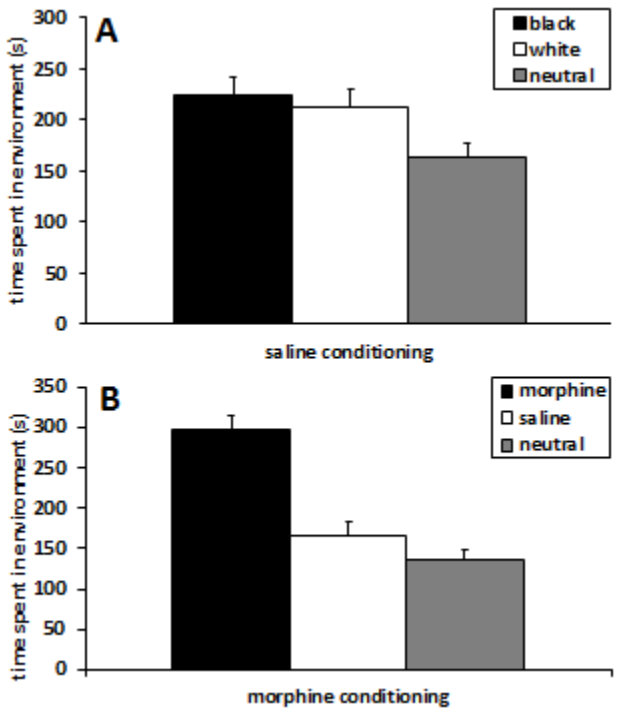
Supplementary Material

Morphine, but not saline, produces a significant place preference in our unbiased place conditioning procedure

Male Wistar rats (N=17) received 8 saline-environment pairings (4 vehicle injections paired with the black environment and 4 vehicle injections paired with the white environment). These subjects showed no preference for either the black or white environment (Supplementary Figure 1A). Conversely, rats showed a significant preference for an environment (either black or white) paired with 10 mg/kg morphine (N=16) as revealed by a one-way ANOVA (drug conditioning) [$F(1, 31) = 4.22$, $p < 0.05$] (Supplementary Figure 1B). In these experiments, there was no significant difference in the quantity of time spent in the neutral gray environment.



Supplementary Figure 1. As opposed to saline control injections, 10 mg/kg morphine produces significant morphine place preferences.

(A) Rats showed no significant preference for either a black or white environment (black and white bars, respectively) after receiving saline control injections in both. (B) Conversely, rats demonstrated a significant preference for an environment paired with 10 mg/kg morphine (irrespective of whether it was black or white). In both cases, there was no significant change in the amount of time spent in the neutral gray compartment. Data represent means +/- SEMs of time spent in the morphine- or saline-paired environment.

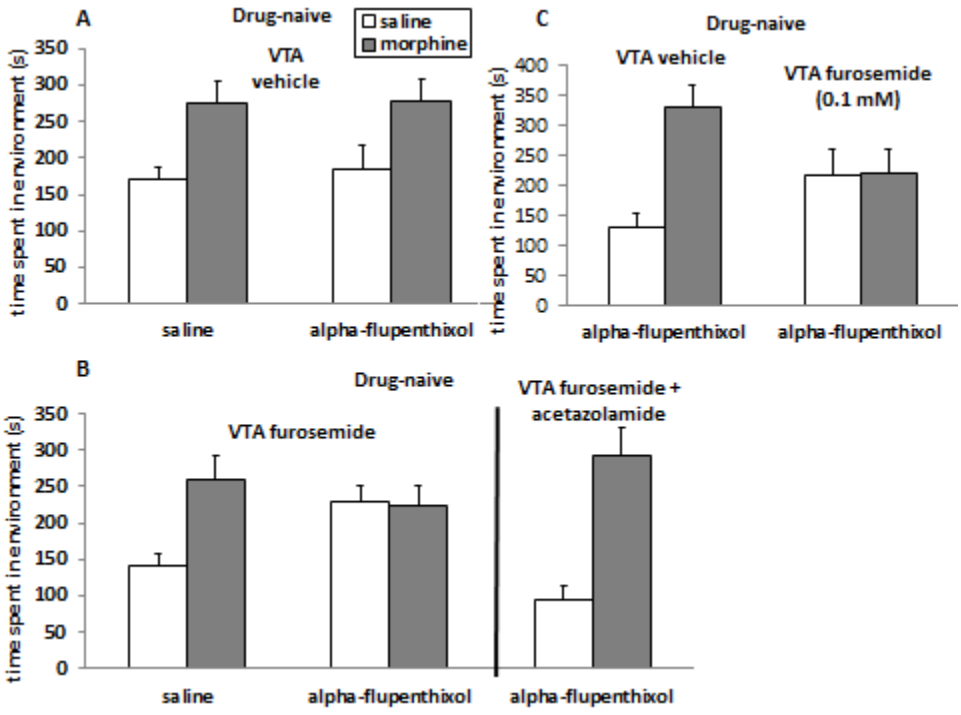
Intra-VTA furosemide switches intra-VTA opiate motivation from dopamine-independent to dopamine-dependent in non-deprived animals

