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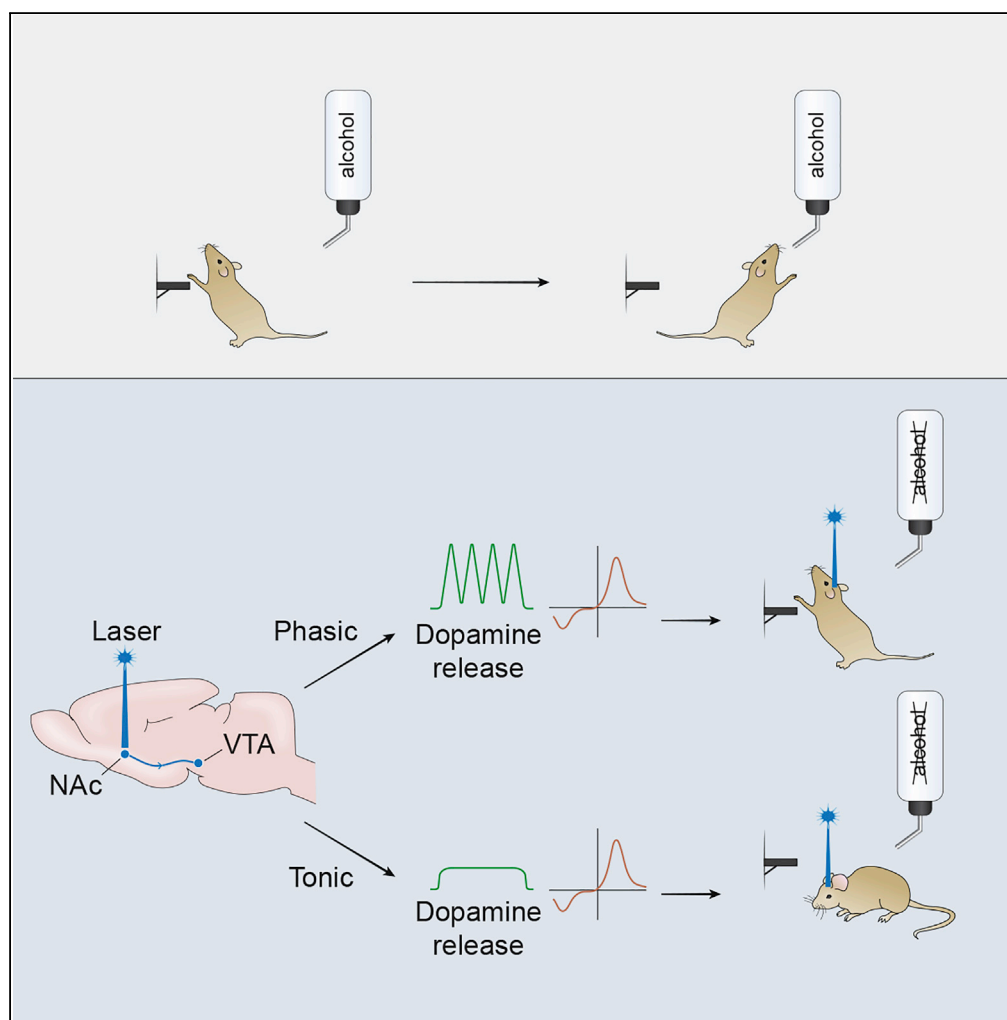
Opposite Consequences of Tonic and Phasic Increases in Accumbal Dopamine on Alcohol-Seeking Behavior

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HIGHLIGHTS

VTA-NAc DA transmission
can bidirectionally
modulate motivated
behavior

Optogenetic increases in
phasic DA release in the
NAc enhance alcohol
seeking

Optogenetic increases in
tonic DA release in the
NAc inhibit alcohol
seeking

Phasic DA release can be
decreased by the
concurrent tonic
activation

Article

Opposite Consequences of Tonic and Phasic Increases in Accumbal Dopamine on Alcohol-Seeking Behavior

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SUMMARY

Despite many years of work on dopaminergic mechanisms of alcohol addiction, much of the evidence remains mostly correlative in nature. Fortunately, recent technological advances have provided the opportunity to explore the causal role of alterations in neurotransmission within circuits involved in addictive behaviors. Here, we address this critical gap in our knowledge by integrating an optogenetic approach and an operant alcohol self-administration paradigm to assess directly how accumbal dopamine (DA) release dynamics influences the appetitive (seeking) component of alcohol-drinking behavior. We show that appetitive reward-seeking behavior in rats trained to self-administer alcohol can be shaped causally by ventral tegmental area-nucleus accumbens (VTA-NAc) DA neurotransmission. Our findings reveal that phasic patterns of DA release within this circuit enhance a discrete measure of alcohol seeking, whereas tonic patterns of stimulation inhibit this behavior. Moreover, we provide mechanistic evidence that tonic-phasic interplay within the VTA-NAc DA circuit underlies these seemingly paradoxical effects.

INTRODUCTION

Alcohol use disorder (AUD) is a widespread neuropsychiatric disease, which can be defined as a pathological motivation to seek and consume alcohol. During the last several decades, researchers have sought to identify the neurobiological substrates responsible for these pathological drinking behaviors. The mesolimbic dopamine (DA) system has, perhaps, received the most attention as a key pathway that may become dysregulated in AUD and other addictive disorders. Indeed, many compelling studies have demonstrated that chronic exposure to all drugs of abuse promotes maladaptive alterations within the DAergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Volkow et al., 2011; Volkow and Morales, 2015; Koob and Volkow, 2016). Moreover, some data support the notion that VTA-NAc DA signaling can be involved in several elements associated with the development of addictive behavior, including reinforcement learning (Day et al., 2007; Flagel et al., 2011; Steinberg et al., 2013; Hart et al., 2014; Chang et al., 2016; Parker et al., 2011; Darvas et al., 2014), motivated drug and reward seeking (Phillips et al., 2003; Stuber et al., 2012; Wassum et al., 2013; Pascoli et al., 2015; Halbout et al., 2019), and drug and reward intake (Volkow et al., 2007; Mikhailova et al., 2016). However, despite more than 30 years of work on this topic, much of this evidence remains mostly correlative in nature with only a paucity of direct evidence that mesolimbic DA signaling regulates alcohol-drinking behaviors.

Fortunately, recent technological advances have provided the opportunity to explore the causal role of alterations in neurotransmission within circuits involved in addictive behaviors. To that end, recent studies have provided the first direct evidence that VTA-NAc DA neurotransmission directly modulates alcohol consumption (Bass et al., 2013; Juarez et al., 2017).

Importantly, alcohol drinking, as with many other addictive behaviors, can be deconstructed into distinct appetitive and consummatory components (Samson and Czachowski, 2003). Appetitive elements are those involved in seeking (motivated) behaviors directly related to the procurement of alcohol, whereas consummatory elements are behaviors associated with the actual act of drinking. Although dysregulation of both these processes likely contributes to the etiology of AUD, appetitive and consummatory behaviors can be differentially regulated and are likely mediated by distinct neural circuits. Notably, the first studies that sought to establish a causal role of mesolimbic DA signaling in alcohol drinking employed a procedure

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that did not allow for the discrete assessment of the seeking component of this behavior (Bass et al., 2013; Juarez et al., 2017). Here, we address this critical gap in our knowledge by integrating optogenetic stimulation of VTA-NAc DA release and an operant alcohol self-administration regimen that can dissociate appetitive and consummatory measures to directly assess how accumbal DA release dynamics influences the appetitive (motivational or seeking) component of alcohol drinking behavior. It should be pointed out that the chosen parameters of optostimulations were previously used to mimic tonic and phasic increases in DA transmission in behaving animals (Tsai et al., 2009; Adamantidis et al., 2011; Bass et al., 2013; Mikhailova et al., 2016). These stimulation protocols did not result in any behavioral indications of nonphysiological conditions such as hypo- or hyperdopaminergic states (Tsai et al., 2009; Bass et al., 2013; Mikhailova et al., 2016).

