Adenosine Transporter ENT1 Regulates the Acquisition of Goal-Directed Behavior and Ethanol Drinking through A_{2A} Receptor in the Dorsomedial Striatum

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Adenosine signaling has been implicated in the pathophysiology of many psychiatric disorders including alcoholism. Striatal adenosine A_{2A} receptors (A_{2A}R) play an essential role in both ethanol drinking and the shift from goal-directed action to habitual behavior. However, direct evidence for a role of striatal A_{2A}R signaling in ethanol drinking and habit development has not been established. In the present study, we found that decreased A_{2A}R-mediated CREB activity in the dorsomedial striatum (DMS) enhanced initial behavioral acquisition of goal-directed behaviors and the vulnerability to progress to excessive ethanol drinking during operant conditioning in mice lacking ethanol-sensitive adenosine transporter ENT1 (ENT1^{-/-}). Using mice expressing β -galactosidase (lacZ) under the control of seven repeated CRE sites in both genotypes (CRE-lacZ/ENT1^{+/+} mice and CRE-lacZ/ENT1^{-/-} mice) and the dominant-negative form of CREB, we found that reduced CREB activity in the DMS was causally associated with decreased A_{2A}R signaling and increased goal-directed ethanol drinking. Finally, we have demonstrated that the A_{2A}R antagonist ZM241385 dampened protein kinase A activity-mediated signaling in the DMS and promoted excessive ethanol drinking in ENT1^{+/+} mice, but not in ENT1^{-/-} mice. Our results indicate that A_{2A}R-mediated CREB signaling in the DMS is a key determinant in enhancing the development of goal-directed ethanol drinking in mice.

Introduction

Striatal adenosine levels play an important role in ethanol sensitivity, withdrawal, and drinking (Gordon and Diamond, 1993; Nagy and DeSilva, 1994; Meng and Dar, 1995; Arolfo et al., 2004; Mailliard and Diamond, 2004; Asatryan et al., 2011). In the CNS, the adenosine A_{2A} receptor (A_{2A}R) is enriched in the striatum and is expressed exclusively in striatopallidal neurons, which may regulate inhibitory behavioral control over drug-rewarding processes through the indirect pathway of basal ganglia circuitry (Graybiel, 2008). Recent evidence suggests a direct role of the striatal A_{2A}R receptor in mediating many of the cellular and behavioral responses underlying ethanol- and heroin-seeking behavior (Arolfo et al., 2004; Yao et al., 2006). In addition, A_{2A}R-dependent synaptic activity in striatopallidal neurons plays an essential role in the shift from goal-directed actions to habitual

behaviors (Yu et al., 2009). However, the molecular mechanisms underpinning striatal A_{2A}R-regulated signaling in goal-oriented ethanol-seeking behaviors has remained unknown.

Ethanol is known to inhibit selectively the type 1 equilibrative nucleoside transporter (ENT1), which is one of the main transporters that regulates adenosine levels in the brain (Dunwiddie and Masino, 2001). Mice lacking ENT1 exhibit reduced ataxic and hypnotic responses to acute ethanol exposure and consume more ethanol than wild-type littermates (Choi et al., 2004; Chen et al., 2010). Our recent studies have revealed that adenosine levels are decreased in the striatum of ENT1^{-/-} mice (Kim et al., 2011; Nam et al., 2011). Because the A_{2A}R has a lower binding affinity (K_d) for adenosine (150 nM) than A₁R (70 nM) (Dunwiddie and Masino, 2001), reduced striatal adenosine levels by ENT1 deletion (< 100 nM) (Kim et al., 2011; Nam et al., 2011) may predominantly affect A_{2A}R signaling (Ciruela et al., 2006), which may contribute to excessive ethanol drinking in ENT1^{-/-} mice.

Recently, an essential role of the dorsal striatum in the development of behaviors related to excessive ethanol drinking, which include the regulation of voluntary movement and acquisition of goal-directed actions and stimulus-driven habits, has been revealed (Yin and Knowlton, 2006; Lovinger, 2010). However, the intracellular mechanisms and subregion-specific contributions of the dorsal striatum to ethanol drinking are poorly understood. Therefore, we investigated the contribution of adenosine signaling on operant behaviors in two subregions of the dorsal striatum, the dorsomedial striatum (DMS; equivalent to the caudate nucleus) and the dorsolateral striatum (DLS; equivalent to the

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putamen), which play differential roles in habit formation. The DMS primarily regulates goal-directed (action-outcome) behavior, which is sensitive to outcome devaluation and instrumental learning, whereas the DLS is more involved in habit (stimulus-response) formation (Yin and Knowlton, 2006; Yin et al., 2009).

Because ethanol appears to impair several striatal functions, including reward evaluation, motor function, and habit formation (Yin et al., 2004; Yin et al., 2007; Corbit et al., 2012), we hypothesized that loss of ENT1 function in the DMS reduces $A_{2A}R$ signaling through dampened protein kinase A (PKA)-driven CREB activity and thereby accelerates the transition from goal-directed to habitual behaviors in ethanol drinking. In the present study, we revealed that DMS $A_{2A}R$ -mediated signaling regulates goal-oriented ethanol-seeking behaviors.

Materials and Methods

Animals. ENT1^{−/−} mice were generated as described previously (Choi et al., 2004). F2-generation hybrid mice with a C57BL/6J × 129X1/SvJ genetic background were used for behavioral experiments as described previously (Silva et al., 1997; Crusio et al., 2009). To examine the CREB activity response by the deletion of ENT1, CRE-lacZ mice in a C57BL/6J background were crossed with ENT1^{−/−} mice in a C57BL/6J background, then the CRE-lacZ/ENT1^{+/−} were crossed with ENT1^{+/−} mice in a 129X1/SvJ background to generate CRE-lacZ/ENT1^{+/+} or CRE-lacZ/ENT1^{−/−} mice, which have the same genetic background as ENT1^{−/−} mice. Animal care and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committees in accordance with NIH guidelines. Eight- to 16-week-old male mice used for all experiments were housed at a constant temperature (24 ± 0.5°C) and humidity (60 ± 2%) under a 12 h light/dark cycle (lights on at 7:00 A.M.).

