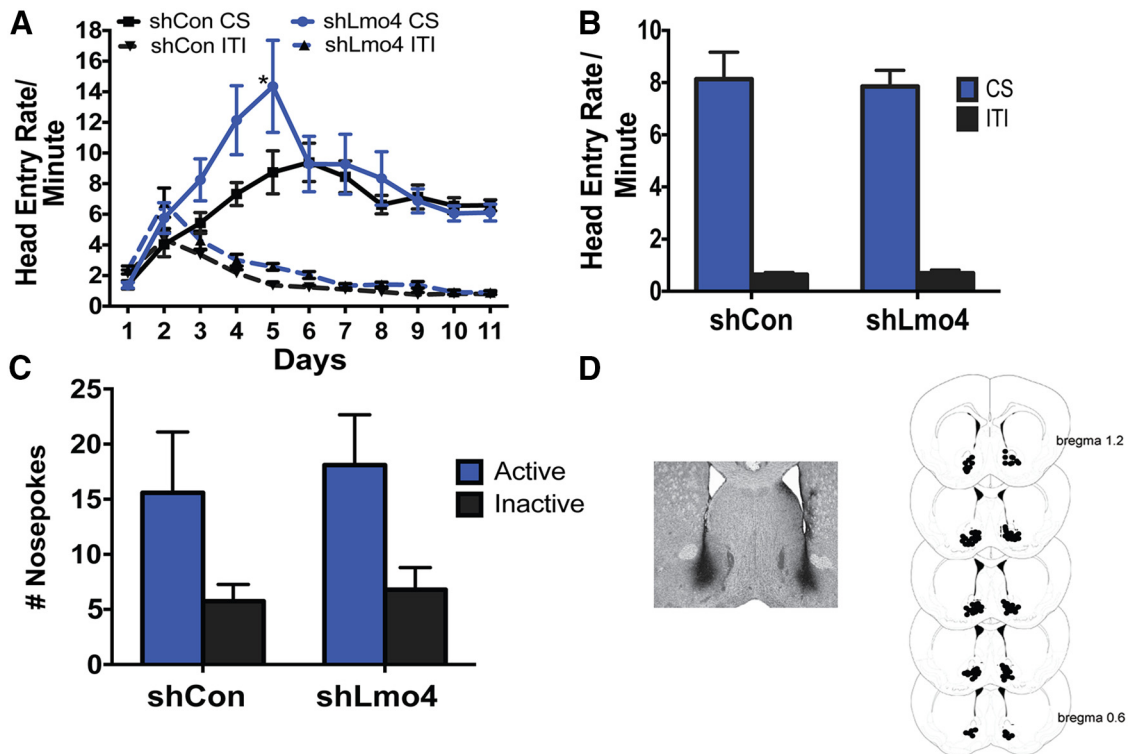


Figure 4. Knockdown of *Lmo4* in the NAc does not affect conditioned reinforcement. **A**, Mice injected with shCon ($n = 12$) or shLmo4 ($n = 10$) made more head entries into the reward port during the CS than during the ITI; shLmo4-injected mice made more head entries than shCon-injected mice only on day 5 ($*p = 0.0146$, Bonferroni test). **B**, During goal tracking sessions, shCon- and shLmo4-injected mice made more head entries during the presentation of the cue than during the ITI. **C**, During conditioned reinforcement, both groups made more active than inactive nosepokes. **D**, Left, GFP staining indicating viral infection in the BLA. Right, Serial reconstruction of microinjection sites.

tests (Fig. 3G). The number of inactive lever presses decreased with training in both groups ($F_{\text{Day}(4,112)} = 4.41$; $p = 0.0024$; $F_{\text{shRNA}(1,28)} = 0.0001$; $p = 0.98$; $F_{\text{shRNA} \times \text{Day}(4,112)} = 0.98$; $p = 0.4226$). Importantly, these analyses did not detect a deficit in the acquisition of instrumental conditioning in shLmo4-injected mice. These results indicate that knockdown of *LMO4* in the BLA is sufficient to selectively impair cue-reward learning and replicate the phenotype we observed in *Lmo4gt/+* mice.

