Title: Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens

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Abstract: Excitatory afferents to the nucleus accumbens (NAc) are thought to facilitate reward seeking by encoding reward-associated cues. Selective activation of different glutamatergic inputs to the NAc can produce divergent physiological and behavioral responses, but mechanistic explanations for these pathway-specific effects are lacking. Here, we compared the innervation patterns and synaptic properties of ventral hippocampus, basolateral amygdala, and prefrontal cortex input to the NAc. Ventral hippocampal input was found to be uniquely localized to the medial NAc shell, where it was predominant and selectively potentiated following cocaine exposure. In vivo, bidirectional optogenetic manipulations of this pathway attenuated and enhanced cocaine-induced locomotion. Challenging the idea that any of these inputs encode motivationally-neutral information, activation of each discrete pathway reinforced instrumental behaviors. Finally, direct optical activation of medium spiny neurons proved to be capable of supporting self-stimulation, demonstrating that behavioral reinforcement is an explicit consequence of strong excitatory drive to the NAc.

Suggested Reviewers:

Opposed Reviewers:
Dear Katja,

Thank you for agreeing to publish our manuscript. Please find attached the revised version. We thank the Reviewers for their comments and have made these changes accordingly.

- To address the concerns of Reviewer #1, we no longer describe *in vivo* ChR2 manipulations as activating specific inputs to the NAc. We now describe how we photostimulated specific axons in the NAc. This change can be found in paragraphs describing figures 7 and 8, as well as in figure legends 6, 7, and 8. We also detail the caveats of these experiments in the 7th paragraph of the discussion. This section starts with “Potential confounds...”. Lastly, we temper our language in the 6th paragraph of the discussion section by replacing the word “suggests” with the phrase “raises the possibility that”. Now the sentence reads “It highlights the similarities of these inputs and raises the possibility that the specific pathway releasing glutamate is not as important as the amount of glutamate that is released.”

- To address the comments of Reviewer #2, we added to the discussion section and elaborated on the methodology behind Figure S2. In the 2nd to last paragraph of the discussion section we included the sentence “One possibility is that the distinction between output pathways might not be as absolute in the NAc as it is in the dorsal striatum (Bertran-Gonzalez et al., 2008).” In the 6th paragraph of the discussion section, we included the sentence “Ventral tegmental area dopamine neurons innervate the NAc, and similar behaviors have been observed when these neurons are selectively stimulated (Witten et al., 2011).” We did not completely remove the discussion of direct and indirect pathway neurons, because it was specifically requested by Reviewer #1 in his/her original review, point #9.

We thoroughly agree with Reviewer #1 that our data suggesting the “amount of glutamate released in the NAc is more important than where it is released from” is highly controversial and goes against the current opinion of the field. While we use appropriately cautious language in the manuscript, we hope that you’ll consider it suitable for a News and Views piece.

Thanks once again for accepting this article.

Yours,

Anto
Response to Reviewers

These Reviewers have been very thoughtful and we greatly appreciate their comments. The manuscript is better now that we have incorporated their suggestions.

Reviewer #1: Overall, I was pleased with the responses from the authors, and I feel that the addition of more raw data (confocal images) and discussion will be very helpful to their broad audience. As before, I think this is a high-quality, fascinating study which has the potential to make a massive impact on the field. I was pleased with most of the point-by-point responses, except for their response to comment #1, where they still insist that they provided "strong evidence" that their in vivo manipulation was input specific, but I did not see this evidence. I also could not find where they explicitly discussed the caveats, and there was no page/line number to help me find it. The authors’ response suggested that they did not understand my concern, and so I will state it again in greater detail, below.

My remaining concerns could be alleviated with several text changes: Specifically, I requested that the authors explicitly demonstrate the input specificity (ruling out the possibility of backpropagating action potentials or even subthreshold depolarization to axon collaterals) or that the authors revise this claim and add an explicit disclaimer. While they did change the wording, the figure legend titles for main figure 6 and 7 are still not completely justified. For example, the authors should revise the legend caption "vHipp Input to the NAc Can Reinforce Instrumental Behaviors" to "Photostimulation of vHipp axons in the NAc Can Reinforce Instrumental Behaviors." This clearly releases them of the responsibility of demonstrating that backpropagating depolarization is not contributing to these behaviors, (nor axons of passage) and encourages follow-up studies to tackle this challenge, which the authors may feel lies beyond the scope of this very substantive and important study. Indeed the authors have chosen to cover a substantial amount of breadth, and I feel that tackling 3 projections to the same depth that the Tye et al., 2011 or Stuber et al., 2011 Nature papers would not be reasonable for a single publication, even in a top-quality journal such as Neuron. If the authors can remove any claims of input-specificity in vivo, I would be happy to support publication of the manuscript in its current form.

One minor point is that the authors might consider citing some more of the relevant literature that has been recently published, but if they are close to their maximum number of references, they might consider citing a recent review of the literature regarding opsins (Mattis et al., Nature Methods, 2011) or the application of them to disease models (Tye and Deisseroth, NRN, 2012).