To demonstrate that morphine can produce motivational effects directly within the VTA, we examined the effect of intra-VTA morphine in previously opiate-naive, non-deprived rats. Rats were pretreated with either saline or alpha-flupenthixol (N=6-9). A 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 26) = 0.071$, $p > 0.05$], a significant effect of drug conditioning (morphine vs. saline) [$F(1, 26) = 10.3$, $p < 0.05$], and no significant interaction [$F(1, 26) = 0.019$, $p > 0.05$], suggesting that non-deprived rats demonstrated intra-VTA morphine place preferences irrespective of alpha-flupenthixol pretreatment (Supplementary Figure 2a).

If an excitatory mode of VTA $GABA_A$ receptor signaling activity is responsible for selecting a dopamine-dependent mechanism of opiate motivation, we hypothesized that intra-VTA infusion of furosemide would promote such a switch. Therefore, in non-deprived rats, the effect of intra-VTA furosemide on intra-VTA morphine's motivational properties was examined. Rats were pretreated with either saline or alpha-flupenthixol (N=5-12). A 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 30) = 0.72$, $p > 0.05$] or drug conditioning (morphine vs. saline) [$F(1, 30) = 3.8$, $p > 0.05$] but a significant interaction effect [$F(1, 30) = 4.2$, $p < 0.05$]. This suggested that after intra-VTA furosemide, opiate motivation was switched to a dopamine-dependent system (Supplementary Figure 2b, left).

In non-deprived animals pretreated with alpha-flupenthixol, we co-infused furosemide and acetazolamide into the VTA of animals conditioned with intra-VTA morphine (N=8 for each group). A t-test revealed a significant effect of intra-VTA morphine [$t(1, 14) = 20.4$, $p < 0.05$], indicating that acetazolamide was able to fully reverse the effect of furosemide and prevent a switch to a dopamine-dependent motivational system (Supplementary Figure 2b, right).

To examine furosemide's specificity, we repeated the above experiment using a 10-fold lower concentration of furosemide (0.1 mM). All rats were pretreated with alpha-flupenthixol (N=6 for each group). A 2x2 ANOVA (VTA treatment x drug conditioning) revealed no effect of VTA treatment (0.1 mM furosemide vs. vehicle) [$F(1, 23) = 0.073$, $p > 0.05$], a significant effect of drug conditioning (morphine vs. saline) [$F(1, 23) = 7.17$, $p < 0.05$] and a significant interaction effect [$F(1, 23) = 6.61$, $p < 0.05$], suggesting that even after intra-VTA infusion of a 10-fold lower concentration of furosemide, opiate motivation was again switched to a dopamine-dependent system (Supplementary Figure 2c). For all other experiments involving furosemide, the higher dose (1 mM) was utilized.