Projections from the BLA to the NAc are implicated in cue-reward association and reward-seeking behavior (Di Ciano and Everitt, 2004; Ambroggi et al., 2008; Stuber et al., 2011). There is also electrophysiological evidence that information about the motivational value of reward-paired cues is encoded in both the BLA and the NAc (Di Ciano and Everitt, 2004; Ambroggi et al., 2008; Stuber et al., 2011). Importantly, medium spiny neurons in the NAc, which are targets of efferent projections from the BLA, also express *Lmo4* (Lasek et al., 2010). Therefore, we investigated the consequences of knockdown of *Lmo4* in the NAc. Our previous study showed that shLmo4 reduced both *Lmo4* mRNA (by 50%) and protein in lentivirus-infected medium spiny neurons in the NAc (Lasek et al., 2010). We found that shLmo4 and shCon-injected mice both learned to associate cue presentation with reinforcer delivery (Fig. 4A), although shLmo4-injected mice appeared to learn the task faster than their shCon-injected counterparts ($F_{\text{shRNA} \times \text{Day}(10,200)} = 2.334$, $p = 0.0127$). However, head entry rates between the groups only differed significantly on day 5 and rates were identical thereafter. In the goal-tracking session, both shCon- and shLmo4-injected mice made significantly more head entries during the presentation of the cue than during the intertrial interval ($F_{\text{Cue}(1,40)} = 157$, $p < 0.0001$; $F_{\text{shRNA}(1,40)} = 0.04$, $p = 0.04$; $F_{\text{shRNA} \times \text{Cue}(1,40)} = 0.08$, $p = 0.77$;

Table 1. Electrophysiological properties of BLA projection neurons from WT and *Lmo4gt/+* mice

	WT	<i>Lmo4gt/+</i>
n , cells (mice)	13 (6)	17 (8)
Resting membrane potential	-68.0 ± 0.6	-68.7 ± 0.5
Input resistance, $M\Omega$	134.3 ± 5.0	186.3 ± 11.8^a
Rheobase current, pA	172 ± 11	120 ± 10^a
Action potential threshold, mV	-41.1 ± 0.8	-41.5 ± 0.7
Action potential amplitude, mV	75.3 ± 2.3	71.9 ± 2.1
Action potential half-width, ms	1.35 ± 0.04	1.42 ± 0.07
AHP amplitude, mV	8.3 ± 0.8	7.2 ± 0.7

Significant difference versus WT ($p < 0.01$) using two-tailed, unpaired t test.

Fig. 4B). Both shCon- and shLmo4-injected mice also displayed conditioned reinforcement ($F_{\text{Nosepokes}(1,40)} = 7.432$, $p = 0.009$; $F_{\text{shRNA}(1,40)} = 0.2117$, $p = 0.65$; $F_{\text{Nosepokes} \times \text{shRNA}(1,40)} = 0.04$, $p = 0.85$; Fig. 4C). Together, these results indicate that downregulation of *Lmo4* in the NAc may accelerate acquisition of cue-reward associations without affecting conditioned reinforcement.

Because knockdown of *LMO4* in the BLA impaired cue-reward learning, we investigated whether electrophysiological properties of BLA neurons in *Lmo4gt/+* mice are altered by performing whole-cell patch-clamp recordings in current-clamp mode. The resting membrane potential and the threshold potential for firing action potentials in response to depolarizing current injection were similar in BLA neurons from WT and *Lmo4gt/+* mice, as were other action potential characteristics, such as amplitude, half-width, and the afterhyperpolarization (AHP) amplitude (Table 1). However, BLA neurons from *Lmo4gt/+* mice exhibited several features of increased excitability, such as greater input resistance and lower rheobase current (the amplitude of

