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Opiate state controls bi-directional reward signaling via $GABA_A$ receptors in the ventral tegmental area

Steven R Laviolette[1], Roger A Gallegos[2], Steven J Henriksen[2] & Derek van der Kooy[1,3]

The neural mechanisms that mediate the transition from a drug-naive state to a state of drug dependence and addiction are not yet known. Here we show that a discrete population of $GABA_A$ receptors in the mammalian ventral tegmental area (VTA) serves as a potential addiction switching mechanism by gating reward transmission through one of two neural motivational systems: either a dopamine-independent (opiate-naive) or a dopaminergic (opiate-dependent or opiate-withdrawn) system. Bi-directional transmission of reward signals through this $GABA_A$ receptor substrate is dynamically controlled by the opiate state of the organism and involves a molecular alteration of the $GABA_A$ receptor. After opiate exposure and subsequent withdrawal, the functional conductance properties of the rat VTA $GABA_A$ receptor switch from an inhibitory to an excitatory signaling mode.

A fundamental question in the neurobiological study of drug addiction is how an organism switches from a drug-naive, non-dependent state to a dependent and addicted state, characterized by compulsive drug seeking behavior and craving. More importantly, are the motivational processes that underlie drug-taking behaviors controlled by the same or separate neural motivational systems during the early acute, non-dependent phase of the addiction process versus the later dependent and withdrawn phase of addiction?

Opiates represent a highly addictive drug class, producing euphoria and rapid dependence followed by intense withdrawal and compulsive drug seeking behaviors[1]. Even after a single exposure to opiates, neurophysiological, motivational and somatic symptoms of withdrawal and dependence may be evident[2,3].

Although opiate dependence and withdrawal have been shown to induce molecular alterations in multiple neural regions[4], the ventral tegmental area (VTA) and associated mesolimbic dopamine system are thought to be critical neural substrates for the motivational and dependence-producing properties of opiates and other drugs of abuse[5]. However, several lines of evidence suggest that the function of dopamine signaling during the addiction process depends on prior drug history[6–8]. Thus, in non-dependent, drug-naive animals, opiates can produce their acute rewarding effects through a dopamine-independent system that is mediated through brainstem reward circuits, including the tegmental pedunculopontine nucleus (TPP)[6,9,10]. Once animals have been chronically exposed to opiates and are in a state of withdrawal, the motivational effects of opiates are dependent on the mesolimbic dopamine system[9,11–13]. In this sense, it has been suggested that dopamine transmission mediates a drug 'wanting' or 'craving' signal, independently of the acute rewarding properties of opiates[7,14].

The divergence between dopamine-independent and dopamine-dependent reward signaling can be seen within the VTA, where opiates produce reward through a dopamine-independent neural system in the drug-naive state, but once chronically exposed to opiates and in withdrawal, VTA opiate reward signaling switches to a dopamine-dependent system[9]. This suggests that the VTA houses both dopamine-dependent and dopamine-independent neural motivational systems[15] and may serve as an anatomical locus that controls a switch from the opiate-naive, dopamine-independent acute drug reward system to the opiate-dependent/withdrawn, dopamine-dependent motivational system.

$GABA_A$ receptors associated with a population of VTA GABAergic neurons control bi-directional reward signaling between separate dopamine-independent and dopamine-dependent reward systems[16]. These VTA GABAergic neurons provide inhibitory input to the dopamine neurons, send descending projections to brainstem regions involved in dopamine-independent reward transmission[17,18] and are activated by reward-related stimuli[19]. The majority of VTA $GABA_A$ receptors are found on non-dopamine neurons[20,21], whereas the majority of VTA $GABA_B$ receptors are found on dopamine neurons[22,23]. *In vitro* or *in vivo* activation of these inhibitory VTA $GABA_A$ receptors with a $GABA_A$ receptor agonist removes the inhibitory, GABAergic input to the VTA dopamine neurons and activates the mesolimbic dopamine system[16,21,24–26]. Antagonist blockade of these $GABA_A$ receptors, on the other hand, serves to increase the activity of VTA GABAergic neurons, activating non-dopamine behavioral systems in the VTA[16,27]. From this unique functional arrangement, we reasoned that $GABA_A$ receptors associated with these cells may serve as an opiate addiction switching mechanism, controlling reward transmission between a non-dopamine, drug-naive neural motivational system, and a dopaminergic, drug-dependent/withdrawn motivational system, as a function of opiate state. Here we show that a molecular alteration in VTA $GABA_A$ receptors induced by chronic opiate exposure and withdrawal serves to dynamically control bi-

[1]Neurobiology Research Group, Department of Anatomy and Cell Biology University of Toronto, Ontario M5S 1A8, Canada. [2]Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037, USA. [3]Department of Medical Genetics and Microbiology, University of Toronto, Ontario M5S 1A8, Canada. Correspondence should be addressed to S.R.L. (laviolette@bns.pitt.edu).

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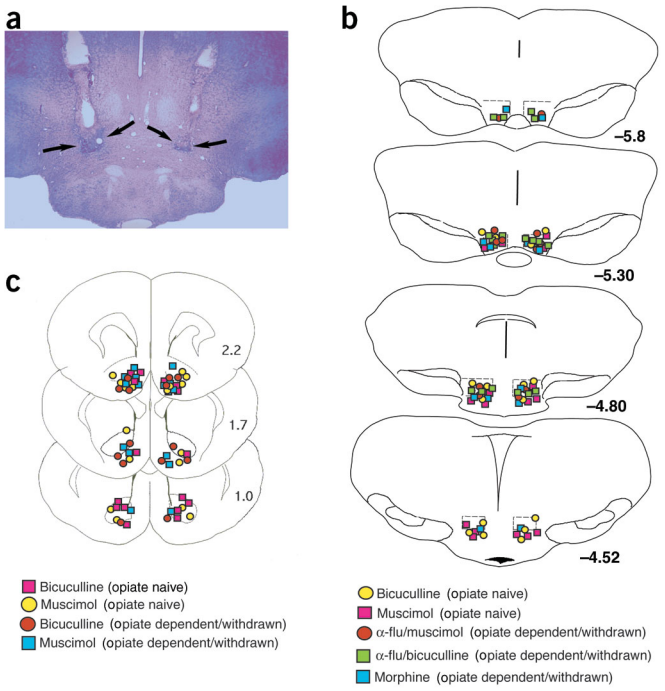

Figure 1 Histological analysis of intra-VTA and intra-NAc bilateral cannulae placements. (**a**) Cresyl Violet stained coronal section of typical bilateral intra-VTA cannulae placements. Arrows indicate cannulae tips. (**b**) Schematic representation of caudal, middle and rostral anatomical VTA levels from which cannulae and immunocytochemical analyses of the VTA were performed. For clarity, representative samples of bilateral cannulae placements are shown for opiate-naive animals receiving either intra-VTA bicuculline (50 ng) or muscimol (50 ng); animals conditioned in an opiate-dependent/withdrawn state and pretreated with α-flu before intra-VTA muscimol (50 ng) or bicuculline (50 ng) and animals receiving intra-VTA morphine (500 ng) and conditioned in a state of opiate dependence and withdrawal. The majority (>90%) of all bilateral intra-VTA cannulae placements were located in the central regions of the VTA (–4.8 to –5.3 mm from bregma). Placements located outside of these anatomical boundaries were excluded from analysis. (**c**) Schematic representation of bilateral intra-NAc cannulae placements for animals that received bilateral intra-NAc α-flu before receiving intra-VTA bicuculline or muscimol.

