BEHAVIORAL NEUROSCIENCE

GABA$_A$ receptors mediate the opposing roles of dopamine and the tegmental pedunculopontine nucleus in the motivational effects of ethanol

Ryan Ting-A-Kee,[1,*] Colleen Dockstader,[2,**] Andrew Heinmiller,[3] Taryn Grieder[1] and Derek van der Kooy[1,2,3]
[1]Institute of Medical Science, University of Toronto, ON, Canada
[2]Neurobiology Research Group, Department of Anatomy and Cell Biology, University of Toronto, ON, Canada
[3]Department of Medical Biophysics, University of Toronto, ON, Canada

Abstract

Recent work has demonstrated that changes in ventral tegmental area (VTA) GABA$_A$ receptor ion conductance properties are responsible for switching morphine's positive reinforcing properties from a dopamine-independent to a dopamine-dependent pathway when an animal transitions from a non-deprived (minimal drug exposure) to a dependent (chronic drug exposure) and withdrawn state. Here we show that a double dissociation of ethanol's positive reinforcing properties is exactly opposite to that seen with morphine. In C57BL/6 mice, ethanol-conditioned place preferences were blocked in dopamine D2 receptor knockout non-deprived mice, but not by a lesion of the tegmental pedunculopontine nucleus (TPP). On the other hand, TPP lesions, but not a D2 receptor mutation, blocked ethanol-conditioned place preferences in ethanol-dependent and withdrawn mice. The opposite effects of ethanol and opiates can be explained by their proposed actions through a common VTA GABA$_A$ receptor switching mechanism.

Introduction

The ethanol addiction field contains puzzling evidence for both dopamine-dependent and dopamine-independent reward modulation. Both *in vivo* (Gessa *et al.*, 1985; Yoshimoto *et al.*, 1992; Weiss *et al.*, 1993) and *in vitro* (Brodie *et al.*, 1990; Brodie & Appel, 1998) studies indicate that ethanol increases dopamine release and/or dopamine firing rates. Furthermore, pharmacological or genetic manipulations that diminish dopaminergic activity inhibit ethanol consumption and preference (Ikemoto *et al.*, 1997; El-Ghundi *et al.*, 1998; Phillips *et al.*, 1998), place conditioning (Cunningham *et al.*, 2000; Risinger *et al.*, 2001), the acquisition of ethanol self-administration (Risinger *et al.*, 2000), and ethanol preference and sensitivity (Phillips *et al.*, 1998). Conversely, perfusion of a dopamine uptake inhibitor increased extracellular nucleus accumbens (NAc) dopamine levels by up to 800% that of baseline, and yet still did not alter ethanol-preferring rat drinking behavior (Engleman *et al.*, 2000). Also, established ethanol self-administration was not disrupted in rats with subsequent 6-hydroxydopamine lesions of the NAc (Rassnick *et al.*, 1993a; Ikemoto *et al.*, 1997), and ethanol place preference was not disrupted in mice given the dopamine receptor antagonist haloperidol (Cunningham *et al.*, 1992a; Risinger *et al.*, 1992).

Ethanol can potently influence the activity of ventral tegmental area (VTA) GABA neurons (Charlton *et al.*, 1997; Gallegos *et al.*, 1999; Melis *et al.*, 2002; Theile *et al.*, 2008), and evidence suggests that its actions on VTA GABA$_A$ receptors renders it capable of modulating ethanol reinforcement (Gatto *et al.*, 1994; Nowak *et al.*, 1998). Recent work by Laviolette *et al.* (2004) demonstrated that these receptors were also responsible for switching the neurobiological substrates mediating opiate reinforcement between dopamine-independent and dopamine-dependent systems. The trigger for this switch occurred when animals transitioned from an opiate-non-deprived (minimal previous drug exposure) to an opiate-dependent (substantial previous drug exposure) and withdrawn state.

On the basis of this idea, we hypothesized that both dopamine-dependent and dopamine-independent neurobiological substrates might also mediate ethanol reinforcement, depending on whether an animal is in an ethanol-non-deprived or an ethanol-dependent and withdrawn state. Therefore, in the present study, we used an unbiased, fully counterbalanced place conditioning paradigm to examine this relationship in an attempt to reconcile conflicting data on the role of dopamine in the motivational effects of ethanol.

Correspondence: Dr R. Ting-A-Kee, *Present address below.
E-mail: r_kee@yahoo.com

*Present address: Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Canada M5S 1A8.

**Present address: Neuroscience and Mental Health Program, The Hospital for Sick Children, Toronto, ON, Canada.

Materials and methods

Animals

All mice used in the D2 receptor genetic experiments were congenic N21 (backcrossed 21 times to C57BL/6) adult (25–35 g) male and

female littermates approximately 4 months of age, obtained from heterozygotic breeding pairs. Offspring were propagated at the University of Toronto and Oregon Health and Science University, and genotyped by polymerase chain reaction (Kelly *et al.*, 1997). The D2 receptor knockout mice manifested only minor observable abnormalities as compared with the D2 receptor wild-type mice. The two genotypes displayed no noticeable differences in postnatal development and, at the beginning of the experiments, the weights were similar for both. There were no deficits in the acquisition or expression of basic motor skills, and mice displayed no abnormal posture or tremor. Detailed locomotor activity testing revealed relatively small decreases in rearing activity in D2 receptor knockout mice (Kelly *et al.*, 1998). For all other experiments, male C57BL/6 mice from Charles River were used.

All experiments were conducted at the University of Toronto. Subjects were housed by gender in groups of four in plastic mouse cages in a sound-attenuated room at a temperature of 22 °C with lights on from 07:00 h to 19:00 h. Access to food and water was *ad libitum*, except during the ethanol-dependent and withdrawn experiments, where mice were restricted to Lieber DeCarli's Regular Ethanol or Control Liquid Diets (Dyets Inc., Bethlehem, PA, USA) (Ritzmann & Tabakoff, 1976). All experiments were approved by the University of Toronto Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines.

Surgery

Lesions of the tegmental pedunculopontine nucleus (TPP) were performed bilaterally under isoflurane anaesthesia by injecting 0.1 M *N*-methyl-D-aspartate (Sigma, Oakville, Canada) in a volume of 0.04 μL of physiological saline, with pH adjusted to 7.4. Sham-lesioned control animals received bilateral injections of the physiological saline vehicle. Microinfusions were performed with a 1-μL Hamilton microsyringe (VWR International, Mississauga, Canada) over a 4-min period. The infusion rate was 0.01 μL/min, after which the injector was left in place for an additional 1 min to allow diffusion of the solution from the injector tip. The injection coordinates for the TPP were as follows: from bregma, AP: −4.4 mm, and L: ±1.1 mm; and from the dural surface, V, −3.8 mm. Subcutaneuous ketoprofen (3.0 mg/kg) was administered as an analgesic. Animals were allowed at least 10 days of recovery time before conditioning.