X-gal staining for measurement of CREB activity. Brains from CRE-lacZ mice were rapidly dissected out and put into a cold fixative solution (2% paraformaldehyde, 0.2% glutaraldehyde in PBS) overnight at 4°C. Coronal cryostat sections (40 μm) were cut and washed 3 times for 5 min each time with PBS and then incubated overnight in a dark tray at 37°C in the reaction solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS). Sections were washed the following day and mounted on microscope slides. The number of X-gal⁺ cells was counted in the regions of interest (0.15 mm²) on a Leica DM4000B microscope.

Western blotting. Mice were anesthetized with carbon dioxide and rapidly decapitated. Brains were quickly removed and dissected to isolate the DMS. Briefly, tissues were homogenized in a solution containing 50 mM Tris buffer, pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.1% SDS, protease inhibitor mixture (Roche), and phosphatase inhibitor mixture type I and II (Sigma). Homogenates were centrifuged at 500 × g at 4°C for 15 min and supernatants were collected. Proteins were separated by 4–12% NuPAGE-ETM Bis-Tris gels at 130 V for 2 h, transferred onto PVDF membranes (Invitrogen) at 30 V for 1 h, and incubated with antibodies against PKA (1:1000, #4782; Cell Signaling Technology), phospho-PKA Thr-197 (1:1000, sc32968; Santa Cruz Biotechnology), CREB (1:1000, #9197; Cell Signaling Technology), phospho-CREB Ser-133 (1:1000, #9198S; Cell Signaling Technology), and GAPDH (1:1000, MAB374; Millipore). Blots were developed using chemiluminescent detection reagents (Pierce). Chemiluminescent bands were detected on a Kodak Image Station 4000R scanner and quantified using NIH ImageJ software. Protein levels were normalized by GAPDH and quantified compared with the control group.

Cannula implantation into the DSM and DLS. Mice were anesthetized with ketamine/xylazine (100 and 15 mg/kg, i.p.) and placed in a digital stereotaxic alignment system (Model 1900; David Kopf Instruments). After the skull was exposed, the mouse was positioned with bregma at the focal point and the skull was leveled using a dual-tilt measurement tool. A 0.5-mm-diameter hole was drilled for placement of the guide cannulas as described previously (Nam et al., 2011). Bilateral guide cannulas (26 Ga Double Guide; Plastics One) were implanted into the DMS (anterior–posterior [AP]: 0.5 mm; medial–lateral [ML]: ± 1.0 mm; dorsal–ventral [DV]: 1.5 mm) or DLS (AP: 0.5 mm; ML: ± 2.5 mm; DV: 2.3 mm). Mice

were given 7 d for recovery after stereotaxic surgery and weights were monitored daily after the surgery to ensure a healthy recovery.

Microinjection of ZM241385 into the DMS and DLS. Mice were given 7 d for recovery after stereotaxic surgery before guide cannulas were implanted. To determine either DMS- or DLS-specific $A_{2A}R$ regulation, 10 μM ZM241385 (Tocris Bioscience), an $A_{2A}R$ -specific antagonist dissolved in 15% DMSO, or vehicle was infused with an infusion pump (Harvard Apparatus) and microinjection needles (26 Ga Double Guide; Plastics One) at a 1 μl/min flow rate separately into each respective target brain region to a total volume of 1 μl. The injection needle was extended 0.5 mm below the tip of the cannula. Because 10 μM ZM241385 is known to inhibit $A_{2A}R$ effectively (Stella et al., 2003) and to show a saturated pharmacological effect for 2 h, behavioral experiments were performed or the mice were decapitated 2 h after the ZM241385 microinjection (Naassila et al., 2002).

Microinfusion of HSV virus into the DSM and DLS. Stereotaxic injections of the HSV viruses (provided by Dr. E. Nestler at Mount Sinai School of Medicine) were conducted as described previously (Carlezon et al., 1998). Mice were anesthetized with ketamine/xylazine (100 and 15 mg/kg, i.p.) and placed in a digital stereotaxic alignment system (Model 1900; David Kopf Instruments). Two holes for bilateral injection were drilled above the target brain region. The injector (33 Ga; Plastics One) was connected to a Hamilton syringe (25 μl) and virus infusion was controlled by automatic pump. To infuse the viruses, 0.5 μl of HSV vectors (HSV-dnCREB or HSV-GFP) was bilaterally injected into either the DMS (AP: 0.5 mm; ML: ± 1.0 mm; DV: 1.5 mm) or DLS (AP: 0.5 mm; ML: ± 2.5 mm; DV: 2.3 mm) at a 0.1 μl/min rate. Injectors remained in place for an additional 5 min per each infusion side.

Operant responding in ENT1^{+/+} and ENT1^{−/−} mice. Mice were placed on a food restriction schedule to maintain their body weight at 85% of their free-feeding weight and trained to get a 20% sucrose reward as described previously (Yin et al., 2004; Hilario et al., 2007). All operant training procedures were performed in computer-controlled mouse operant chambers (Model ENV-307W; Med Associates) equipped with a rear 3W house light. Opposite the house light were two nose poke response holes, each of which was separated by a solution-dispensing trough. All operant training programs, schedules of reinforcement, cue lights, and syringe pump activations (Model PHM-100; Med Associates) were controlled by Med PC-IV (Med Associates). The active nose poke response hole was equipped with a cue light that turned on for 1 s after a nose poke and then immediately turned off, resulting in syringe pump activation and effectively associating the cue light with solution reinforcement. Syringe pumps delivered the reward solution (0.1 ml) by one reinforcer in response to active nose poking. Responses on the similarly equipped inactive nose poke response hole were recorded but resulted in no consequences. All groups of mice were subjected to 30 min magazine-training sessions to establish an association between the solution trough and a randomly delivered 20% sucrose solution reinforcer (0.1 ml/reinforcement on average every 60 s) as described previously (Yin et al., 2004; Hilario et al., 2007). Subsequently, the mice were shaped by successive approximation to elicit a nose poke response for sucrose solution and this operant was maintained by fixed ratio (FR1) until mice received 30 reinforcers. To examine the acquisition of goal-directed behaviors, a group of mice were given 2 d of training on a random ratio schedule (RR10: one reinforcer delivered on average every 10 nose pokes) and then 3 d of training on an RR20 schedule, which produces goal-directed behaviors (Hilario et al., 2007). To examine habitual responding, a separate group of mice were given 2 d of training on a random interval (RI15: one reinforcer delivered on average every 15 s after the last reinforcer) schedule and then 3 d of training on an RI60 schedule (Hilario et al., 2007).