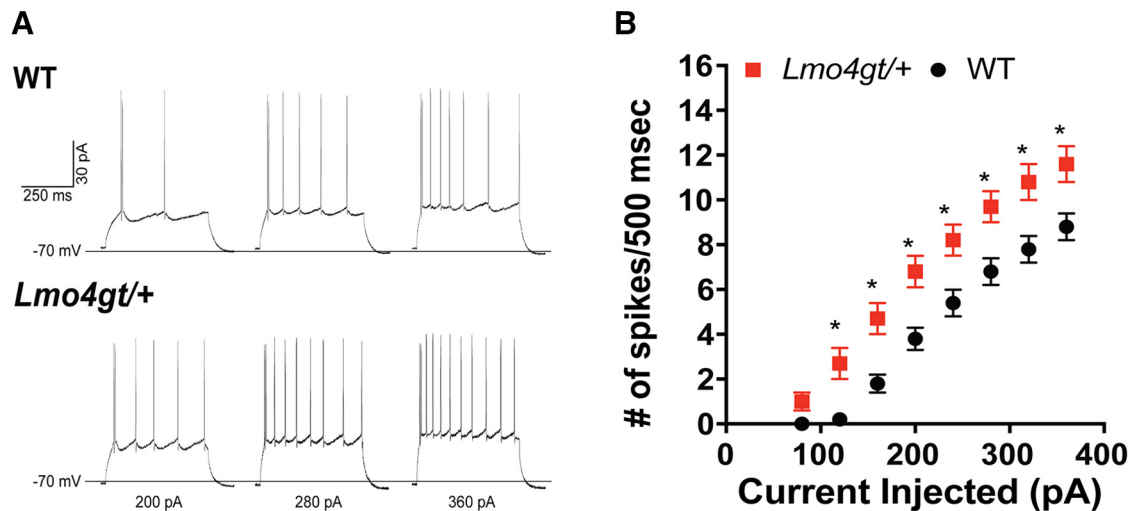


Figure 5. BLA pyramidal neurons from *Lmo4gt/+* mice show increased excitability. **A**, Representative traces for neurons from WT (top) and *Lmo4gt/+* (bottom) mice showing action potentials evoked by 200, 280, and 360 pA current steps. **B**, Neurons from *Lmo4gt/+* mice ($n = 17$) showed greater action potential firing in response to increasing amplitudes of 500 ms depolarizing current injections relative to WT ($n = 13$) ($*p < 0.05$, Bonferroni test).

depolarizing current injection necessary to evoke action potential firing; Table 1). Moreover, the escalation in action potential firing in response to increasing depolarizing steps ($F_{\text{Amplitude}(9,252)} = 255.7$, $p < 0.0001$) was not the same for both genotypes ($F_{\text{Amplitude} \times \text{Genotype}(9,252)} = 6.087$, $p < 0.0001$) and, overall, *Lmo4gt/+* BLA neurons displayed greater spike firing ($F_{\text{Genotype}(1,28)} = 10.61$, $p = 0.003$; Fig. 5A,B). Together, these findings suggest that *Lmo4gt/+* BLA neurons are more responsive than WT BLA neurons to excitatory inputs.

The ability of cues to act as conditioned reinforcers and support responding under second-order schedules of reinforcement is dependent upon dopamine signaling in the BLA. Bilateral infusions of D1, D2, or D3R antagonists into the BLA impair second-order conditioning (Di Ciano and Everitt, 2004; Di Ciano and Everitt, 2005; Di Ciano, 2008) and block cue-induced reinstatement to cocaine seeking (Berglind et al., 2006). Therefore, we compared *Drd1*, *Drd2*, and *Drd3* expression in the BLA of WT and *Lmo4gt/+* mice by quantitative PCR (qPCR). We observed a striking reduction in *Drd2* mRNA, but no change in *Drd1* mRNA in the BLA of *Lmo4gt/+* mice (Fig. 6A). We were unable to detect *Drd3* mRNA using commercial qPCR assays in the BLA of either WT or *Lmo4gt/+* mice.