Drugs

Anhydrous ethanol (Commercial Alcohols, Brampton, Canada) was dissolved in 0.9% sterile saline to make a 20% (v/v) solution; the dose was adjusted by altering the volume of this solution and injecting intraperitoneally. Morphine sulfate (Almat Pharmachem Inc., Concord, Canada) was dissolved in a 0.9% saline solution and injected intraperitoneally at 1 mL/kg. (+)-Bicuculline (Sigma) was dissolved in a 0.9% saline solution in the presence of two drops of glacial acetic acid (EMD Chemicals Inc., Gibbstown, NJ, USA), and injected intraperitoneally at 10 mL/kg. Cocaine hydrochloride (BDH Inc., Toronto, Canada) was also dissolved in a 0.9% saline solution and injected intraperitoneally at 10 mL/kg.

Place conditioning apparatus

The place conditioning apparatus consisted of two environments that differed in color and texture, each measuring 15 × 15 × 15 cm. One environment consisted of a black box with a smooth, black plexiglas floor, and the other consisted of a white box with a jagged white plastic floor. Before each conditioning session, the black box was scented with a 3% acetic acid solution (EMD Chemicals Inc.). A removable metal wall separated the two boxes, each side being painted with the corresponding color. The ceilings of the boxes were made of clear, removable plexiglas. Time and activity levels were recorded using three pairs of photobeams set 4 cm apart. There are no baseline biases for either of the two conditioning environments, and there are no biases with the injection procedure itself under saline conditions (Dockstader *et al.*, 2001).

Place conditioning procedure

Mice undergoing ethanol conditioning received 24 conditioning trials (12 alternating drug and vehicle pairings) over 24 days. This treatment regimen does not produce significant signs of withdrawal, and hence can be considered to model an ethanol-non-deprived motivational state. Immediately prior to conditioning, mice were given an intraperitoneal injection of either drug or saline; they were then exposed to one of the two conditioning environments for a 5-min period. For bicuculline experiments, animals were given an intraperitoneal injection of bicuculline (1.0 mg/kg) 1–2 min prior to both ethanol and saline injections. Both treatment compartment and order of drug presentation were fully counterbalanced within all groups. Morphine and cocaine conditioning followed a similar protocol.

Animals in the ethanol-non-deprived groups were given access to Lieber DeCarli's Control Liquid Diet 4 days prior to the commencement of conditioning, replacing rodent chow and water. This access was maintained for the duration of the experiment. Animals in the ethanol-dependent and withdrawn groups were given access to Lieber DeCarli's Ethanol Liquid Diet for 4 days prior to the commencement of conditioning, replacing rodent chow and water. For all of the subsequent conditioning trials, the ethanol liquid diet was removed and replaced with a control liquid diet, 8:00 h prior to each conditioning trial. After 8:00 h without the ethanol liquid diet, animals demonstrate moderate somatic symptoms of withdrawal and show a conditioned place aversion to the withdrawal-paired environment (data not shown). Approximately 1:00 h after conditioning, the ethanol diet was reintroduced and the control diet was removed. This cycle continued for the duration of conditioning.

After the final conditioning trial, mice were allowed to rest uninterrupted in their home cage for 1 week until the test day, and any liquid diets were permanently replaced with rodent chow and water. On the test day, under drug-free conditions, mice were given equal access to both boxes simultaneously by removing the shared wall and introducing the animal into the center of the test area. Time and activity in each environment were recorded over a 10-min period.

Recording somatic signs of withdrawal

In pilot studies, somatic withdrawal signs were observed at 2, 4, 6, 8 and 24 h after removal of ethanol liquid diets, prior to conditioning. Mice expressed the highest degree of somatic withdrawal at 8:00 h post-ethanol removal. To quantify physical dependence, a scaled description was implemented. Dependence and withdrawal were assessed by assigning the following scores in reaction to handling: 0, little or no reaction; 1, piloerection or jerk; 2, weak tremor; 3, severe tremor; 4, seizure; and 5, death while in seizure (Ritzmann & Tabakoff, 1976).

Histology

At the end of the experiments, animals that had undergone lesion surgery were deeply anesthetized with sodium pentobarbital (Animal Resources

Centre, Montreal, Canada) and perfused transcardially with 30 mL of physiological saline followed by 30 mL of 4% formaldehyde. Brains were rapidly removed, and stored for at least 24:00 h in a 25% sucrose/4% formaldehyde post-fixative. Brains were then flash frozen at −80 °C, sliced in a freezing microtome into 40-μm-thick sections, and mounted on gelatin-coated slides. TPP and sham lesions were verified with cresyl violet staining and light microscopy with reference to the atlas of Hof *et al.* (2000). Investigators were blind to the behavioral performance of the animals during lesion analyses. A total of 126 mice (successful sham and lesioned mice) were included in the behavioral analyses. Mice were excluded from the analyses if their lesions were situated outside of the TPP or only encompassed one hemisphere. In these cases, it was observed that mice with misplaced lesions did not differ from the sham-lesioned animals (data not shown). Examination of cresyl violet-stained sections revealed that most lesions (detected via extensive cell gliosis) were localized in the TPP region and resulted in ablation of >50% of the TPP (our minimal definition to be included as a successful lesion). TPP lesions affected both medial and lateral aspects of the TPP, such that cholinergic (Rye *et al.*, 1987), glutamatergic and GABAergic (Nakano, 2000) neurons were ablated.

Statistical analysis

Data were analysed using one-way or two-way ANOVA or Student's *t*-tests, where appropriate ($\alpha = 0.05$). *Post hoc* analysis was performed using the Student–Neuman–Keuls multiple comparison test where appropriate.

Results

The D2 receptor is critical in mediating non-deprived ethanol reinforcement

C57BL/6 mice showed low-dose (0.2 g/kg) preferences for, as well as high-dose (4.0 g/kg) aversions to, the ethanol-paired environments as compared with the saline environments on the test day. An ANOVA revealed a main effect of dose ($F_{4,39} = 6.637$, $P < 0.05$). *Post hoc* Newman–Keuls analyses revealed significant preferences at 0.2 g/kg ($P < 0.05$) and significant aversions at 4.0 g/kg ($P < 0.05$) (Fig. 1).