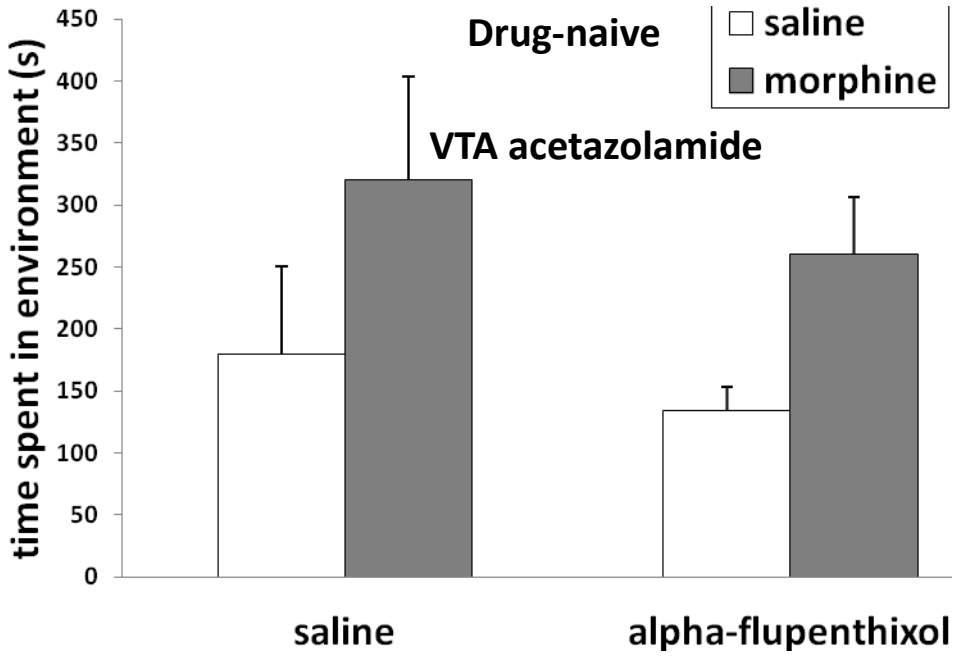


Supplementary Figure 2. In non-deprived rats, intra-VTA morphine place preferences are attenuated by dopamine receptor antagonism only after intra-VTA furosemide infusion

(a) Intra-VTA morphine (gray bars) induced conditioned place preferences in rats receiving intra-VTA vehicle irrespective of pretreatment with alpha-flupenthixol. (b) Conversely, in rats receiving intra-VTA furosemide, morphine place preferences were attenuated by alpha-flupenthixol pretreatment (left). Despite an infusion of furosemide, significant intra-VTA morphine conditioned place preferences were present in rats receiving alpha-flupenthixol after intra-VTA infusion of acetazolamide (right). (c) Intra-VTA infusion of a 10-fold lower dose of furosemide also made intra-VTA morphine place preferences sensitive to alpha-flupenthixol pretreatment. Data represent means +/- SEMs of time spent in the morphine- or saline-paired environment.

In drug naive animals, intra-VTA acetazolamide has no effect on systemic opiate motivation

To demonstrate that acetazolamide did not possess any non-specific effects, we examined the effect of acetazolamide on systemic morphine place preferences in previously opiate-naive, non-deprived rats (N=4 per group). A 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 12) = 0.76$, $p > 0.05$], a significant effect of drug conditioning (morphine vs. saline) [$F(1, 12) = 4.9$, $p < 0.05$], and no significant interaction [$F(1, 12) = 0.015$, $p > 0.05$], suggesting acetazolamide did not have any effect in non-deprived rats (Supplementary Figure 3).