Operant responding for sucrose and ethanol in response to $A_{2A}R$ inhibition. All operant training procedures were as described previously. To examine the behavioral effect of $A_{2A}R$ inhibition (ZM241385), both the RR10 and RR20 schedules were trained toward sucrose and ethanol reward. To perform the operant responding for ethanol (10%), sucrose substitution was used to measure the ethanol response when the mice had reached a stable baseline of sucrose reward. To examine the effect of ZM241385 treatment during each response on the RR schedule, ZM241385 (20 mg/kg, i.p.) was injected 2 h before the daily operant

experiment. Operant experiments with ZM241385 treatments (3 d) were compared with baseline responding (2 d) for sucrose or ethanol solution.

Operant responding for ethanol in response to HSV-dnCREB. To assess goal-directed behavior and the transition to habitual behavior leading to ethanol seeking in response to dnCREB, C57BL/6J mice were trained to self-administer a 20% sucrose solution at FR1 followed by RR10, as described above (Dickinson et al., 2002; Hilario et al., 2007). Next, mice were microinjected with the dominant-negative form of CREB-expressing viral vector (HSV-dnCREB) bilaterally to the DMS (AP: 0.5 mm; ML: ± 1.0 mm; DV: 1.5 mm) or DLS (AP: 0.5 mm; ML: ± 2.5 mm; DV: 2.3 mm), whereas control mice were injected with an HSV-GFP vector in the DMS. One day after the microinjection, baseline nose poking for sucrose reinforcement (RR10) was measured for 2 d to ensure that the microinjections did not impair operant responding. Once this was verified, 10% ethanol was added to the sucrose solution reinforcer to measure ethanol self-administration (2 d). To find evidence of habitual responding behavior, devaluation of responding for ethanol by aversion conditioning was conducted (2 d), followed by extinction of responding for ethanol (2 d), in addition to a final reacquisition (2 d) of responding behavior. Figure 4C shows operant task progression. Aversion conditioning was induced by a 20 mg/kg intraperitoneal injection of 0.15 M LiCl after each operant conditioning session and before being returned to the home cage. Each extinction session had a duration of 8 min when responding was not reinforced. For the final reacquisition sessions, responding for ethanol was again reinforced. To compare the efficacy of the HSV-dnCREB treatment and its behavioral effects, all mice were retested 1 month later in each of the operant conditioning paradigms described above (Fig. 4C).

Histology. After the behavioral experiments, mice implanted with cannulas were examined for accuracy of these placements. Brains were perfused with 4% PFA and a 30% sucrose solution, cut in coronal sections measuring 50 μ m thick, and stained with hematoxylin and eosin. Mice in which cannula placements were not in the target region were removed from data analysis and $\sim 10\%$ of the data were thus excluded. We also confirmed the location of viral-mediated gene expression using HSV-GFP histologically (Fig. 4F).

Two-bottle choice ethanol self-administration. Oral alcohol self-administration and preference were examined using a two-bottle choice experiment. Mice were given 24 h access to two bottles, one containing plain tap water and the other containing an ethanol solution. The concentration of ethanol was raised every fourth day, increasing from 3–6 to 10% (v/v) ethanol. Ethanol consumption was calculated by dividing the average ethanol consumption (standardized for evaporation) during each ethanol concentration and vehicle or $A_{2A}R$ ligand injection period by body weight. To examine the effect of ZM241385 or CGS21680 on ethanol drinking, the volume of ethanol consumed in response to saline, 10 and 20 mg/kg intraperitoneal ZM241385, or 1 mg/kg saline, and 2 mg/kg CGS21680 for 4 d after the 10% ethanol period in the two-bottle drinking experiment were compared. Ethanol preference was calculated at each ethanol concentration and treatment by dividing the total ethanol solution consumption at each ethanol concentration or treatment (standardized for evaporation) by the total fluid (ethanol plus water) consumed. Taste differences were assessed using saccharin (0.03%) or quinine (15 μ M) drinking compared with water drinking.

Statistical analysis. Data are presented as the mean $\pm$ SEM. Statistical analyses were performed using unpaired two-tailed *t* test (Prism 5 software; GraphPad), one- or two-way ANOVA (SigmaStat Version 3.1 software; Systat). Results were considered significantly different when $p < 0.05$.

Results

$A_{2A}R$ regulates PKA signaling in the DMS of ENT1^{−/−} mice

We first examined whether decreased adenosine levels as a result of ENT1 deletion (Kim et al., 2011; Nam et al., 2011) reduces $A_{2A}R$ -mediated PKA activity in the striatum of ENT1^{−/−} mice (Fig. 1A). Levels of the active form of PKA, phospho-PKA (Thr197), were significantly decreased in the DMS of ENT1^{−/−} mice compared with ENT1^{+/+} mice (Fig. 1B; $t = 2.9$, $p < 0.05$),

whereas no significant change was observed in the DLS (Fig. 1C). To verify whether reduced $A_{2A}R$ signaling decreases pPKA levels, pPKA levels in response to an $A_{2A}R$ antagonist (ZM241385, 20 mg/kg, i.p.) in the DMS were examined. Inhibition of $A_{2A}R$ decreased pPKA levels in ENT1^{+/+} mice, which was similar to the basal level of pPKA in ENT1^{−/−} mice ($t = 3.9$, $p < 0.05$). In contrast, no additional reduction of pPKA levels was observed in ENT1^{−/−} mice (Fig. 1B). Total PKA protein expression was similar between genotypes and in response to ZM241385 treatment in the DMS (Fig. 1B) and the DLS (data not shown). In addition, the possibility of alterations in other extracellular neurotransmitter levels was investigated. Extracellular glutamate (Nam et al., 2010), dopamine, and serotonin levels (data not shown) measured by microdialysis were similar between genotypes in the dorsal striatum. Therefore, our results suggest that the lack of adenosine transport by ENT1 deletion results in the reduction of $A_{2A}R$ -mediated PKA activity in the DMS.

