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Infusion of brain-derived neurotrophic factor into the ventral tegmental area switches the substrates mediating ethanol motivation

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Abstract

Recent work has shown that infusion of brain-derived neurotrophic factor (BDNF) into the ventral tegmental area (VTA) promotes a switch in the mechanisms mediating morphine motivation, from a dopamine-independent to a dopamine-dependent pathway. Here we showed that a single infusion of intra-VTA BDNF also promoted a switch in the mechanisms mediating ethanol motivation, from a dopamine-dependent to a dopamine-independent pathway (exactly opposite to that seen with morphine). We suggest that intra-VTA BDNF, via its actions on TrkB receptors, precipitates a switch similar to that which occurs naturally when mice transit from a drug-naive, non-deprived state to a drug-deprived state. The opposite switching of the mechanisms underlying morphine and ethanol motivation by BDNF in previously non-deprived animals is consistent with their proposed actions on VTA $GABA_A$ receptors.

Introduction

Studies of the substrates mediating ethanol motivation demonstrate support for both dopamine-dependent and dopamine-independent mechanisms. Ethanol induces dopamine release and increases dopamine cell firing rates (Gessa *et al.*, 1985; Yoshimoto *et al.*, 1992; Weiss *et al.*, 1993), and pharmacological or genetic manipulations that reduce dopaminergic activity have been demonstrated to inhibit various measures of ethanol-preferring behavior (Ahlenius *et al.*, 1973; Ikemoto *et al.*, 1997; El-Ghundi *et al.*, 1998; Phillips *et al.*, 1998). Conversely, other reports of dopaminergic system depletion have failed to produce any such changes (Kiianmaa *et al.*, 1979; Cunningham *et al.*, 1992a; Rassnick *et al.*, 1993; Engleman *et al.*, 2000). Our previous work posited a potential solution to this dilemma (Ting-A-Kee *et al.*, 2009). We proposed that the motivational state of the organism [whether the animal was in a drug non-deprived (minimal previous drug exposure) or an ethanol-deprived state (substantial previous drug exposure)] determined whether or not ethanol motivation was dopamine-dependent (Fig. 1). However, the mechanisms behind this switch remain elusive.

Similar to ethanol, opiates have also been demonstrated to possess motivational properties that are dependent on either dopamine-based or non-dopamine-based mechanisms (Amalric & Koob, 1985; Olmstead *et al.*, 1998; Hnasko *et al.*, 2005). Recent work demonstrated that ventral tegmental area (VTA) $GABA_A$ receptors, located primarily on GABA neurons, are responsible for switching the mechanisms mediating opiate motivation between dopamine-independent and dopamine-dependent systems (Laviolette *et al.*, 2004). Whereas normally this switch is triggered when an animal changes from a non-deprived to an opiate-deprived state, a single infusion of brain-derived neurotrophic factor (BDNF) into the VTA can mimic this transformation (Vargas-Perez *et al.*, 2009).

Ethanol also potently influences the activity of VTA GABA neurons (Gallegos *et al.*, 1999; Melis *et al.*, 2002; Theile *et al.*, 2008) and evidence suggests that VTA $GABA_A$ receptors may mediate ethanol motivation (Gatto *et al.*, 1994; Nowak *et al.*, 1998). This suggests the possibility that ethanol might utilize a similar VTA $GABA_A$ receptor-based 'switching' apparatus for drug motivation as seen with opiates. Therefore, we tested whether intra-VTA infusions of BDNF also regulated the mechanisms mediating ethanol's motivational properties. We predicted that, whereas intra-VTA BDNF

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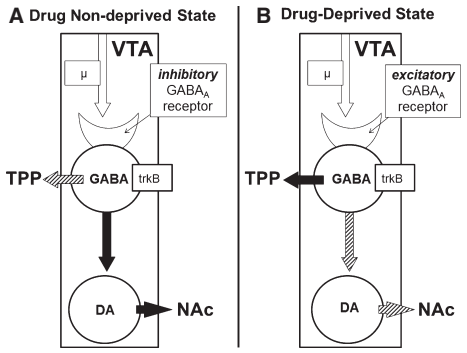

FIG. 1. A hypothesized VTA $GABA_A$ receptor switch model. $GABA_A$ receptors associated with VTA GABAergic neurons switch the substrates mediating opiate and ethanol motivation between a brainstem tegmental pedunculopontine nucleus (TPP)-dependent pathway and a dopamine (DA)-dependent mesolimbic pathway to the nucleus accumbens (NAc). (A) In drug non-deprived animals, ethanol acts on $GABA_A$ receptors to produce dopamine-dependent positive reinforcement (black arrows) and opiates act pre-synaptically at μ-opioid receptors, producing TPP-dependent positive reinforcement (striped arrow). (B) $GABA_A$ receptors switch from hyperpolarizing (inhibitory) to depolarizing (excitatory) in drug-deprived animals or animals treated with intra-VTA BDNF (possibly acting via GABAergic TrkB receptors to mediate these effects). Consequently, the mechanisms mediating opiate (striped arrows) and ethanol (black arrow) reinforcement are reversed. Modified from Ting-A-Kee *et al*. (2009).

would switch morphine motivation to a dopamine-dependent mechanism, this same manipulation would switch ethanol motivation to a dopamine-independent mechanism. We further predicted that this switch required BDNF's high-affinity TrkB receptor and that intra-VTA infusion of TrkB small interference RNAs (siRNAs) would therefore prevent this switch. Last, given that the motivational effects of morphine and ethanol depend upon separate substrates in either a non-deprived or deprived state, we hypothesized that their motivational effects were additive in non-deprived mice. Our results are consistent with a VTA $GABA_A$ receptor switching model that is responsible for the selection of both opiate and ethanol output motivational pathways.