We reveal that phasic and tonic VTA-NAc DA release patterns bidirectionally modulate an appetitive alcohol drinking-related behavior. Specifically, we demonstrated that a high-frequency stimulation pattern (50 Hz) that evoked DA transients with temporal and concentration features similar to real-time DA fluctuations observed during drug-seeking behavior (Phillips et al., 2003; Owesson-White et al., 2009) resulted in an escalation of alcohol seeking. In contrast, applying low-frequency stimulation (5 Hz), and therefore shifting DA release into the tonic mode, where cells simultaneously fire at their basal frequency (Hyland et al., 2002; Floresco et al., 2003), suppressed this behavior. We also provide mechanistic evidence that tonic-phasic interplay within the VTA-NAc DA circuit underlies these seemingly puzzling effects on alcohol seeking.

RESULTS

### Viral Infusion and Operant Alcohol Self-Administration

Adult male rats (n = 7) were trained to complete a daily 30 lever press response requirement to gain 20-min access to a sipper tube containing a 10% ethanol solution. After subjects displayed stable drinking behavior (Figure S1), they were anesthetized and received intra-VTA injections of an adeno-associated viral (AAV) construct, which restricted ChR2 expression to tyrosine hydroxylase (TH)-positive neurons (Fox et al., 2016; Mikhailova et al., 2016). Two days following the surgery, rats were returned to the operant drinking procedure, where they quickly resumed their original levels of alcohol intake (≈1.0 g/kg/20 min). These values were normally distributed (p = 0.204, Shapiro normality test). No significant difference was revealed between alcohol consumption measured as the weekly average per animal before and after the surgery (0.99 ± 0.18 g/kg versus 1.14 ± 0.14 g/kg, p = 0.527, paired t test). Four weeks after AAV infusion, robust colocalization of ChR2-EYFP and TH expression was observed in the VTA (Figure 1A, 97.2% ± 1.5% of ChR2-positive cells were also TH positive; n = 5) along with ChR2-positive terminals (Figure S2).

Operant Responding during Extinction Trial and Reinforced Sessions

To isolate a measure of alcohol seeking devoid of any consummatory behaviors, extinction probe trials were conducted once per week. During these trials, subjects were presented with all the alcohol-related cues, but completion of the lever press response requirement was not reinforced. Total lever presses during these 20-min extinction sessions represent a well-validated measure of appetitive or seeking behavior (Samson and Czachowski, 2003). As previously shown, lever press responding increased almost 3-fold during extinction trials (Figure 1B). As expected, the values obtained in the two reinforcing sessions that preceded the extinction trial were not normally distributed (p < 0.0003, Shapiro normality test) due to the ceiling effect of the 30 press requirement, whereas the data collected during the trial and the next session passed the normality test (p = 0.297 and p = 0.089). Therefore, statistical differences between the responses during the reinforced session and the extinction trial were confirmed using non-parametric analyses (Kruskal-Wallis and Wilcoxon matched-pairs signed rank test). The non-parametric one-way ANOVA on ranks revealed a significant difference between the sessions (p = 0.0001), whereas pairwise non-parametric tests confirmed significant differences between the extinction trial and reinforced sessions (p < 0.05).

Relationship between Alcohol Consummatory and Seeking Behaviors

Whether extinction trials can modify alcohol intake during subsequent reinforced sessions was also studied (Figure 1C). These values were normally distributed (p > 0.222, Shapiro normality test) and therefore analyzed with a repeated measures ANOVA test. No effect of the extinction trial on subsequent alcohol intake was found ($F_{(2.445, 14.67)}$ = 0.9918; p > 0.05).
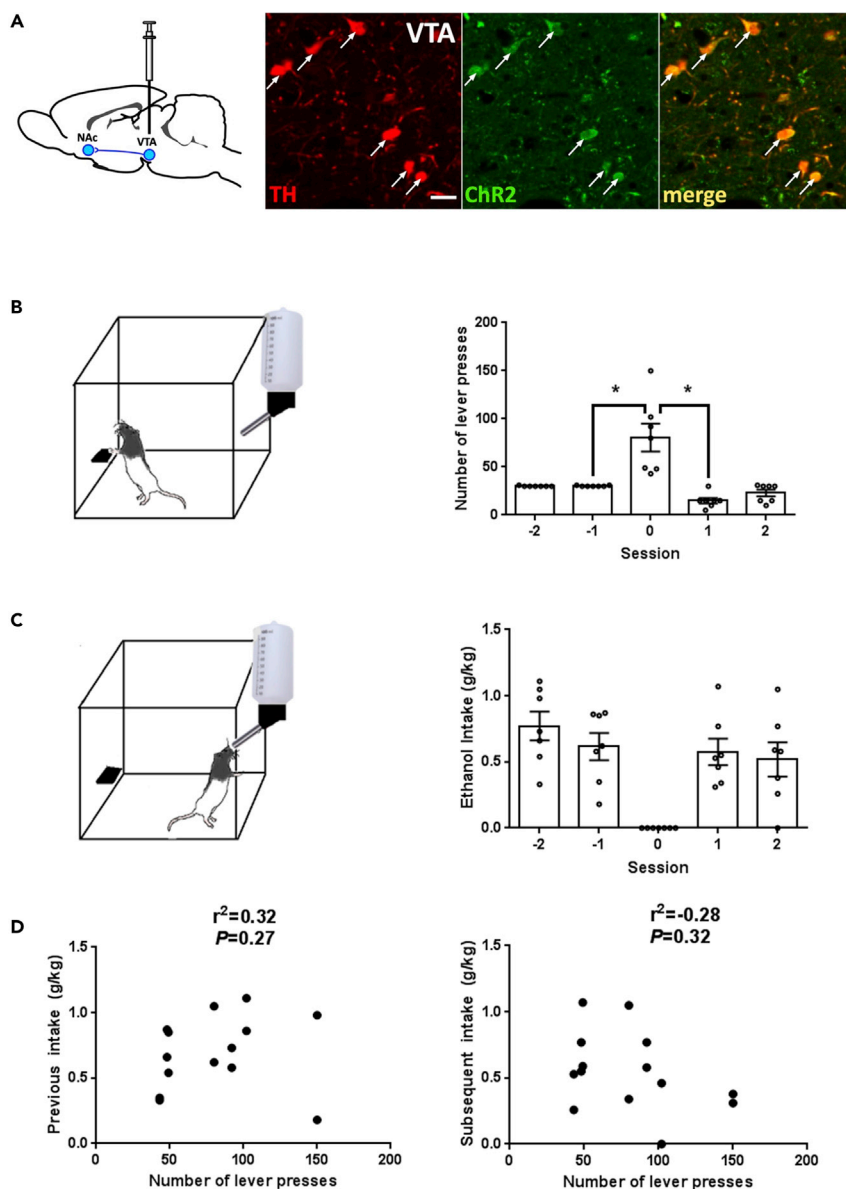
Figure 1. Optogenetically Targeting DA Cells within the VTA of Rats Consuming Ethanol in an Operant Behavior Test

(A) Rats, previously trained to press a lever daily to gain access to a 10% ethanol solution for 20 min (see Figure S1), were anesthetized and injected in the VTA with a combination of DIO-ChR2-EYFP-AAV2/10 and TH-iCRE-AA2/10 via a Hamilton syringe (left panel). Immunohistochemical analysis confirmed that ChR2-EYFP was largely restricted to TH-positive cells in the targeted area (right panel). Scale bar = 25 μm. See Figure S2 for the nucleus accumbens.

(B) Individual data from seven rats are presented. Following virus injection, subjects acquired the 30 lever press response requirement. As expected, rats exhibited a significant increase in the number of lever presses when their action was not reinforced throughout the session (session 0). *p < 0.05 relative to reinforced session (Kruskal-Wallis test and Wilcoxon matched-pairs signed rank test), which was performed before (−1) or after (1) the non-reinforced session (session 0). Data are presented as mean ± SEM.

(C) Ethanol consumption was not significantly different between daily reinforced tests conducted prior to and following the extinction probe trials (p > 0.05). Data are mean ± SEM.

(D) No significant correlation was observed between lever responding during a 20-min non-reinforced session (extinction probe trial) and the amount of ethanol consumed on the two reinforced sessions preceding (left figure) or following (right figure) an extinction test (p > 0.05).

Using Spearman correlation analysis, no relationship was observed between the number of lever presses completed during extinction trials and alcohol intake on the two reinforced sessions immediately preceding, or following, extinction probe trials (p > 0.05) (Figure 1D). Therefore, our data support the notion that consummatory and appetitive behaviors are not correlated with each other and are likely mediated by distinct neural circuits. It should also be noted that prior studies using this model have reported blood ethanol concentrations in excess of 60 mg% in cohorts with a similar range of intakes (ranged from 0.2 to 1.2 g/kg), blood alcohol levles that fall within the intoxicating range (Samson and Czachowski, 2003).