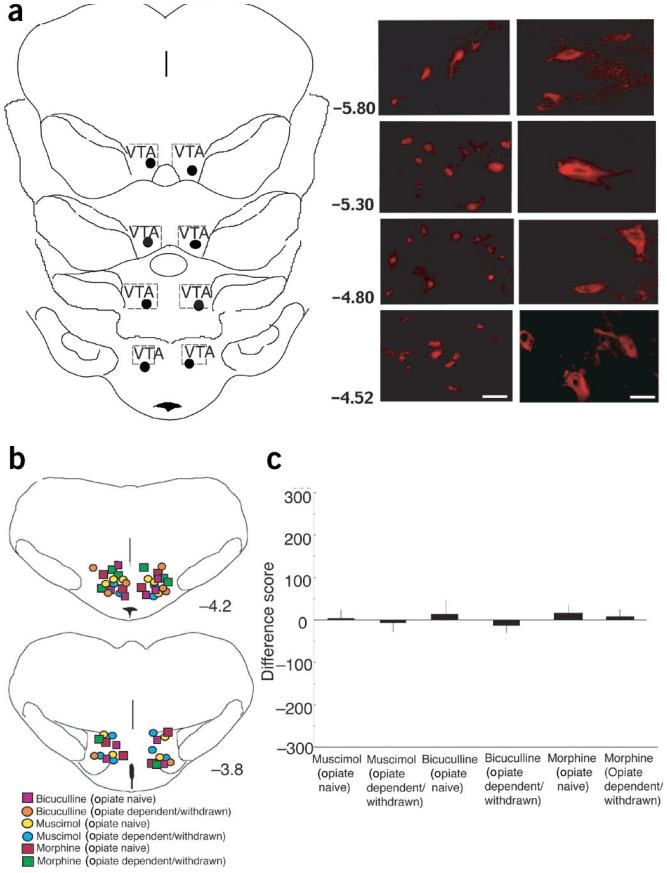

Figure 2 To verify the presence of dopaminergic A10 neurons within the vicinity of VTA cannulae tips, alternate VTA sections were processed for TH immunoreactivity. (**a**) TH$^+$ dopaminergic neurons were found in the immediate vicinity (within 50 μm) of VTA injector tips at all four levels of the VTA schematically presented in this figure (right side). Black circles represent the locations of VTA injector cannulae from which the adjacent microphotographs were taken (left side). Darkfield microphotographs show TH$^+$ neurons (red) at a lower (column immediately adjacent to VTA schematic, scale bar = 100 μm) and higher magnification (column on far right, scale bar = 20 μm). (**b**) Locations of anatomical control placements in posterior/lateral hypothalamic areas for bicuculline (50 ng), muscimol (50 ng) or morphine (500 ng) conditioned in either opiate-naive or opiate-dependent/withdrawn states. (**c**) Microinfusions of bicuculline, muscimol or morphine into the posterior/lateral hypothalamic regions did not produce motivational effects in either opiate-naive or opiate-dependent/withdrawn animals.

directional reward signaling through separate VTA reward output systems by switching the signaling properties of the $GABA_A$ receptor from inhibitory to excitatory.

RESULTS

Opiate state controls bi-directional VTA reward signaling

Bilateral intra-VTA microinfusions of the $GABA_A$ receptor agonist muscimol (5–50 ng/μl) or the $GABA_A$ receptor antagonist bicuculline (5–50 ng/μl) into the VTA produced rewarding effects as measured in the conditioned place preference paradigm (**Figs. 1a,b** and **2**). This dose range (5–50 ng) represents the motivationally active intra-VTA concentration range for both bicuculline and muscimol. Lower doses do not produce motivational effects and higher doses produce seizures or motor incapacitation[16]. The rewarding effects of either bicuculline or muscimol were similar in both naive and opiate-dependent/withdrawn animals (**Fig. 3a,b**) and were evident regardless of whether cannulae were located in the caudal, intermediate or rostral portions of the VTA (**Figs. 1b** and **2**). Immunohistochemical analysis of intra-VTA cannulae placements confirmed that tyrosine-hydroxylase (TH)-positive, dopaminergic neurons were present within a 50-μm range of the VTA injector tips at all levels of the VTA included in behavioral analysis (**Fig. 2a**). Anatomical control experiments revealed that microinfusions of bicuculline (50 ng), muscimol (50 ng) or morphine (500 ng) directly into the posterior/lateral hypothalamic regions rostral to the VTA (**Fig. 2b**) produced no motivational effects in either opiate-naive or opiate-dependent/withdrawn animals (**Fig. 2c**; bicuculline: naive, $t_4 = 0.38$, $P > 0.05$; dependent/withdrawn, $t_4 = 0.76$, $P > 0.05$; muscimol: naive, $t_4 = 0.71$, $P > 0.05$; dependent/withdrawn, $t_3 = 0.18$, $P > 0.05$; morphine: naive, $t_4 = 0.74$, $P > 0.05$; dependent/withdrawn, $t_4 = 0.18$, $P > 0.05$). A three-way ANOVA comparing difference scores between times spent in saline relative to bicuculline or muscimol-paired environments revealed no effects of group (opiate-naive versus opiate-dependent/withdrawn; $F_{1,49} = 0.14$; $P > 0.05$), treatment (muscimol or bicuculline, $F_{1,49} = 1.43$; $P > 0.05$) or dose (5 or 50 ng; $F_{1,49} = 3.67$; $P > 0.05$) on difference scores between times spent in drug versus saline-paired environments. Thus, regardless of previous opiate history and withdrawal state, the rewarding effects of bicuculline (5 ng: naive, $n = 9$; with-

drawn, $n = 8$; 50 ng: naive, $n = 8$; withdrawn, $n = 6$; **Fig. 3a**) or muscimol (5 ng: naive, $n = 7$; withdrawn, $n = 9$; 50 ng: naive, $n = 8$; withdrawn, $n = 8$; **Fig. 3b**) were quantitatively similar over an order of magnitude dose range (5–50 ng).

We compared the role of dopamine signaling in the mediation of intra-VTA bicuculline or muscimol reward both in previously opiate-naive animals and in animals after 21 h of opiate withdrawal. We blocked dopamine transmission either systemically (0.8 mg/kg; intraperitoneal (i.p.) injection) or directly in the mesolimbic dopamine terminal field with bilateral nucleus accumbens (NAc) microinfusions of α-flupenthixol, a broad-spectrum dopamine receptor antagonist[28] (α-flu, **Fig. 1c**; 3 μg/0.5 μl). Neither of these treatments alone produce any motivational effects[29], but they block dopamine-dependent opiate and amphetamine reward[13,30]. For systemic α-flu, a three-way ANOVA revealed a significant interaction among group (bicuculline or muscimol) treatment (α-flu or saline) and opiate state (dependent/withdrawn or naive) on difference scores between times spent in saline versus bicuculline- or muscimol-paired environments ($F_{1,54} = 11.1$; $P < 0.05$). In opiate-naive animals, α-flu blocked the rewarding effects of muscimol ($n = 7$, *post-hoc* test, $P < 0.05$) but not bicuculline ($n = 8$, *post-hoc* test, $P > 0.05$; **Fig. 3c**). However, in opiate-withdrawn animals (21 h), dopamine receptor blockade reversed the dissociation between intra-VTA muscimol and bicuculline reward transmission: dopamine receptor blockade now blocked the rewarding effects of bicuculline ($n = 9$, *post-hoc* test, $P < 0.05$) but not muscimol ($n = 6$, *post-hoc* test, $P > 0.05$; **Fig. 3d**). Direct blockade of dopamine receptors in the NAc similarly dissociated dopamine-dependent and dopamine-independent intra-VTA reward signaling as a function of opiate state. A three-way ANOVA revealed a significant interaction among state (opiate naive or withdrawn) group (muscimol or bicuculline) and treatment (α-flu or saline) on difference scores between times spent in saline versus muscimol- or bicuculline-paired environments ($F_{1,32} = 42.02$, $P < 0.05$). In opiate-naive animals, intra-NAc dopamine receptor blockade blocked intra-VTA muscimol reward ($n = 9$, *post-hoc* test, $P < 0.05$), but not bicuculline reward ($n = 8$, *post-hoc* test, $P > 0.05$). In contrast, in opiate-withdrawn animals, this same treatment completely blocked intra-VTA bicuculline reward ($n = 7$, *post-hoc* test, $P < 0.05$), but had no effect on muscimol reward ($n = 9$, *post-hoc* test, $P > 0.05$; **Fig. 3c**,**d**). These results demonstrate a double-dissociation between $GABA_A$ receptor–mediated VTA reward transmission through either a non-dopamine reward pathway or dopamine-dependent reward pathway as a function of the opiate state of the animal.