Materials and methods

Animals

For all experiments, male C57BL/6 mice (Charles River) were used. Subjects were housed four per cage in a sound-attenuated room (lights on 07:00–19:00 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. A total of 278 mice were used for these experiments.

Drugs

The BDNF (Sigma) was dissolved in phosphate-buffered saline (pH 7.4). Lentiviral-TrkB-siRNAs were obtained from Bahi *et al*. (2008) (United Arab Emirates University, Al Ain, UAE). Alpha-flupenthixol (0.8 mg/kg) (Sigma), morphine sulfate (10 mg/kg) (Almat Pharmachem Inc., Concord, Canada) and anhydrous ethyl alcohol (0.2 g/kg) (Commercial Alcohols, Brampton, Canada) were dissolved in 0.9% saline and injected i.p. Previous work indicated that, in C57BL/6 mice, this low dose of ethanol was most effective at generating a conditioned place preference (Ting-A-Kee *et al.*, 2009). Alcohol was diluted into a 20% (v/v) solution. For experiments involving both morphine and ethanol, both drugs were mixed together and co-administered.

Blood ethanol levels

Separate groups of previously drug-naive mice received acute i.p. injections of saline or 0.2 or 0.5 g/kg ethanol. At 5 min post-injection, mice were killed under CO_2 anesthesia and blood collected in duplicate in Eppendorf tubes via cardiac puncture. The samples were centrifuged (3000 ***g***) and serum (0.1 mL) drawn off. After the addition of acetonitrile, enzymatic detection was performed to determine blood ethanol levels (detection limit 1.0 mM).

Surgery

The BDNF infusions were performed bilaterally (0.05 μL of a 0.1 μg/μL solution) with a 1 μL Hamilton syringe (VWR) over 1 min, plus an additional 1 min to allow for diffusion. Preliminary experiments indicated that this dose of BDNF, although small in comparison with other rat studies (Lu *et al.*, 2004; Vargas-Perez *et al.*, 2009), was effective. A small volume was infused to minimize spread to surrounding areas. Sham controls received phosphate-buffered saline. Infusions of lentiviral-green fluorescent protein (GFP) or lentiviral-TrkB-siRNAs were performed bilaterally (0.1 μL), using a procedure identical to that stated above. These injections produce either GFP expression or TrkB mRNA depletions throughout the VTA (unpublished results). The VTA injection coordinates were (mm from bregma): AP, −3.0; ML, ±0.6; DV, −4.1 (Hof *et al.*, 2000). The VTA dorsal controls used DV, −3.1. Ketoprofen (3.0 mg/kg, s.c.) was administered as an analgesic. Animals were given 1 week of recovery time before conditioning.

Place conditioning apparatus

The place conditioning apparatus (SOF-700RA-25 Two Chamber Place Preference Apparatus) was obtained from Med Associates Inc. (VT, USA). One environment was black with a metal rod floor and the other was white with a wire mesh floor. An intermediate gray area housed a removable partition.

Place conditioning procedure

Mice undergoing ethanol conditioning received 24 conditioning trials (12 alternating drug and saline vehicle pairings) on separate days. We found that this treatment regimen produced robust ethanol place preferences but no significant signs of withdrawal and can be considered to model a non-deprived motivational state (Ting-A-Kee *et al.*, 2009).

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 subsequent conditioning trials, the ethanol liquid diet was removed and replaced with a control liquid diet at 8 h prior to each conditioning trial. After 8 h without the ethanol liquid diet, animals demonstrate moderate somatic symptoms of withdrawal and show a conditioned place aversion to the withdrawal-paired environment (Fig. S1). At approximately 1 h after conditioning, the ethanol diet was reintroduced and the control diet removed. This cycle

continued for the duration of conditioning, during both drug and vehicle conditioning sessions. This ensured that withdrawal was felt over both compartments and allowed us to focus our analysis on the reinforcing effects of ethanol.

Alpha-flupenthixol (0.8 mg/kg i.p.) or saline pre-treatments occurred at 1 h prior to conditioning, for both drug and vehicle pairings, in order to balance any motivational effects of the treatments (although alpha-flupenthixol does not possess any motivational effects of its own at this dose) (Laviolette & van der Kooy, 2003; Grieder *et al.*, 2010). Mice received i.p. injections of drug or saline and were then exposed to one of the conditioning environments for 5 min. Morphine conditioning followed a similar protocol of eight conditioning trials of 15 min each. All conditioning was unbiased and fully counterbalanced for treatment compartment and order of drug presentation. There were no baseline preferences for any environment, nor did we observe any treatment-induced preferences for the black or white environments (Fig. S2).