We subsequently examined the behavior of D2 receptor wild-type and knockout mice in the same paradigm, to determine the role of the dopamine D2 receptor in mediating the motivational effects of ethanol (referred to in this paper as 'ethanol motivation'). Wild-type and mutant mice were conditioned while in an ethanol-non-deprived state. A two-way ANOVA (ethanol dose × genotype) on the place preference scores revealed a significant interaction between genotype and dose ($F_{1,27} = 15.736$, $P < 0.05$) (Fig. 2, left). *Post hoc* Newman–Keuls analyses revealed significant preferences at 0.2 g/kg ($P < 0.05$) and significant aversions at 4.0 g/kg ($P < 0.05$) for only the D2 receptor wild-type mice. D2 receptor mutant mice showed neither 0.2 g/kg ethanol place preferences nor 4.0 g/kg ethanol place aversions (both $P > 0.05$). There was a significant difference between the knockout and wild-type groups at both ethanol doses (both $P < 0.05$). The female/male ratios were approximately the same for each group, and no effects of gender were observed. These results show that the dopamine D2 receptor is critical in mediating both the positive and negative reinforcing effects of ethanol in previously ethanol-naive, non-deprived mice.

Ethanol motivation is D2 receptor-independent in ethanol-dependent and withdrawn mice

To induce ethanol dependence, we gave mice constant access to Lieber DeCarli's Ethanol Liquid Diet, replacing both rodent chow and water.
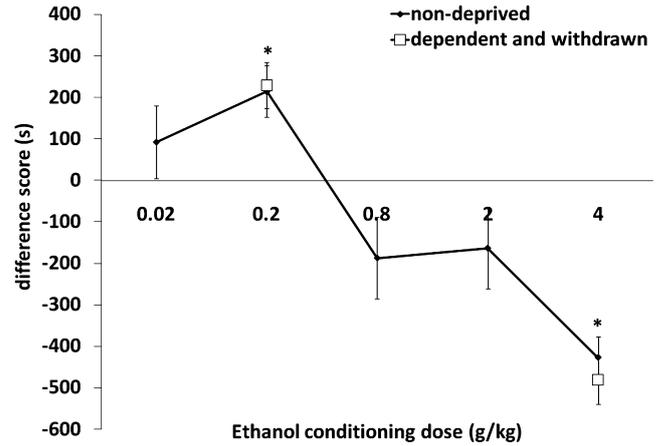

FIG. 1. C57BL/6 mice show conditioned place preferences and aversions to ethanol in both the ethanol-non-deprived and ethanol-dependent and withdrawn states. Conditioned place preferences (positive scores) and aversions (negative scores) were induced by ethanol at a range of doses in previously ethanol-naive, non-deprived (black diamonds) and ethanol-dependent and withdrawn (white squares) C57BL/6 mice. Data represent means of times spent in ethanol-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free and withdrawal-free conditions. *Significant preference for or aversion to the ethanol-paired vs. the saline-paired environment ($P < 0.05$).
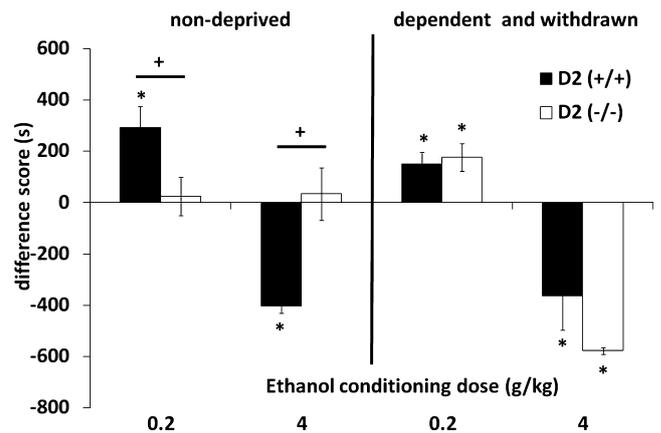

FIG. 2. D2 receptor wild-type, but not knockout, mice show low-dose-conditioned place preferences and high-dose-conditioned place aversions when in the ethanol-non-deprived state, whereas both D2 receptor wild-type and knockout mice show low-dose-conditioned place preferences and high-dose-conditioned place aversions in the ethanol-dependent and withdrawn state. Conditioned place preferences were induced by a low dose of ethanol, and conditioned place aversions by a high dose of ethanol, in ethanol-non-deprived D2 receptor wild-type mice, but not in D2 receptor knockout mice, after a series of intermittent exposures to ethanol. Conversely, when treated in a state of ethanol dependence and withdrawal, both D2 receptor wild-type and knockout mice exhibited conditioned place preferences for a low dose of ethanol and conditioned place aversions to a high dose. Data represent means of times spent in ethanol-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free and withdrawal-free conditions. *Significant preference for or aversion to the ethanol-paired vs. the saline-paired environment ($P < 0.05$). + indicates a significant difference between the two groups ($P < 0.05$).

After 4 days of liquid diet exposure, all animals had regained the weight loss that they incurred immediately after the liquid diet was introduced. At this time, the amount of liquid diet consumed on a daily basis was similar for both D2 receptor wild-type and knockout mice (means ± SEM = 10.0 ± 1.0 mL and 11.3 ± 1.7 mL, respectively)

($t_{1,28} = -0.718$, $P > 0.05$). Similarly, gas chromatography analyses showed that blood ethanol levels of both genotypes were also similar (means ± SEM = 186.51 ± 5.59 mg/dL for wild-type mice and 193.08 ± 7.05 mg/dL for knockout mice) ($t_{1,28} = -0.712$, $P > 0.05$). During the subsequent conditioning trials, mice were in a state of ethanol withdrawal, and demonstrated somatic signs of withdrawal. A two-way ANOVA (genotype × motivation state) revealed that both D2 receptor genotypes demonstrated significantly more somatic signs of withdrawal when in an ethanol-dependent and withdrawn state than when in an ethanol-non-deprived state ($F_{1,29} = 54.092$, $P < 0.05$) and that signs were seen equivalently in both D2 receptor genotypes ($F_{1,29} = 0.866$, $P > 0.05$) (data not shown). There was no significant interaction between genotype and motivation state.