To address this concern, we no longer describe in vivo ChR2 manipulations as activating specific inputs to the NAc. We now describe how we photostimulated specific axons in the NAc. This change can be found in paragraphs describing figures 7 and 8, as well as in figure legends 6, 7, and 8. We also detail the caveats of these experiments in the 7th paragraph of the discussion section. This section starts with “Potential confounds...”.

I should emphasize to both the editors and the reviewers that this added disclaimer should not necessarily be viewed as a weakness of the manuscript, so much as an opportunity to evoke follow-up studies from the rest of the field. Indeed, adding such a explicit description of the caveats would only
serve to increase my enthusiasm (and decrease my skepticism) for this paper, and I would not be surprised if a clear invitation for future experiments would actually increase reader interest and subsequent citations...

A final text concern that I would strongly recommend the authors revise is the following sentence: "It highlights the similarities of these inputs and suggests the specific pathway releasing glutamate is not as important as the amount of glutamate that is released." First of all, this is a highly contradictory statement, and if the authors wish to challenge this dogma, then they would need to demonstrate a number of things experimentally: 1) That it is indeed input-specific (i.e. that there is not back-propagation, with pharmacological or other challenging approaches) 2) A quantitative demonstration of the amount of glutamate released by each illumination parameter and viral expression strategy (axon source) 3) That axons of passage are not contributing 4) That there are not axo-axonal connections of these regions that might also contribute to these behaviors. The claim that the input does not matter is a very serious claim that certainly goes against the current opinion of the field: while this finding would be of great interest if it were true, the authors would need to a great deal more work experimentally to be able to make this claim. The authors are, of course, welcome to speculate on this topic. However they should either clearly state that this is pure speculation, or phrase it as a question for future studies to address.

To address this concern, we temper our language in the 6th paragraph of the discussion section by replacing the word “suggests” with the phrase “raises the possibility that”. Now the sentence reads “It highlights the similarities of these inputs and raises the possibility that the specific pathway releasing glutamate is not as important as the amount of glutamate that is released.”

As I stated above, the data in this study are very interesting, and the authors do not need to over-interpret their data to ensure that it will be of high impact. Again, I would predict that the more conservative discussion/interpretation of their results, and the more openly they delineate the future experiments that this studies inspires, the more lasting an impact it will have.

Reviewer #2: The authors were quite responsive to the earlier critiques in this substantial revision of the manuscript. The study is much improved and a major flaw in the previous version has been corrected.

The major concern we had in the previous version were in part confirmed by the additional experiments. We argued that one reason vHIP activation could promote cocaine's effect was because more ChR2 fibers came from this area and thus more glutamate could be released from this input. Indeed, the authors now find that if the other inputs where activated with stronger stimulation they
could evoke approach behavior similar to the vHIP. Thus the role of the vHIP-input is not unique, but instead is due to the ability of the light to elicit sufficient glutamate release to activate MSNs (presumably) in the accumbens.

The involvement of nucleus accumbens in reinforcement is already well established. This is a weakness of the study and the results with vHIP-stimulation and cocaine could be a direct consequence of this technical limitation. Yet, the experiments with the silencing of the vHIP with halorhodopsin are more convincing and intriguing. Together with the in vitro findings we think that the authors show that signals from vHIP have an important role in cocaine's action.

Thus, the major flaw in the previous version has been adequately addressed. There are still some points in the discussion that need to be addressed.

1) At the end of the Discussion, the authors discuss the direct and indirect pathway and motivational responding: however, these two pathways are poorly defined in the nucleus accumbens. For example, D1/D2 dopamine receptors are more often colocalized in the nucleus accumbens, in contrast to the dorsal striatum (Meredith 1999. We suggest that the authors rather discuss that the nucleus accumbens shell is specifically innervated the VTA neurons (Zahm 1999), which could contribute more specifically to the rewarding effect of nucleus accumbens activation they observe.

2) The authors should include a more detailed methodological description of quantification is performed the figure legend of Figure S2. I find it difficult to draw any conclusions of this figure without information on how the experiment was performed.

To address the comments of Reviewer #2, we added to the discussion section and elaborated on the methodology behind Figure S2. In the 2nd to last paragraph of the discussion section we included the sentence “One possibility is that the distinction between output pathways might not be as absolute in the NAc as it is in the dorsal striatum (Bertran-Gonzalez et al., 2008).” In the 6th paragraph of the discussion section, we included the sentence “Ventral tegmental area dopamine neurons innervate the NAc, and similar behaviors have been observed when these neurons are selectively stimulated (Witten et al., 2011).” We did not completely remove the discussion of direct and indirect pathway neurons, because it was specifically requested by Reviewer #1 in his/her original review, point #9.
**Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens**

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**SUMMARY**

Excitatory afferents to the nucleus accumbens (NAc) are thought to facilitate reward seeking by encoding reward-associated cues. Selective activation of different glutamatergic inputs to the NAc can produce divergent physiological and behavioral responses, but mechanistic explanations for these pathway-specific effects are lacking. Here, we compared the innervation patterns and synaptic properties of ventral hippocampus, basolateral amygdala, and prefrontal cortex input to the NAc. Ventral hippocampal input was found to be uniquely localized to the medial NAc shell, where it was predominant and selectively potentiated following cocaine exposure. In vivo, bidirectional optogenetic manipulations of this pathway attenuated and enhanced cocaine-induced locomotion. Challenging the idea that any of these inputs encode motivationally-neutral information, activation of each discrete pathway reinforced instrumental behaviors. Finally, direct optical activation of medium spiny neurons proved to be capable of supporting self-stimulation, demonstrating that behavioral reinforcement is an explicit consequence of strong excitatory drive to the NAc.