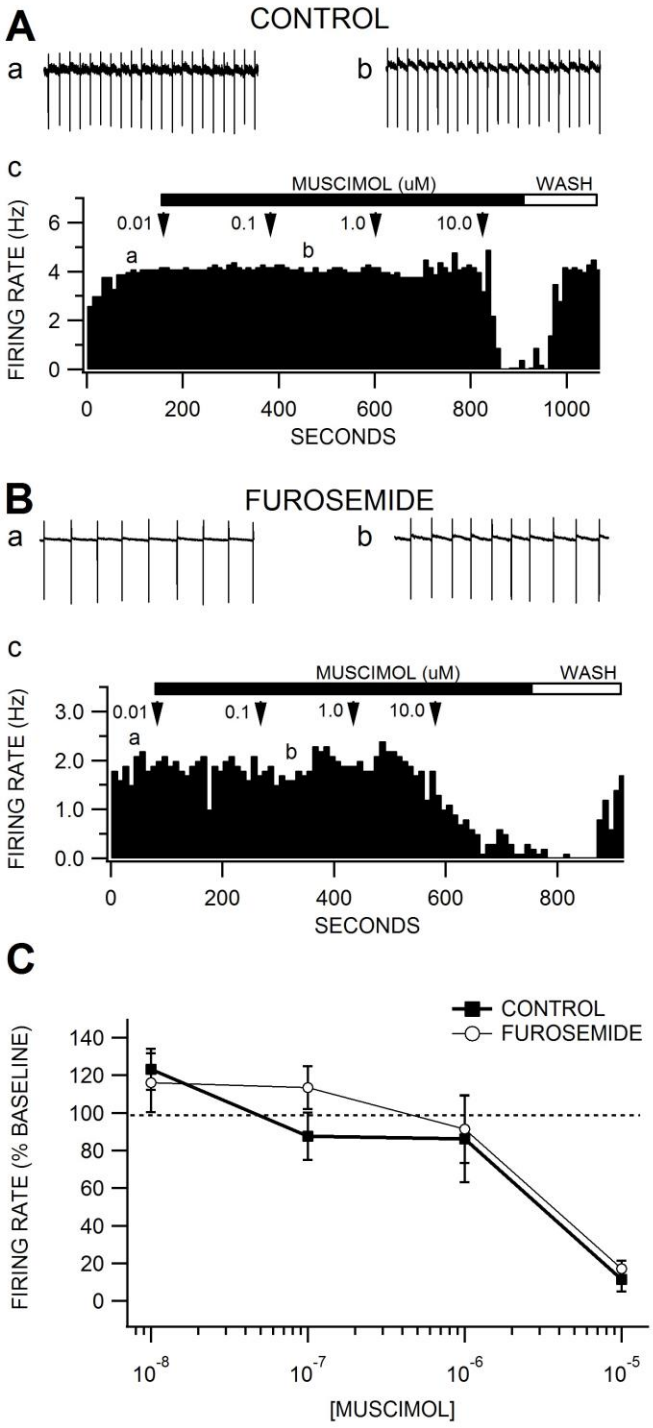
Supplementary Figure 3. Intra-VTA acetazolamide has no effect on systemic morphine motivation in drug naive animals

In naive, non-deprived rats, intra-VTA acetazolamide had no effect on the place preferences produced by systemic morphine in either saline- or alpha-flupenthixol-pretreated rats. Data represent means +/- SEMs of time spent in the morphine- or saline-paired environment.

Furosemide has no effect on muscimol-induced inhibition of VTA dopamine neuron firing rates

Putative VTA dopamine neurons identified by location, regular firing activity compared to GABA neurons and by the lack of GFP fluorescence in GAD GFP mice. The average firing rate of VTA dopamine neurons recorded in control mice was 6.6 ± 2 Hz (N=8), and in the presence of 100 µM furosemide was 4.5 ± 0.8 Hz (N=9). Furosemide did not significantly affect the firing rate of VTA dopamine neurons [$F(1, 16) = 1.04$, $p > 0.05$]. Muscimol (0.01 – 10.0 µM) inhibited putative VTA dopamine neuron firing rate in control

mice, but only at the 10.0 µM level (Supplementary Figure 4a), which was not significantly altered in furosemide-treated slices (Supplementary Figure 4b). Supplementary Figure 4c summarizes the effects of muscimol (0.01 – 10.0 µM) on putative VTA dopamine neurons in control vs. furosemide. A two-way ANOVA (treatment vs. muscimol concentration) revealed a significant main effect of muscimol concentration [$F(3, 50) = 17.7$, $p < 0.05$] but no effect of treatment (control vs. furosemide) [$F(1, 50) = 0.49$, $p > 0.05$], nor any significant treatment x concentration interaction [$F(3, 50) = 0.44$, $p > 0.05$]. Tukey HSD test revealed that the 10.0 µM concentration of muscimol significantly inhibited the firing rates of the dopamine cells as compared with all other muscimol concentrations ($p < 0.05$).