After the final conditioning trial, mice were allowed to rest uninterrupted in their home cage for 1 week until test day and any liquid diets were permanently replaced with rodent chow and water. On test day, under drug-free conditions, mice were given access to all conditioning environments simultaneously by removing the shared partition and introducing the animal into the intermediate gray area separating the two conditioning environments. The time and activity in each environment was recorded for 10 min.

Histology

Mice that underwent control or drug infusions were anesthetized with 180 mg/kg sodium pentobarbital (Animal Resources Centre, Montreal, QC, Canada) and perfused with 30 mL each of 0.9% saline and 4% formaldehyde. Brains were removed and stored in a 25% sucrose/4% formaldehyde post-fixative at 4 °C, sliced using a cryostat into 30 μm sections, and mounted on gelatin-coated slides. Correct placements were verified with cresyl violet staining and light microscopy (Hof *et al.*, 2000). Mice were excluded if any infusions were outside the VTA; these mice did not differ from sham controls.

Statistical analysis

ANOVA or two-tailed Student's *t*-tests were used to detect significance ($P < 0.05$) where appropriate.

Results

Blood ethanol levels

At 5 min after acute i.p. injection of 0.2 g/kg ethanol, we observed blood ethanol levels of approximately 44 ± 5 mg/dL ($n = 10$). For comparison, saline injections produced no detectable levels of ethanol ($n = 4$) and a higher ethanol dose (0.5 g/kg) produced levels of approximately 74 ± 3 mg/dL ($n = 8$). Data represent mean ± SEM.

Dopamine does not mediate non-deprived opiate motivation

Mice without prior exposure to opiates received intra-VTA phosphate-buffered saline and underwent morphine place conditioning (10 mg/kg). At 1 h prior to conditioning, mice were pre-treated with either saline ($n = 16$) or the broad-spectrum dopamine receptor antagonist alpha-flupenthixol (0.8 mg/kg) ($n = 21$). A two-way

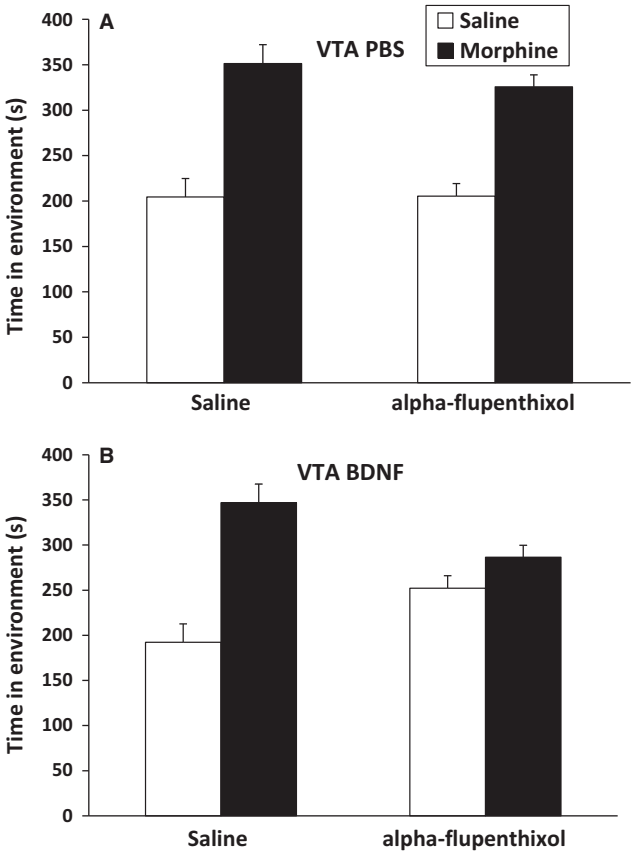

FIG. 2. Morphine place preferences are attenuated by dopamine receptor antagonism only after intra-VTA BDNF infusion. (A) Morphine induced conditioned place preferences in mice receiving intra-VTA phosphate-buffered saline (PBS) irrespective of pre-treatment with alpha-flupenthixol ($n = 16$–21). (B) Conversely, in mice receiving intra-VTA BDNF, morphine place preferences were attenuated by alpha-flupenthixol pre-treatment ($n = 8$–9). Data represent mean ± SEM of time spent in the drug-paired environment.

2×2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,35} = 0.34$, $P = 0.56$), a significant effect of conditioning drug (morphine vs. saline) ($F_{1,35} = 39.9$, $P < 0.0001$), and no significant interaction ($F_{1,35} = 0.40$, $P = 0.533$), suggesting that these mice showed morphine place preferences irrespective of whether or not they were pre-treated with alpha-flupenthixol (Fig. 2A).