**INTRODUCTION**

The NAc plays a major role in the generation of motivated behaviors (Berridge, 2007; Ikemoto, 2007; Nicola, 2007). It is thought to facilitate reward-seeking by integrating dopaminergic reinforcement signals with glutamate-encoded environmental stimuli (Brown et al., 2011; Day et al., 2007; Flagel et al., 2011; Phillips et al., 2003; Stuber et al., 2008). A prominent idea is that the glutamate input to the NAc encodes the context, cues, and descriptive features that characterize any given moment in time (Berke and Hyman, 2000; Everitt and Wolf, 2002; Kelley, 2004; Pennartz et al., 2011). Together, glutamate and dopamine can promote synaptic plasticity, which is thought to be a crucial neural mechanism in the NAc by which pertinent environmental cues become more salient than other stimuli (Kheirbek et al., 2009; Sun et al., 2008; Wolf and Ferrario, 2010). This may occur following addictive drug use, when a hypersensitivity to drug-associated cues coincides with strengthened glutamatergic synapses in the NAc (Di Chiara, 2002; Schmidt and Pierce, 2010; Schultz, 2011).

Prominent glutamate input to the NAc comes from the ventral hippocampus (vHipp), basolateral amygdala, and prefrontal cortex (Friedman et al., 2002; Phillipson and Griffiths, 1985). Pathway-specific activation of these fibers has been demonstrated to elicit distinct physiological and behavioral responses (Goto and Grace, 2008; Sesack and Grace, 2010). For example, vHipp input is particularly capable of stably depolarizing NAc neurons, allowing prefrontal cortex input to generate spike firing in these cells (O'Donnell and Grace, 1995). Basolateral amygdala input, unlike prefrontal cortex input, readily supports optogenetic self-stimulation (Stuber et al., 2011). To elucidate the mechanistic underpinnings
of these types of pathway-specific effects, we examined the innervation patterns and synaptic properties of vHipp, basolateral amygdala, and prefrontal cortex input to the NAc. In addition, we assayed each pathway for cocaine-induced synaptic plasticity and subjected each one to optogenetic manipulations in vivo.

RESULTS

To examine the innervation patterns of excitatory input to the NAc, we targeted enhanced yellow fluorescent protein (EYFP) expression to projection neurons in the vHipp, basolateral amygdala, and prefrontal cortex (Figure 1A; additional images are shown in Figure S1). When EYFP expression was measured in the NAc in images captured with identical settings, the brightest fluorescent signal was observed in vHipp fibers located in the medial NAc shell (Figure 1B). In the NAc core and lateral shell, the fluorescence coming from vHipp axons was relatively modest. In contrast, EYFP expression in the amygdala and prefrontal cortex input, while not as pronounced in the medial shell, was more apparent throughout other subregions of the ventral striatum. The innervation patterns of these two pathways were considerably uneven, yet not as localized to any specific subregion as the vHipp fibers were to the medial shell (Figure 1 and S1).

To substantiate the indication that vHipp fibers predominate in the medial NAc shell, we injected the retrograde tracer Fluoro-Gold into this region (Figure 2A). This approach enabled the identification of NAc shell-projecting neurons throughout the brain (Brog et al., 1993). We identified large populations of retrogradely-labeled cells in several regions, including the hippocampus (ventral subiculum and entorhinal cortex), basolateral amygdala and prefrontal cortex (Figure 2B). Using slices from each region that contained dense populations of NAc-projecting cells, we counted more medial NAc shell-projecting neurons in the vHipp than in either the basolateral amygdala or prefrontal cortex (Figure 2C). These manual cell counts highly correlated with the anti-Fluoro-Gold fluorescent signal in each slice (Figure S2; $R^2 = 0.86; p < 0.01$), and quantifying this signal throughout each region demonstrated that more inputs to the medial NAc shell come from the vHipp than from the other examined regions (Figure 2D).

To compare the functional strength and synaptic properties of each of these afferent pathways, we employed an optogenetic approach and targeted channelrhodopsin-2 (ChR2) expression to projection neurons in these areas (Mattis et al., 2012). Brain slice, whole cell recordings were then obtained in areas of conspicuous fluorescence within the medial NAc shell (Figure 1A). The fluorescence in these targeted hotspots, relative to the average signal from vHipp fibers in the medial NAc shell, was $1.4 \pm 0.2$, $0.9 \pm 0.1$, and $0.7 \pm 0.1$ for the vHipp, amygdala, and PFC pathways, respectively. Irrespective of which pathway was optically stimulated, excitatory postsynaptic currents (EPSCs) were observed in more than 95% of recorded neurons (Figure 3A). This result suggests that each medium spiny neuron subtype in the NAc shell is innervated by each of these pathways, and that single neurons in this region receive input from multiple sources (Finch, 1996; French and Totterdell, 2002, 2003; Groenewegen et al., 1999; McGinty and Grace, 2009).