The effects of dependence and withdrawal on ethanol motivation were studied prior to the experiments in genetically modified mice. C57BL/6 mice conditioned while ethanol-dependent and in withdrawal demonstrated a significant preference for the low dose of ethanol (0.2 g/kg) and a significant aversion to the high dose (4 g/kg) (Fig. 1). The behavior of D2 receptor wild-type and knockout mice was subsequently examined in the ethanol-dependent and withdrawn state. A two-way ANOVA (ethanol dose × genotype) on the place preference scores revealed a significant effect of dose ($F_{1,22} = 84.331$, $P < 0.05$) but no effect of genotype ($F_{1,22} = 1.836$, $P > 0.05$) or any interaction between dose and genotype ($F_{1,22} = 2.996$, $P > 0.05$) (Fig. 2, right). *Post hoc* Newman–Keuls analyses revealed significant preferences at 0.2 g/kg ($P < 0.05$) and significant aversions at 4.0 g/kg ($P < 0.05$) for both D2 receptor wild-type and knockout mice. There were no significant differences between the two groups in terms of the sizes of their preferences or aversions ($P > 0.05$). The male/female ratio was approximately the same for each group, and no gender effects were observed. These data demonstrate that the activity of the D2 receptor is not critical in mediating ethanol motivation in ethanol-dependent and withdrawn mice.

Ethanol reinforcement is TPP-independent in ethanol-non-deprived mice

We examined the effect of TPP lesions on ethanol motivation in previously ethanol-naive, non-deprived mice. A two-way ANOVA (ethanol dose × presence or absence lesion) on the place preference scores of ethanol-non-deprived mice with sham or TPP lesions revealed a main effect of dose ($F_{1,45} = 30.569$, $P < 0.05$) but no effect of lesion ($F_{1,45} = 0.0747$, $P > 0.05$) or any interaction effect ($F_{1,45} = 0.0628$, $P > 0.05$). *Post hoc* Newman–Keuls analyses revealed significant preferences at 0.2 g/kg ($P < 0.05$) and significant aversions at 4.0 g/kg ($P < 0.05$) for both sham-lesioned and TPP-lesioned mice (Fig. 3A, left). There were no significant differences between the two groups in terms of the sizes of their preferences or aversions ($P > 0.05$). These data demonstrate that the TPP does not mediate either the positive or negative reinforcing effects of ethanol when animals are conditioned in the ethanol-non-deprived state.

The positive reinforcing effects of ethanol are TPP-dependent in ethanol-dependent and withdrawn mice

As past work has suggested that motivation state influences which neurobiological substrates are important for drug reinforcement (Laviolette *et al.*, 2004), we examined the effect of TPP lesions on ethanol motivation in ethanol-dependent and withdrawn mice. A two-way ANOVA (ethanol dose × presence or absence of lesion) on the
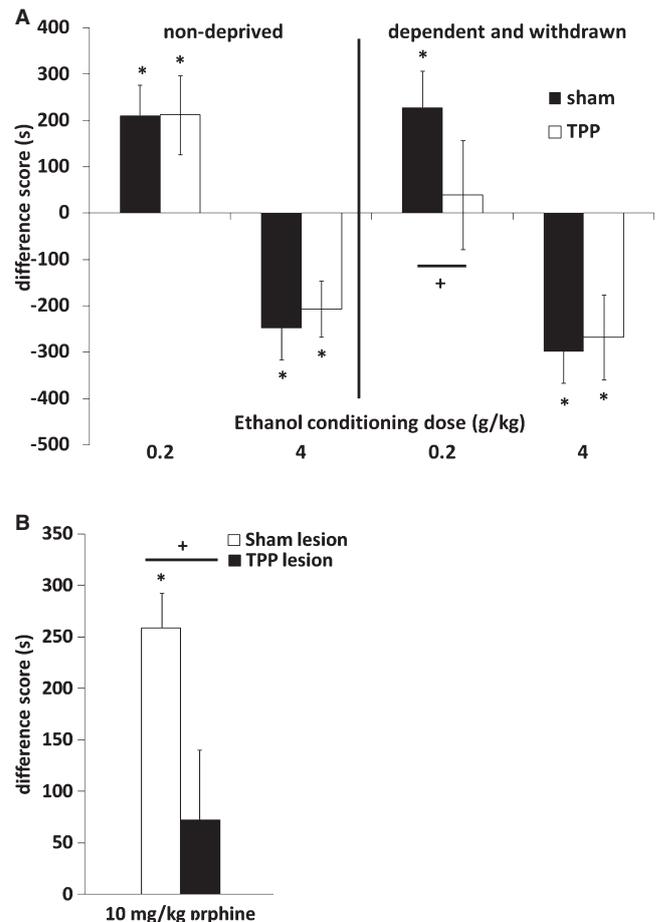

FIG. 3. (A) Sham and tegmental pedunculopontine nucleus (TPP)-lesioned mice show conditioned place aversions in both the ethanol-non-deprived and ethanol-dependent and withdrawn states, and conditioned place preferences in the ethanol-non-deprived state, but only sham-lesioned mice show conditioned place preferences in the ethanol-dependent and withdrawn state. Conditioned place preferences and aversions were induced by low and high doses of ethanol, respectively, in both sham-lesioned and TPP-lesioned non-deprived mice. Separate groups of both sham-lesioned and TPP-lesioned mice were made dependent and withdrawn with ethanol, until dependence developed and somatic withdrawal signs were observed. The sham-lesioned group demonstrated conditioned place preferences when conditioned in a state of withdrawal with low doses of ethanol; however, the TPP-lesioned animals did not. Both groups demonstrated conditioned place aversions to the high dose of ethanol when conditioned while ethanol-dependent and in withdrawal. Data represent means of times spent in ethanol-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free and withdrawal-free conditions. *Significant preference for or aversion to the ethanol-paired vs. the saline-paired environment ($P < 0.05$). + indicates a significant difference between the two groups ($P < 0.05$). (B) TPP-lesioned mice do not show morphine place preferences in the non-deprived state. Conditioned place preferences were induced by morphine (10 mg/kg) in sham-lesioned but not TPP-lesioned mice when conditioned in the non-deprived state. Data represent means of times spent in morphine-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free conditions. *Significant preference for the morphine-paired vs. the saline-paired environment ($P < 0.05$). + indicates a significant difference between the two groups ($P < 0.05$).

place preference scores of ethanol-dependent and withdrawn mice with sham or TPP lesions revealed an interaction between dose and lesion ($F_{1,47} = 3.75$, $P = 0.05$). *Post hoc* Newman–Keuls analyses revealed a significant preference for the sham-lesioned group at 0.2 g/kg of ethanol ($P < 0.05$) but no preference for the TPP-lesioned group ($P > 0.05$), and a significant difference between the two groups

themselves ($P < 0.05$). Both sham-lesioned and TPP-lesioned animals demonstrated significant aversions at 4.0 g/kg (both $P > 0.05$) (Fig. 3A, right).