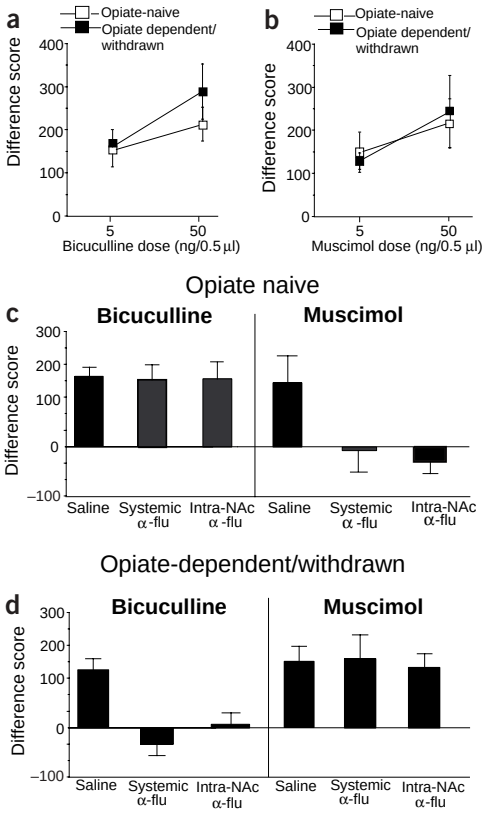

Figure 3 Motivational effects of intra-VTA muscimol or bicuculline in the opiate-naive versus opiate-dependent/withdrawn state: role of dopamine signaling. (**a**) Intra-VTA bicuculline (5–50 ng) produces robust rewarding effects in either opiate-naive or opiate-dependent/withdrawn animals. (**b**) Intra-VTA muscimol (5–50 ng) produces robust rewarding effects in both opiate-naive and opiate-dependent/withdrawn animals. (**c**) In opiate-naive animals, systemic (0.8 mg/kg) or intra-NAc dopamine receptor blockade did not attenuate intra-VTA bicuculline reward (50 ng), but it completely blocked intra-VTA muscimol (50 ng) reward. (**d**) In opiate-dependent/withdrawn animals, these identical dopamine receptor blockade treatments now completely block the rewarding effects of intra-VTA bicuculline (50 ng), but have no effect on intra-VTA muscimol reward (50 ng).

$GABA_A$ and opiate VTA reward switching operate in parallel

Within the VTA, the rewarding effects of opiates switch to a dopamine-dependent reward pathway by 21 h of opiate withdrawal[9]. We asked whether opiate exposure alone is sufficient to induce this switch, or whether the animal needs to be in a state of opiate withdrawal for the VTA switching mechanism to be operational. We examined the role of dopamine signaling in the mediation of intra-VTA bicuculline (50 ng), muscimol (50 ng) or morphine (500 ng) reward in animals that received the identical heroin pre-exposure protocol (see Methods) but were taken out of withdrawal with a single dose of heroin (0.5 mg/kg) 3.5 h before conditioning. To establish the timeline of opiate withdrawal aversions, we used a single-sided conditioned place aversion (CPA) procedure (see Methods) and examined the motivational effects of opiate withdrawal at 3.5 h and 21 h post-injection. When conditioned at 3.5 h since the last heroin exposure, animals showed no CPA to the withdrawal-paired environment ($t_4 = 0.11$, $P > 0.05$, $n = 5$; **Fig. 4a**). In contrast, animals exposed to environments at 21 h after opiate injection ($n = 8$) showed strong CPA to environments paired with opiate withdrawal ($t_7 = 5.61$, $P < 0.05$; **Fig. 4a**), demonstrating that the development of opiate withdrawal aversion is dependent on the time elapsed since the previous opiate exposure. We examined the timeline by which intra-VTA opiate reward signaling switches from a non-dopamine reward system to a dopamine-dependent reward system following the last heroin exposure in an opiate-dependent animal. Animals received systemic α-flu (0.8 mg/kg) or saline 2.5 h prior to intra-VTA morphine (500 ng/0.5 μl). A two-way ANOVA comparing difference scores between times spent in saline or morphine-paired sides revealed a significant interaction between group (3.5 or 21 h withdrawal) and treatment (α-flu or saline) ($F_{1,19} = 10.5$; $P < 0.05$). Whereas the rewarding effects of morphine were not blocked by α-flu at 3.5 after injection ($n = 6$, *post-hoc* test, $P > 0.05$), they were completely blocked at 21 h after opiate injection ($n = 6$, *post-hoc* test, $P < 0.05$; **Fig. 4b**). Thus, 3.5 h after heroin injection, CPA is absent, and intra-VTA opiate reward signaling can be mediated independently of dopamine. In contrast, at 21 h, the aversive effects of withdrawal are strongly present, and the withdrawal-alleviating effects of intra-VTA opiates are dopamine-dependent (**Fig. 4a**,**b**).

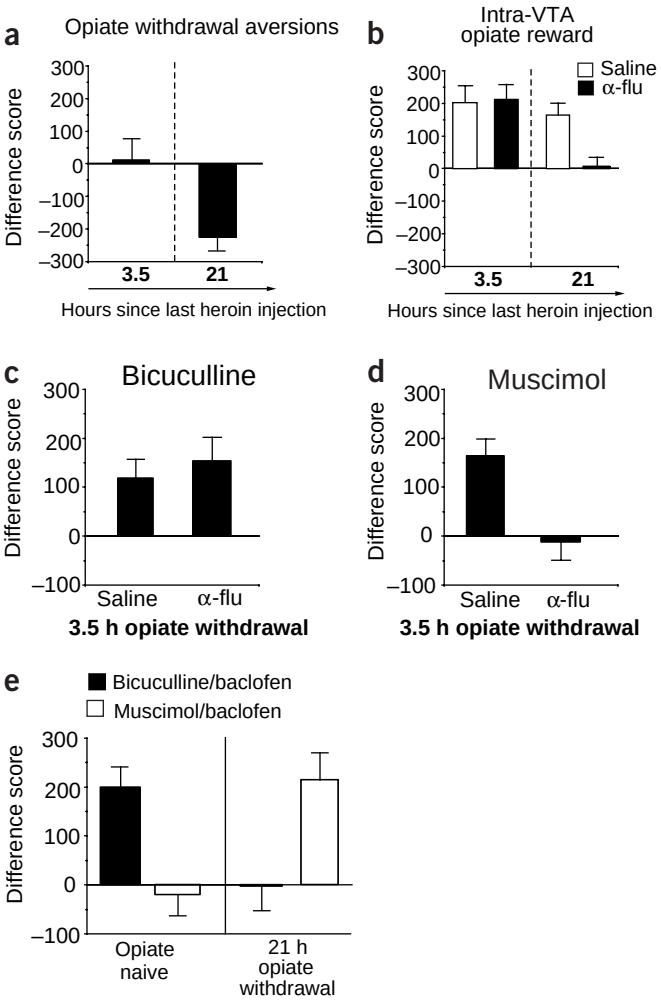

Figure 4 Time-dependent effects on opiate withdrawal aversions and role of dopamine signaling; effects of $GABA_B$ receptor activation on VTA $GABA_A$ reward transmission. (**a**) The aversive effects of opiate withdrawal are present at 21 h post-opiate exposure, but not at 3.5 h of withdrawal. (**b**) Intra-VTA morphine reward (500 ng) signaling is independent of dopamine transmission at 3.5 h of opiate withdrawal, but by 21 h, dopamine receptor blockade completely blocks intra-VTA morphine (500 ng) reward. (**c**) In opiate-dependent animals that had been taken out of withdrawal (3.5 h post-opiate exposure) intra-VTA bicuculline (50 ng) reward signaling was dopamine-independent, similar to opiate-naive animals. (**d**) In opiate-dependent animals that had been taken out of withdrawal (3.5 h post-opiate exposure) intra-VTA muscimol (50 ng) reward signaling was dopamine-dependent, similar to opiate-naive animals. (**e**) Activation of VTA $GABA_B$ receptors double dissociates intra-VTA $GABA_A$ receptor–mediated reward transmission. In opiate-naive animals, activation of $GABA_B$ receptors with baclofen (70 ng) had no effect on bicuculline reward signaling (50 ng) but completely blocked muscimol reward signaling (50 ng). Conversely, in opiate-dependent/withdrawn animals, baclofen now completely blocks the bicuculline reward signal, but does not effect the muscimol reward signal.

We next explored the role of dopamine signaling in $GABA_A$ receptor reward transmission in animals conditioned 3.5 h after opiate injection. A two-way ANOVA revealed a significant interaction between group (muscimol or bicuculline) and treatment (saline or α-flu) on difference scores between times spent in saline versus muscimol or bicuculline-paired environments ($F_{1,29} = 7.3$, $P < 0.05$). Whereas the rewarding effects of intra-VTA muscimol were blocked by dopamine receptor blockade ($n = 8$, *post-hoc* test, $P < 0.05$), the effects of bicuculline were not ($n = 8$, *post-hoc* test, $P > 0.05$), even though these animals had received chronic opiate exposure (**Fig. 4c**,**d**). Thus, similar to intra-VTA opiate reward, functional switching between $GABA_A$ receptor-mediated dopamine-dependent and dopamine-independent motivational systems in the VTA depends on the presence of opiate withdrawal, occurring between 3.5 and 21 h from the last opiate exposure.