Optical stimulations with a maximum amount of light proved vHipp fibers could elicit the largest excitatory currents in postsynaptic neurons (Figure 3B). This pathway was also unique in its ability to drive post-synaptic action potentials in “physiological” brain slice recordings (Figure S3A). This was an apparent consequence of the hyperpolarized resting membrane potential of medium spiny neurons, typically around -85 mV, in conjunction with a pervasive feed forward inhibitory circuit. Conditions in brain slices are such that postsynaptic spiking was only reliably observed when both the vHipp input was optically stimulated and the corresponding EPSCs were greater than 600 pA. To eliminated the influence of feed forward inhibition in all voltage clamp experiments, we included picrotoxin (100 µM) in recording solutions.
These electrophysiological results, in conjunction with the retrograde labeling and EYFP expression data, suggest vHipp input is predominant in the medial NAc shell. Technical considerations, however, particularly related to the extent of viral infection and ChR2-expression, are important to consider. To test if ChR2-EYFP expression was similar between virally-infected brain regions, we measured fluorescence intensity in representative animals at the center of each injection site. This signal was comparable between brain regions, suggesting ChR2 expression levels were not significantly different between injection sites (Figure S3B). Another consideration is the spread of viral particles, which can potentially differ between brain regions. Viral infection did often occur in regions immediately outside the targeted structures, but our concern was with the relative infection rate in areas that contained NAc-projecting cells. To gauge the proportion of NAc-projecting cells infected in each region, we measured fluorescence in 3-dimensions explicitly where NAc-projecting cells were typically located. The prefrontal cortex had a higher average fluorescence than the other two regions, likely due to the relatively small size and simple cytoarchitecture of this structure (Figure S3C). This result indicates that a relatively greater proportion of NAc shell-projecting neurons in the prefrontal cortex, compared to the other two regions, expressed ChR2. A third technical consideration is the time it takes for expressed ChR2 to diffuse the length of an axon, which can be weeks to months. To test if ChR2-expression was comparable in the axons of each pathway, we recorded optically-evoked EPSCs using different intensities of stimulus light (Figure S3D). The presumption is that differential ChR2 expression levels would make axons more or less sensitive to light. The input-output curves show normalized EPSC amplitudes were virtually identical between pathways, suggesting ChR2 levels were comparable in each group of axons in the NAc (Figure S3E).

The extent to which these pathways innervate the medial NAc shell could entirely underlie the disparate maximal EPSC amplitudes, but differences in probability of vesicle release and postsynaptic responsiveness to glutamate could also be consequential. Vesicle release probability has been shown to relate to the ratio of EPSC amplitudes obtained with paired pulse stimulation (Silver et al., 1998), so we calculated a paired pulse ratio for each pathway. In contrast to the prefrontal cortex and vHipp fibers, which showed comparable amounts of paired pulse depression, amygdala input exhibited paired pulse facilitation (Figure 3C and S3F). This indicates amygdala fibers have a relatively low probability of transmitter release, which would have been a factor in the measured EPSC responses. To assess pathway-specific postsynaptic responsiveness to glutamate, we measured quantal amplitude by replacing calcium with strontium to desynchronize transmitter release (Figure 3D) (Goda and Stevens, 1994). Asynchronous EPSC amplitudes did not differ between pathways (Figure 3E), which suggest that the divergent maximum EPSC amplitudes reflect differences in number of transmitter release sites and vesicle release probability.

EPSCs elicited from the optical stimulation of vHipp fibers appeared to have relatively slow decay kinetics, an indication that the excitatory receptors mediating these currents were distinct. To test if there were pathway-specific differences in the composition of postsynaptic glutamate receptors, as has been observed in other cell types (Good and Lupica, 2010; Kumar and Huguenard, 2003; Smeal et al., 2008), we determined the current-voltage relationship of AMPA and NMDA receptors in each pathway. AMPAR-mediated currents obtained with optical stimulation exhibited a linear current-voltage relationship that did not differ between pathways (Figure 4A). This result indicates the postsynaptic receptors apposing these inputs have comparable amounts of GluR2 subunits (Gittis et al., 2011; McCutcheon et al., 2011). A pathway-specific difference was found, however, in the voltage dependence of NMDARs (Figure 4B). Medium spiny neurons held at hyperpolarized membrane potentials passed a proportionally large peak inward current through NMDARs at vHipp to NAc shell synapses. This result indicates these specific NMDARs are composed of subunits relatively less sensitive to Mg2+ blockade (Hull et al., 2009). Consequently, even at resting membrane potentials they can make significant contributions to excitatory transmission. This would have contributed to the larger overall
EPSC amplitudes elicited from vHipp fibers. It also might explain why this pathway is especially capable of eliciting stable depolarized states in NAc neurons (O'Donnell and Grace, 1995).