Optogenetic Manipulation of DA Release in the NAc

To determine the effect of VTA-NAc DA release on extinction trial responding, optical stimulations were delivered directly to the NAc during the first 10 min of these non-reinforced sessions (Figure 2A). To confirm that the optogenetic manipulations of DA release were effective, separate experiments, using *in vivo* fast scan cyclic voltammetry (FSCV), were conducted on another cohort of anesthetized rats (n = 7). Both phasic and tonic activation of the VTA-NAc circuitry resulted in substantial increases in accumbal DA concentrations (Figures 2B, 2C, and S3). These data passed the Shapiro-Wilk normality test (p > 0.868). The lower-frequency stimulation (5 Hz) applied for longer periods (60 s) mimicked tonic increase in DA dynamics (Figure 2B). However, brief (1 s) high-frequency (50 Hz) stimulations induced phasic patterns of NAc DA release (Figure 2C). As expected, the maximal amplitude of phasic DA efflux was significantly greater than the amplitude of tonic release (p = 0.002, unpaired t test) (Figure S3).

Bidirectional Modulation of Alcohol-Seeking Behavior through Tonic or Phasic Stimulations

Neither pattern significantly altered the latency to the first lever press (p > 0.05, Mann-Whitney test) (Figure 2D). However, driving accumbal DA transmission into a tonic pattern dramatically decreased the number of lever presses (p = 0.0008, Mann-Whitney test), whereas phasic activation significantly increased this measure of seeking behavior (p = 0.034, Mann-Whitney test) (Figure 2E). Noticeably, both latency and lever press values were not normally distributed in the non-reinforced session without optogenetic stimulation (p = 0.008 and p = 0.036, respectively; Shapiro-Wilk test).

Our previous study demonstrated that phasic optoactivation of DA neurons with ChR2 expression facilitates reward-seeking behavior in operant tests (Adamantidis et al., 2011). Expectedly, no changes in this behavior following the same stimulation were observed in control animals, which were injected with AAV:eYFP (Adamantidis et al., 2011). This is in good agreement with multiple reports where light stimulation of mesolimbic DA neurons did not modify operant responses in the absence of opsin expression (Steinberg et al., 2013, 2014; Fischbach and Janak, 2019; Ilango et al., 2014). Therefore, it is unlikely that the bidirectional regulation of alcohol-seeking behavior observed following contrasting stimulation patterns resulted from artifactual activation of the brain tissue. Nevertheless, we explored the possibility that photostimulation itself (in the absence of ChR2 expression) could affect DA level (Figure S4). Repeated measures one-way ANOVAs found no significant effect of time on the signal during either high- (50 Hz; $F(2.911, 11.64) = 0.7586$, $p = 0.536$) or low- (5 Hz; $F(3.498, 13.99) = 1.192$, $p = 0.354$) frequency optical stimulation. However, profound DA release was triggered with an electrical stimulation under the same experimental conditions ($F(1.807, 7.228) = 12.03$, $p < 0.01$).

Exploring the Interplay between Optically Induced Tonic and Phasic DA Release in the NAc

Why do tonic and phasic stimulation of VTA-NAC DA release have opposite effects on extinction probe trial responding? The finding that optically generating phasic patterns of accumbal DA activity promotes alcohol-seeking behavior is actually consistent with the findings of many prior studies that have revealed correlative relationships between subsecond phasic DA release and appetitive behavior for other reinforcers. If phasic DA release promotes alcohol seeking, one possible explanation for the decrease in extinction probe trial responding observed following tonic stimulation of accumbal DA terminals is that the sustained, low-frequency stimulation may prevent DA terminals from engaging in the phasic release that drives appetitive behavior. To explore this possibility, we combined *in vivo* optogenetic and FSCV methods to examine directly real-time interactions between tonic and phasic patterns of DA release in the NAc. Two optical fibers from separate lasers were inserted above the VTA of anesthetized rats (n = 7) that had been injected with the TH-ChR2 virus and a microelectrode for voltammetric detection was positioned in the NAc (Figure 3A). This configuration allowed for the measurement of real-time, subsecond NAc DA changes evoked by tonic or phasic patterns of VTA DA cell stimulation or by simultaneous delivery of both patterns.
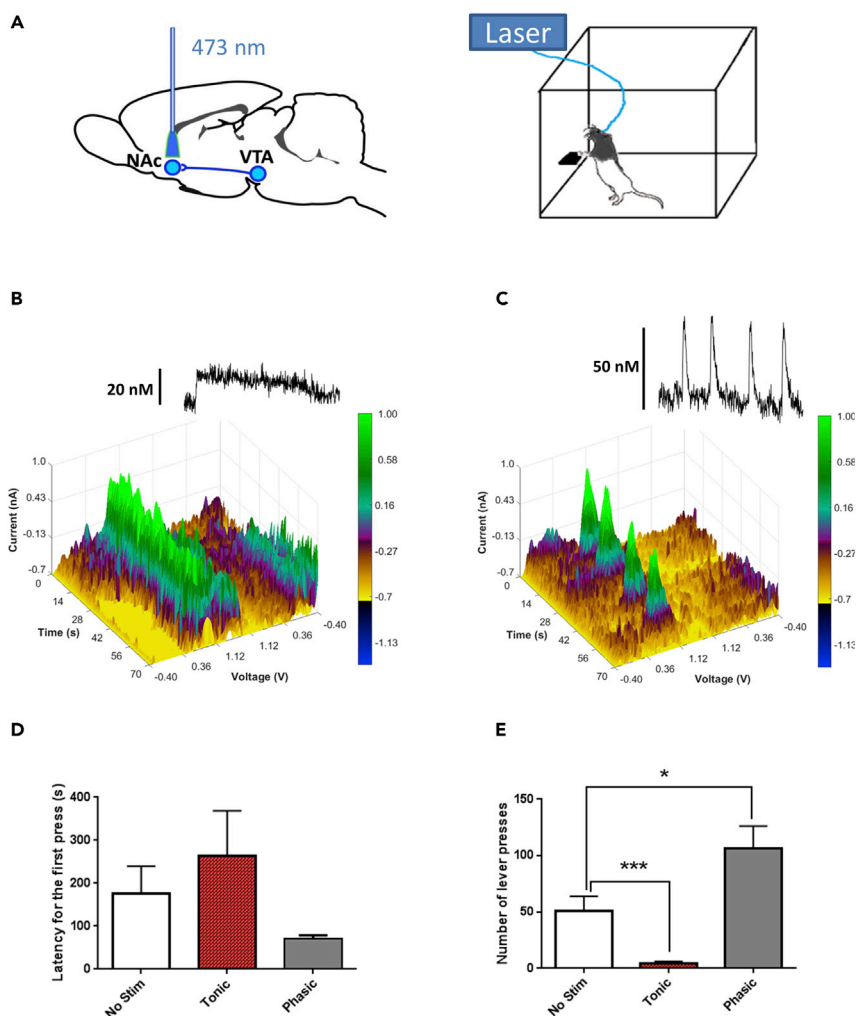
Figure 2. Optogenetically Manipulating DA Release in the Rat NAc during Ethanol-Seeking Behavior and under Anesthetized Conditions

(A) Schematic configuration for optical activation of accumbal DA terminals that was used to trigger tonic and phasic patterns of DA transmission.

(B) Tonic DA release patterns were triggered by blue light applied for 1 min at 5 Hz with 1-s intervals between stimulations (10 min total). A three-dimensional color plot topographically represents electrochemical changes detected with voltammetry in the rat NAc over 70 s.

(C) Phasic DA release patterns were induced by blue light stimulation (1 s, 50 Hz) with 15-s intervals between each pulse train for the first 10 min of the operant session. A color plot depicts the increase in DA release (uninterrupted green rise) that is time locked to optical activation. Four divided green spikes denote DA transients, which are time-locked to optical stimulations. Therefore, two main patterns of DA transmission, phasic and tonic, could be optogenetically mimicked during the operant behavior test. See also Figures S3–S5.