Decreased $A_{2A}R$ -mediated CREB activity in the DMS

CREB transcriptional activity, one major transcription factor downstream of PKA, was investigated by measuring the active form of CREB, which is phosphorylated on serine 133 (pCREB). Interestingly, pCREB protein levels were reduced in ENT1^{−/−} mice (Fig. 1D), whereas total CREB protein expression was similar between genotypes. To further investigate *in vivo* CREB activity in the DMS, mice expressing β -galactosidase (lacZ) under the control of seven-repeated CRE sites in both genotypes (CRE-lacZ/ENT1^{+/+} mice and CRE-lacZ/ENT1^{−/−} mice) were used. Consistent with the observation of decreased pCREB protein levels in the DMS, lacZ expression was significantly reduced in the DMS of CRE-lacZ/ENT1^{−/−} mice compared with that of CRE-lacZ/ENT1^{+/+} mice (Fig. 1E; $t = 4.3$, $p < 0.05$). To confirm that this reduction in CREB activity was a result of decreased $A_{2A}R$ activation, an $A_{2A}R$ antagonist (ZM241385) was microinjected into the DMS to determine whether this would reduce the CREB activity in CRE-lacZ mice. CREB activity was significantly reduced in the DMS 2 h after ZM241385 microinjection (10 μ M) compared with vehicle microinjection (Fig. 1F; $t = 3.4$, $p < 0.05$), whereas no change was observed in the DLS or nucleus accumbens after microinjection to the DMS. These *in vivo* results indicate that decreased CREB activity in the DMS is a result of reduced $A_{2A}R$ activation, which may be an important component contributing to the behavioral phenotype observed in ENT1^{−/−} mice.

$A_{2A}R$ inhibition increases initial acquisition of operant conditioning

Because the DMS has been suggested to be mainly involved in the action-outcome contingency (Yin and Knowlton, 2006), $A_{2A}R$ -mediated CREB activity in the DMS was investigated in goal-directed behavior or the development of habitual behavior, which could underlie excessive ethanol drinking. As described previously (Yin et al., 2004; Hilario et al., 2007), mice were placed in an operant chamber and trained to nose poke for 20% sucrose using an RR schedule, which measures goal-directed instrumental performance. Compared with their wild-type littermates, ENT1^{−/−} mice had a higher baseline rate of nose poking for sucrose during early training (RR10) and developed a significantly faster rate during the late stage of training (RR20), indicating an increased motivation for the ENT1^{−/−} mice to perform behaviors leading to reinforcement even as the probability for this reinforcement decreased (Fig. 2A). Two-way ANOVA indicated a significant effect of genotype ($F_{(1,95)} = 6.74$, $p < 0.05$) and

Figure 1. Decreased $A_{2A}R$ -mediated CREB activity in the DMS of $ENT1^{-/-}$ mice. **A**, Schematic showing brain regions in which tissue samples were taken from the DMS and DLS for Western blot analysis. **B**, Decreased pPKA (T197) in the DMS ($t = 2.9, p < 0.05$) of $ENT1^{-/-}$ mice. No change in total PKA protein expression was observed. $A_{2A}R$ antagonist (ZM241385, 20 mg/kg, i.p.) decreased pPKA levels in $ENT1^{+/+}$ mice to a level similar to that of $ENT1^{-/-}$ mice ($t = 2.7, p < 0.05$), normalizing PKA activity between genotypes ($n = 8$). * $p < 0.05$ compared with $ENT1^{+/+}$ mice after normalization by GAPDH (unpaired, two-tailed t test). **C**, No significant pPKA or total PKA levels change were detected in the DLS. **D**, pCREB (Ser133; $t = 3.4, p < 0.05$) levels were reduced in the DMS of $ENT1^{-/-}$ mice ($n = 8$). * $p < 0.05$ compared with $ENT1^{+/+}$ mice after normalization by GAPDH (unpaired, two-tailed t test). **E**, Decreased CRE-driven lacZ expression in the DMS of CRE-lacZ/ $ENT1^{-/-}$ mice compared with CRE-lacZ/ $ENT1^{+/+}$ mice ($n = 6; t = 4.3, p < 0.05$). **F**, DMS-specific $A_{2A}R$ inhibition (ZM241385, microinjection, 10 μ M, 2 h) decreased the CREB activity in CRE-lacZ mice compared with that of vehicle control ($n = 5; t = 3.2, p < 0.05$). Representative DMS coronal brain sections of lacZ expression are shown by X-Gal staining. Scale bar, 100 μ m. All data are presented as the mean $\pm$ SEM.

Figure 2. Inhibition of $A_{2A}R$ enhances the initial acquisition of operant responding for sucrose solution. **A, B**, ENT1^{-/-} mice showed an increased rate of initial acquisition in both the RR (**A**) and RI (**B**) schedules compared with that of ENT1^{+/+} mice ($n = 16$). * $p < 0.05$ by two-way measures ANOVA followed by Tukey *post hoc* analysis; # $p < 0.05$ for the main effect of genotype. **C**, The rate of initial acquisition (RR10) was significantly increased by ZM241385 treatment (ZM241385, 20 mg/kg, i.p.) in ENT1^{+/+} mice ($t = 2.2$, $p < 0.05$), whereas no significant change was observed in ENT1^{-/-} mice. **D**, Neither genotype showed any changes during an extended RR (RR20) schedule in response to $A_{2A}R$ antagonist ($n = 8$). All data are presented as mean $\pm$ SEM.