The amount of Liquid Diet consumed on a daily basis was similar for sham-lesioned and TPP-lesioned mice (means ± SEM = 13.6 ± 2.1 mL and 14.4 ± 1.5 mL) ($t_{1,13} = -0.319$, $P > 0.05$). Gas chromatography analyses showed that blood ethanol levels of both groups were also similar (means ± SEM = 207.33 ± 8.074 mg/dL and 201.59 ± 11.03 mg/dL for sham and TPP-lesioned mice, respectively) ($t_{1,13} = 0.428$, $P > 0.05$). In a separate group of sham-lesioned and TPP-lesioned mice, a two-way ANOVA indicated that significantly more somatic signs were seen when animals were ethanol-dependent and in withdrawal than when they were when non-deprived ($F_{1,29} = 17.387$, $P < 0.05$) and that symptoms were seen equivalently in sham-lesioned and TPP-lesioned mice ($F_{1,29} = 0.597$, $P > 0.05$). These data demonstrate that the TPP mediates the positive reinforcing effects of ethanol only when animals are in an ethanol-dependent and withdrawn state.

The TPP mediates the positive reinforcing effects of morphine in drug-non-deprived mice

To confirm that our lesions were functionally effective, we conditioned separate groups of drug-non-deprived sham-lesioned and TPP-lesioned animals with 10 mg/kg morphine. Whereas sham-lesioned mice showed significant preferences for the morphine-paired environment on the test day, lesions of the TPP blocked these place preferences ($t_{1,21} = 7.576$, $P < 0.05$, and $t_{1,15} = 1.020$, $P > 0.05$, respectively) (Fig. 3B). An ANOVA (sham vs. TPP lesions) on the place preference scores revealed a significant difference between the two surgical treatments ($F_{1,37} = 6.663$, $P < 0.05$). Representative schematic coronal sections showing TPP lesions are presented in Fig. 4.

GABA_A receptors mediate the positive reinforcing effects of ethanol

Within the VTA, GABA_A receptors are primarily localized on GABAergic neurons (Churchill *et al.*, 1992; Kalivas, 1993), a site upstream of both the dopamine and TPP reward output systems. We examined whether systemic blockade of these receptors by a GABA_A receptor antagonist, bicuculline, would have an effect on ethanol reinforcement. Ethanol-non-deprived and ethanol-dependent and withdrawn C57BL/6 mice were pretreated with intraperitoneal bicuculline (1.0 mg/kg) prior to ethanol place conditioning. Bicuculline alone had no motivational effects itself, as determined in a simple place conditioning procedure ($t_{1,7} = -0.27611$, $P > 0.05$) (Fig. 5A, right). A two-way ANOVA (saline or bicuculline pretreatment × motivation state) revealed a main effect of drug treatment ($F_{1,28} = 14.173$, $P < 0.05$) but no significant interaction between drug treatment and motivational state ($F_{1,28} = 3.693$, $P > 0.05$). *Post hoc* Newman–Keuls analyses revealed that bicuculline pretreatment blocked conditioned place preferences for a low dose of ethanol in both ethanol-non-deprived and ethanol-dependent and withdrawn mice (both $P > 0.05$) (Fig. 5A). There was a significant difference between bicuculline-pretreated and saline-pretreated groups in both non-deprived and dependent and withdrawn states (both $P < 0.05$).

To determine whether GABA_A receptor blockade was producing a general learning deficit, we examined the effects of bicuculline pretreatment on cocaine-conditioned (5 mg/kg) place preferences in drug-non-deprived mice. Both saline-pretreated and bicuculline-pretreated mice displayed robust conditioned place preferences for the
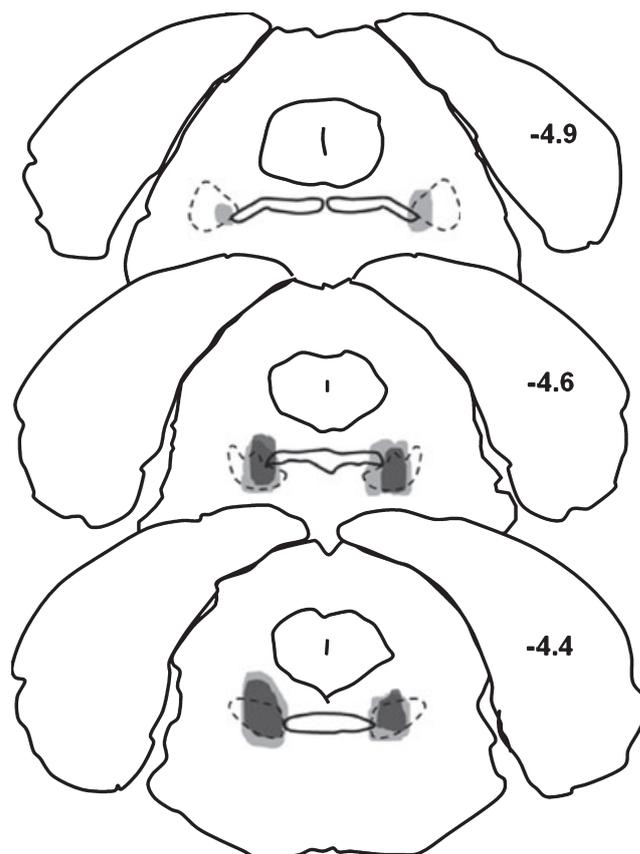

FIG. 4. Schematic coronal sections showing representative lesions of the tegmental pedunculopontine nucleus (TPP). Distance caudal to bregma (mm) is listed for each cross-section. The TPP is shown as the structured outlined with dotted lines. Shaded areas overlapping the TPP indicate sites of representative smallest and largest lesions.

cocaine-paired environment on the test day ($t_{1,7} = 2.530$, $P < 0.05$, and $t_{1,7} = 4.50$, $P < 0.05$, respectively) (Fig. 5B). There was no significant difference between the two groups ($F_{1,14} = 0.819$, $P > 0.05$). Therefore, blockade of the GABA_A receptor did not impede general learning, motor or motivational processing in our mice. Thus, the reinforcing effects of ethanol in both ethanol-non-deprived and ethanol-dependent and withdrawn motivational states are mediated by GABA_A receptors.