$GABA_B$ receptors dissociate VTA reward switching

VTA $GABA_B$ receptors are functionally and anatomically localized to dopamine neurons[21,23]. If the observed functional switch at the $GABA_A$ receptor takes place at a common VTA substrate, upstream of the mesolimbic dopamine system, then activation of inhibitory VTA $GABA_B$ receptors associated with these dopamine neurons should double dissociate (in a manner analogous to neuroleptic dopamine receptor blockade) bi-directional VTA reward transmission as a function of opiate state. To examine this, we co-administered the $GABA_B$ agonist baclofen (70 ng/0.5 μl) with either intra-VTA muscimol (50 ng/0.5 μl) or bicuculline (50 ng/0.5 μl) in both naive animals and animals that were opiate dependent and in 21 h of withdrawal. This dose of baclofen produces no motivational effects[16], and similar concentrations of intra-VTA baclofen attenuate cocaine self-administration and decrease dopamine release in the mesolimbic pathway[31,32]. A two-way ANOVA revealed a significant interaction between group (opiate-naive or withdrawn) and treatment (baclofen/muscimol or baclofen/bicuculline) on difference scores between times spent in saline or drug-paired environments ($F_{1,27} = 22.7$; $P < 0.05$). In opiate-naive animals, baclofen blocked the rewarding effects of intra-VTA muscimol ($n = 7$, *post-hoc* test, $P < 0.05$), but not bicuculline ($n = 7$, *post-hoc* test, $P > 0.05$; **Fig. 4e**). However, in opiate-dependent/withdrawn animals, baclofen blocked the rewarding effects of bicuculline ($n = 7$, *post-hoc* test, $P < 0.05$), but not muscimol ($n = 7$, *post-hoc* test, $P > 0.05$; **Fig. 4e**). Thus, inactivation of VTA dopamine neurons with a $GABA_B$ agonist serves to double-dissociate $GABA_A$ receptor–mediated VTA reward transmission through a non-dopamine or dopamine-dependent reward pathway, as a function of opiate state.

Due to the functional arrangement of VTA GABAergic neurons, differential activation of inhibitory $GABA_A$ receptors associated with these neurons will either activate the VTA GABA neurons (as with the $GABA_A$ antagonist) or inactivate these neurons, and in turn activate the VTA dopamine neurons (as with the $GABA_A$ agonist). This pattern of results was seen in previously opiate-naive animals (**Fig. 3c**). In opiate-dependent/withdrawn animals, we observed a reversal of this pattern (**Fig. 3d**). These results are paradoxical because if the same $GABA_A$ receptor compounds act through an inhibitory $GABA_A$ receptor in opiate-withdrawn animals, the same pattern of results would be expected in both opiate states. However, if the conductance properties of the $GABA_A$ receptor were switched from inhibitory to excitatory in the opiate-dependent/withdrawn state, then agonist or antagonist actions at the receptor would produce opposite effects at VTA GABAergic neurons from that observed in the naive state: muscimol would now activate an excitatory $GABA_A$ receptor, activating the dopamine-independent reward pathway, whereas blocking this excitatory receptor with bicuculline would have a net inhibitory effect on the GABAergic neuron, removing inhibition to the dopamine neurons and activating the dopamine-dependent reward pathway. We tested this hypothesis at the molecular, physiological and behavioral levels of analysis.

Opiate state modulates CREB through $GABA_A$ receptors

To examine whether opiate exposure and withdrawal induces a molecular switch in the signaling properties of VTA $GABA_A$ receptors

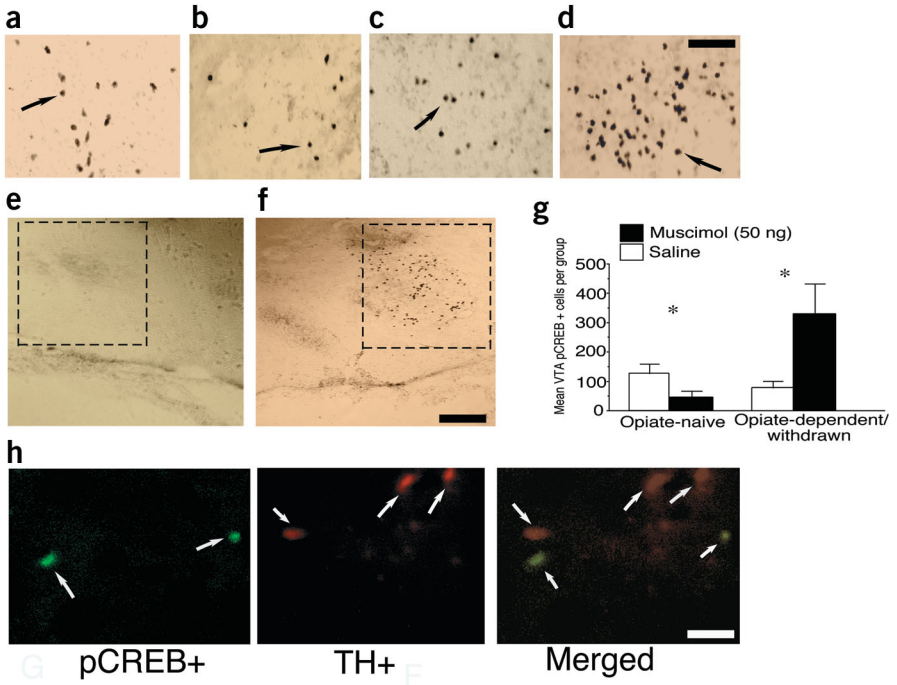

Figure 5 Agonist activation of VTA $GABA_A$ receptors decreases CREB phosphorylation in the opiate-naive state, but increases CREB phosphorylation in the opiate-dependent/withdrawn state. (**a**) Microphotograph of a VTA section from an opiate-naive, intra-VTA saline treated animal. Arrows indicate representative pCREB positive cells, in this and subsequent panels. (**b**) VTA section from an opiate-naive, intra-VTA muscimol (50 ng) treated animal. (**c**) VTA section from an opiate-dependent/withdrawn (21 h), intra-VTA saline treated animal. (**d**) VTA section from an opiate-dependent/withdrawn, intra-VTA muscimol (50 ng) treated animal. (**e**) VTA section (about –5.3 mm from bregma, see **Fig. 1b**) from an opiate-naive, intra-VTA muscimol (50 ng) treated animal. pCREB expression is not evident. Black hatched box surrounds the VTA region in this panel and in **f**. (**f**) VTA section (about –5.3 mm from bregma, see **Fig. 1b**) from an opiate-dependent/withdrawn, intra-VTA muscimol (50 ng) treated animal. VTA pCREB expression is strongly expressed in the VTA following $GABA_A$ receptor activation. (**g**) Quantification of pCREB expression in the VTA following intra-vTA saline or muscimol (50 ng) in opiate-naive or opiate-dependent/withdrawn animals. Bars represent mean pCREB-positive cells within the VTA per group. $*P < 0.05$ (see Methods). (**h**) $pCREB^+$ (green) cells in the VTA. (**i**) TH^+ (red) cells in the VTA. (**j**) Merged image showing separate distributions of $pCREB^+$ (non-dopamine) and TH^+ (dopamine) neurons within the VTA. Scale bars: 40 μm (**a**–**d**); 200 μm (**e**,**f**); 30 μm (**h**–**j**).

from inhibitory to excitatory signaling[33], we examined the effects of VTA $GABA_A$ receptor activation on the phosphorylation of CREB in either opiate-naive or opiate-dependent/withdrawn animals. Phosphorylation of CREB at Ser133 correlates with both inhibitory (decreased CREB phosphorylation) or excitatory (increased CREB phosphorylation) post-synaptic events associated with $GABA_A$ receptor signaling[34,35]. Previous reports have shown that activation of $GABA_A$ receptors with muscimol results in fewer pCREB-positive cells in several brain regions when the $GABA_A$ receptor is in inhibitory mode but causes increased pCREB expression when activation of the $GABA_A$ receptor mediates a depolarizing response[34,35]. We examined whether muscimol (50 ng) activation of VTA $GABA_A$ receptors would cause a decrease in pCREB-positive cell numbers in previously opiate-naive animals, and, by contrast, an increase in pCREB-positive cell numbers in opiate-dependent/withdrawn animals. In animals that were opiate-naive, intra-VTA muscimol ($n = 6$; 50 ng) caused a significant decrease in VTA pCREB-positive cells relative to control animals receiving intra-VTA saline ($n = 5$; $t_8 = 2.28$; $P < 0.05$; **Fig. 5a–g**). In contrast, in opiate-dependent/withdrawn animals (21 h), intra-VTA muscimol ($n = 5$; 50 ng) caused a significant increase in pCREB-positive VTA cells relative to saline control ($n = 5$; $t_8 = 3.1$; $P < 0.05$; **Fig. 5g**). The highest concentrations of pCREB-positive neurons (**Fig. 5a–f**) were localized in the immediate vicinity of the intra-VTA injector tips, with fewer at farther distances from the injector locations. This expression pattern was present regardless of whether the injector tips were localized in the caudal, middle or rostral VTA sections, and pCREB-positive neurons were observable at all levels of the VTA that were analyzed (**Fig. 1b** and **Fig. 2**).