Figure 3. DMS-specific inhibition of $A_{2A}R$ enhances the initial acquisition of operant responding for sucrose solution. **A**, Schematic representation of the microinjection cannula placements within the DMS and DLS in coronal sections (Franklin and Paxinos, 2007). The numbers on each illustration indicate millimeters from bregma. The location of the microinjector tips is represented by circles for DMS and rhombuses for DLS. **B**, DMS-specific $A_{2A}R$ inhibition using ZM241385 (10 μ M microinjection for 3 d) enhanced the nose poking for initial acquisition of goal-directed response (RR10) in C57BL/6J mice (Tukey *post hoc* analysis, two-way ANOVA), whereas no significant nose-poking change was observed after ZM241385 treatment in the DLS ($n = 6$). All data are presented as mean $\pm$ SEM.

schedule ($F_{(4,95)} = 3.6$, $p < 0.05$) on the nose-poking rate, with no interaction between genotype and schedule. The effect of ENT1 deletion on the acquisition of habitual responding was also examined using an RI schedule during operant conditioning (Fig. 2B). Two-way ANOVA indicated an effect of genotype ($F_{(1,83)} =$

20.4, $p < 0.05$) and schedule ($F_{(4,83)} = 21.4$, $p < 0.05$) on the nose-poking rate, with a significant interaction between genotype and schedule ($F_{(4,83)} = 8.9$, $p < 0.05$). Tukey *post hoc* test revealed that ENT1^{-/-} mice showed a higher nose-poking rate for sucrose compared with ENT1^{+/+} mice during the late stage of the RI60 schedule. This pattern of reinforcement may suggest an increased motivation for ENT1^{-/-} mice to actively engage in compulsive-like responding in both goal-directed and habitual behaviors (Fig. 2B).

To investigate the role of $A_{2A}R$ inhibition in the development of instrumental acquisition and goal-directed action, the ability of ZM241385 to enhance the instrumental performance of ENT1^{+/+} mice to a level similar to that of ENT1^{-/-} mice was assessed. ZM241385 treatment increased the nose-poking rate for sucrose during the early period of an RR schedule (RR10) in ENT1^{+/+} mice ($t = 2.1$, $p < 0.05$), whereas ENT1^{-/-} mice showed no significant changes in response to the treatment (Fig. 2C). The late period of responding during an RR schedule (RR20) was unchanged by ZM241385 (Fig. 2D). To confirm the role of DMS-specific attenuation of $A_{2A}R$ -mediated signaling on the enhancement of the early stage of goal-directed action, the nose-poking rate for sucrose after the DMS-specific ZM241385 microinjection was investigated using C57BL/6J mice and compared with that in response to DLS microinjection (Fig. 3A). Two-way ANOVA showed an effect of brain region ($F_{(1,71)} = 14.0$, $p < 0.05$), treatment ($F_{(8,71)} = 10.6$, $p < 0.05$), and the interaction between brain region and treatment ($F_{(8,71)} = 3.8$, $p < 0.05$). Tukey *post hoc* analysis only revealed significantly increased nose poking for sucrose after DMS-specific ZM241385 microinjection (d 7–9), whereas no significant changes in nose poking were observed during ZM241385 treatment in the DLS (Fig. 3B). Overall, these findings demonstrate that the absence of ENT1 or inhibition of $A_{2A}R$ signaling in the DMS enhances the initial acquisition of goal-directed action and may facilitate the transition to habitual behavior.

Attenuation of $A_{2A}R$ -mediated CREB activity in the DMS promotes goal-directed ethanol intake

Whether excessive ethanol intake was under the control of goal-oriented behavior through $A_{2A}R$ -mediated CREB activity was also investigated. Similar to what was observed when sucrose solution was used as the reinforcer, ZM241385 treatment increased nose poking for 10% ethanol solution during the early period of an RR schedule (RR10) in ENT1^{+/+} mice ($t = 2.2$, $p < 0.05$; Fig. 4A), whereas there was no significant change in response to treatment in ENT1^{-/-} mice. Consistently, the late period of responding during an RR schedule (RR20) was unchanged by ZM241385 (Fig. 4B). Overall, these data indicate that the increased ethanol intake is causally related to decreased $A_{2A}R$ activation during the initial acquisition of goal-directed behavior.

Because *in vivo* CREB activity in the DMS was decreased by both ENT1 deletion and $A_{2A}R$ signaling attenuation, the association of decreased CREB activity in the DMS with ethanol-seeking behaviors was examined using operant conditioning (Dickinson et al., 2002; Hilario et al., 2007). To generalize the

behavioral role of CREB in the DMS, C57BL/6J mice, a commonly used inbred strain for operant behaviors (Lederle et al., 2011), were used. The operant conditioning experiment (RR10) was performed after viral expression of HSV-dnCREB or control (HSV-GFP) and was repeated in the same mice 1 month later (clearance of virus) to confirm that the observed behaviors were really mediated by CREB activity (Fig. 4C). Initially, mice were trained to nose poke for a 20% sucrose solution reinforcer. Mice were then bilaterally microinjected with HSV-dnCREB into the DMS and control mice were injected with HSV-GFP to confirm viral expression location, as shown in Figure 4F. To identify whether the ethanol seeking was associated with decreased CREB activity, basal sucrose seeking was measured and 10% ethanol was added into the sucrose solution to measure operant responding for ethanol. Then, to assess the effect of dnCREB on habit formation, the mice were subjected to aversion conditioning (Dickinson et al., 2002), followed by extinction sessions during which responding was not reinforced. One-way ANOVA demonstrated that control mice (HSV-GFP expression in the DMS) showed a significant devaluation effect on ethanol seeking after aversion conditioning using LiCl during the extinction period ($F_{(3,48)} = 3.5, p < 0.05$; Fig. 4D). Consistently, inhibition of CREB activity with dnCREB (HSV-dnCREB) in the DMS precluded habitual responding because these mice displayed a normal extinction on operant responding ($F_{(3,87)} = 6.1, p < 0.05$). Multiple-comparison analysis by Tukey *post hoc* analysis revealed that dnCREB significantly increased the nose-poking rate for ethanol compared with basal sucrose responding (Fig. 4D). To confirm that CREB mediated the observed behaviors in DMS, operant conditioning was repeated after the clearance of HSV-dnCREB gene expression in the DMS. These mice displayed a significant extinction effect on operant responding ($F_{(3,39)} = 8.4, p < 0.05$) without enhanced ethanol seeking. Overall, these data provide strong evidence in favor of the claim that dampened CREB activity in the DMS is causally related to increased goal-directed ethanol intake in C57BL/6J mice.