(D and E) Changes in alcohol-seeking behavior of rats (n = 7) were evaluated by measures of the latency to the first lever press and total number of lever presses during non-reinforced sessions. (D) No significant changes in the latency to the first press were found between sessions with (tonic and phasic) and without stimulations ($p > 0.05$). (E) Shifting DA release in the NAc into a tonic mode resulted in a significant decrease in the number of lever presses. However, phasic activation of the NAc significantly increased this measure.

*$p < 0.05$; ***$p < 0.001$ (Mann-Whitney). Data are mean $\pm$ SEM.

As values of DA response to tonic stimulation were not normally distributed ($p = 0.0004$), a non-parametric test (Kruskal-Wallis) was used for the statistical analysis of these results (Figure 3B). As previously shown, phasic stimulation elicited higher elevations in DA concentrations relative to tonic optical activation ($p < 0.001$, Dunn's multiple comparisons test). Importantly, tonic stimulation delivered concurrently with phasic
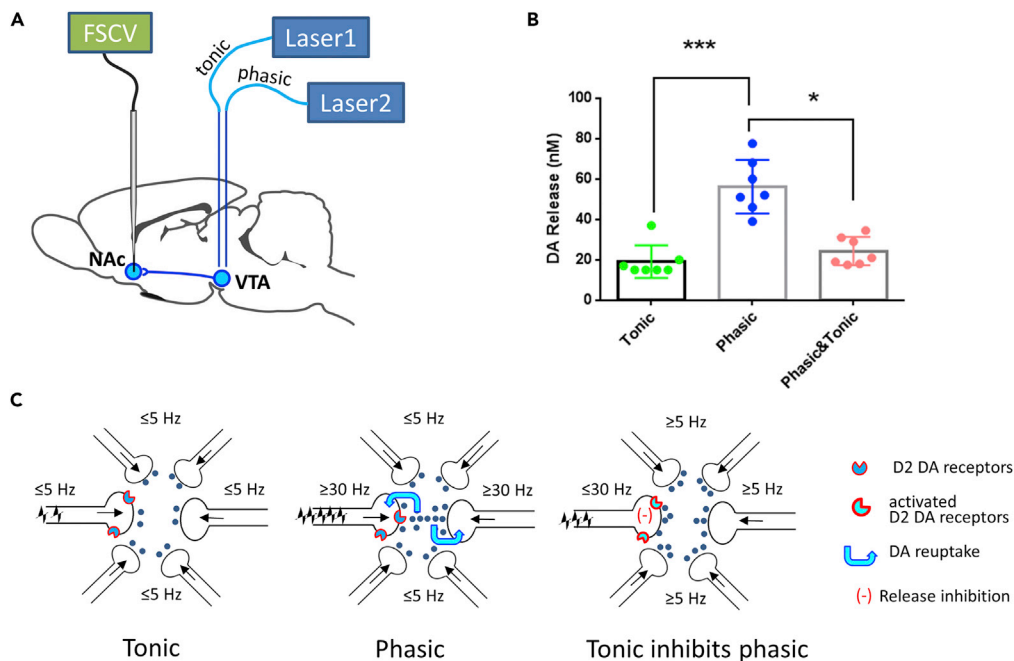
Figure 3. The Interaction between Phasic and Tonic DA Release Triggered by Optogenetic Activation of VTA-NAc DA Synapses

(A) Schematic representation of experiments, which combined real-time DA measurements performed with FSCV in the NAc and light activation of the VTA. Two optical fibers connected with separate lasers were inserted above the VTA of anesthetized rats (n = 7) to mimic coinciding tonic and phasic patterns of accumbal DA transmission with 5- and 50-Hz stimulations, respectively. See also Figures S3 and S4.

(B) The amplitude of phasic DA release was significantly higher than tonic DA elevation. However, combining tonic and phasic stimulations resulted in a reduced amplitude of DA release compared with that which was triggered by phasic activation alone. $*p < 0.05$; $***p < 0.001$ (Kruskal-Wallis test and Dunn's multiple comparison test). Data are mean ± SEM.

(C) A simplified model of neurochemical events that take place at the level of presynaptic terminals when DA releases with tonic (left), phasic (middle), or overlapping (right) patterns. Tonic: when cells fire with low frequency (≤ 5 Hz), DA releases with a distinct pattern, which results in a relatively low but stable DA concentration in the extrasynaptic space. Phasic: when some cells begin to fire with higher frequency (≥ 30 Hz) and this firing is synchronized, higher DA concentrations are released. These DA transients exist briefly (from subseconds to 1–2 s), because the re-uptake mechanism through the DA transporter rapidly eliminates them (blue arrows), taking DA inside terminals. Phasic DA activity can be downregulated by a presynaptic feedback mechanism. This happens when DA transmission is shifting into the tonic mode (the majority of cells fire at a frequency of 5 Hz). The tonic DA concentrations activate D_2 DA autoreceptors, and this action results in an inhibition of high frequency-induced phasic DA release.

stimulation inhibited the large phasic NAc DA responses, yielding a response that was not different from tonic stimulation alone ($p > 0.05$, Dunn's multiple comparisons test).

A simplified model of the interaction between tonic and phasic modes of DA transmission is illustrated in Figure 3C. This model illustrates our interpretation of how optogenetically induced changes in alcohol-seeking behavior arise because of changes in DA dynamics at the level of accumbal terminals. Under basal conditions, VTA DA cells fire at a low frequency (4–6 Hz), resulting in a modest, but stable, DA concentration. Brief increases in the firing rate (≥ 30 Hz) lead to rapid DA transients, which are quickly terminated via re-uptake mechanisms. Importantly, this phasic pattern of DA activity has previously been observed in association with drug- and reward-seeking behaviors (Phillips et al., 2003; Owesson-White et al., 2009; Stuber et al., 2012; Wassum et al., 2013). Reproducing this pattern by high-frequency (50 Hz) optogenetic stimulation of accumbal DA terminals during an operant test leads to an increase in alcohol seeking. However, optogenetic delivery of 5-Hz stimulation during the period of lever presses for alcohol forces DA transmission into the tonic mode. Under this condition, more cells simultaneously fire with their maximal rate limited to the tonic firing rate of these cells, providing DA levels sufficient to activate presynaptic DA autoreceptors that regulate release of the neurotransmitter. The activation of this presynaptic feedback mechanism results in an inhibition of phasic DA release and, as a consequence, aclohol-seeking behavior is suppressed.

DISCUSSION

Here, we show that appetitive reward-seeking behavior in rats trained to self-administer alcohol can be causally shaped by VTA-NAc DA neurotransmission. Our findings reveal that phasic patterns of subsecond accumbal DA release within this circuit enhance a discrete measure of alcohol seeking, whereas tonic patterns of stimulation within this pathway inhibit this behavior. We also demonstrate that tonic photoactivation of these VTA-NAc DA terminals occludes concurrent phasic DA release, which is likely needed to drive motivated behavioral responding for alcohol.

The fact that DA is released in the NAc through two distinct temporal patterns has been known for many years (Grace, 1991, 2000; Wightman and Robinson, 2002; Zweifel et al., 2009). However, for a variety of technical reasons, most studies have ignored these distinct temporal dynamics of DA neurotransmission and focused primarily on how the relative magnitude of DA release modifies behavioral output. Our findings reveal that, in addition to the importance of the magnitude of NAc dopamine release behaviors, the pattern through which this enhancement of DA occurs also plays a crucial role in shaping motivation to obtain abused drugs. To this point, we found that optogenetically increasing NAc DA release leads to opposite effects on alcohol seeking, depending on whether this increased release is generated by tonic or phasic patterns of synaptic activity. These findings have important implications for efforts related to developing new therapeutic strategies aimed at restoring normal synaptic DA activity, as the interventions may need to pay attention to the patterns through which DA signaling is modulated, not solely the magnitude of the DA release events.