There is a substantial amount of literature implicating NAc synaptic plasticity in drug abuse disorders, so we assayed each pathway for cocaine-induced synaptic plasticity (Figure 5A) (Kourrich et al., 2007; Koya and Hope, 2011; Wolf and Tseng, 2012). Synaptic potentiation can be mediated by increases in either the number of AMPARs per synapse or current flux per AMPAR (Luscher and Malenka, 2011). Both outcomes have been observed in the NAc following cocaine use, and both cause increases in quantal amplitude (Conrad et al., 2008; Dobi et al., 2011; McCutcheon et al., 2011; Pascoli et al., 2012). Comparing asynchronous EPSCs as an index of quantal amplitude, in saline- and cocaine-treated mice (15 mg/kg intraperitoneal), we found a significant cocaine-induced increase in synaptic strength selectively in vHipp input (Figure 5B). To corroborate this result, we employed a second, independent measure of synaptic potentiation, the ratio of currents mediated by AMPA and NMDA receptors. This measure derives from data suggesting potentiated synapses exhibit increases in AMPA, but not NMDA, receptor responses (Bredt and Nicoll, 2003; Ungless et al., 2001), although changes in NMDAR responses have also been observed (Kombian and Malenka, 1994). AMPA/NMDA receptor response ratios were determined in both cocaine- and saline-treated mice for each pathway by recording optically-evoked currents at +40 mV (Figure 5C). Consistent with the strontium data, a significant effect of cocaine on AMPA/NMDA receptor response ratios was only observed in the vHipp input (Figure 5D). Together, these findings show that cocaine use selectively strengthens vHipp synapses in the medial NAc shell. It is important to note that considering the sparseness of vHipp input to the NAc core and lateral shell, it is unlikely this pathway-specific effect underlies drug-induced synaptic changes that have been observed in those regions.

There is an emerging consensus that cocaine-induced changes in synaptic strength can involve calcium permeable AMPARs, which have been linked to drug craving, but only if cocaine is self-administered over many days (Conrad et al., 2008; Wolf and Tseng, 2012). Our intraperitoneal cocaine injections did not alter the current-voltage relationship of AMPAR-mediated currents in vHipp to NAc synapses, which is consistent with this notion (Figure 5E). The current-voltage relationship was linear in both drug naïve and cocaine-treated mice, indicating this synaptic potentiation did not reflect increases in calcium-permeable AMPARs. Since the hippocampus has been implicated in the recognition of novel environments, which is where mice show the most pronounced locomotor responses to cocaine (Badiani et al., 2011; Chun and Phelps, 1999; Vezina and Leyton, 2009), we tested if the same cocaine injection schedule administered in animals’ home cages could also potentiate vHipp to NAc synapses. AMPA/NMDA receptor response ratios were similarly elevated in home cage cocaine-treated mice, suggesting location of drug use is not the sole determinant of this effect (Figure 5A).

The pathway-specificity of this synaptic potentiation raised the possibility that vHipp input to the NAc drives behavioral responses to cocaine. To test this idea, we used a viral approach to target halorhodopsin 3.0 (NpHR) expression bilaterally to the vHipp and, during the same surgery, implanted optical fibers just dorsal to the NAc shell. Six weeks post-surgery, expressed NpHR-EYFP had diffused throughout vHipp infected cells and was observed in axon terminals in the NAc (Figure 5A). Control mice were treated identically, except they were infected with a virus that only coded for EYFP expression. For 30 minute periods over five consecutive days, these mice were attached to optical tethers and placed in an unfamiliar environment where they were given intraperitoneal cocaine injections (10 mg/kg). Immediately after each of the first five injections, laser light was used to attenuate transmitter release from NpHR-expressing axon terminals (Stuber et al., 2011; Tye et al., 2011). A difference was observed in distance traveled between NpHR and EYFP groups, with the NpHR group showing significantly less cocaine-induced locomotion on days 2-9 (Figure 6A). Differences in locomotor responses expanded over time and were slow to dissipate during sessions that were not paired with laser light. On the last day, there was no difference between groups. In cocaine-naïve mice,
inhibition of vHipp input did not affect locomotion, as tested in an open field chamber (Figure 6B). The proportion of time spent in the center of the open field chamber during the first visit, a measure of anxiety-related behavior, also did not differ between groups (Figure S5B). Thus, inhibiting vHipp input to the NAc selectively attenuates cocaine-induced locomotion. This demonstrates that endogenous activity in this pathway contributes to behavioral responses to cocaine.

To test if augmenting activity in this pathway could enhance cocaine-induced locomotion, we repeated the experiment in mice that expressed ChR2 in the vHipp, instead of NpHR (Figure S5A). We also changed the location where cocaine was administered to the animal’s home cage, presuming vHipp activity would be relatively low in this familiar setting and more amenable to ChR2-induced increases in activity. As anticipated, ChR2 activation increased cocaine-induced locomotion (Figure 6C). On the last day laser light was not used and no significant differences between groups were observed. In cocaine-naïve mice, neither locomotion (Figure 6D) nor anxiety-related measures (Figure S5B) were affected by the activation of this pathway. This result indicates that the light stimulus enhancement of cocaine-induced locomotion was an emergent property of vHipp input related to the drug. Presumably, cocaine-associated dopamine signaling transforms the impact of glutamatergic transmission in the NAc.