Conversely, decreased CREB activity by HSV-dnCREB in the DLS showed no significant effect on ethanol seeking behavior. However, no significant reduction in nose poking for ethanol was observed during the extinction period. This may

Figure 4. Attenuation of $A_{2A}R$ -mediated CREB activity in the DMS promotes goal-directed behavior in operant responding for ethanol solution. **A**, Early acquisition (RR10) for 10% ethanol solution was increased by ZM241385 treatment (20 mg/kg, i.p.) in ENT1^{+/+} mice ($t = 2.2, p < 0.05$), whereas no significant change was observed in ENT1^{-/-} mice ($n = 7$). **B**, Neither genotype showed any changes from ZM241385 during an extended RR (RR20) schedule in operant conditioning using ethanol solution ($n = 10$). All data are presented as mean $\pm$ SEM. **C**, Schematic illustrating the experimental procedures for operant conditioning for ethanol seeking in response to HSV-dnCREB. Mice were trained to respond on sucrose reward and then HSV-dnCREB was infused into DMS or DLS using stereotaxic surgery. After surgery, operant responding for sucrose solution measured basal nose poking (S) and then compared with response for 10% ethanol in sucrose solution (S + E), extinction (Ex) and reacquisition (Re). The operant conditioning was repeated after 1 month to compare the effect of dnCREB clearance in operant responding. **D**, Mice injected with dnCREB (HSV-dnCREB) in the DMS showed significantly increased ethanol seeking and decreased nose poking during the extinction period, whereas control mice with control (HSV-GFP) or inactive dnCREB in the DMS showed significantly decreased nose poking during the extinction. **E**, Mice injected with dnCREB (HSV-dnCREB) in the DLS showed habitual nose poking during the extinction, whereas mice injected with inactive dnCREB showed significantly decreased nose poking during the extinction ($n = 8-12$). **F**, Schematic representation of viral vector microinjection coordinates (Franklin and Paxinos, 2007) with HSV-GFP expression in coronal sections for DMS. HSV-GFP expression in the DMS is marked by arrows. The AP number on each illustration indicates millimeters from bregma. The placements of the injection needle are represented by circles for DMS and rhombuses for DLS. Scale bar, 200 μ m. LV indicates lateral ventricles; LSN, lateral septa nuclei. All data are presented as mean $\pm$ SEM.

Figure 5. $A_{2A}R$ inhibition increases ethanol self-administration similar to that of $ENT1^{-/-}$ mice. **A**, Ethanol consumption was increased in $ENT1^{+/+}$ mice with ZM241385 treatment, whereas no changes in $ENT1^{-/-}$ mice were observed. **B**, Ethanol preference was also increased in $ENT1^{+/+}$ mice with 20 mg/kg ZM241385 treatment ($n = 13$). S indicates saline; 10, 10 mg/kg, i.p.; and 20, 20 mg/kg, i.p. * $p < 0.05$ for the main effect of genotype; * $p < 0.05$ for the main effect of each treatment. **C**, Taste preference for saccharin or quinine was not changed in response to ZM241385 treatment (20 mg/kg, i.p.) in either genotype ($n = 13$). **D**, Ethanol consumption was significantly decreased in $ENT1^{-/-}$ mice with a 2 mg/kg concentration of $A_{2A}R$ agonist (CGS21680), whereas $ENT1^{+/+}$ mice showed no response to treatment. **E**, Treatment with 2 mg/kg CGS21680 decreased ethanol preference in $ENT1^{-/-}$ mice ($n = 15$ – 18). **F**, Taste preference for saccharin or quinine was not changed by CGS21680 treatment (2 mg/kg, i.p.) in either genotype. All data are presented as mean $\pm$ SEM.

implicate increased habitual or compulsive behavior by decreased CREB activity in the DLS. After the expiration of HSV-dnCREB gene expression (1 month later) in the DLS, one-way ANOVA demonstrated that these mice displayed a significant extinction effect after aversion conditioning using LiCl during the extinction period ($F_{(3,39)} = 8.1$, $p < 0.05$), similar to that of control HSV-GFP and HSV-dnCREB in the DMS (Fig. 4E).

These results demonstrate that reduced CREB activity in the DMS results in excessive ethanol seeking, whereas no difference was observed during either the extinction or the reacquisition periods. However, reduced CREB activity in the DLS when mice were trained with an RR10 operant schedule appears to result in increased habitual or compulsive behavior during the extinction period. Therefore, decreased $A_{2A}R$ -mediated CREB signaling in the DMS may be essential in promoting ethanol drinking through the control of goal-directed behaviors.