Non-deprived opiate motivation is switched to a dopamine-dependent mechanism after infusion of intra-ventral tegmental area brain-derived neurotrophic factor

A separate group of mice without prior exposure to opiates received intra-VTA BDNF and underwent morphine place conditioning (10 mg/kg). The mice were pre-treated with either saline ($n = 8$) or alpha-flupenthixol ($n = 9$). A two-way 2×2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,15} = 0.0001$, $P = 0.996$), a significant effect of conditioning drug (morphine vs. saline) ($F_{1,15} = 11.3$, $P = 0.0043$), and a significant interaction ($F_{1,15} = 4.58$, $P = 0.0491$), suggesting that only after intra-VTA BDNF was pre-treatment with alpha-flupenthixol able to fully attenuate morphine place preferences (Fig. 2B), replicating previous results in rats (Vargas-Perez *et al.*, 2009).

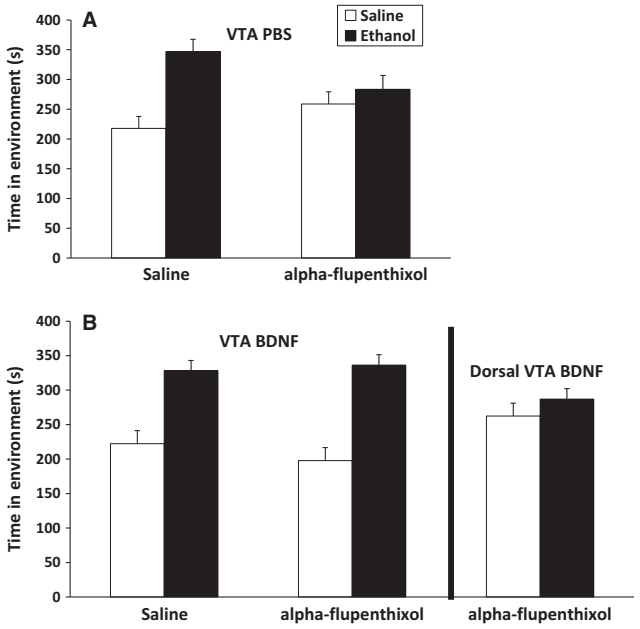

FIG. 3. Ethanol place preferences are no longer attenuated by dopamine receptor antagonism after intra-VTA BDNF infusion. (A) Ethanol induced conditioned place preferences in mice receiving intra-VTA phosphate-buffered saline (PBS), which were attenuated by pre-treatment with alpha-flupenthixol (n = 13–27). (B) Conversely, in mice receiving intra-VTA BDNF, ethanol produced place preferences that were now insensitive to alpha-flupenthixol (n = 8–15) (left). Pre-treatment with alpha-flupenthixol was able to attenuate an ethanol place preference in mice receiving BDNF dorsal to the VTA (n = 7) (right). Data represent mean ± SEM of time spent in the drug-paired environment.

Dopamine mediates non-deprived ethanol motivation

Mice without prior exposure to ethanol received intra-VTA phosphate-buffered saline and underwent ethanol place conditioning (0.2 g/kg). The mice were pre-treated with either saline (n = 27) or alpha-flupenthixol (n = 13). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,38}$ = 0.224, P = 0.639), a significant effect of conditioning drug (ethanol vs. saline) ($F_{1,38}$ = 9.88, P = 0.0033), and a significant interaction ($F_{1,38}$ = 5.43, P = 0.025), suggesting that these mice showed ethanol place preferences that were attenuated by pre-treatment with alpha-flupenthixol, similar to previous results using dopamine receptor-2 knockout animals (Ting-A-Kee *et al.*, 2009) (Fig. 3A).

Non-deprived ethanol motivation is switched to a dopamine-independent mechanism after infusion of intra-ventral tegmental area brain-derived neurotrophic factor

A separate group of mice without prior exposure to ethanol received intra-VTA BDNF and underwent ethanol place conditioning (0.2 g/kg). The mice were pre-treated with either saline (n = 8) or alpha-flupenthixol (n = 15). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,21}$ = 0.104, P = 0.75), a significant main effect of conditioning drug (ethanol vs. saline) ($F_{1,21}$ = 26.5, P < 0.0001), and no significant interaction ($F_{1,21}$ = 0.023, P = 0.88), suggesting that, after intra-VTA BDNF, these mice demonstrated ethanol place preferences irrespective of whether or not they were pre-treated with alpha-flupenthixol (Fig. 3B, left).