As many prior studies have shown that phasic NAc DA release is associated with seeking behaviors for a wide range of reinforcers (Jones et al., 2010; Phillips et al., 2003; Wassum et al., 2013), it seems likely that this pattern of accumbal DA activity may promote alcohol seeking if the cause-effect relationship between these chemical changes and behavior exists. In fact, previous work has shown that electrically evoked phasic DA transients can promote a lever press for cocaine (Phillips et al., 2003). However, because electrical stimulation of the VTA can result in the release of multiple neurotransmitters in several brain areas, conclusions regarding a causal role of DA in the changes in drug seeking were limited. More direct evidence for a role of phasic DA in promoting motivated behavior comes from voltammetric studies showing that repeated cocaine exposure may enhance cue-evoked incentive motivation through augmented phasic DA signaling (Ostlund et al., 2014) and that these chemical alterations were positively correlated with lever press activity for reward, whereas slow DA changes (tonic) were not related to this activity (Wassum et al., 2013). Although these data strongly support a role for accumbal DA in reward-seeking behaviors, which originally was based mainly on pharmacological manipulations and lesion data (Berridge and Robinson, 1998; Berridge, 2007; Berridge et al., 2009), this evidence remains correlative in nature. In fact, based on compelling data, it was postulated that phasic VTA-NAc DA signaling serves a teaching function, encoding the association between cues that predicted rewarding events and reporting errors in reward prediction (Schultz, 2002; Bayer and Glimcher, 2005; Day et al., 2007; Zweifel et al., 2009; Steinberg et al., 2013; Hart et al., 2014). In this regard, our finding that, in rats trained to self-administer ethanol, lever press responding can be optogenetically manipulated through VTA-NAc DA transmission provides causal support for a distinct role of DA for seeking behavior in addition to DA's well established role for reinforcement learning. Moreover, the fact that tonic and phasic activation of this circuit has opposite effects on this measure of alcohol-seeking behavior emphasizes the importance of temporal DA release dynamics in modulating this measure of motivated behavior.

What might explain these divergent behavioral responses promoted by tonic and phasic increases in accumbal DA release? One likely hypothesis is that driving accumbal DA transmission in a tonic mode may occlude phasic DA release that normally encodes motivated behaviors. This hypothesis has existed for many years, based primarily on results obtained with pharmacological challenges (Grace, 2000; Phillips et al., 2003; Oleson et al., 2009). To test this idea directly, we integrated *in vivo* optogenetics and FSCV methods to examine the interaction between tonic and phasic patterns of accumbal DA neurotransmission. We found that both tonic and phasic stimulation of the VTA-NAc pathway increased DA release, with the magnitude of DA release being significantly greater with phasic stimulation. However, simultaneous delivery of tonic and phasic patterns of stimulation led to a marked inhibition of the large DA responses observed with phasic stimulation alone. Thus, sustained tonic stimulation of accumbal DA terminals may decrease extinction probe trial responding by preventing

DA terminals from engaging in phasic signaling patterns that normally promote alcohol-seeking behaviors. In fact, a previous microdialysis study found that changes in tonic DA release are negatively correlated with the number of lever presses required to obtain reward (Ostlund et al., 2011). This is also consistent with a series of studies that have demonstrated that (−)-OSU6162, a monoamine stabilizer that increases tonic dopamine release in the NAc (Feltmann et al., 2016), significantly decreased alcohol-seeking behaviors in rats and suppressed craving measures in alcohol-dependent individuals (Steensland et al., 2012; Khemiri et al., 2015).

At first glance, the results of this study may seem inconsistent with our prior findings that tonic photoactivation of VTA DA neurons decreased alcohol intake in an intermittent two-bottle choice procedure, whereas phasic stimulation of these cells did not significantly modulate alcohol drinking (Bass et al., 2013). However, there is strong evidence that seeking and consummatory elements of alcohol drinking behavior are distinct processes. For example, a meta-analysis of the drinking behavior of 234 rats using the same operant procedure employed in this study found no correlation between prior day alcohol intake and extinction probe trial responding (Samson and Czachowski, 2003). Moreover, intra-NAc infusion of raclopride, a D2 receptor antagonist, significantly reduced extinction probe trial responding while having no effect on alcohol intake, if the operant response requirement was removed (Samson and Chappell, 2004). Here, we provide additional support for the dissociation between appetitive and consummatory alcohol-drinking behaviors, as we found no relationship between extinction responding and alcohol drinking on the session preceding or following the extinction sessions.

In summary, our findings provide causal evidence that tonic-phasic interplay within the VTA-NAc DA circuit can significantly modulate an alcohol-seeking behavior. Our data demonstrate that shifting accumbal DA transmission into a tonic mode, while rats are engaged in alcohol-seeking behavior, diminishes further motivation to obtain alcohol. We further show that this effect is likely mediated by an inhibition of phasic DA release that normally promotes this behavior. These findings may have important clinical implications for studies seeking to manipulate mesolimbic DA release as a treatment for AUD. Given recent evidence that NAc stimulation shows some promise in the treatment of AUD (Azevedo and Mammis, 2018; Salib et al., 2018), these results may guide optimal therapeutic parameters of stimulation that could be particularly effective in eliminating maladaptive drinking behaviors.

Limitations of the Study

Our neurochemical experiments with dual stimulation of the VTA were designed to test empirically a long-existing general hypothesis regarding the interaction between tonic and phasic DA release patterns in the NAc. Although this experiment was conducted on alcohol-naive subjects, it should be noted that in our initial observations, we did not find any significant alcohol intake-related changes in optogenetically evoked DA signals. Nevertheless, future studies, using models of alcohol use disorder (e.g., chronic intermittent alcohol vapor), should be conducted to determine whether prolonged alcohol exposure affects tonic-phasic interplay. Furthermore, this study was focused on the appetitive component of alcohol-drinking behavior. Previously, we and others provided evidence that VTA-NAc DA transmission modulates alcohol consumption in an intermittent two-bottle choice procedure. Whether this is also the case in operant models of alcohol self-administration should be clarified in future experiments.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100877.

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AUTHOR CONTRIBUTIONS

E.A.B., C.E.B., and K.D.B. designed electrochemical experiments and analyzed the data. V.P.G. and A.L.D. performed electrochemical and ethanol self-administration studies. E.A.B., K.D.B., and J.L.W. designed optogenetic experiments and drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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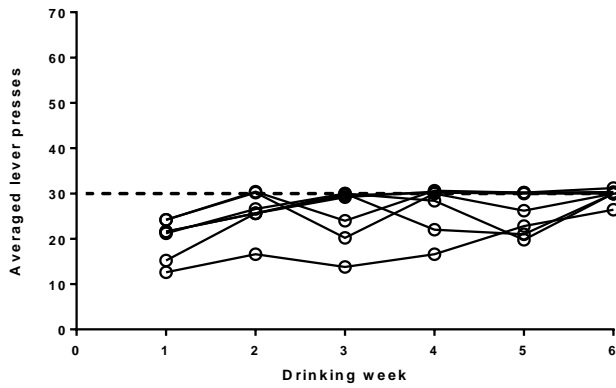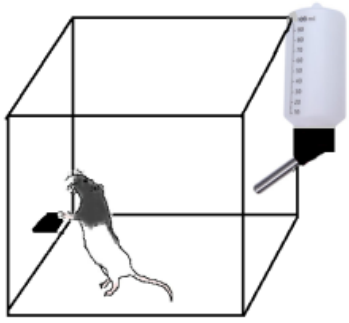
Supplemental Information

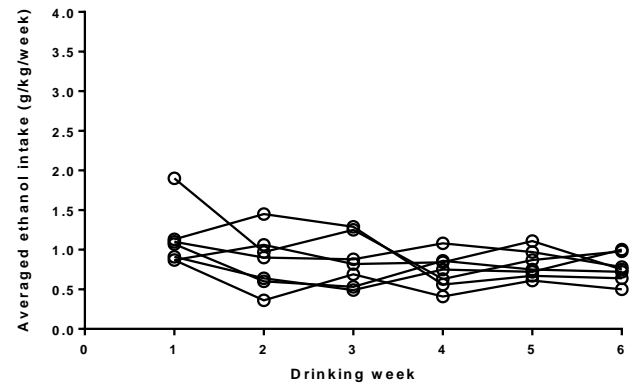# Opposite Consequences of Tonic and Phasic

Increases in Accumbal Dopamine

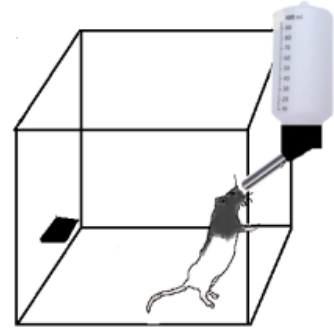# on Alcohol-Seeking Behavior

Evgeny A. Budygin, Caroline E. Bass, Valentina P. Grinevich, Alex L. Deal, Keith D. Bonin, and Jeff L. Weiner