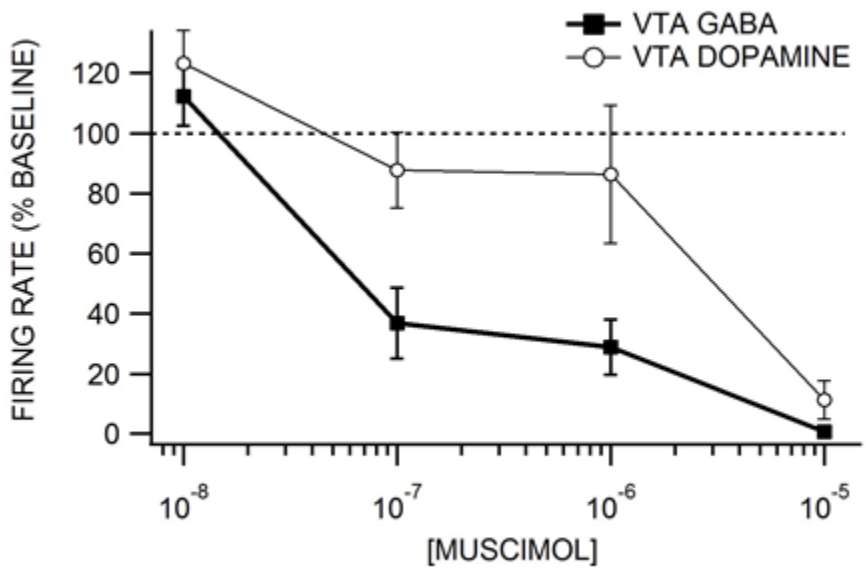


Supplementary Figure 4. *Relative lack of muscimol effects on VTA DA neurons*. (Aa,b) These are representative 5 sec traces of putative DA neuron spike activity recorded before and after muscimol. (Ac) This ratemeter shows the firing rate of this

neuron (traces in a,b recorded at times indicated on graph), which was approximately 4 Hz, before and after application of 0.01 – 10.0 µM muscimol, which was inhibited by muscimol, but only at the 10 µM concentration. (Ba,b) These are representative 5 sec traces of the spike activity of another putative DA neuron spike activity recorded before and after muscimol in the presence of 100 µM furosemide. (Bc) This ratemeter shows the firing rate of this neuron (traces in a,b recorded at times indicated on graph), which was approximately 2 Hz before and after application of 0.01 – 10.0 µM muscimol. Only 10 µM muscimol inhibited the firing rate of this neuron in the presence of furosemide. (C) Comparison of muscimol (0.01 – 10.0 µM) effects on putative VTA DA neuron firing rate for control and furosemide treatment conditions. Muscimol inhibited VTA DA neurons, but only at the 10 µM concentration, which was not altered by furosemide.

Comparison of muscimol effects on VTA GABA and dopamine neurons.

VTA GABA neurons were significantly more inhibited by 0.01- 10.0 µM muscimol than putative DA neurons (Supplementary Figure 5). A two-way ANOVA (cell type vs. muscimol concentration) revealed a significant main effect of muscimol concentration [$F(3, 57) = 24.8$, $p < 0.05$] and cell type (GABA vs. dopamine) [$F(1, 57) = 14.2$, $p < 0.05$] but no significant treatment x concentration interaction [$F(3, 50) = 2.14$, $p > 0.05$]. Post-hoc Tukey HSD revealed a significant difference between the lowest concentration (10 nM) of muscimol and all other muscimol concentrations, as well as a difference between the highest concentration (10 µM) and all other concentrations ($p < 0.05$).