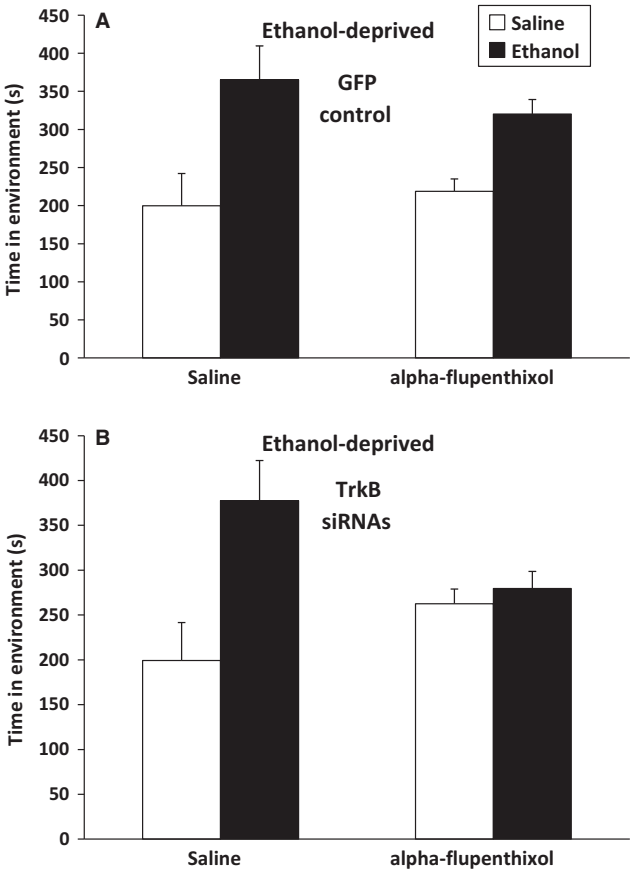

FIG. 4. In ethanol-deprived mice, BDNF TrkB receptors are necessary for the switch to a dopamine-independent reinforcement mechanism. (A) In ethanol-deprived mice that had received control intra-VTA lentiviral-GFP infusions, ethanol induced conditioned place preferences irrespective of pre-treatment with alpha-flupenthixol (n = 6–23). (B) Conversely, in ethanol-deprived mice that received intra-VTA lentiviral-TrkB-siRNAs, ethanol place preferences were attenuated by pre-treatment with alpha-flupenthixol (n = 12–13). Data represent mean ± SEM of time spent in the drug-paired environment.

To confirm the VTA as the site of BDNF's actions, a separate group of mice without prior exposure to ethanol (n = 7) received a BDNF infusion 1 mm dorsal to the VTA and underwent ethanol place conditioning (0.2 g/kg). The mice were pre-treated with alpha-flupenthixol. A t-test revealed no main effect of ethanol ($t_{1,6}$ = 0.517, P = 0.624), suggesting that, unless BDNF was infused into the VTA, alpha-flupenthixol pre-treatment was sufficient to attenuate an ethanol place preference (Fig. 3B, right).

Ethanol-deprived ethanol motivation is dopamine-independent

Mice received an intra-VTA infusion of control lentiviral-GFP prior to being made ethanol-dependent via exposure to an ethanol liquid diet and undergoing subsequent ethanol place conditioning (0.2 g/kg). The mice were pre-treated with either saline (n = 6) or alpha-flupenthixol (n = 23). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,27}$ = 0.1, P = 0.753), a significant effect of conditioning drug (ethanol vs. saline) ($F_{1,27}$ = 10.8, P = 0.0028), and no significant interaction ($F_{1,27}$ = 0.61, P = 0.44), suggesting that these mice showed ethanol place preferences irrespective of whether or not they were pre-treated with alpha-flupenthixol (Fig. 4A).

Brain-derived neurotrophic factor TrkB receptors are required for the switch to a dopamine-independent mechanism in ethanol-deprived mice

A separate group of mice received an intra-VTA infusion of lentiviral-TrkB-siRNAs prior to being made ethanol-dependent via exposure to an ethanol liquid diet and undergoing subsequent ethanol place conditioning (0.2 g/kg). This mixture of lentiviral-TrkB siRNAs has been reported to produce up to 93% TrkB receptor silencing (Bahi *et al.*, 2008). The mice were pre-treated with either saline ($n = 12$) or alpha-flupenthixol ($n = 13$). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,23} = 0.067$, $P = 0.8$), a significant effect of conditioning drug (ethanol vs. saline) ($F_{1,23} = 7.05$, $P = 0.014$), and a significant interaction ($F_{1,23} = 4.93$, $P = 0.037$), suggesting that ethanol-deprived mice infused with intra-VTA TrkB siRNAs showed ethanol place preferences that were now attenuated by pre-treatment with alpha-flupenthixol (Fig. 4B).

The motivational properties of opiates and ethanol are additive via distinct neurobiological pathways

Given that morphine and ethanol produce their motivational effects via different mechanisms in non-deprived, drug-naive animals (dopamine-independent and dopamine-dependent, respectively), we examined whether their motivational effects were additive. To test this, morphine (1 mg/kg) and ethanol (0.2 g/kg) were co-administered using a procedure identical to the one described previously for ethanol.

We paired one conditioning environment with both drugs and the other with morphine alone. The mice were pre-treated with either saline ($n = 15$) or alpha-flupenthixol ($n = 12$). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,25} = 0.0002$, $P = 0.99$), a significant effect of conditioning drug (morphine and ethanol vs. morphine) ($F_{1,25} = 7.38$, $P = 0.012$), and a significant interaction ($F_{1,25} = 4.79$, $P = 0.038$), suggesting that mice only showed a preference for both drugs over morphine alone when they were not pre-treated with alpha-flupenthixol (Fig. 5A).

In a separate group of mice, we paired one environment with both drugs and the other with ethanol alone. Mice were pre-treated with either saline ($n = 16$) or alpha-flupenthixol ($n = 12$). A two-way 2 × 2 ANOVA (pre-treatment × conditioning drug as a repeated measure) revealed no effect of pre-treatment ($F_{1,26} = 0.0023$, $P = 0.96$), a significant main effect of conditioning drug (morphine and ethanol vs. ethanol) ($F_{1,26} = 9.96$, $P = 0.0004$), and no significant interaction ($F_{1,26} = 0.163$, $P = 0.69$), suggesting that mice always preferred an environment paired with both drugs over one paired with ethanol alone (Fig. 5B).