A

B

Figure S1. Alcohol seeking and taking behaviors for the 6-week training procedure, related to Figure 1. Following an abbreviated sucrose-substitution protocol, rats (n=7) were provided with 10% ethanol solution that was available after the completion of a response requirement. The requirement was individualized for each rat depending on previous behavior (from 10 to 30 presses) in order to obtain alcohol regularly. By the end of the training period, all rats met the final requirement criterion of 30 lever presses to receive 10% ethanol. The number of lever presses (A) and alcohol intake (g/kg) (B) were averaged per animal per week.
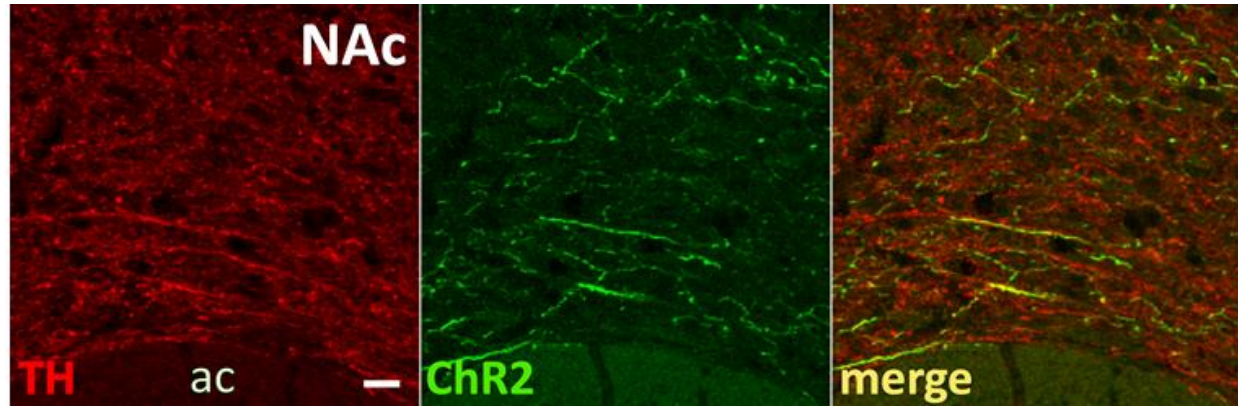
Figure S2. Optogenetically targeting DA terminals within the nucleus accumbens of rats consuming ethanol in an operant behavior test, related to Figure 1. Rats, previously trained to press a lever to obtain access to a 10% ethanol solution for 20 minutes, were anesthetized and injected in the VTA with a combination of DIO-ChR2-EYFP-AAV2/10 and TH-iCRE-AA2/10 via a Hamilton syringe. Immunohistochemical analysis confirmed that ChR2-EYFP was restricted to TH-positive cells in the targeted area. The scale bar is 20 microns.
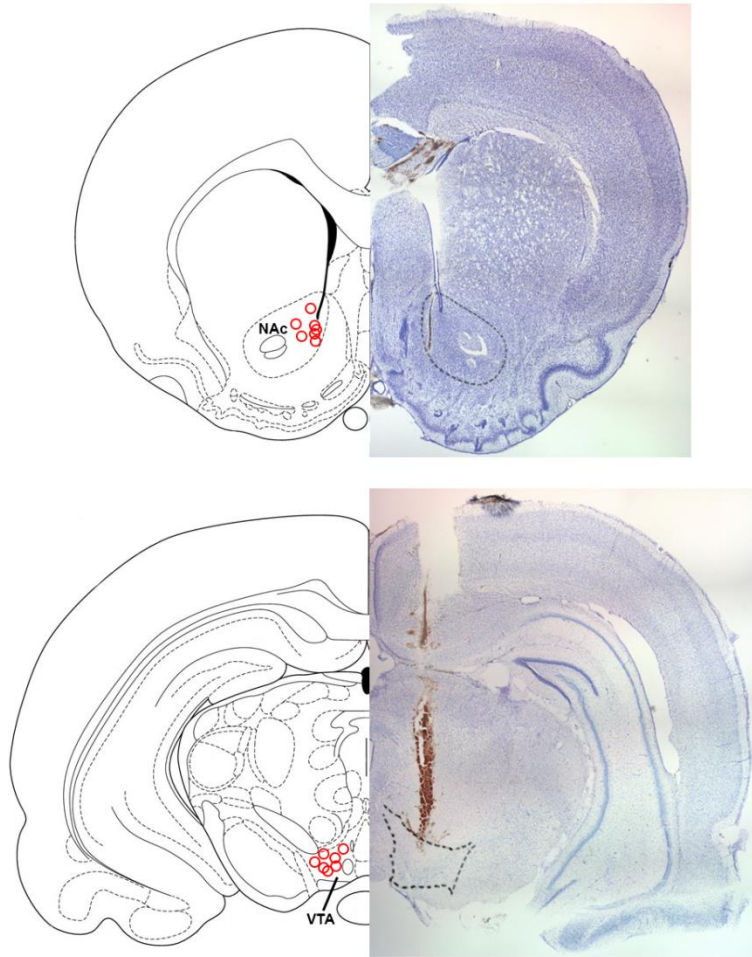
Figure S3. Schematic depictions (left) and representative images (right) of recording electrode (top) and optical fiber (bottom) placements (n=7), related to Figures 2 and 3. Note that all electrochemical recordings were performed on anesthetized animals. Red circles indicate placements of ventral tip of electrodes and fibers.
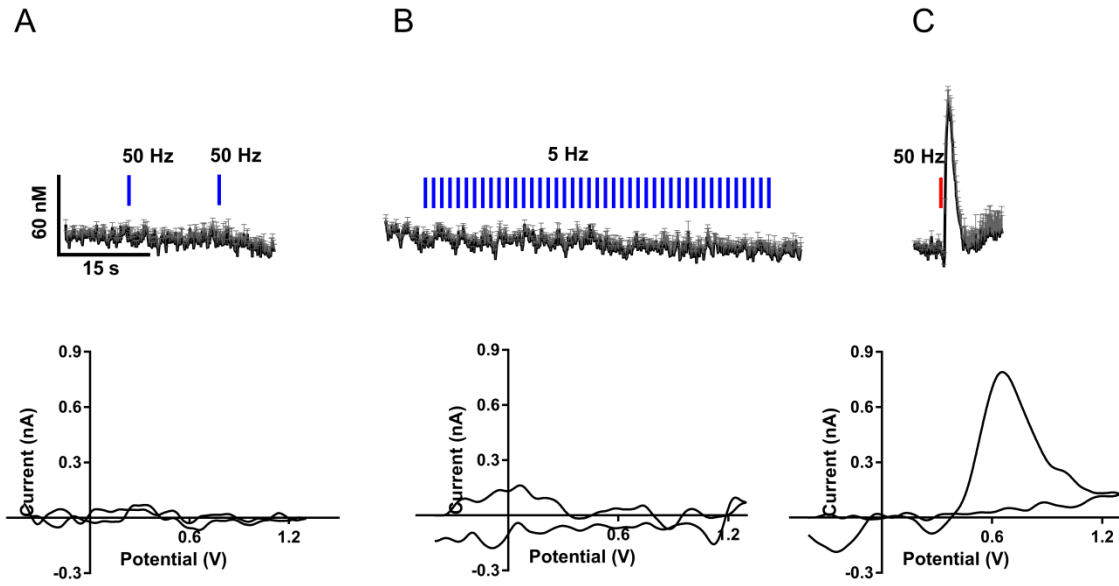
Figure S4. Optogenetic and electrical activation of the VTA in anesthetized rats without light sensitive opsin, related to Figures 2 and 3. Rats were injected in the VTA with viral construct referred in Figures 1 and S2 but with no ChR2. Four weeks later, animals were anesthetized and FSCV recordings in the NAc were performed. Optoactivation of the VTA at 50 Hz frequency for 1 s did not did not alter baseline recordings in the NAc (A). Identically, no effect of the stimulation at 5 Hz for 60 s was found (B). However, when an electrical stimulation (50 Hz, 1 s) was applied, a patent DA release was detected in the same rats (C). These data are presented as a mean ± s.e.m. from 5 rats. Blue bars indicate light stimulation, while the red bar shows an electrical stimulation. The lower panel demonstrates background-subtracted cyclic voltammograms obtained during stimulations. The electrical stimulation only (C, lower panel) induced the signal that was identified as DA based on its electrochemical signature.