To distinguish non-dopamine from dopaminergic VTA cells relative to pCREB expression, a subsequent experimental group ($n = 5$) that was opiate- dependent/withdrawn received intra-VTA muscimol (50 ng) at 21 h after opiate withdrawal. VTA sections from these animals were analyzed with double immunocytochemical labeling using markers for tyrosine hydroxylase (TH) and pCREB. Comparing numbers of single-labeled pCREB-positive VTA cells and single-labeled TH-positive VTA cells relative to cells double-labeled for both pCREB and TH, revealed that the majority (83%) of cells in the VTA expressing pCREB after $GABA_A$ agonist activation were of non-dopaminergic, presumably GABAergic, identity (**Fig. 5h–j**). A small percentage of VTA cells (17%) showed double labeling for both TH and pCREB, thus demonstrating that some VTA dopaminergic neurons also increase pCREB expression after $GABA_A$ receptor activation. Thus, consistent with previous reports, excitatory and inhibitory $GABA_A$ receptor activation produce opposing effects on pCREB expression[34,35]. Within the VTA, this $GABA_A$ receptor–mediated regulation of pCREB expression can be controlled by the opiate state of the animal. No pCREB-positive neurons were observed outside the anatomical boundaries of the VTA, indicating anatomical specificity of these microinfusions within the A10 region.

A subset of VTA GABAergic neurons switch to excitatory signaling

To test *in vivo* for an opiate withdrawal–induced switch from an inhibitory to excitatory $GABA_A$ receptor conductance in VTA GABAergic neurons, we performed iontophoretic recordings of electrophysiologically identified GABAergic neurons in the VTA[36] and compared responses in these neurons to application of muscimol in the opiate-naive versus opiate-dependent/withdrawn states. In saline-treated controls tested in an opiate-naive state, GABAergic VTA neurons ($n = 8$) showed typical response patterns to the $GABA_A$ receptor agonist, with 100% of the cells showing a significant decrease in activity relative to pre-drug firing rates ($t_7 = 8.8$, $P < 0.05$; 37% firing frequency decrease from baseline) in response to muscimol (**Fig. 6a**,**b**,**e**,**g**). However, in opiate-dependent/withdrawn animals, a subset of GABAergic VTA neurons (4/9, 44%) showed significantly

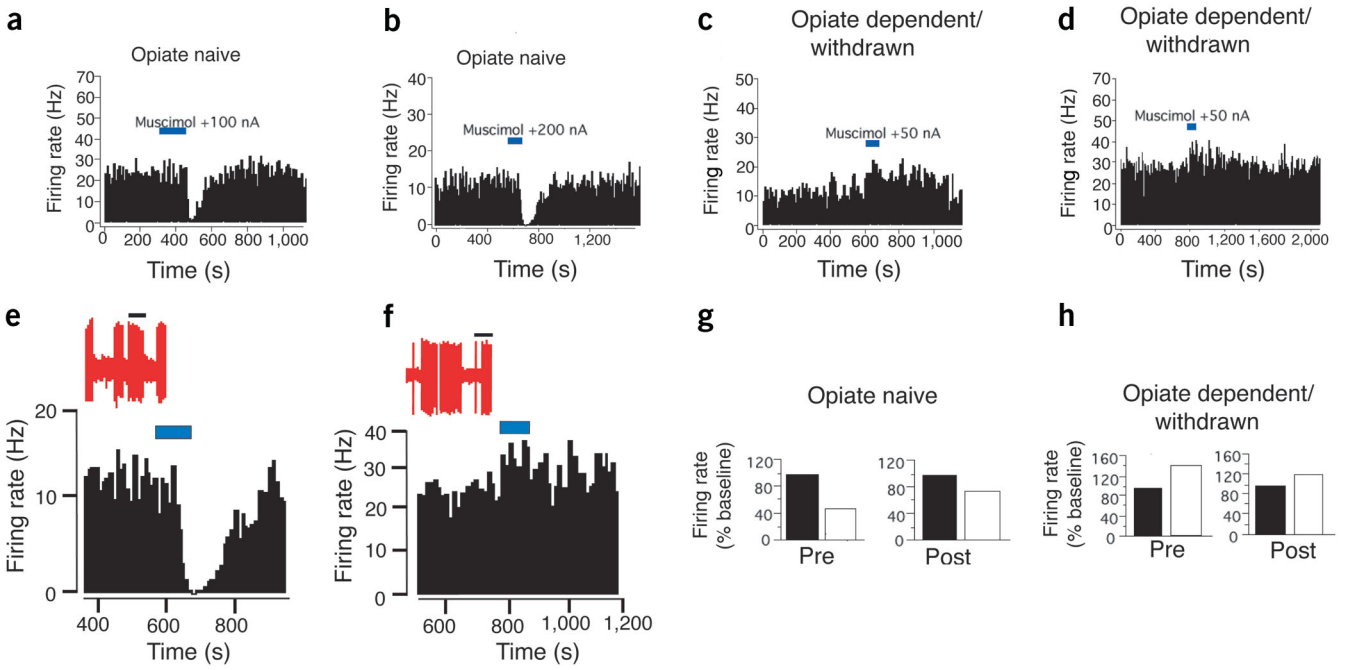

Figure 6 Single-unit extracellular recordings of VTA GABAergic neurons shows that a subpopulation of these neurons respond with excitatory depolarization to receptor activation in the opiate-dependent/withdrawn state. (**a**–**d**) Blue bar trace shows onset and duration of muscimol application. (**a**,**b**) VTA GABAergic neuronal activity in response to muscimol application recorded from opiate-naive animals showing typical inhibitory firing patterns. (**c**,**d**) VTA GABAergic neuronal activity in response to muscimol application recorded from opiate-dependent/withdrawn (21 h) animals showing excitatory firing pattern. (**e**) The inhibitory response to iontophoretic muscimol application (blue bar) from the opiate-naive neuron in panel **b** showing in greater detail the 200-s pre- and post-recording epochs. Raw firing rate is shown in red with a black bar indicating muscimol application. (**f**) The excitatory response to iontophoretic muscimol application (blue bar) from the opiate-withdrawn neuron shown in panel **d** showing in greater detail, the 200-s pre- and post-recording epochs. Raw firing rates are shown in red with a black bar indicating muscimol application. (**g**,**h**) GABAergic neuronal activity change in firing activity for the 5-min recording period following muscimol application as a percentage of baseline firing rate recorded from two neurons from two opiate-naive animals (**g**) and from two neurons from two opiate-dependent/withdrawn animals (**h**).

increased activity relative to pre-drug firing rates ($t_3 = 6.4$, $P < 0.05$; 34% firing frequency increase; **Fig. 6c**,**d**,**f**,**h**) in response to muscimol, whereas 5/9 (56%) showed the typical inhibitory response. Comparing these pre- and post-muscimol firing rate differences across opiate-naive versus opiate-dependent/withdrawn groups revealed a significant difference between groups ($t_6 = 2.63$; $P < 0.05$), demonstrating that a subset of VTA GABAergic neurons undergo a functional switch from inhibitory to excitatory, in the opiate-dependent/withdrawn state.