$A_{2A}R$ inhibition promotes ethanol drinking

The ability of an $A_{2A}R$ antagonist, ZM241385, which decreases $A_{2A}R$ signaling and CREB-mediated gene expression, to regulate overall ethanol consumption and preference using a two-bottle choice self-administration experiment was examined. $ENT1^{-/-}$ mice showed increased basal ethanol consumption ($t = 3.3$, $p < 0.05$) compared with $ENT1^{+/+}$ mice, as described previously (Choi et al., 2004; Chen et al., 2010; Nam et al., 2011). A 20 mg/kg ZM241385 treatment induced excessive ethanol drinking in $ENT1^{+/+}$ mice (Fig. 5A). Two-way ANOVA showed an effect of genotype ($F_{(1,87)} = 21.9$, $p < 0.05$) and ZM241385 dose ($F_{(2,87)} = 3.4$, $p < 0.05$) with no interaction. One-way ANOVA analysis revealed that ZM241385 increased ethanol consumption in a

dose-dependent manner ($F_{(2,52)} = 4.0$, $p < 0.05$) in $ENT1^{+/+}$ mice because the Tukey *post hoc* analysis revealed increased ethanol drinking in $ENT1^{+/+}$ mice by 20 mg/kg intraperitoneal ZM241385, whereas no significant effect was observed in $ENT1^{-/-}$ mice ($F_{(2,35)} = 0.8$, $p = 0.45$; Fig. 5A). Similarly, ZM241385 increased ethanol preference in $ENT1^{+/+}$ mice ($F_{(2,47)} = 4.9$, $p < 0.05$), because the Tukey *post hoc* analysis showed increased ethanol preference in $ENT1^{+/+}$ mice by 20 mg/kg intraperitoneal ZM241385, but not in $ENT1^{-/-}$ mice ($F_{(2,35)} = 0.2$, $p = 0.84$; Fig. 5B). Taste preference for saccharin or quinine was similar between genotypes during ZM241385 treatment (Fig. 5C). These results suggest that $A_{2A}R$ inhibition increases ethanol drinking in $ENT1^{+/+}$ mice and this was similar to basal drinking levels of $ENT1^{-/-}$ mice.

Whether $A_{2A}R$ activation using a specific agonist, CGS21680, decreases ethanol consumption or preference via $A_{2A}R$ activation was also investigated. $ENT1^{-/-}$ mice showed increased basal ethanol consumption compared with wild-type littermates ($t = 2.5$, $p < 0.05$) and showed a dose-dependent decrease in ethanol consumption when treated with CGS21680 (Fig. 5D). Two-way ANOVA showed an significant effect of genotype ($F_{(1,89)} = 6.6$, $p < 0.05$) and CGS21680

dose ($F_{(2,89)} = 3.5$, $p < 0.05$) with no interaction. One-way ANOVA analysis revealed that CGS21680 decreased ethanol consumption in a dose-dependent manner ($F_{(2,39)} = 7.2$, $p < 0.05$) in $ENT1^{-/-}$ mice, because the Tukey *post hoc* analysis revealed decreased ethanol drinking in $ENT1^{-/-}$ mice by 2 mg/kg intraperitoneal CGS21680, whereas $ENT1^{+/+}$ mice showed no response to CGS21680 treatment ($F_{(2,49)} = 2.3$, $p = 0.11$; Fig. 5D). One-way ANOVA analysis revealed that CGS21680 treatment decreased ethanol preference in $ENT1^{-/-}$ mice ($F_{(2,32)} = 9.4$, $p < 0.05$), because the Tukey *post hoc* analysis revealed decreased ethanol preference in $ENT1^{-/-}$ mice by 2 mg/kg intraperitoneal CGS21680, but not in $ENT1^{+/+}$ mice ($F_{(2,56)} = 2.7$, $p = 0.08$; Fig. 5E). Taste preference for saccharin or quinine was similar between genotypes during CGS21680 treatment (Fig. 5F). These results support the notion that reduced striatal $A_{2A}R$ activation underlies excessive ethanol consumption and preference in response to decreased striatal adenosine levels by the deletion of $ENT1$. Therefore, $A_{2A}R$ regulation may be a possible pharmacological strategy to prevent the escalation of excessive ethanol drinking.

Discussion

In the present study, we have demonstrated that increased ethanol drinking and faster acquisition of goal-directed behavior in $ENT1^{-/-}$ mice are correlated with decreased adenosine-mediated $A_{2A}R$ signaling in the DMS. Interestingly, inhibiting DMS $A_{2A}R$ promoted goal-directed behavior and increased sucrose or ethanol seeking in an operant self-administration experiment. Hypo- $A_{2A}R$ function in the DMS of $ENT1^{-/-}$ mice may lead to increase goal-oriented behavior, which is an early step

toward habit formation in response to positive reinforcers and may result in an irreversible habit (Graybiel, 2008).

The DMS appears to play a role in outcome devaluation and instrumental learning (Yin et al., 2004, 2005), critical components in the evaluation of actions and anticipation of the consequences of a particular behavior (i.e., an action–outcome relationship). Interestingly, ethanol can reverse the direction of long-term plasticity in the DMS (Yin et al., 2007), which implies that altered signaling in the DMS is involved in the acquisition of action–outcome contingencies or ethanol-related behaviors. In this study, we demonstrated that $ENT1^{-/-}$ mice more readily acquire instrumental responding for sucrose via RR and RI schedules of reinforcement compared with $ENT1^{+/+}$ mice, even though there was no changes in taste preference. Because the ratio schedule delivers rewards by a contingent number of responses, it produces the development of an action–outcome association. In contrast, because interval schedules deliver rewards in a specific time interval after response, it produces the development of behaviors similar to habit (Yin et al., 2004; Yin and Knowlton, 2006; Wiltgen et al., 2012). Because we observed that $ENT1^{-/-}$ mice exhibited enhanced goal-directed behavior (RR) and subsequent habit formation (RI), decreased adenosine concentration in the striatum may contribute to both faster establishment of goal-directed behavior and premature transition or susceptibility to habit formation.