Discussion

Ethanol produces both positive and negative reinforcing effects in C57BL/6 mice

A clear dose–response curve for the positive and negative reinforcing effects of ethanol was seen in C57BL/6 mice with the use of our place conditioning paradigm. Historically, ethanol place conditioning in this strain has been difficult to establish, and we suggest that this is due partially to an insufficient exploration of lower ethanol doses. In the current experiments, not only did C57BL/6 mice both approach and avoid environments paired with ethanol, but also, the sensitivity of C57BL/6 mice to ethanol's positive reinforcing effects was an order of magnitude lower than that seen in the DBA/2J mouse strain typically used in ethanol reinforcement paradigms (Cunningham *et al.*, 1992b). Conversely, we found that the 4.0 g/kg dose of ethanol produced strong negative reinforcing effects in C57BL/6 mice. This is
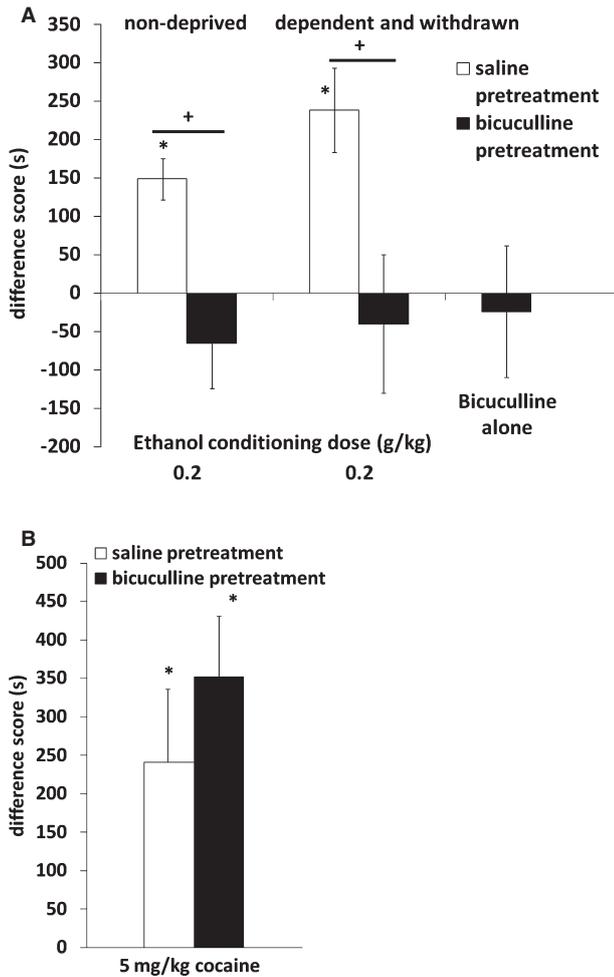
FIG. 5. (A) C57BL/6 mice do not show ethanol place preferences when pretreated with a GABA$_A$ receptor antagonist. Conditioned place preferences were induced by a low dose of ethanol in C57BL/6 mice pretreated with saline, but not in those pretreated with intraperitoneal bicuculline (1.0 mg/kg), a GABA$_A$ receptor antagonist, for both ethanol-non-deprived and ethanol-dependent and withdrawn mice. Bicuculline alone had no motivational effects as measured in an independent place conditioning experiment (right). Data represent means of times spent in ethanol-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free conditions. *Significant preference for the ethanol-paired vs. the saline-paired environment ($P < 0.05$). + indicates a significant difference between the two groups ($P < 0.05$). (B) Non-deprived cocaine-conditioned place preferences are not blocked by bicuculline pretreatment. Intraperitoneal cocaine (5.0 mg/kg)-conditioned place preferences were induced in cocaine-non-deprived C57BL/6 mice pretreated with saline or bicuculline. Data represent means of times spent in cocaine-paired minus saline-paired environments (difference scores ± SEM) when animals were tested under drug-free conditions. *Significant preference for the cocaine-paired vs. the saline-paired environment ($P < 0.05$).

in contrast to the results of Cunningham *et al.* (1992b), who found that this dose produced no motivational effects in the same strain of mouse. The reason for this difference is not clear, although it is possible that different experimental paradigms (for example, we used more conditioning trials and no habituation phase) offer some explanation.

Non-deprived ethanol motivation is D2 receptor-dependent

The present data show that the D2 receptor is critical in mediating both the rewarding and aversive effects of ethanol in non-deprived mice. These results are consistent with work showing that D2 knockout mice

display decreased alcohol consumption (Phillips *et al.*, 1998) and ethanol place preferences (Cunningham *et al.*, 2000). The fact that both ethanol reward and ethanol aversion were blocked in D2 receptor knockout mice is not entirely surprising, given that dopamine has been linked to both rewarding and aversive aspects of drug motivation (Salamone, 1994; Schultz, 2000; Laviolette & van der Kooy, 2003; Wise, 2004).

As knockout mice were used to obtain these data, it is possible that developmental compensation or gene linkage may also have influenced our results. Although we cannot entirely rule out these possibilities, we note that these same mice displayed no morphine-conditioned place preferences when conditioned while in a morphine-dependent and withdrawn state (Dockstader *et al.,* 2001), and that this result has been replicated using the broad-spectrum dopamine receptor antagonist α-flupenthixol in wild-type mice (unpublished results), suggesting that the crucial feature of the knockout was indeed the lack of a functional D2 receptor and not some other compensatory effect.

Ethanol-dependent and withdrawn motivation is dopamine-independent

The current experiments are the first to demonstrate place conditioning in the ethanol-dependent and withdrawn animal. The present data show that, in contrast to ethanol-non-deprived D2 receptor knockout mice, ethanol-dependent and withdrawn D2 receptor knockout mice showed normal ethanol-conditioned place preferences (0.2 g/kg dose) and aversions (4.0 g/kg dose). Ethanol withdrawal produces a decrease in dopamine release (Diana *et al.*, 1993; Weiss *et al.*, 1996; Shen, 2003), and this result is consistent with reports suggesting that the importance of dopamine may be limited to the acquisition of ethanol reinforcement, and not its maintenance (Rassnick *et al.*, 1993a; Ikemoto *et al.*, 1997). Our data support the idea that once mice are ethanol-dependent and in withdrawal, dopamine is no longer responsible for mediating ethanol's positive reinforcing properties.

Interestingly, we saw no apparent differences between the place conditioning scores in non-deprived mice and those in mice conditioned while in an ethanol-dependent and withdrawn state. One might have predicted either an increase or decrease in these scores, due to sensitization or tolerance to ethanol's motivation effects, but this was not observed. Indeed, increased aversive responses to naloxone were reported previously in opiate-dependent as compared with opiate-non-deprived animals (Mucha *et al.*, 1982), whereas opposite responses were seen with nicotine antagonists in nicotine-dependent as compared with nicotine-non-deprived animals (Mucha, 1997). Furthermore, it is entirely possible that, although the place conditioning scores are similar in the present study, different processes are responsible for producing them. For instance, the place preferences in non-deprived mice may be primarily mediated by ethanol's inherent reinforcing properties, whereas in mice conditioned while in a state of ethanol dependence and withdrawal, place preferences may be mediated more by the alleviation of withdrawal, similar to the case with opiates (Bechara & van der Kooy, 1992a).