Carbonic anhydrase inhibition reverses VTA reward switching

To examine behaviorally whether the observed switch in bi-directional reward transmission between dopaminergic and non-dopaminergic reward substrates through VTA $GABA_A$ and opiate receptors is dependent upon a switch from inhibitory to excitatory $GABA_A$ receptor signaling, we co-administered the carbonic anhydrase (CA) inhibitor acetazolamide (10–50 μM; 5–25 nmol/0.5 μl). Acetazolamide blocks selectively depolarizing $GABA_A$ receptor–mediated potentials by inhibiting CA[33]. If the observed switch in VTA reward transmission involves a transformation from inhibitory to excitatory signaling at the $GABA_A$ receptor, blockade of excitatory potentials through the $GABA_A$ receptor should prevent the functional switch from dopamine-independent to dopamine-dependent reward signaling at $GABA_A$ receptors associated with VTA GABAergic neurons. Co-administration of acetazolamide (10 or 50 μM) with either intra-VTA bicuculline (50 ng) or muscimol (50 ng) prevented the switch induced by opiate withdrawal between dopamine-dependent and dopamine-independent reward transmission (**Fig. 7a**,**b**). In the presence of acetazolamide, systemic α-flu (0.8 mg/kg) blocked the rewarding effects of muscimol in opiate-dependent/withdrawn animals (21 h) (**Fig. 7a**), but not bicuculline (**Fig. 7b**). A two-way ANOVA revealed a significant interaction between group (bicuculline or muscimol) with treatment (saline or α-flu) on difference scores between times spent in saline or drug-paired environments ($F_{1,29} = 12.3$; $P < 0.05$). α-flu blocked the rewarding effects of muscimol ($n = 9$, *post-hoc* test, $P < 0.05$) relative to saline-pretreated animals ($n = 7$). In contrast, animals showed a significant preference for bicuculline-paired environments ($n = 7$, *post-hoc* test, $P < 0.05$) relative to saline controls ($n = 7$). Thus, muscimol reward signaling was switched back to the dopamine-dependent reward pathway, whereas bicuculline reward signaling was switched back to the dopamine-independent reward pathway. In opiate-dependent/withdrawn animals, this switching of motivational substrates indicates that $GABA_A$ receptor signaling reverted to the opiate-naive state, in the presence of acetazolamide. Furthermore, acetazolamide (50 μM) had no effect on the rewarding effects of either bicuculline or muscimol, as both saline control groups (non-α-flu-pretreated) displayed CPP for drug-paired environments (*post-hoc* tests, all $P < 0.05$). A lower concentration of acetazolamide (10 μM) similarly prevented the functional switching of $GABA_A$ reward transmission in opiate-withdrawn animals: intra-VTA muscimol reward was blocked by systemic α-flu ($t_6 = 0.31$; $P > 0.05$) whereas intra-VTA bicuculline reward was not ($t_7 = 4.54$; $P < 0.05$; **Fig. 7a**,**b**). In two separate, opiate-naive control groups, acetazolamide (50 μM) was co-administered with intra-VTA bicu-

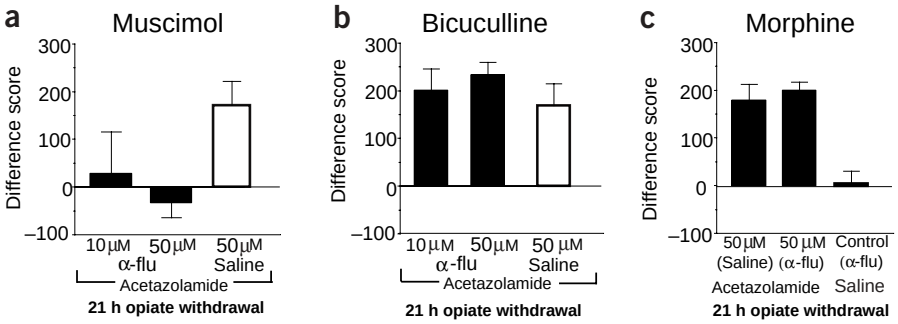

Figure 7 The functional switch in $GABA_A$ receptor–mediated reward transmission is dependent upon carbonic anhydrase activity in the opiate-withdrawn state. (**a**) In opiate-dependent/withdrawn (21 h) animals, blockade of VTA CA activity with acetazolamide (10 or 50 μM) restored intra-VTA muscimol reward transmission (50 ng) to a dopamine-dependent neural motivational pathway, as dopamine receptor blockade now completely blocks intra-VTA muscimol reward. (**b**) In opiate-dependent/withdrawn (21 h) animals, blockade of VTA CA activity with acetazolamide (10 or 50 μM) restored intra-VTA bicuculline reward transmission (50 ng) to a dopamine-independent neural motivational pathway, as dopamine receptor blockade now has no effect on intra-VTA bicuculline reward transmission. (**c**) Blocking CA activity similarly prevented the intra-VTA opiate reward signaling switch to a dopamine-dependent pathway in the opiate-dependent/withdrawn state. In the presence of acetazolamide (50 μM), dopamine receptor blockade had no effect on VTA morphine (500 ng) reward transmission, but in the absence of acetazolamide, this same dopamine receptor antagonism completely blocked VTA morphine reward signaling.

culline (50 ng, $n = 6$) or muscimol (50 ng, $n = 7$). Both the muscimol group ($t_6 = 4.6$; $P < 0.05$) and bicuculline group ($t_5 = 8.1$; $P < 0.05$) showed robust CPP for the drug-paired environments, indicating that acetazolamide was not inducing any non-specific behavioral impairments (data not shown). Thus, the observed functional switching between dopaminergic and non-dopaminergic VTA reward substrates during the opiate-withdrawn state depends on the activity of CA, suggesting an opiate-withdrawal-induced switch between an inhibitory, Cl^--mediated inhibitory $GABA_A$ receptor conductance to a $HC0_3^-$-mediated, excitatory $GABA_A$ receptor conductance.

We next examined whether the switch in intra-VTA opiate reward signaling to a dopamine-dependent reward system that occurs in states of opiate withdrawal is dependent upon a functional molecular alteration occurring at the level of the VTA $GABA_A$ receptor, similar to $GABA_A$ receptor–mediated reward. Thus, we tested whether acetazolamide (50 μM) would similarly prevent the switch to a dopamine-dependent, VTA opiate reward pathway in opiate-withdrawn animals (**Fig. 4b**). We co-administered acetazolamide (50 μM) with intra-VTA morphine (500 ng). This dose of intra-VTA morphine is a just suprathreshold rewarding dose[9] in opiate-withdrawn animals (21 h). We also pretreated separate groups with either saline or α-flu (0.8 mg/kg). An additional group of animals (in 21 h opiate withdrawal) was pretreated with α-flu in the absence of acetazolamide.

In the presence of acetazolamide, the rewarding effects of intra-VTA morphine were not blocked by α-flu in animals that were in a state of opiate withdrawal. However, in the control group of animals conditioned in the absence of acetazolamide, α-flu blocked the rewarding effects of morphine (500 ng; **Fig. 7c**). A one-way ANOVA revealed a significant effect of treatment group (acetazolamide/morphine/saline ($n = 8$) or acetazolamide/morphine/α-flu ($n = 9$) or morphine/α-flu ($n = 7$)) on difference scores between times spent in saline or morphine-paired environments ($F_{2,23} = 15.7$; $P < 0.05$). *Post-hoc* analysis revealed that animals receiving intra-VTA morphine and neuroleptic pretreatment in the absence of acetazolamide spent significantly less time in the morphine-paired environment than either group conditioned in the presence of acetazolamide (**Fig. 7c**). A separate control group conditioned in an opiate-naive state in the presence of acetazolamide (50 μM) showed normal morphine reward (data not shown). Thus, similar to the effects observed with $GABA_A$ receptor reward transmission, acetazolamide in the VTA reversed the opiate withdrawal–induced switch to a dopamine-dependent opiate reward signaling pathway.

DISCUSSION

Our behavioral, molecular and electrophysiological results indicate that VTA $GABA_A$ receptors localized to a subset of GABAergic neurons can act as a functional switching mechanism to direct neural motivational signals through pharmacologically and anatomically separate brain reward systems. This switching involves a transformation of VTA $GABA_A$ receptors from inhibitory to excitatory signaling modes and is dynamically regulated by opiate state.