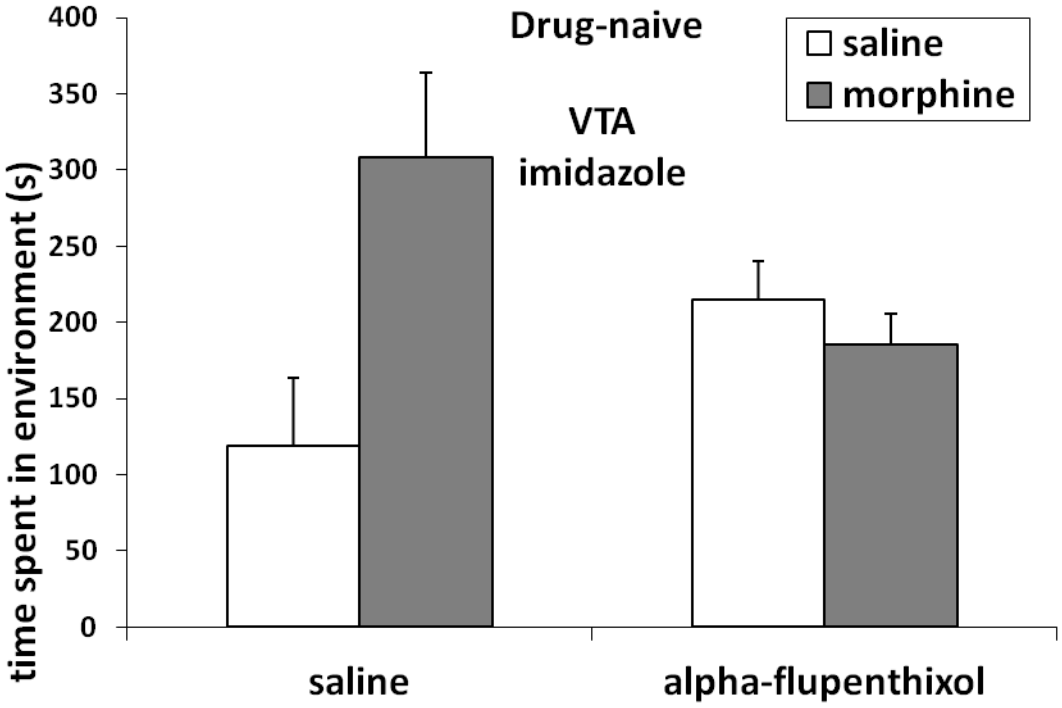


Supplementary Figure 5. GABA vs. dopamine neuron sensitivity to muscimol. Ventral tegmental area GABA neurons were significantly more sensitive to muscimol than putative dopamine neurons.

Intra-VTA imidazole switches intra-VTA opiate motivation from dopamine-independent to dopamine-dependent in non-deprived animals

To test whether an alternative to furosemide also would be effective in producing a switch in the mechanisms underlying opiate motivation, we utilized the carbonic anhydrase enzyme activator imidazole. We hypothesized that imidazole would produce an increase in bicarbonate ion production which would, in turn, result in a switch to a depolarizing mode of VTA $GABA_A$ receptor activity and consequently, a switch to a dopamine-dependent opiate motivational system. In non-deprived rats receiving intra-VTA imidazole and pretreated with either saline or alpha-flupenthixol (N=7-11), a 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 35) = 0.157$, $p > 0.05$], a significant effect of drug conditioning (morphine vs. saline) [$F(1, 35) = 5.28$, $p < 0.05$], and a significant interaction [$F(1, 35) = 9.94$, $p < 0.05$], suggesting that non-deprived rats receiving intra-VTA imidazole demonstrate morphine place

preferences that are now blocked by alpha-flupenthixol pretreatment (Supplementary Figure 6).



Supplementary Figure 6. In non-deprived rats, intra-VTA morphine place preferences are attenuated by dopamine receptor antagonism after intra-VTA imidazole infusion

In rats receiving intra-VTA imidazole, intra-VTA morphine place preferences (gray bars) were unaffected by saline pretreatment but were attenuated by alpha-flupenthixol pretreatment. Data represent means +/- SEMs of time spent in the morphine- or saline-paired environment.