The TPP is important for the positive reinforcing effects of ethanol in ethanol-dependent and withdrawn, but not non-deprived, mice

Lesions of the TPP nucleus block non-deprived opiate motivation in the place conditioning paradigm (Bechara & van der Kooy, 1989; Olmstead & Franklin, 1993, 1994; Nader *et al.*, 1995). We wished to investigate the effects of TPP lesions on ethanol-non-deprived and

ethanol-dependent and withdrawn ethanol motivation. As in previous work, TPP lesions were effective in blocking morphine place preferences in opiate-non-deprived mice. These lesions did not block ethanol place preferences in ethanol-non-deprived mice. However, in ethanol-dependent and withdrawn mice, these same TPP lesions were effective in blocking ethanol place preferences. Therefore, it appears that the dopamine D2 receptor and TPP systems play diametrically opposing roles in ethanol reinforcement: the dopamine D2 receptor is responsible for mediating the positive reinforcing effects of ethanol in non-deprived mice, and the TPP is responsible for mediating the positive reinforcing effects of ethanol in dependent and withdrawn mice.

In our paradigm, mice were always conditioned while in a state of ethanol withdrawal. Therefore, our place preference data can be interpreted as: (i) preferences for the ethanol-paired, withdrawal-alleviating environment; or (ii) aversions to the withdrawal-paired environment. From the present data, we cannot easily distinguish between these two possibilities, although we note that investigation of similar circumstances with morphine showed that morphine place preferences in the dependent and withdrawn state were primarily driven by the alleviation of morphine-induced withdrawal (Bechara and van der Kooy, 1992a). TPP lesions did not block the aversive effects of ethanol in the ethanol-dependent and withdrawn state. This is not surprising, as TPP lesions have yet to be shown to play a direct role in the aversive effects of any stimulus, although they are important for the induction of withdrawal aversions following administration of acute morphine (Vargas-Perez *et al.*, 2007). D2 receptor knockout mice also demonstrated a high-dose ethanol aversion. It is unclear what neurobiological system(s) is responsible for this particular aversive effect. It is possible that it is simply due to non-specific systemic effects of the ethanol. It is also unclear whether this ethanol aversion in the dependent and withdrawn state is $GABA_A$ receptor-dependent.

The positive reinforcing effects of ethanol are $GABA_A$ receptor-dependent

Our results suggest that ethanol's positive reinforcing effects are $GABA_A$ receptor-dependent. However, it is unclear which specific $GABA_A$ receptors are responsible for mediating these effects, as our study utilized systemic administrations of bicuculline. Indeed, previous work has suggested the amygdala and NAc (Hyytia & Koob, 1995; Eiler & June, 2007) or TPP (Samson & Chappell, 2001) as important sites for $GABA_A$ receptor modulation of ethanol reinforcement. Certainly, the present results support these possibilities. Another possibility – which is not mutually exclusive – is the VTA (Gatto *et al.*, 1994; Nowak *et al.*, 1998). Indeed, past work has implicated VTA $GABA_A$ receptors located on GABAergic neurons as the locus of a motivational switching mechanism for opiates (Laviolette *et al.*, 2004). On the basis of this, we hypothesize that these same VTA $GABA_A$ receptors may also be crucial for the positive reinforcing effects of ethanol. Indeed, pretreatment of both ethanol-non-deprived and ethanol-dependent and withdrawn groups with bicuculline – at a dose that produced no motivational effects of its own – was enough to completely abolish all ethanol place preferences, whether they were mediated via a dopamine-dependent (in the case of ethanol-non-deprived mice) or a TPP-dependent (in the case of ethanol-dependent and withdrawn mice) mechanism. These results are in agreement with studies showing decreases in ethanol self-administration after pre-treatment with $GABA_A$ receptor antagonists (Rassnick *et al.*, 1993b; Petry, 1997; Nowak *et al.*, 1998). However, our results are in contrast

to those of other investigators, including Chester & Cunningham (1999), who showed that certain doses of $GABA_A$ receptor antagonists actually potentiated ethanol-conditioned place preferences. It is possible that procedural differences are responsible for these conflict-
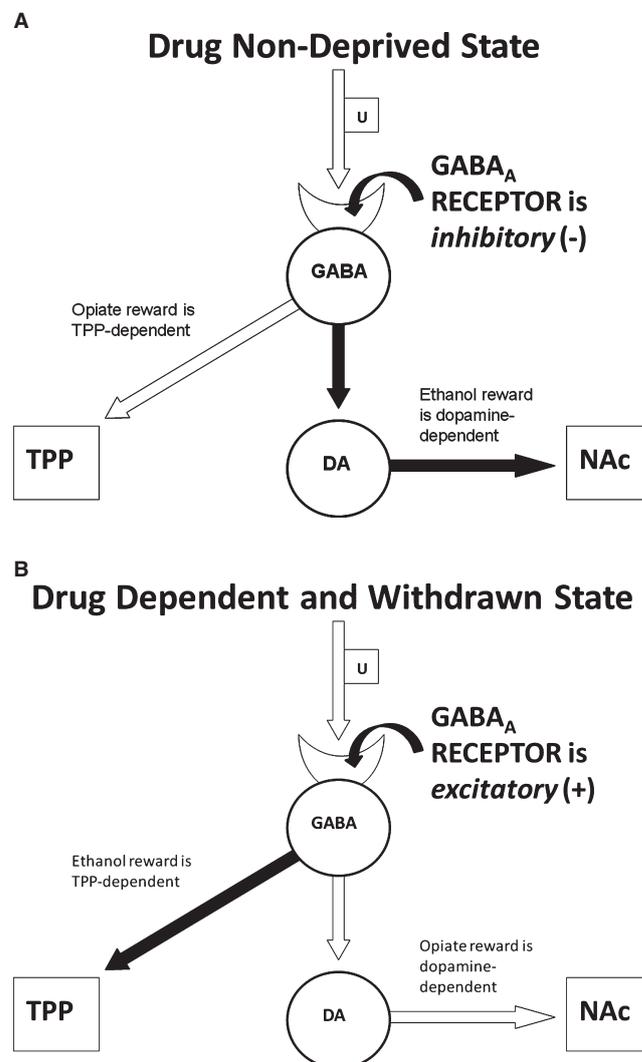