Opiate exposure and withdrawal did not induce any observable changes in the reward sensitivity of VTA $GABA_A$ receptors. However, we observed a double dissociation in the bi-directional VTA $GABA_A$ reward signaling as a function of opiate state. Given that activation or inhibition of inhibitory VTA $GABA_A$ receptors produces reward through a common receptor substrate[16], a switch in the bi-directional transmission of dopaminergic and non-dopaminergic VTA reward signals as a function of opiate state seems highly paradoxical. If VTA $GABA_A$ receptors remain inhibitory during opiate-dependence/withdrawal, the same pattern of results observed in opiate-naive animals would be expected: agonist activation of $GABA_A$ receptors would produce dopamine-dependent reward by removing inhibition on the mesolimbic dopamine system, and antagonist blockade would produce a dopamine-independent reward signal by increasing the activity of VTA GABAergic neurons and the associated non-dopamine reward pathway. The finding that $GABA_A$ receptors associated with a subset of VTA GABAergic neurons switch their conductance properties from an inhibitory to an excitatory conductance mode can provide a functionally parsimonious explanation for the bi-directional reward switching seen in the present study. This reversal in $GABA_A$ receptor conductance can account for the present results because agonist activation or antagonist blockade of excitatory VTA $GABA_A$ receptors would produce the opposite pattern of activation of VTA GABAergic neurons to that produced by an inhibitory $GABA_A$ receptor: agonist activation of an excitatory receptor would increase GABAergic neuronal firing activating the non-dopamine reward pathway, whereas antagonist blockade would decrease the activity of these neurons, removing inhibition of the dopamine system and activating a dopamine-dependent reward signal.

Functional VTA $GABA_A$ receptors are predominantly localized to GABAergic neurons (although some $GABA_A$ receptors are also localized to the dopamine neurons). These GABAergic neurons send inhibitory inputs to the dopamine neurons but also send ascending projections to the forebrain[37] and descending efferents to brainstem structures, including the TPP[17,38]. In the opiate-naive state, agonist activation of inhibitory VTA $GABA_A$ receptors will remove inhibitory input to dopamine neurons, and activate the mesolimbic dopamine system, producing dopamine-dependent reward transmission. In

contrast, antagonist blockade of VTA $GABA_A$ receptors produces activation of the GABAergic neurons and associated efferent pathways, presumably to dopamine-independent, brainstem reward circuits[16,27]. The TPP represents a likely reward output for the non-dopamine reward pathway, as lesions of this nucleus block dopamine-independent bicuculline reward but not dopamine-dependent muscimol reward in the opiate-naive state, and this pattern is reversed in opiate-dependent/withdrawn animals (data not shown). Alternatively, it is possible that within the VTA, separate GABAergic neuronal populations could signal dopamine-dependent versus dopamine-independent reward signals such that GABAergic neurons with greater projections to the TPP may be preferentially involved in bicuculline (dopamine-independent) reward signaling in the opiate-naive state, whereas GABAergic neurons with preferential input to the VTA dopamine neurons may be preferentially involved in the dopamine-dependent muscimol reward signal. Nevertheless, such an arrangement would not explain the functional switching in $GABA_A$ receptor signaling observed in the present study, as inhibition of dopamine neurons by activation of VTA $GABA_B$ receptors[31,32,39] also dissociated $GABA_A$ receptor–mediated reward signaling as a function of opiate state (**Fig. 4e**).

VTA reward switching controlled by a common mechanism

We observed several functional similarities between the VTA reward switching phenomena for $GABA_A$ and μ-opiate receptors. First, the timelines over which opiate withdrawal induces a qualitative switch between dopamine-independent (opiate-naive) and dopamine-dependent (opiate-dependent/withdrawn) reward signaling for $GABA_A$ and opiate receptor signaling appear to operate in parallel (**Fig. 4**). Second, dopamine-dependent and dopamine-independent reward transmission through VTA $GABA_A$ receptors follows the same reward output switching pattern for both opiate and $GABA_A$ VTA reward signaling. Even after chronic opiate exposure, when animals are taken out of withdrawal, the reward output parameters of VTA $GABA_A$ receptor–mediated transmission are restored to the opiate-naive state, demonstrating considerable plasticity in this neurobiological switching mechanism. Third, acetazolamide similarly blocks the switch to the opiate-dependent/withdrawn dopamine system for both opiate and $GABA_A$ reward signaling, even in the presence of 21 h opiate withdrawal.

How might $GABA_A$ versus opiate receptor populations in the VTA both produce their rewarding motivational effects through a common VTA molecular switching mechanism? Although present evidence suggests that VTA dopamine neurons lack functional opiate receptors[24,40], a large concentration of μ-opiate receptors associates directly with non-dopamine, GABAergic VTA neurons[24,40] and modulates the inhibitory VTA GABA inputs to the mesolimbic dopamine system[40,41]. There are also presynaptic VTA μ-opiate receptors that form synapses with both dopamine and non-dopamine VTA neurons[42,43]. In addition, μ-opiate receptor–containing GABAergic terminals in the VTA form inhibitory synapses with dendrites of VTA GABAergic neurons, which may inhibit the inhibitory GABAergic input to these neurons[42].This implies that opiates may activate non-dopamine VTA neurons (in a manner analogous to bicuculline's post-synaptic effects on VTA GABA neurons), which in turn could activate a non-dopamine VTA reward signal. However, opiates could also act directly upon the VTA GABAergic neurons, inhibiting these cells (in a manner analogous to muscimol), thus activating a dopamine-dependent reward signal. Finally, opiates could act further upstream of the VTA GABAergic neurons during withdrawal, to produce a dopamine-dependent reward effect by inhibiting a now excitatory GABAergic input to the GABAergic neurons by removing inhibition to the dopamine reward pathway.

Inhibition of VTA CA with acetazolamide reversed the opiate withdrawal–induced, bi-directional reward switch in the VTA $GABA_A$ receptor substrate, restoring the functional parameters of both VTA $GABA_A$ receptor–mediated signaling and opiate reward signaling substrates to the opiate-naive state. Although other molecular alterations in the VTA cannot be ruled out, our results suggest an important role for HCO_3^- conductance in $GABA_A$ receptor–mediated motivational switching, as well as a corresponding alteration in transmembrane [Cl^-] conductance as a result of chronic opiate exposure and withdrawal. Furthermore, intra-VTA administration of muscimol produced differential effects on VTA CREB phosphorylation as a function of opiate state, consistent with previous reports linking CREB phosphorylation levels with either inhibitory or excitatory signaling through $GABA_A$ receptor substrates[34,35].

The intra-VTA alterations produced by chronic opiate exposure and withdrawal that may lead to the transformation in $GABA_A$ receptor conductance properties are not presently understood. According to one report, levels of GABA release in the VTA were highly elevated during opiate withdrawal[44]. Greater release of GABA during opiate withdrawal could lead to a breakdown in the transmembrane $GABA_A$ receptor Cl^- gradient at GABAergic neurons, resulting in a shift towards HCO_3^- mediated $GABA_A$ receptor depolarization, and a subsequent switch in motivational signaling to the dopamine-dependent VTA reward pathway. Alternatively, opiate dependence and withdrawal may induce a pathological elevation of intracellular CA levels, leading to an imbalance between Cl^- and HCO_3^- transmembrane gradients and a shift to HCO_3^- efflux, resulting in a net depolarizing response at the $GABA_A$ receptor on VTA GABAergic neurons during opiate withdrawal in response to receptor activation.

Extracellular recording of VTA GABAergic neurons in opiate-dependent/withdrawn animals revealed that a subpopulation (44%) of these neurons switched to excitatory $GABA_A$ receptor signaling, a finding we never observed in the opiate-naive state. The precise mechanism by which the transformation of a subpopulation of neurons from inhibitory to excitatory signaling can lead to a directional switch in a neuronal circuit pathway is not known. According to a recent study, however, a switch in only 22% of $GABA_A$ synapses in the human temporal lobe is sufficient to cause interictal epileptic seizure activity[45]. In addition, a shift from inhibitory to excitatory $GABA_A$ receptor signaling in neurons of suprachiasmatic nucleus could account for functional circadian switching[46], demonstrating that molecular alterations leading to a switch from inhibitory to excitatory $GABA_A$ receptor signaling may underlie important behavioral phenotypes.

From a motivational perspective, the present results suggest that fundamentally distinct neural systems mediate opiate reward in the early, acute phase versus the dependent/withdrawn stage of opiate exposure. The $GABA_A$ receptor–mediated neural mechanism that controls the switch between these systems shows remarkable plasticity: it is dynamically regulated by the opiate state of the animal over the course of hours. One implication is that, rather than opiate addiction producing a static, adapted or sensitized neural motivational state, the switch from the opiate-naive, non-dependent state to the opiate-dependent/withdrawn state involves a discrete molecular switching event that defines a dynamic, neurobiological boundary between the acute phase of drug taking versus the dependent and withdrawn phases of addiction.