To determine whether reductions in *Drd2* mRNA levels lead to functional deficits, we compared D2R-mediated signaling in the BLA of WT and *Lmo4gt/+* mice. Stimulation of the D2-like receptors rapidly increases phosphorylation of AKT at Thr³⁰⁸ of D2R in primary cultures and *in vivo* in the NAc (Brami-Cherrier et al., 2002; Mannoury la Cour et al., 2011). In contrast, prolonged elevation of dopaminergic tone in the NAc, reduces Thr³⁰⁸AKT phosphorylation (Beaulieu et al., 2005; Beaulieu et al., 2007; Beaulieu et al., 2011). The effect of D2R activation on AKT phosphorylation in the BLA is not known. We examined P-Thr³⁰⁸ levels in the BLA of WT and *Lmo4gt/+* mice 30 min after a systemic administration of the selective D2/3R agonist quinpirole (Fig. 6B,C; Brami-Cherrier et al., 2002; Mannoury la Cour et al., 2011). P-Thr³⁰⁸ AKT levels were similar in BLA tissue from saline-treated WT and *Lmo4gt/+* mice (P-Thr³⁰⁸/Total AKT ratio values for WT = 0.46 ± 0.05 , *Lmo4gt/+* = 0.513 ± 0.07 , $p = 0.5626$, Student's *t* test, $n = 8-10/\text{genotype}$). However, quinpirole significantly enhanced P-Thr³⁰⁸ levels in WT, but not

in *Lmo4gt/+* mice (Figs. 6B,C), indicating a deficit in D2/3R signaling in *Lmo4gt/+* mice.

Dopamine activates BLA neurons through a mechanism that requires both D1Rs and D2Rs (Kröner et al., 2005). Because both D2R expression and D2/3R-induced activation of Akt were reduced in the BLA of *Lmo4gt/+* mice, we predicted that dopamine-induced activation of *Lmo4gt/+* BLA neurons would also be reduced. Brief (~4 min) bath application of dopamine (10 μM) significantly increased the firing frequency of BLA pyramidal neurons in response to depolarizing current injection in both genotypes, although the effect was much greater in WT neurons than in *Lmo4gt/+* neurons ($F_{\text{Dopamine}(10,110)} = 7.2$, $p < 0.0001$; $F_{\text{Genotype}(1,11)} = 5.3$, $p = 0.041$; $F_{\text{Dopamine} \times \text{Genotype}(10,110)} = 2.8$, $p = 0.0039$; Fig. 6D–F). Therefore, the ability of BLA pyramidal neurons to respond dynamically to dopamine is compromised in *Lmo4gt/+* mice.

Discussion

Our results reveal a novel role for the transcriptional regulator *Lmo4* in attributing motivational significance to reward-paired cues. *Lmo4gt/+* mice are as adept as their WT counterparts in learning the association between cue presentations and reinforcer delivery. However, they are deficient in subsequently using these reward-paired cues as conditioned reinforcers to learn a novel instrumental task. *Lmo4gt/+* mice are not impaired in instrumental conditioning, so their deficit in conditioned reinforcement is due to their inability to assign motivational significance to reward-paired cues. Knockdown of *Lmo4* in the BLA recapitulated this selective deficit in conditioned reinforcement, indicating that this phenotype is not due to a developmental abnormality in *Lmo4gt/+* mice. This phenotype is likely related to a change in BLA pyramidal cell function because electrophysiological studies indicated enhanced excitability, but impaired dopamine-induced excitation of BLA neurons in *Lmo4gt/+* mice. This impaired response to dopamine is likely due to the striking reduction in *Drd2* expression and D2-mediated signaling in the BLA.