A
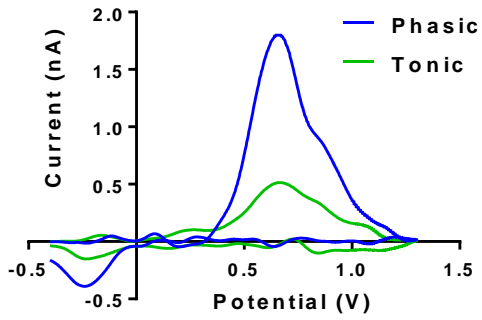
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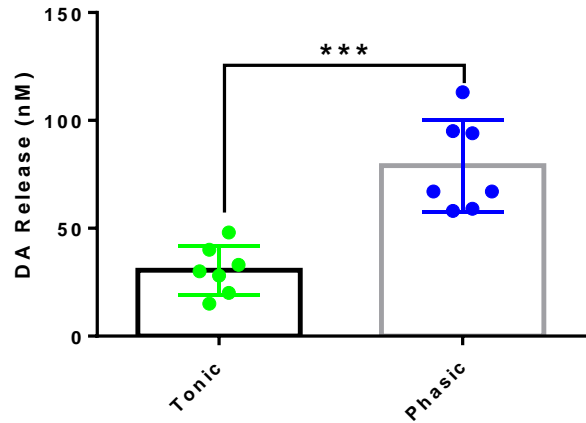
Figure S5. Effect of optogenetic stimulations on DA release *in vivo*, related to Figure 2. DA concentration changes were recorded in the nucleus accumbens of anesthetized rats (n=7) using FSCV. DA was identified by its oxidation (≈0.6 V) and reduction (≈-0.2 V on the negative going scan) features. (A) Representative background-subtracted cyclic voltammograms from tonic (green) and phasic (blue) DA responses are presented. (B) The amplitude of phasic DA release was significantly higher than tonic DA elevation. ***$P<0.001$ (unpaired t test). These data are presented as a mean ± s.e.m.

Supplemental Information

Transparent methods

Animals. Adult (90-150 days old) male Long-Evans rats, weighing 300-380g, were individually housed in a temperature-controlled vivarium in acrylic cages on a 12/12 h light/dark cycle (lights out at 6:00 PM). Food and water were available ad libitum, except during experimental sessions (30 min), conducted during the light cycle (8:30 AM – 12:30 PM) from Monday to Friday. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

Viral constructs and packaging. Virus packaging and titering were previously described (Bass et al., 2013; Fox et al., 2016; Mikhailova et al., 2016). A standard triple transfection packaging protocol was used to package viruses to generate pseudotyped AAV2/10 (Xiao et al., 1998). All viruses were titered by quantitative PCR and were 1012 vector genome copies per ml. The three plasmids were an AAV2 plasmid, which contained the transgene to be packaged, pHelper (Stratagene, La Jolla, CA) provided adenoviral helper functions, and an AAV2/10 rep/cap plasmid containing the AAV2 replicase and AAV10 capsid genes (Gao et al., 2002; De et al., 2006). The EF1α-DIO-ChR2-EYFP-pAAV and TH-iCRE-pACP plasmids were previously described (Gompf et al., 2015). Briefly, Cre recombinase expression is driven by a rat tyrosine hydroxylase (TH) promoter, which restricts expression to TH+ neurons (Oh et al., 2008; Gompf et al., 2015). In the second construct, ChR2-EYFP is driven by a strong, generalized promoter (EF1α), but this transgene is oriented in a DIO configuration that requires Cre recombinase to reorient it to an active, drivable position in relation to the EF1α promoter. When co-infused into the VTA, the Cre is expressed only in TH+ (dopaminergic) neurons that are then the only cells that express ChR2-EYFP.

Stereotaxic virus injection. Subjects were anesthetized using ketamine hydrochloride (80 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.). Once placed in a stereotaxic frame, the scalp was shaved and wiped with iodine. The skull was uncovered by making an incision centrally along the scalp. Two small drill holes were fashioned for two skull screws to stabilize a cement cap. Two final holes were drilled on the right side above the VTA (from bregma: anterior 5.8 mm; lateral, 0.7 mm) for the virus injection and for the NAc (from bregma: anterior 1.3 mm; lateral, 1.3 mm), into which an optic-fluid cannula (OFC) (Doric Lenses, Canada) was implanted (DV, 7.0 mm). Next, a combination of DIO-ChR2-EYFP-AAV2/10 and TH-iCRE-AAV2/10 were coinjected (1.2 µl total) gradually into the VTA (DV, 7.3mm) over 13 min through the OFC via a Hamilton syringe. Previously we have shown that this combinatorial system restricts ChR2 expression to dopaminergic neurons in the VTA (Gompf et al., 2015; Mikhailova et al., 2016). Dental cement stabilized by skull screws was used to cover the exposed skull. Subjects were returned to their home cages for recovery once the cement was dry.

Operant Self-Administration. *Training*. Daily sessions were performed in commercially available, sound-attenuated operant chambers (Med Associates, East Fairfield, VT) as previously described (Samson et al., 1999). Each chamber contained a house light to signal the beginning of a session, a retractable lever and a sipper tube that extended into the chamber. The system was computer controlled and appetitive and consummatory responses were collected at 2 Hz and analyzed using MedPC software (Med Associates, St. Albans, VT).

To train rats to self-administer alcohol (ethanol), we used an abbreviated sucrose substitution protocol based on the method of Samson (Samson, 1986). Briefly, on the first day of shaping, animals were acclimated to chambers for two hours using a 10% sucrose solution on a fixed ratio (FR1) schedule that resulted in 45-second access to the sipper. Over the next consecutive days, session time, fixed ratio, and sipper access time were modified and the sucrose concentration was decreased by 1% each day and replaced with increasing concentrations of ethanol. The fixed ratio schedule was increased from a FR1 to a FR4 over an 8-day period. On day 9, the schedule was changed such that completion of a response requirement of eight lever presses (RR8) resulted in a twenty minute presentation of the reinforcer. Over the next consecutive sessions at 10% ethanol, the response requirement was gradually increased from RR10 to RR15, 20, 25 and finally 30 for at least 2 days at each response requirement (individualized for each rat's capability). This type of individual training allowed for all animals to reach the criterion of 30 presses per session by the end of the six week period. Appetitive and consummatory behaviors were monitored daily and data were collected using Med Associates software. Animals had 20 minutes to complete the RR30.

Extinction Probe Trials. To obtain an appetitive measure devoid of any consummatory behaviors, extinction probe trials were conducted. On the days when extinction probe trials were performed, rats were placed into the operant

chamber and allowed to respond on the lever for the entire 30 minutes, regardless of the number of lever presses completed. Extinction probe trials were conducted on each animal, at least two weeks apart.

Optical Stimulation. Optical stimulations were performed during extinction tests at least 4 weeks following viral transfection. This interval was necessary to obtain the level of ChR2 expression that is adequate for effective triggering of DA release in terminals (Bass et al., 2010, 2013; Mikhailova et al., 2016). It is important to note that when optical stimulation experiments were performed they were randomized across animals so that some received phasic and some received tonic stimulation. Days of stimulation during the extinction trial were staggered across days of the week and no single animal was stimulated on more than one day per two week period. The optical setup had a laser at wavelength 473 nm (Beijing Viasho Technology Co., Ltd, Beijing, China) with a 100 mW maximum power output. A programmable function generator (Hewlett-Packard model 8116A) provided control signals to modulate the laser via the TTL input control port on the laser power supply. Parameters of the light presentation for tonic stimulation were 3000 pulses at 5 Hz (total light exposure of 10 minutes) and 50 pulses at 50 Hz with 30 s intervals for phasic stimulation (Bass et al., 2013). These light paradigms were applied for the first 10 minutes of the session. The optical pulse procedure began manually by firing a pulse generator (Systron Donner Model 100C), which activated a digital delay generator (SRS Model DG535). The digital delay generator was used to ensure the function generator was appropriately gated to select a finite number of pulses from the continuous waveforms typically produced by the function generator. The function generator produced a series of 5 Hz and 50 Hz square pulses. The total number of pulses in one data stream was gated by the digital delay generator because the temporal length of a gate pulse received by the function generator dictated the number of square pulses produced for each trigger by the function generator. Individual pulses had a temporal width of 4 ms within each series of pulses. A commercial power meter (Thorlabs Model S121C, Newton, New Jersey) was used to measure the laser power output.