Fig. 6. (A) A hypothesized ventral tegmental area (VTA) $GABA_A$ receptor switch model for animals in a non-deprived state. This model proposes that signaling through $GABA_A$ receptors associated with VTA GABAergic neurons controls a functional divergence point between a dopamine (DA)-dependent mesolimbic pathway to the nucleus accumbens (NAc) and a dopamine-independent motivational pathway to the tegmental pedunculopontine nucleus (TPP). We propose that $GABA_A$ receptors switch from being inhibitory in the drug-non-deprived animal to being excitatory in the drug-dependent and withdrawn animal. Ethanol activation of inhibitory $GABA_A$ receptors in a drug-non-deprived animal would inhibit the activity of these GABAergic neurons, releasing the inhibition of the dopamine neurons (mediated through a $GABA_B$ receptor) and resulting in an increase in dopaminergic neuron activity. Opiates, acting presynaptically to the GABA neuron on GABA afferent terminals (as indicated by the μ receptor), would reduce GABA release onto the $GABA_A$ receptors themselves and therefore have effects exactly opposite to those of ethanol. (B) A hypothesized VTA $GABA_A$ receptor switch model for animals in a drug-dependent and withdrawn state. Ethanol activation of excitatory $GABA_A$ receptors in the ethanol-dependent and withdrawn animal would potentiate the activity of the GABA neuron, potently increasing the inhibitory effect of the GABAergic neurons on dopamine neurons and also activating a non-dopaminergic, TPP-dependent motivational pathway. Opiates, again acting presynaptically to the GABA neuron on GABA afferent terminals (as indicated by the μ receptor), would reduce GABA release onto the $GABA_A$ receptors themselves and, again, have effects exactly opposite to those of ethanol.

ing results. For example, in our study we utilized more conditioning trials, performed bicuculline injections in both ethanol and saline-paired environments, and used the C57BL/6 strain of mouse (as opposed to DBA/2J). Alternatively, although highly speculative, it is possible that the results of Chester & Cunningham (1999) are due to activation of an alternative, dopamine-independent pathway (Laviolette & van der Kooy, 2001) that, when combined with ethanol's own dopamine-dependent reinforcing actions, produces a synergistic effect on reinforcement (bicuculline alone being insufficient to produce motivational effects).

GABA$_A$ receptors form a motivational switching mechanism

Anatomically, VTA GABA$_A$ receptors are well positioned to mediate a switch between dopamine-dependent and TPP-dependent reward systems (Swanson, 1982; Grace & Bunney, 1985; Bechara & van der Kooy, 1989, 1992b; Kalivas, 1993; Laviolette *et al.*, 2004). We hypothesize that these receptors form a motivational switch that directs both opiate and ethanol reward into TPP or dopamine outputs, albeit in opposite directions, during switching from a non-deprived to a dependent and withdrawn motivational state. Ethanol withdrawal is known to produce changes in VTA neuron properties (Gallegos *et al.*, 1999; Brodie, 2002; Hopf *et al.*, 2007) and GABA$_A$ receptor subunit composition (Charlton *et al.*, 1997; Cagetti *et al.*, 2003), and it is possible that these changes are important for precipitating this switch in the neurobiological substrates mediating ethanol reinforcement. Functionally, the switch is caused by a change in the ion conductance properties of the GABA$_A$ receptors themselves (Laviolette *et al.*, 2004).

Past work has shown that when animals are in a non-deprived state, intra-VTA injections of muscimol, a GABA$_A$ receptor agonist, elicit reward via a dopamine-dependent mechanism, and injections of bicuculline, a GABA$_A$ receptor antagonist, elicit reward via a TPP-dependent mechanism (Laviolette & van der Kooy, 2001). We propose that ethanol, a positive allosteric modulator of GABA$_A$ receptors, activates VTA GABA$_A$ receptors in a similar manner to muscimol, thereby (i) inhibiting GABAergic neuron activity and, consequently, (ii) alleviating the GABAergic inhibition of dopamine release, resulting in a dopamine-dependent reward signal (Fig. 6A).

In non-deprived animals, opiate motivation is contingent upon the TPP and not dopamine. Given this, we propose that opiates must act in a similar way to bicuculline, blocking activation of GABA$_A$ receptors and thereby increasing the activity of GABAergic neurons. This would, in turn, (i) maintain inhibition of the dopamine neurons, and (ii) evoke a reward signal via the dopamine-independent TPP pathway (Fig. 6A). Recent studies have shown that presynaptically localized μ-opiate receptors that synapse with VTA GABA neurons exist within the VTA (Garzon & Pickel, 2001; Svingos *et al.*, 2001). Were opiates to act on these receptors (and not those located on the cell bodies of the GABA neurons themselves), they would inhibit GABA release onto the GABA$_A$ receptors themselves – in effect, acting functionally as GABA$_A$ receptor antagonists, similar to bicuculline, and producing reward via an identical TPP-dependent mechanism.

When mice were conditioned in an ethanol-dependent and withdrawn state, ethanol reinforcement shifted from being dopamine-dependent to TPP-dependent. This shift can be explained by postulating a change in the ion conductance properties of the GABA$_A$ receptors (Staley *et al.*, 1995; Stein & Nicoll, 2003; Laviolette *et al.*, 2004). If these receptors switch from producing a net hyperpolarizing effect (in the non-deprived state), to producing a net depolarizing effect (in the dependent and withdrawn state), then a GABA$_A$ receptor agonist such as ethanol would have an opposite effect in the ethanol-dependent and withdrawn state (Fig. 6B). Activation of an excitatory receptor would increase the activity of the GABAergic neurons, maintain the inhibition on the dopamine cells, and send a reward signal to the TPP. Conversely, inhibition of this same receptor (with opiates) would decrease the activity of the GABAergic neurons, decrease the inhibition on the dopamine cells, and produce a dopamine-dependent reward signal. Recent iontophoretic recordings of VTA GABAergic neurons demonstrated that 100% of these neurons showed an inhibitory response to a GABA$_A$ receptor agonist when the animal was in a non-deprived state. When animals became opiate-dependent and withdrawn, approximately one-half of these same neurons then showed an excitatory response to a GABA$_A$ receptor agonist (Laviolette *et al.*, 2004).

In conclusion, we demonstrate that dopamine D2 receptor and TPP systems show doubly dissociable roles in the mediation of ethanol's positive reinforcing effects. D2 receptors are critical for ethanol reinforcement in the non-deprived state, and the TPP is critical for ethanol reinforcement in the dependent and withdrawn state. Furthermore, we hypothesize that this relationship is dependent upon a switching mechanism mediated by VTA GABA$_A$ receptors. Our findings help to elucidate the currently unclear role of dopamine in the motivational effects of ethanol.

Abbreviations

NAc, nucleus accumbens; SEM, standard error of the mean; TPP, tegmental pedunculopontine nucleus; VTA, ventral tegmental area.

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