In the context of cue–reward learning, the BLA facilitates the association of reward-paired cues with the motivational properties of the reward. This information is then transmitted from the

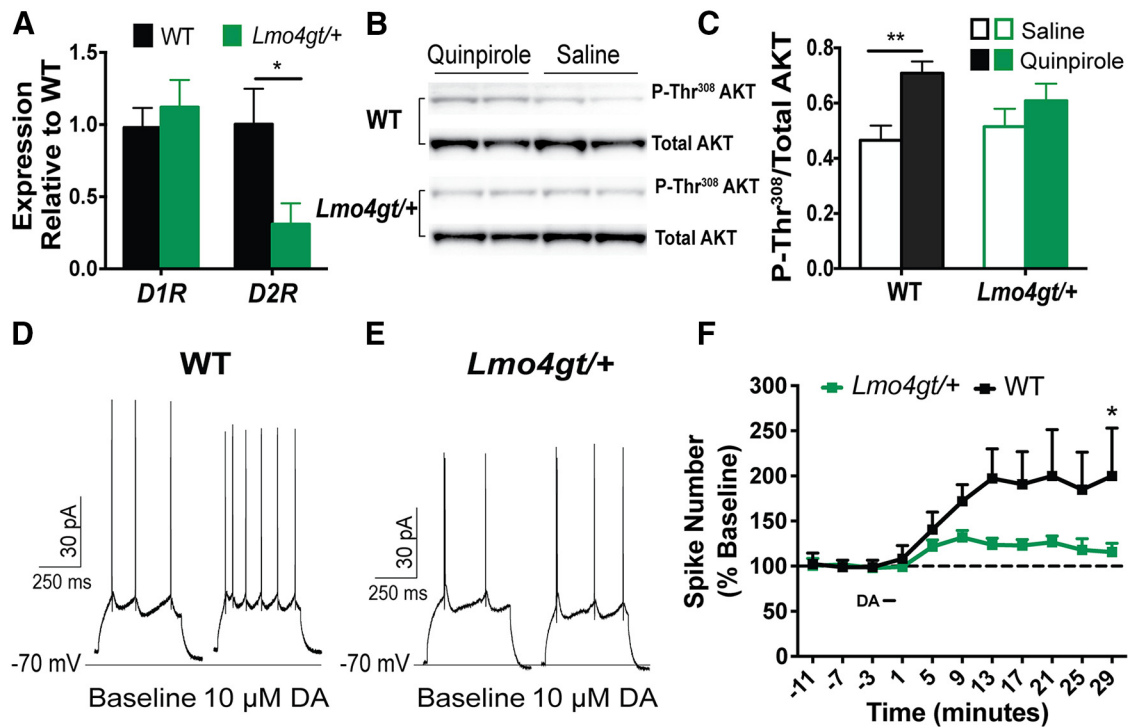


Figure 6. *D2R* abundance and signaling are reduced in the BLA of *Lmo4gt/+* mice. **A**, BLA of WT ($n = 6$) and *Lmo4gt/+* ($n = 8$) mice showed similar levels of *Drd1* mRNA, but there was a striking reduction in *Drd2* mRNA the BLA of *Lmo4gt/+* mice compared with WT mice ($*p = 0.04$, two-tailed t test). **B**, **C**, Systemic administration of 1 mg/kg quinpirole increased P-Thr³⁰⁸ AKT immunoreactivity in the BLA of WT, but not *Lmo4gt/+* mice ($**p = 0.0045$, two-tailed t test; $n = 10$ –11 WT and $n = 8$ –9 *Lmo4gt/+* mice). **D**–**F**, BLA pyramidal neurons from *Lmo4gt/+* mice are less responsive to dopamine. Representative traces for WT (**D**) and *Lmo4gt/+* (**E**) neurons show action potentials evoked by 500 ms depolarizing current injection before and after bath application of dopamine. **F**, Time course for the effect of dopamine (DA) on spike firing. Data shown are mean \pm SEM values derived from the current amplitude that elicited the maximal change in spike number relative to the baseline average, and are from 5 (WT) and 8 (*Lmo4gt/+*) cells ($*p = 0.02$, Bonferroni test).