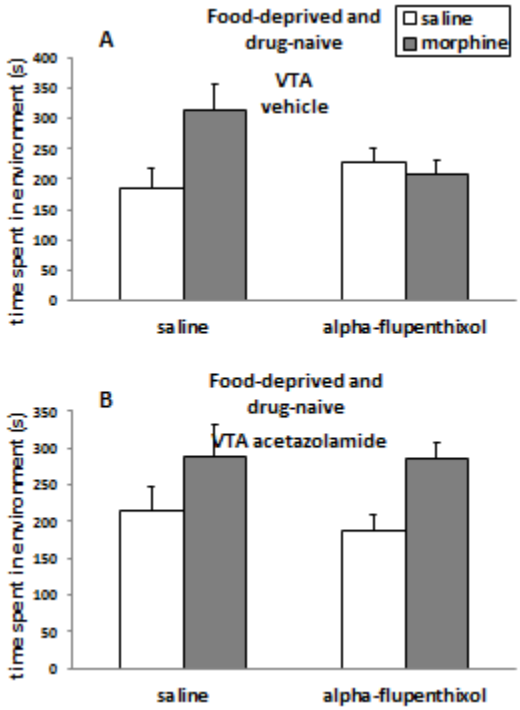
Intra-VTA acetazolamide switches opiate motivation from dopamine-dependent to dopamine-independent in opiate-naive, food-deprived animals

Previous work has indicated that a state of food deprivation is capable of switching naive opiate motivation to a dopamine-dependent state (Nader and van der Kooy 1994). We postulated that the basis for this effect might be due to interactions at a common

substrate, namely, VTA $GABA_A$ receptors. Therefore, we examined intra-VTA morphine's motivational properties in previously opiate-naive, but food-deprived rats.

For food-deprivation studies, eight conditioning sessions were spaced evenly over 16 days and each session occurred after 16 hours of food deprivation (food was returned approximately 1 hour after conditioning). Rats were pretreated with either saline or alpha-flupenthixol (N=11-17). A 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 52) = 1.05$, $p > 0.05$] or drug conditioning (morphine vs. saline) [$F(1, 52) = 3.3$, $p > 0.05$] but a significant interaction effect [$F(1, 52) = 6.2$, $p < 0.05$], suggesting that in opiate-naive, food-deprived animals, morphine place preferences were dopamine-dependent (Supplementary Figure 7a), an effect opposite to that normally observed in purely opiate-naive animals (Figure 2a).

If food deprivation causes a bicarbonate ion-dependent shift in the signaling properties of VTA $GABA_A$ receptors, then intra-VTA acetazolamide should be able to reverse this effect. Therefore, we examined the effect of intra-VTA acetazolamide on intra-VTA morphine's motivational properties in previously opiate-naive, but food-deprived, rats. Rats were pretreated with either saline or alpha-flupenthixol (N=15 for both groups). A 2x2 ANOVA (pretreatment x drug conditioning) revealed no effect of pretreatment [$F(1, 56) = 0.22$, $p > 0.05$], a significant effect of drug conditioning (morphine vs. saline) [$F(1, 56) = 7.9$, $p < 0.05$], and no significant interaction [$F(1, 56) = 0.13$, $p > 0.05$]. This suggested that food deprivation produced a shift in $GABA_A$ receptor signaling and that intra-VTA acetazolamide was able to overcome this effect, returning morphine place preferences to a dopamine-independent state (Supplementary Figure 7b).



Supplementary Figure 7. Intra-VTA morphine place preferences are attenuated by dopamine receptor antagonism in opiate-naive, but food-deprived, animals

(a) In opiate-naive, food-deprived animals, intra-VTA morphine conditioned place preferences (gray bars) were blocked by pretreatment with alpha-flupenthixol. (b) However, in rats receiving intra-VTA acetazolamide, intra-VTA morphine place preferences were observed even in the presence of alpha-flupenthixol pretreatment. Data represent means +/- SEMs of time spent in the morphine- or saline-paired environment.