To explore if vHipp input encodes neutral contextual information or rather the incentive properties of the environment, we examined if optical activation of vHipp axons in the NAc could bias where mice spent their time in a three-room chamber (Tyé and Deisseroth, 2012). Mice had complete freedom of movement in these chambers. Optical stimulation was paired with one side of the chamber on days 2-4. Whenever mice entered and remained in the laser-paired context, light was pulsed in the NAc activating ChR2-positive vHipp fibers. With this instrumental protocol, mice spent more time in the laser-paired side of the chamber as soon as optical stimulation was available (Figure 7A). This preference for the laser-paired side persisted throughout the experiment, even on the ‘probe’ test day when laser light was not employed. Interestingly, this bias reflected a reduced probability that mice would exit from the laser-paired side of the chamber (Figure 7B and S6A), which contrasts with the behavior of animals in classical conditioned place-preference experiments (German and Fields, 2007). Neither the speed nor distance traveled by these mice increased across sessions (Figure S6B).

The artificial nature of the optically-induced neuronal activity would conceivably disrupt any discrete contextual information processing. If this consequence is what produced the place preference observed above, optical inhibition of this pathway might produce similar results. To test this idea, we mimicked the experimental design, but used NpHR and optical inhibition instead of ChR2. This context-specific inhibition of vHipp axons in the NAc did not influence where mice spent their time (Figure S6C). Thus, in a relatively neutral environment, physiological activity in this pathway does not significantly influence basic exploratory behavior.

To investigate the possibility that brief bursts of optical stimulation were sufficient to reinforce instrumental behavior, we gave mice the opportunity to optogenetically self-stimulate vHipp axons in the NAc. Within 15 minutes of entering operant chambers for the first time, mice expressing ChR2 in the vHipp began persistently nose poking to obtain bursts of light into the NAc (Figure 7C). Inactive nose poke holes were largely ignored and EYFP control mice did not regularly nose poke into either hole (Figure 7D). The rate of behavioral responding in the ChR2-expressing mice steadily increased upon subsequent sessions and was similarly robust when tested 70 days later (Figure S6D). Together with the place preference experiment, this work shows that both short and long stimulations of vHipp axons in the NAc are rewarding.

Our examination of pathway-specific synaptic differences suggested the only reason prefrontal cortex input to the NAc might not support self-stimulation is because it is a relatively weak input. We reasoned that if the optogenetic stimulus was robust enough, it might be possible that each excitatory input to NAc could reinforce instrumental behavior. We first tested this in the three-room chambers and, using a 6 Hz pulse frequency contingent on mice being in the laser-paired room, we found that
mice preferred to spend time on the side of the chamber paired the optical stimulation, regardless of which afferent pathway to the NAc was activated in each mouse (Figure 8A). In these same mice, optogenetic self-stimulation was also observed, although importantly we increased the strength of the light stimulus to compensate for the weaker inputs (Figure 8B). We used 30, 60, and 90 pulses for the vHipp (20 Hz), basolateral amygdala (20 Hz), and prefrontal cortex fibers (30 Hz), respectively. These results raise the possibility that the specific excitatory pathway activated is not as important as how much glutamate is released into the NAc, at least in terms of generating motivated behaviors.

It is surprising that discrete glutamate release facilitated reward seeking, since it has been hypothesized that the inhibition of NAc neurons is what encodes reward (Carlezon and Thomas, 2009; Carlezon and Wise, 1996; Roitman et al., 2005; Taha and Fields, 2006). To directly test the role of NAc cell activity in modulating these behaviors, we gave mice the opportunity to self-stimulate medium spiny neurons. We indiscriminately targeted ChR2 expression to NAc projection neurons in the medial NAc shell and implanted optical fibers just above this area (Figure 8C). Robust self-stimulation was observed in these mice (Figure 8D), demonstrating that mice will work to obtain a non-selective activation of neurons downstream of dopamine signaling. This result shows that indiscriminate bulk activation of NAc neurons is sufficient to reinforce instrumental behavior.

DISCUSSION

By comparing the innervation patterns and synaptic properties of vHipp, basolateral amygdala, and prefrontal cortex input to the NAc, we identified vHipp fibers as being uniquely concentrated in the medial NAc shell. In this region vHipp input was predominant and selectively strengthened following cocaine injections. We also employed bidirectional optogenetic manipulations in vivo to demonstrate that vHipp input to the NAc drives cocaine-induced locomotion. Optical stimulations designed to offset the differential potency of each input proved that activation of each afferent pathway could reinforce instrumental behavior. We also found that mice will work for the direct stimulation of NAc neurons.

Pathway-specific stimulation of excitatory input to the NAc has been shown to elicit disparate physiological and behavioral responses (O'Donnell and Grace, 1995; Stuber et al., 2012). In search of pathway-specific synaptic differences that might underlie these types of effects, we unexpectedly found vHipp fibers were predominant in the medial NAc shell. Correspondingly, retrograde tracing demonstrated a greater abundance of medial NAc shell-projecting neurons in the vHipp than in either the basolateral amygdala or prefrontal cortex. Brain slice electrophysiological recordings in the medial NAc shell confirmed that vHipp input was uniquely effective in exciting these postsynaptic neurons.