Fast-scan cyclic voltammetry recordings. Extracellular DA concentrations, before and after optical stimulation of the VTA, were measured using FSCV. The experiments were performed at least 4 weeks following viral infusion. All subjects were secured in a stereotaxic frame following urethane (1.5 g/kg, i.p.) anesthesia. After the scalp was shaved and cleaned, the skull was uncovered by making a central incision along the scalp. One hole was fashioned above the NAc (from bregma: anterior, 1.3 mm; lateral, 1.3 mm) and another hole above the ipsilateral VTA (from bregma: posterior, 5.8 mm; lateral, 0.7 mm). A final hole was placed on the contralateral hemisphere for the implantation of an Ag/AgCl reference electrode connected to a voltammetric amplifier (UNC Electronics Design Facility, Chapel Hill, NC). A carbon fiber microelectrode (exposed fiber length: 100 µm; diameter: 6 µm) connected to the voltage amplifier and secured to the stereotaxic frame arm was lowered into the hole drilled above the NAc (ventral, 7.4 mm). Photostimulation was achieved via an optical fiber (200 µm diameter) inserted above the VTA and connected to a laser (Viasho, China). To generate tonic and phasic patterns of DA increases, we applied the protocol used in previous studies (Bass et al., 2013; Mikhailova et al., 2016). In the experiment with tonic-phasic interplay (Fig.3), two optical fibers connected to different lasers were placed above the VTA with 750-800 µm distance between tips. A calculation based on the cones of light emanating from the two sources indicated that 93-95 % of the light exposed volume was not overlapped by these two sources of photostimulation. In addition it is the edges of the two separated light cones that overlap, and the intensities are weaker in each of the beams near their peripheries than in their central regions, making the effects of the small overlap even weaker than the 7% volumetric overlap would suggest. This overlap was calculated using the following formula for a cone cut by a plane parallel to the cone's symmetry axis

$$V = \frac{H}{3R}\left[R^3 \cos^{-1}\left(\frac{x}{R}\right) - 2Rx\sqrt{R^2 - x^2} + x^3 \ln\left(\frac{R + \sqrt{R^2 - x^2}}{x}\right)\right],$$

where H is the cone height, R is the radius at the base of the cone, and x is the distance from the center of one of the cones to the bisecting plane. We have two of these zones contributing to our overlap region, so the overlap volume is $V_{overlap} = 2V$. In our case, $H = 1.13$ mm, which is estimated based on stereotaxic coordinates for the VTA (Paxinos and Watson, 2009), $R = 0.478$ mm, based on the numerical aperture of each fiber (NA = 0.39) and its diameter (200 µm), and $x = 0.275$ mm, is the distance to a point midway between the nearest edges of the two fibers.

Therefore, given the small overlap volume and the weaker intensities in the overlap regions, we can conclude that the majority of DA cell bodies were separately activated with the tonic (5 Hz) or phasic (50 Hz) protocol without any overlap. The light paradigm consisted of 50 light pulses at 50 Hz with 10 s intervals for phasic stimulation and 100 light pulses at 5 Hz for tonic stimulation. It is important to highlight that tonic stimulation was applied 5 s before the first phasic stimulation and ended 5 s after the second phasic stimulation, when these two patterns were combined. Under this circumstance, the increase in tonic DA release should result in an activation of DA autoreceptors that triggers a negative feedback mechanism of DA regulation. Therefore, when the high frequency stimulation was delivered, the presynaptic state was tuned to diminish phasic DA response. The averaged amplitude from two phasic transients, triggered with a 10-second interval, was used for the evaluation of changes in DA release.

Voltammetric recordings occurred at the carbon fiber electrode every 100 ms by applying a triangular waveform (-0.4 to +1.3V, 400 V/s). Oxidation and reduction peaks were observed at +0.6 V and -0.2 V respectively (vs. Ag/AgCl reference) identifying DA as the released chemical. Data were digitized (National Instruments, Austin, TX) and stored on a computer. Calibration of the carbon fiber electrodes was performed with known concentrations of DA (5, 10 µM) *in vitro* (Mateo et al., 2004; Oleson et al., 2009).

Nissl staining. Following the end of the experiment, rats were transcardially perfused with 4% paraformaldehyde and their brains were removed. The brains were then sliced and underwent Nissl staining to confirm proper placement of the recording and stimulating electrodes. Briefly, brains were removed and submerged in sucrose solutions of increasing concentrations (10%, 20%, and 30%) for at least 24h each, allowing the brain to sink before moving to the next concentration and kept at 4°C until further processing. Slices containing the NAc and VTA were taken using a vibratome (50 µm thickness; Vibratome 1000 Plus, The Vibratome Company, St. Louis, MO, USA) and mounted onto slides. After allowing the slides to dry at room temperature overnight, they were then processed for Nissl staining. Slides were submerged in deionized water (DI H_2O) and a series of ethanol solutions (50%, 70%, 90%, and 100%) for 3 min each rinse. They were then placed into 2 separate baths of xylene for 10 and 15 min each, respectively. Following this, the sections were rehydrated through a series of descending ethanol concentrations (100%, 95%, 70%, and 50%) followed by DI H_2O for 3 min each rinse. Next, the slices were submerged and stained in the cresyl violet solution (cresyl violet acetate, 0.1% aqueous, Electron Microscopy Sciences #26089-01) until the desired staining intensity had been achieved (typically 5-10 submersions) and then moved to a differentiation solution (0.5% glacial acetic acid) for 1-2 quick rinses. Then following 3 rapid DI H_2O rinses, the slices were dehydrated through a series of increasing ethanol concentrations (50%, 70%, 95%, and 100%) and 2 additional rinses of 100% ethanol for 3 min each concentration. Finally, the slides were submerged in xylene for 10 min before being placed in a final bath of xylene from which they were taken and coverslipped using DePeX mounting medium (Serva #18243.01). After allowing to dry overnight, slides were visualized and the locations of the recording and stimulating electrode tips were noted (see Fig. S3).

Immunohistochemistry. Rats were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then transcardially perfused with 10% normal buffered formalin. After removal, brains were soaked overnight in fixative at 4° C and then incubated in a 25% sucrose solution overnight until the brains sank. Fifty µm thick sections were obtained on an American Optical 860 sliding microtome. Free-floating coronal sections, which contained the midbrain, were processed for immunohistochemistry. Briefly, sections were washed in PBS for 5 min followed by 3 x 10 min rinses in PBS + 0.5% triton X-100. Primary antibody diluted in PBS + 0.3% triton X-100 was applied overnight at 4ºC while shaking. Primary antibodies used were mouse anti-tyrosine hydroxylase (ImmunoStar #22941) at a 1:4000 dilution and a rabbit anti-GFP (Invitrogen #A6455, also cross reacts with EYFP) at a 1:2000 dilution. The following day, sections underwent 3 x 10 min PBS rinses and then were incubated with secondary antibodies of Alexa 555 donkey anti-mouse (Invitrogen, #A31570, 1:4000) and Alexa 488 goat anti-rabbit (Invitrogen #A11034, 1:2000) at room temperature for 2 hours while shaking. A last set of 3 x 10 min PBS rinses were applied to the sections that were then mounted onto slides and coverslipped with Prolong Gold media. Slides were visualized via a Zeiss LSM 710 confocal microscope.

Statistical analysis. Data were analyzed in GraphPad Prism (GraphPad Software, San Diego, CA). The sample size (n=7 rats per each experimental group) was based on power analyses and was similar to those reported in previous studies (Bass et al., 2013, Fox et al., 2016; Mikhailova et al., 2016). The Shapiro-Wilk normality test was used to attest to the normality of the data sets. Based on results of this evaluation, the data were further analyzed with parametric (one-way ANOVA with multiple comparison test) or nonparametric approaches (Kruskal-Wallis,

Wilcoxon or Mann-Whitney test) to determine statistical significance. Spearman correlation analysis was used to evaluate the relationship between lever pressing behavior (alcohol seeking) and alcohol consumption. The data were presented as mean ± s.e.m. and the criterion of significance was set at $P<0.05$.

Supplemental References

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