BLA to downstream neural structures including the NAc and OFC, which aid in the selection of appropriate behavioral responses (Everitt et al., 1999; Mead and Stephens, 2003). Animals with BLA lesions are unable to use first-order conditioned stimuli as secondary reinforcers for the acquisition of a novel operant response, display deficits in conditioned reinforcement, and are insensitive to the current value of the reinforcer in outcome devaluation experiments (Everitt et al., 1989; Hatfield et al., 1996; Málková et al., 1997; Baxter et al., 2000; Mead and Stephens, 2003). Further, disconnection lesions and electrophysiological studies suggest that acquisition of reinforcing value by reward-paired cues is dependent upon projections from the BLA to the NAc and OFC (Di Ciano and Everitt, 2004; Ambroggi et al., 2008). BLA lesions also impair the ability of cocaine-paired cues to reinstate cocaine seeking without altering cocaine self-administration (Meil and See, 1997). These studies suggest an important role for the BLA and its efferent projections in encoding the reinforcing properties of reward-paired cues rather than the primary reward itself. The molecular mechanisms in the BLA that govern this learned behavior have been relatively unexplored. Our finding that LMO4 in the BLA is required for the attribution of motivational significance to reward-paired stimuli provides an important advance in understanding these molecular mechanisms.

LMO4 can affect synaptic plasticity by modulating activity dependent gene expression and neurite outgrowth (Vu et al., 2003; Kashani et al., 2006). We demonstrated previously that, within the BLA, LMO4 is present predominantly in pyramidal neurons, where it functions to negatively regulate fear learning (Maiya et al., 2012). Mice with global or BLA-specific reduction

in LMO4 display enhanced contextual and cued fear conditioning. Further, *Lmo4gt/+* mice display increased cFos in the BLA after fear learning, suggesting a role for *Lmo4* in negatively regulating the expression of genes in the BLA that are important for fear memory formation (Maiya et al., 2012). Therefore, it is intriguing that global or BLA-specific reduction in LMO4 impairs conditioned reinforcement. Studies in rats and monkeys suggest that separate populations of neurons in the BLA encode cues paired with appetitive and aversive outcomes (Paton et al., 2006; Shabel and Janak, 2009). We do not yet know whether the transcriptional targets of LMO4 are different in these subpopulations or if different transcriptional targets mediate the effect of LMO4 on these different behaviors.

How does LMO4 affect the function of BLA pyramidal neurons? Our data point to at least two potential mechanisms. First, the results of our electrophysiological experiments implicate a novel role for LMO4 in limiting the excitability of BLA principal neurons, because neurons from *Lmo4gt/+* mice exhibited greater membrane excitability than WT neurons. This increase in excitability was associated with a $>20\%$ increase in membrane resistance without a change in resting membrane potential, action potential threshold potential, amplitude half-width, or AHP amplitude. There are numerous voltage-dependent and synaptic conductances that LMO4 might contribute to increased excitability of BLA principal neurons (Faber et al., 2001; Sah et al., 2003; Kröner et al., 2005). In the hippocampus, loss of LMO4 in CA3 neurons downregulates the type 2 ryanodine receptor (RyR2), thereby compromising calcium-induced calcium release and the induction of CA3–CA1 synaptic plasticity (Qin et al., 2012), whereas, in the hypothalamus, selective ablation of LMO4

in single-minded 1 (*Sim1*) neurons decreases voltage-dependent calcium channels Cav2.1 and Cav2.3 and reduces neuronal excitability (Zaman et al., 2014). Such mechanisms are predicted to dampen neuronal function and therefore would not explain enhanced excitability in BLA principal neurons of *Lmo4gt/+* mice. It appears, therefore, that disruption of LMO4-regulated gene transcription can lead to diverse functional outcomes that are cell-type specific.