Furthermore, our results indicate that $A_{2A}R$ plays an essential role in the early acquisition phase of instrumental responding, because ZM241385 treatment in $ENT1^{+/+}$ mice mimicked the faster initial acquisition of instrumental conditioning (RR10) in $ENT1^{-/-}$ mice to both natural reward by sucrose and hedonic reward by ethanol. Therefore, our results support an essential role of $A_{2A}R$ signaling in both habit formation for natural reward and addiction, suggesting similar behavioral control through corticostriatal circuit in both processes. Recent studies have shown that habit formation for natural reward is regulated by striatopallidal neurons of the “indirect pathway” in the basal ganglia circuit (Fuxe et al., 2007; Lovinger, 2010). Striatopallidal-specific $A_{2A}R$ deletion increased goal-directed behavior for food (Yu et al., 2009) and reduced striatopallidal cAMP levels, which are correlated with faster acquisition of the reinforcer and a higher rate of lever pressing with extensive training in an instrumental conditioning experiment in mice (Lobo et al., 2007). Moreover, in our study, DMS-specific $A_{2A}R$ attenuation promotes initial acquisition for sucrose reward in habit formation, which highlights a role of $A_{2A}R$ regulation in natural reward seeking operant behavior. In addition, the “indirect pathway” also regulates inhibitory control over drug reward processes (Durieux et al., 2009) and alteration of glutamatergic synaptic plasticity may be essential for habit formation in addiction. Intriguingly, because $A_{2A}R$ activation facilitates glutamatergic synaptic plasticity in striatopallidal neurons and acute ethanol reverses glutamatergic synaptic plasticity in the DMS (Yin et al., 2007; Yu et al., 2009), $A_{2A}R$ attenuation appears to be important in positive reinforcement in the early stage of habit formation toward ethanol reward. Conversely, because striatal $A_{2A}R$ signaling is also associated with dopaminergic innervations through its interaction with dopamine D_2 receptors in striatopallidal neurons (Durieux et al., 2009; Fuxe et al., 2010), investigating how $A_{2A}R$ -mediated neuroadaptation regulates ethanol-specific behaviors or involves in other drugs of abuse in altered circuits with other brain regions is warranted. Nonetheless, $A_{2A}R$ signaling in striatopallidal neurons is essential to the regulation of behaviors directed toward the acquisition of natural and ethanol reward.

Figure 6. Schematic representation of the possible mechanism in the DMS for goal-directed ethanol seeking behaviors through $A_{2A}R$ –CREB-mediated signaling.

Agreeing with our findings, striatal-specific $A_{2A}R^{-/-}$ mice have been found previously to show increased goal-directed behavior (Yu et al., 2009) and to consume more alcohol compared with wild-type littermates (Naassila et al., 2002), which further supports that the dampening of $A_{2A}R$ signaling in the DMS plays an essential role in increased alcohol drinking in $ENT1^{-/-}$ mice. Consistently, daily treatment of ZM241385 (20 mg/kg, i.p., for 4 d) increased ethanol consumption and preference in $ENT1^{+/+}$ mice to a level similar to that of $ENT1^{-/-}$ mice in a two-bottle choice experiment, suggesting that $ENT1$ deletion or $A_{2A}R$ inhibition promotes increased goal-directed ethanol drinking, which may be implicated in “binge alcohol drinking.” Repeated ethanol exposure during goal-directed alcohol drinking may lead to the development of habitual ethanol-seeking behavior as a result of enhanced pleasurable effects, which outweigh the aversive effects of ethanol.

Interestingly, the adenosine receptor antagonist caffeine is known to increase ethanol drinking (Kunin et al., 2000) and a recent co-crystal structural study revealed that ZM241385 and caffeine have similar pharmacological binding properties to $A_{2A}R$ (Dore et al., 2011). Because caffeine is known to block the $A_{2A}R$ -mediated PKA signaling pathway, but not the A_1R (Lindskog et al., 2002; Huang et al., 2005), our observations demonstrate the importance of the interaction of ethanol with the $A_{2A}R$ system and explain why concurrent intake of ethanol and caffeine may lead to heavier drinking patterns and greater prevalence of goal-directed binge alcohol drinking (Butler and Prendergast, 2012). Further investigation is needed to elucidate the pharmacological

regulation by caffeine and brain-region-specific A_{2A}R regulation in alcohol use disorders and related behavioral disorders.

Because CREB is downstream of A_{2A}R signaling, viral-mediated CREB gene regulation was examined to investigate DMS-specific ethanol related behaviors. To determine how the hedonic effect of ethanol is associated with goal-directed ethanol intake, we performed operant conditioning for ethanol seeking and extinction during the active HSV-dnCREB viral expression period (Carlezon et al., 1998). We measured extinction after aversion conditioning by pairing it with LiCl (Dickinson et al., 2002) instead of reinforcer devaluation in the home cage. Although ethanol has a hedonic component, its aversive effect in taste might dampen the initial motivation to consume ethanol in the home cage during the HSV virus expression period. Decreased CREB activity in the DMS promotes excessive ethanol seeking and goal-directed responding during the ethanol acquisition phase, which was not observed in the HSV-GFP control and after the clearance of viral expression. Decreased CREB activity in the DLS significantly increased nose poking during the extinction period compared with DMS controls but not after the clearance of viral expression, which suggests that decreased CREB in the DLS may promote habitual or compulsive seeking behaviors that are not regulated by A_{2A}R. Consistent with our findings, the decreased CREB-mediated gene product in the DLS was shown to promote ethanol drinking, whereas sucrose intake was shown to be regulated by the DMS in mice (Jeanblanc et al., 2009). Interestingly, chronic ethanol exposure has been shown to induce hypo-CREB activity in the striatum and may lead to the development of habitual ethanol seeking through reduced CREB activity in both the DMS and DLS (Yang et al., 1998; Wand et al., 2001; Lonze and Ginty, 2002).

In summary, our findings demonstrate that reduced A_{2A}R-mediated CREB activity in the DMS is associated with increased vulnerability to both goal-directed behavior and excessive ethanol drinking (Fig. 6). Our results reveal a novel A_{2A}R molecular signaling pathway in the DMS that may contribute to the vulnerability of developing habitual ethanol consumption.

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