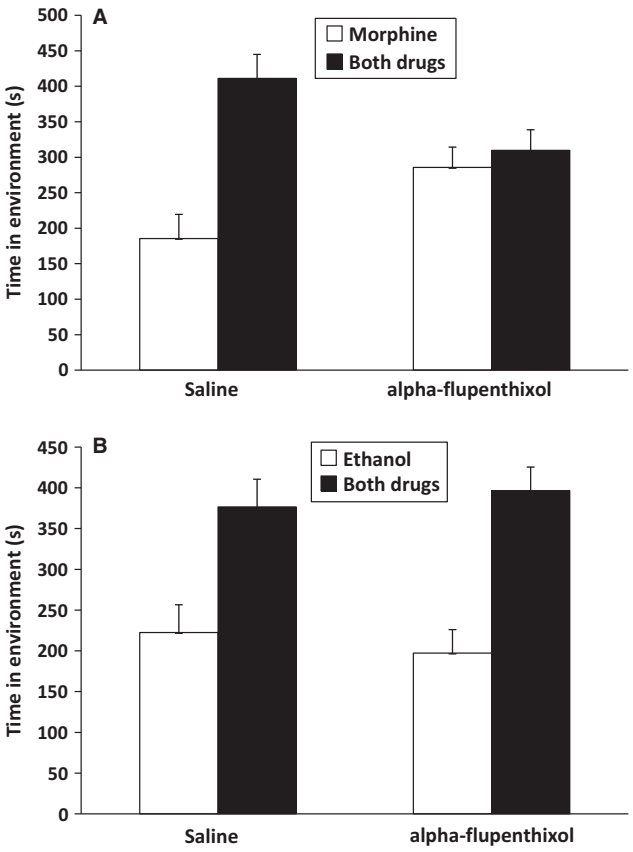

FIG. 5. The positive reinforcing effects of morphine and ethanol are additive via distinct mechanisms. (A) Previously drug non-deprived mice showed strong preferences for an environment paired with both drugs over an environment paired with morphine alone. This preference was abolished by pre-treatment with alpha-flupenthixol ($n = 12$–15). (B) Conversely, previously drug non-deprived mice showed strong place preferences for an environment paired with both morphine and ethanol over an environment paired with ethanol alone, regardless of alpha-flupenthixol pre-treatment ($n = 12$–16). Data represent mean ± SEM of time spent in the drug-paired environment.

Discussion

The switch in substrates underlying opiate and ethanol motivation is consistent with a switch in $GABA_A$ receptor activity

Work with opiates and ethanol, using both associative and operant paradigms, suggests that the substrates responsible for their motivational properties are not absolute but rather change based on the current physiology of the animal (Olmstead *et al.*, 1998; Laviolette *et al.*, 2004; Sabino *et al.*, 2009; Ting-A-Kee *et al.*, 2009). We have proposed a 'switch' hypothesis of motivation that posits that the drug history of an animal determines the mechanisms responsible for drug motivation. According to this model, $GABA_A$ receptors on VTA GABA neurons play a central role in the selection of either dopamine-independent or dopamine-dependent output mechanisms (Laviolette *et al.*, 2004). The ion conductance properties of these receptors [whether they produce hyperpolarization (inhibition) or depolarization (excitation) of VTA GABA neurons] determines whether a dopamine-independent or dopamine-dependent output pathway is selected.

Ethanol motivation can be explained by postulating that, as a positive allosteric modulator of $GABA_A$ receptors (located primarily on GABA neurons in the VTA), ethanol induces an inward chloride ion flow in VTA GABA neurons. This would hyperpolarize these cells, alleviate their inhibition of dopamine release, and result in a dopamine-dependent signal (Fig. 1A). Conversely, the opposing effects of morphine can be explained by postulating that morphine acts on pre-synaptic GABA-releasing terminals that synapse onto GABA neurons in the VTA (Svingos *et al.*, 2001; Xia *et al.*, 2011). According to the model, morphine binding to pre-synaptic μ-opioid receptors would reduce GABA release onto $GABA_A$ receptors in a manner analogous to a $GABA_A$ receptor antagonist (Laviolette *et al.*, 2004). The loss of a hyperpolarizing $GABA_A$ receptor input would depolarize VTA GABA cells, maintain their inhibition of

VTA dopamine cells, and produce positive reinforcement via a tegmental pedunculopontine nucleus-dependent process (Laviolette & van der Kooy, 2001; Vargas-Perez *et al.*, 2009).

The transit to a drug-deprived state produces a functional change in VTA $GABA_A$ receptors, which 'switch' to mediate depolarization of GABA cells upon activation, instead of hyperpolarization (Laviolette *et al.*, 2004). Now, according to the model, if ethanol were to act on these same VTA $GABA_A$ receptors, it would have an opposite effect, increasing the activity of VTA GABAergic neurons and resulting in continued inhibition of the dopamine cells and a tegmental pedunculopontine nucleus-based signal (Fig. 1B). Conversely, (indirect) inhibition of VTA $GABA_A$ receptors with morphine would result in the loss of a depolarizing GABAergic input, leading to decreased GABAergic activity. Consequently, VTA dopamine cells would be disinhibited, producing a dopamine-dependent signal for opiates.