Postsynaptic responsiveness to glutamate (quantal amplitude) and AMPAR compositions were comparable between pathways, but vesicle release probability and NMDAR compositions were not. Paired pulse stimulation experiments indicated that amygdala fibers have a relatively low probability of vesicle release. Accordingly, these synapses may function in a manner similar to a high pass filter, which implies burst firing patterns in this pathway could be necessary to drive postsynaptic neurons. NMDARs at vHipp to NAc synapses were found to be relatively less sensitive to Mg$^{2+}$ blockade. Consequently, these NMDARs pass significant current at resting membrane potentials. Considering how the slow decay kinetics of NMDAR-mediated currents can encourage synaptic summation, in conjunction with the relatively abundant synaptic contacts of this input, this property could explain why vHipp input has a superior ability to stably depolarize medium spiny neurons (O'Donnell and Grace, 1995). Additionally, due to the importance of NMDARs in synaptic plasticity, this feature could render vHipp synapses especially mutable.

We did observe vHipp selective synaptic plasticity following intraperitoneal cocaine injections. This was unexpected because cocaine-induced synaptic plasticity has been observed throughout the NAc, and vHipp innervation of the NAc is extraordinarily localized to the medial shell (Lee and Dong,
In response to seconds of the chamber, they will also work to terminate behavior, it is likely that vHipp input is predominant in the medial NAc shell. Different inputs may show more plasticity where they are most robust.

Additionally, activity of vHipp axons in the NAC proved consequential to cocaine-induced locomotion. Optogenetic inhibition of this pathway attenuated this behavior, while optogenetic stimulation enhanced it. These data demonstrate that activity in vHipp axons in the NAc drive cocaine-induced locomotion, and the context-dependence of this behavior might be attributable to activity in this pathway (Badiani et al., 2011; Vezina and Leyton, 2009). Since neither activation nor inactivation of this pathway influenced basal locomotion, the differential effects following cocaine injections are presumably related to drug-induced dopamine signaling. Dopamine may bias postsynaptic activity towards one cell type or another and interactions with glutamate likely control the extent of cocaine-induced locomotion. These findings contradict the idea that a decrease in NAC neuron excitability promotes cocaine-induced locomotion (Dong et al., 2006), but are consistent with evidence that striatal c-fos induction is much stronger if cocaine injections are given in a novel environment (Uslaner et al., 2001). The impact of attenuating vHipp input on cocaine-induced locomotion grew over repeated injections, which raises the possibility that vHipp-induced locomotion during cocaine use is related to behavioral sensitization to cocaine. Overall, however, the sensitizing effect of repeated cocaine injections was observed in spite of the optogenetic manipulations.

The most notable finding presented here might be that photostimulation of each of the different afferent pathways to the NAC reinforced instrumental behavior. Admittedly, the bulk stimulations used were not physiological, but the fact that activity in each pathway can support these behaviors is a critical characteristic of the network. It highlights the similarities of these inputs and raises the possibility that the specific pathway releasing glutamate is not as important as the amount of glutamate that is released. Additionally, the information encoded in these inputs clearly has motivational value, which supports the theory that dopamine in the NACs acts to amplify or regulate the incentive properties of environmental stimuli that are presumably encoded in glutamatergic signals (Berridge, 2007). Ventral tegmental area dopamine neurons innervate the NAc, and similar behaviors have been observed when these neurons are selectively stimulated (Witten et al., 2011). A challenge now is in determining when each glutamatergic pathway is physiologically active and consequential in shaping behavior.

Potential confounds of the in vivo ChR2 data include the back propagation of ChR2-induced action potentials as well as activation of fibers that simply pass through the illuminated region of the brain. With our optical equipment, photostimulation could have occurred in the NAc as well as more medial nuclei, including the intermediate lateral septal nucleus and the nucleus of the vertical limb of the diagonal band. We do not rule out the potential contribution of fibers of passage or back-propagating action potentials for each in vivo ChR2 effect. All together, however, with three excitatory afferent pathways and medium spiny neurons themselves all proving capable of eliciting the same behavior, it is likely that glutamate release in the NAc was the main determinant.