METHODS

Animals and surgery. Male Wistar rats (Charles River, Quebec; weighing 300–350 g at the start of experiments) were anesthetized with Somnotol (0.8 mg/ml; i.p.) and placed in a stereotaxic device. Twenty-two gauge, stainless steel guide cannulae (Plastics One) were bilaterally implanted 2 mm dorsal to the VTA at a 10° angle using the following stereotaxic coordinates: from bregma, A/P –5.0, L ±2.3; from the dural surface, V –8.0. For animals receiving bilateral intra-NAc microinfusion of α-flu, the following NAc stereotaxic coordinates were used (angled at 10°): from bregma, A/P +1.8, L ±3.1; from the dural surface V –6.8. Rats trained in a state of opiate dependency and withdrawal received daily 0.5 mg/kg subcutaneous injections of heroin commencing 4 d before the start of conditioning. Animals show strong motor sensitization to heroin by the third injection (unpublished observations). Animals were conditioned 3.5 or 21 h after their last heroin injection. During conditioning, this dose of heroin was administered as a maintenance dose 3.25 h after the termination of training. Over the course of the experiments, animals thus received a total of 20 heroin injections. The aversive effects of withdrawal induced by this regimen are qualitatively similar to those observed after a 3-week regimen of morphine administration, which produces aversive motivational effects as well as somatic withdrawal signs. All experimental protocols were approved by the Animal Care Committee guidelines of the University of Toronto and the Government of Canada.

Histology and immunocytochemistry. To anatomically verify intra-VTA or intra-NAc cannulae placements, brains were cut into 40-μm sections and stained with Cresyl Violet then examined with light microscopy. For immunocytochemical analysis of pCREB expression levels in the VTA, four randomly selected groups of animals taken from previously opiate-naive VTA GABA experimental groups with intra-VTA cannulae were used at the completion of prior experiments. Thirty minutes after receiving either intra-VTA muscimol or saline, animals were anesthetized with Somnotol (0.8 mg/ml) and perfused transcardially with phosphate-buffered saline (PBS, 0.1 M) followed by paraformaldehyde (4%) in phosphate buffer (0.1 M). Brains were post-fixed for 16 h, and transferred to 25% sucrose in phosphate buffer (0.1 M) solution for 72 h. 40-μm sections were mounted on gelatin coated slides and frozen at –20 °C before staining. Sections were washed 3 × 10 min with PBS and pre-incubated with 5% normal goat serum (NGS) + 0.3% Triton-X in PBS for 15 min. Sections were incubated for 30 min with 10% NGS followed by 1% NGS for 30 min and incubated overnight at 4 °C with the pCREB primary antibody (1:1,000 dilution, rabbit polyclonal, Upstate Biotechnology) in 1% NGS. The pCREB antibody is specific to Ser133-phosphorylated CREB. After primary incubation, sections were washed 3 × 5 min with PBS and incubated at 4 °C in biotinylated goat-anti-rabbit IgG (1:200) in 0.05% Triton-X for 120 min. Sections were then rinsed 3 × 5 min in PBS and incubated with the avidin-biotinylate-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; diluted 1:55) for 2 h at 4 °C. Sections were washed 3 × 5 minutes in PBS and reacted with 0.05% diaminobenzadine and 0.05% H_20_2 in PBS (Vector Laboratories). The reaction was stopped for all sections as soon as positive staining of pCREB was observed. For immunofluorescent double labeling of tyrosine hydroxylase (TH) and pCREB, an additional experimental group that were heroin-dependent and withdrawn ($n = 5$) received intra-VTA muscimol (50 ng) and then perfused as described above. VTA sections were prepared as described above. Sections were incubated overnight in the presence of both the TH (1:1,000; mouse monoclonal; Upstate) and pCREB (1:1,000; rabbit monoclonal; Upstate) primary antibodies in PBS (0.1 M) in 1% NGS at 4 °C. Sections were then washed in PBS (3 × 10 min) and then incubated for 60 min in PBS (0.1 M) with Triton (0.05 %) and NGS (10%) to which the secondary antibodies (FITC-conjugated, 1:200 and TRITC-conjugated, 1:200) were added. Immunofluorescence detection of either pCREB- or TH-positive cells or cells positive for both markers, was performed on a Nikon fluorescent microscope with a 40× objective. A 10 × 10 mm ocular grid was used to delimit the counting field. To correct for possible double counting of cells in serial sections, the optical dissector method[47] was used to estimate pCREB-immunoreactive cell numbers in the VTA. Counts were made of pCREB-positive cells within 1 mm^2 ocular grid areas superimposed on the sections at 100× magnification. pCREB-positive cells were counted within three defined VTA sections for each animal, representing rostral, middle and caudal VTA areas (see **Fig. 2**).

Drugs and microinjection procedure. Muscimol hydrobromide, baclofen, cis-flupenthixol (Research Biochemicals), bicuculline methiodide (Sigma), morphine (BDH) or acetazolamide (Sigma) were dissolved in physiological saline (pH adjusted to 7.4). Bilateral VTA or NAc microinjections (0.5 μl volume per infusion) were performed over 1 min. Injectors were left in place for a further 1 min to ensure adequate diffusion from the injector tip. Animals were placed in the conditioning environments immediately after injection. Systemic α-flu saline vehicle was injected intraperitoneally 2.5 h before intracerebral microinjections. For intra-NAc α-flu experiments, animals received bilateral intra-NAc microinfusions of α-flu 15 min before intra-VTA muscimol or bicuculline. For intra-VTA co-administration experiments with baclofen or acetazolamide, animals received these drugs simultaneously (in the same suspension) with a specific drug (muscimol, bicuculline or morphine).

Conditioning procedures. All animals were conditioned using a standard place conditioning procedure as previously described[9,13]. Conditioning took place in one of two distinct environments that differed in color, texture and smell. Animals received four drug-environment and four saline-environment conditioning sessions, and exposure to conditioning environments was counterbalanced in all experiments. Each conditioning session lasted 40 min. At testing, 1 week after the end of conditioning (all animals were tested drug-free), animals were placed on a narrow, neutral gray zone that separated the two test compartments. Times spent in each environment were scored separately for each animal for a total of 10 min. To examine the aversive effects of opiate withdrawal, a single-sided withdrawal conditioning procedure was used. The single-side-withdrawal procedure involves exposing animals to only one of the two conditioning environments with the environment paired with opiate withdrawal counterbalanced within groups and this conditioning cycle is repeated until each animal has been exposed to the conditioning environment four times over an 8-d period as described previously[13].

Ionotophoretic recording of VTA GABAergic neurons. Animals were chronically treated with either heroin (0.5 mg/kg, subcutaneously) or saline once daily for 8 d prior to the recording experiments. Approximately 21 h after the last heroin or saline injection, animals were anesthetized, tracheotimized and placed in a stereotaxic apparatus. The anesthetized state was maintained by delivering halothane (0.5–0.9%) in air for the duration of the experiment. Single-unit extracellular potentials (spikes) corresponding to VTA GABAergic neuron identity were identified according to previously established criteria[36] and recorded using a glass micropipette containing 3 M NaCl glued to a multibarrel pipette containing the drug solutions. Spikes were amplified and filtered (Axoprobe 1A, Axon Instruments), discriminated (Mentor N-750 Spike Analyzer) and stored to disk using National Instruments NB-MIO-16 A/D hardware and custom LabView software. Raw spike trains were recorded at 20 kHz. Muscimol (5 Mm, Sigma) was dissolved in physiological saline. Muscimol was retained with –10 nA holding currents and ejected with cationic currents (ranging from 20–200 nA) using a Neurophore BH-2 iontophoresis unit (Medical Systems Corp.) while recording neuronal activity. Different iontophoretic (20–200 nA) currents were applied to the majority of recorded single neuronal units in a counterbalanced manner. Currents producing the most consistent and stable extracellular responses were selected for iontophoretic recording epochs. Pipette assemblies were lowered into the VTA using a pizoelectric micropositioner (Burleigh).

Data analysis. All data were analyzed with one-, two- or three-way ANOVA, or Student's *t*-tests where appropriate. *Post-hoc* analysis was performed with Newman-Keuls tests.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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