Previous work in C57BL/6 mice showed that the low dose of ethanol used in the present study (which possessed positive reinforcing properties that were $GABA_A$-receptor-dependent and were blocked by systemic bicuculline) was the most reinforcing dose observed over the course of a full ethanol dose–response curve (Ting-A-Kee *et al.*, 2009). This dose is an order of magnitude lower than that often used in the DBA/2J mouse strain traditionally employed in ethanol reinforcement paradigms (Cunningham *et al.*, 1992b). The combination of this lower ethanol dose, differing mouse strain, and a greater number of conditioning trials may help to explain our differing results. Indeed, it is possible that these methodological differences may also be important factors that explain why our findings differ from those of other investigators who have observed no effect of various dopamine antagonists on ethanol place preference behavior (Cunningham *et al.*, 1992a; Risinger *et al.*, 1992). Furthermore, it should be noted that the ethanol place preferences observed here can be interpreted as preferences for the ethanol-paired environment, aversions to the withdrawal-paired environment, or some combination of these two possibilities. Future work will be needed to adequately differentiate between these options.

In the present study, we found that this low ethanol dose produced small but detectable blood ethanol levels. Although less frequently studied, previous work has demonstrated that low concentrations of ethanol (similar to those used in the present study) are capable of producing demonstrable changes in the brain. For example, low doses of ethanol can inhibit *N*-methyl-D-aspartate-induced currents (Lovinger *et al.*, 1989), produce changes in VTA GABA cell activity (Gallegos *et al.*, 1999), and impair memory on certain behavioral paradigms (Melchior & Ritzmann, 1996).

Intra-ventral tegmental area brain-derived neurotrophic factor is necessary and sufficient for the transit to a dopamine-independent ethanol motivational system

As the present work utilized systemic injections of both morphine and ethanol, it is unclear exactly where these drugs are producing their effects. Indeed, although we suggest a VTA-based $GABA_A$ receptor model, $GABA_A$ receptors in multiple parts of the brain have been proposed as critical mediators of ethanol reinforcement (Hyytia & Koob, 1995; Eiler & June, 2007). However, the present work suggests that a single infusion of intra-VTA BDNF is sufficient to produce a complete switch in the mechanisms underlying both morphine and ethanol motivation. This suggests that, wherever their direct action lies, morphine and ethanol motivation is mediated at least in part through interactions within the VTA. In both cases, intra-VTA BDNF was sufficient to precipitate a switch to a 'drug-deprived state' in spite of the fact that the mice received minimal drug exposure for the entirety of the experiment. Indeed, BDNF mRNA and protein levels are found to be increased in opiate-deprived rats (Vargas-Perez *et al.*, 2009).

Further evidence that the VTA is an important site for the motivational effects of ethanol comes from our work with TrkB siRNAs. In mice conditioned with ethanol while in an ethanol-deprived state, we observed that ethanol's motivational effects were no longer dopamine-dependent. However, in ethanol-deprived mice that received intra-VTA siRNAs targeted to the BDNF TrkB receptor [which is located on both GABA and dopamine neurons (Numan & Seroogy, 1999; Vargas-Perez *et al.*, 2009)] no switch to a dopamine-independent pathway was observed. This suggests that BDNF signaling through VTA TrkB receptors is necessary for the change to a drug-deprived motivational state and a corresponding switch in opiate and ethanol motivational mechanisms. Future work is necessary to determine whether levels of TrkB receptors are altered in drug-deprived animals.

There is a wealth of data suggesting that BDNF plays an important role in drug addiction. Levels of this neurotrophic factor are increased in various brain regions in response to drug administration and/or withdrawal (Meredith *et al.*, 2002; Vargas-Perez *et al.*, 2009). Furthermore, intra-VTA BDNF enhances the behavioral effects of cocaine (Lu *et al.*, 2004) and BDNF appears to be necessary for the development of cocaine addictive behavior (Graham *et al.*, 2007; Lobo *et al.*, 2010), although it also has been linked to decreases in the behavioral effects of various drugs of abuse (McGough *et al.*, 2004; Lobo *et al.*, 2010). Our work differs from these perspectives in that it suggests that BDNF is not causing a change in the quantitative effects of either morphine or ethanol *per se* but rather a change in the qualitative substrates mediating these effects.