Second, our results demonstrate reduced *Drd2* expression, D2R-mediated signaling, and dopamine-induced excitability in the BLA of *Lmo4gt/+* mice. A wealth of evidence points to a role for dopaminergic signaling in the BLA in cue–reward learning and the subsequent ability of reward-paired cues to guide goal-directed behaviors. Antagonism of D1Rs and D2Rs in the BLA impairs cue-induced reinstatement to cocaine seeking (Berglind et al., 2006). Infusions of selective D3R antagonists into the BLA prevent second-order conditioning and cue-induced reinstatement to nicotine seeking in rats (Khaled et al., 2014). Pharmacological disconnection procedures performed by unilateral injections of the nonselective dopamine receptor antagonist α -flupenthixol into the BLA and the AMPA-kainate receptor antagonist LY293558 into the NAc on the contralateral side impair second-order conditioning (Di Ciano and Everitt, 2004). Further, electrophysiological studies reveal that dopamine-induced excitability of BLA projection neurons is dependent upon D1R and D2R activation (Kröner et al., 2005). Because LMO4 is a transcriptional regulator, the molecular mechanism by which it modulates conditioned reinforcement may involve changes in gene expression and our findings implicate the D2R as a transcriptional target of LMO4 in this behavior.

The signaling pathways functioning downstream of D2R in the BLA are not known. Here, we provide evidence that D2-like activation enhances phosphorylation of AKT at Thr³⁰⁸ in WT mice. This result is consistent with published literature using primary cultured striatal neurons, heterologous expression systems, and *in vivo* in the NAc (Brami-Cherrier et al., 2002; Nair and Sealson, 2003; Mannoury la Cour et al., 2011). Importantly, our results indicate that quinpirole-induced phosphorylation of Thr³⁰⁸ AKT is substantially blunted in *Lmo4gt/+* mice. Although quinpirole activates both D2R and D3R subtypes, we were unable to detect *Drd3* mRNA by qPCR in the BLA of either WT or *Lmo4gt/+* mice. These assays gave us a robust signal for *Drd3* mRNA in the NAc, leading us to conclude that *Drd3* mRNA is not expressed or is expressed at very low levels in the BLA. Based on this evidence, we conclude that impaired D2R signaling contributes to deficits in AKT phosphorylation in *Lmo4gt/+* mice. Further, electrophysiological studies revealed a striking deficit in dopamine-modulation of BLA projection neuron excitability in *Lmo4*-deficient mice that is likely due to reduced D2R expression and D2-mediated signaling in *Lmo4gt/+* mice.

LMO4 could modulate *Drd2* expression directly by nucleating a transcription complex that activates *Drd2* transcription or it could mediate its effects indirectly by modulating the expression of other transcriptional regulators of *Drd2*. ChIP assays are widely used to identify transcription factor binding sites on promoter elements. However, LMO4 does not bind directly to promoter elements (Heberlein et al., 2009), making it difficult to discriminate between direct versus indirect effects of LMO4 on gene expression. Whole genome approaches such as ChIP-Seq, which do not rely on sequence information to identify binding sites, may provide an alternative strategy. Finally, nongenomic effects of LMO4 might also contribute to the observed deficits in conditioned reinforcement. A recent study found that LMO4

inhibits protein tyrosine phosphatase 1B (PTP1B) in the BLA, leading to upregulation of endocannabinoid signaling (Qin et al., 2015). However, an effect of LMO4-PTP1B signaling on D2R would need to be indirect because tyrosine phosphorylation is not known to regulate D2R abundance or function.

A recent study suggested that only cues that are imbued with incentive properties can activate cFos in the BLA, NAc, and OFC (Flagel et al., 2011). It is possible that knockdown of LMO4 in the BLA disrupts a transcriptional network that impairs neuronal activation in these brain regions, leading to deficits in goal-directed behavior. Future studies using whole genome approaches such as RNA-Seq to identify additional transcriptional targets of LMO4 in the BLA should be informative in this regard. Identification of these targets may not only reveal molecular mechanisms by which LMO4 functions in the BLA, but also could enable discovery of new drug targets for the treatment of psychopathologies such as addiction, in which aberrant cue–reward learning plays an important role.

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