A growing body of work has focused on the non-traditional depolarizing properties of $GABA_A$ receptors (Stein & Nicoll, 2003). Indeed, the fact that direct infusion of intra-VTA BDNF was successful in precipitating a switch in the mechanisms mediating drug motivation suggests that BDNF itself is capable of altering the ion conductance properties of $GABA_A$ receptors (Rivera *et al.*, 2002; Coull *et al.*, 2005). However, it is still to be explained exactly how this change is accomplished. One possibility is that BDNF alters the levels and/or activity of the co-transporter potassium chloride channel, which is responsible for removing intra-cellular chloride ions (Rivera *et al.*, 2002; Wake *et al.*, 2007). Downregulation of this transporter on VTA GABA cells would result in an intra-cellular buildup of chloride and, in turn, might allow for another ion flow to dominate (such as the extrusion of bicarbonate through the $GABA_A$ receptor) and produce a depolarizing response (Staley *et al.*, 1995). Similarly, BDNF might influence $GABA_A$ receptor cell surface expression (Brunig *et al.*, 2001), although our work suggests that it is unlikely that this alone could account for our results, as ethanol (which is proposed to act on these same receptors) produces similar-sized $GABA_A$ receptor-dependent place preferences in both drug-naive and drug-dependent and withdrawn motivational states (Ting-A-Kee *et al.*, 2009). Other possible actions of BDNF [such as its role in cortical excitability (Rutherford *et al.*, 1997) and the determination of $GABA_A$ receptor subunit composition (Thompson *et al.*, 1998)] cannot be discounted and warrant investigation.

Morphine and ethanol motivation is additive

The interactions between opioids and ethanol are well characterized. Indeed, ethanol is known to interact with endogenous opioids such

that naltrexone, an opioid receptor antagonist, decreases ethanol self-administration in animals and reduces relapse in human alcoholics (Altshuler *et al.*, 1980; Volpicelli *et al.*, 1992). Our work builds on this research and suggests that, although intimately related, it is still nevertheless possible to separate the positive reinforcing properties of morphine and ethanol at the level of dopamine receptor antagonism. Morphine and ethanol produce their motivational effects via different output systems, which we suggest are selected by a common upstream mediator, namely VTA $GABA_A$ receptors. The simplest such model, with both drugs acting on the same $GABA_A$ receptor located on a GABA neuron, is pictured in Fig. 1. As these drugs cause opposing effects on $GABA_A$ receptors, we speculated that they might actually 'cancel' each other out and, paradoxically, produce zero net motivational effects when co-administered. Alternatively, morphine and ethanol could produce additive effects by acting in a sequential manner, or on separate populations of VTA $GABA_A$ receptors entirely.

In drug non-deprived mice, strong preferences for environments paired with both drugs were observed over environments paired with a single drug alone, consistent with an additive-effects hypothesis. Pre-treatment with a dopamine receptor antagonist blocked this preference only when the single drug was morphine. This is consistent with the explanation that an animal given the choice between (i) morphine and the blocked motivational effects of ethanol vs. (ii) morphine alone, cannot differentiate between them. Conversely, an animal given the choice between (i) morphine and the blocked motivational effects of ethanol vs. (ii) the blocked motivational effects of ethanol alone, will still choose the morphine-paired environment. Taken together, these data indicate that the positive motivational effects of morphine and ethanol are additive and fully separable. Although the biological basis for this result is unknown, the data are consistent with the idea that morphine and ethanol are acting on the same VTA $GABA_A$ receptor in a sequential manner, or on different subpopulations of VTA $GABA_A$ receptors.

In conclusion, we demonstrate that intra-VTA BDNF precipitates a switch in the mechanisms mediating the motivational properties of morphine and ethanol, mimicking the process that naturally occurs when an animal becomes drug-deprived. Furthermore, we showed that, in drug non-deprived animals, the reinforcing effects of morphine and ethanol are additive and dissociable. We predict that this same result also holds true in drug-deprived animals, as the reinforcing effects of both drugs would still be subserved by different mechanisms. Our results point to BDNF as a potentially crucial component responsible for selecting the motivational output systems for both morphine and ethanol.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. C57BL/6 mice avoid an environment paired with ethanol withdrawal. After 4 days of exposure to Lieber DeCarli's Ethanol Liquid Diet (replacing all rodent chow and water), the diet was removed and replaced with a control liquid diet at 8 h prior to a 5-min conditioning session for C57BL/6 mice (no drug injections) ($n = 8$). At 1 h after conditioning, the ethanol diet was reintroduced and the control diet removed. This cycle continued for 4 days before the mice were given 1 week to recover with regular rodent chow and water. A 10 min conditioning test revealed a significant conditioned place aversion to the ethanol withdrawal-paired environment vs. the neutral environment ($t_{1,7} = 2.58$, $P = 0.036$). Data represent mean + SEM of time spent in the environments.

Fig. S2. C57BL/6 mice show no baseline preferences in our place conditioning paradigm. Mice received a saline injection prior to a 5 min conditioning session in one of two distinct conditioning environments (see Materials and methods) ($n = 15$). This continued for 8 days (with exposures to alternating conditioning environments) after which the mice were given 1 week prior to testing. A 10 min conditioning test revealed no significant preference or aversion to either the black or white conditioning environments ($t_{1,14} = 0.532$, $P = 0.603$). Data represent mean + SEM of time spent in the environments.

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Disclosure

R.F.T. owns shares and participates in Nicogen Research Inc., a company focused on novel smoking cessation treatment approaches. No Nicogen funds were used in this work and no other Nicogen participants reviewed the manuscript.

Abbreviations

BDNF, brain-derived neurotrophic factor; GFP, green fluorescent protein; siRNAs, small interference RNAs; VTA, ventral tegmental area.

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