Comparisons between optical and electrical brain stimulation reward are intriguing. While rats will work to initiate electrical stimulation of the NAc, they will also work to terminate it after a few seconds (Olds and Olds, 1963), suggesting that the stimulation becomes aversive some time after onset. In response to the low-frequency optical stimulations used here, mice would remain in the laser-paired side of the chamber for minutes at a time. Another difference with classical brain stimulation reward is that the optical stimulations used here did not necessarily result in increased movement (Glickman and Schiff, 1967). These distinctions may relate to the specificity of the optical manipulations.
The fact that bulk activation of NAc shell neurons can also reinforce instrumental behavior underscores the idea that that motivated behavioral responding can be a direct consequence of excitatory drive in the NAc. How this finding relates to the selective stimulation of direct and indirect output pathways of the NAc is unclear. As in the dorsal striatum, these two output pathways have been shown to encode conflicting behavioral signals (Kravitz et al., 2012; Lobo et al., 2010). Indiscriminate stimulation of NAc shell neurons, however, appears to elicit behavioral effects that would conceivably be produced by selective direct pathway stimulation. One possibility is that the distinction between output pathways might not be as absolute in the NAc as it is in the dorsal striatum (Bertran-Gonzalez et al., 2008). It could also be that the anatomical nature of the direct pathway is such that it has a leading role in downstream circuits and is the default option for some behaviors encoded by the NAc. Alternatively, activity in the indirect pathway might not necessarily be a reward-opposing, demotivating force, but it could simply encode a separate dimension of this behavior. In any case, it is important to remember that the artificiality of the optical stimulations, being both massive and instantaneous, can presumably overwhelm inhibitory circuits that might balance activity in these pathways.

In conclusion, the data presented here show that in the medial NAc shell vHipp input is predominant, selectively strengthened following cocaine injections, and of consequence to acute cocaine-induced locomotion. Also, discrete activation of three different excitatory inputs to the NAc, as well as NAc neurons themselves, was shown to reinforce instrumental behavior. Overall, this work contributes to our understanding of excitatory input to the NAc shell, as well as the contribution of this region to reward-related behaviors.

EXPERIMENTAL PROCEDURES

Experimental subjects
Adult male C57BL/6J mice (Jackson Laboratory) were acclimatized to the animal facility for more than two weeks before undergoing surgery and maintained on a 12 h:12 h light:dark cycle. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the National Institute on Drug Abuse animal care and use committee.

Surgeries
Microinjection needles (29-gauge) were connected to a 2-µl Hamilton syringe and filled with purified, concentrated adeno-associated virus (~10^{12} infectious units ml^{-1}) encoding EYFP, ChR2-EYFP or NpHR-EFYP under control of the αCaMKII promoter. Mice were anesthetized with 150mg kg^{-1} ketamine and 50 mg kg^{-1} xylazine and placed in a stereotaxic frame. Microinjection needles were bilaterally placed into the vHipp, basolateral amygdala, prefrontal cortex or NAc shell and 0.5 µl virus was injected over five minutes. The needles were left in place for an additional five minutes to allow for diffusion of virus particles away from injection site. Mice used for in vivo optogenetic experiments had 200 µm core optical fibers, threaded through 1.25 mm wide zirconia ferrules, implanted directly above the NAc shell (+1.4 AP, ±1.5 ML, -3.7 DV at an 11° angle). Optical fibers were secured in place using skull screws and acrylic cement. Wounds of mice destined for confocal imaging or slice electrophysiology were sealed with cyanoacrylate tissue glue.

EYFP Confocal Images
Mice were anesthetized with Euthasol 6-12 weeks after surgery and perfused with ice-cold PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde, and sectioned in 100 µm coronal slices on a VT-1200 vibratome (Leica). Sections were mounted using Mowiol with DAPI. Slides were scanned on a confocal microscope (Olympus) with a 10X objective,
isolating a single Z-plane. To enable comparisons, all images quantified and presented in Figures 1B, S3B, and S3C were captured and processed using identical settings.

**Fluoro-Gold Experiments**
Glass capillary pipettes were pulled to a tip diameter of 30-40 µm and filled with 1% Fluoro-Gold (Fluorochrome) in 100 mM sodium cacodylate, pH 7.5. This micropipette was unilaterally placed in the medial NAc shell of anesthetized mice in a stereotaxic frame. A current of 2 µA was applied in 5-second pulses over 20 minutes. The micropipette was left in place for an additional five minutes to prevent flow of tracer back through the needle track. Seven days after surgery mice were anesthetized and perfused, as described above. Immunohistochemistry and imaging details are available in supplemental information.

**Drug Treatment Prior to Electrophysiology**
Starting four weeks after surgery, mice in this group either remained in their home cage or were placed in an activity box (38 cm by 30 cm) for 40 minutes each day over five consecutive days. At the same time each day, or ten minutes after entering this chamber, mice received intraperitoneal injections of either cocaine (15 mg/kg) or saline (0.9% NaCl). They were prepared for electrophysiological recordings 10-14 days later.

**Electrophysiology**
Six to eight weeks after surgery, mice were anesthetized with Euthasol and perfused with ice-cold, artificial cerebrospinal fluid (ACSF). A detailed description of the solutions, equipment, and recording procedures can be found online in supplemental information. In brief, EYFP expression was examined in slices containing the virus injection sites to assess placement accuracy. If the targeted region was adequately infected with virus, 200 µm thick coronal sections containing the NAc shell were transferred to the recording chamber and superfused with the 32°C ASCF. Medium spiny neurons were voltage clamped at -80 mV, unless otherwise noted. A 200 µm core optical fiber coupled to a diode-pumped solid state laser and positioned above the slice was aimed at the recorded cell. Optically-evoked EPSCs were obtained every 20 s with paired pulses of 473 nm wavelength light (30 mW, 3 ms) using 50 and 100 ms inter-pulse intervals.

All measurements of quantal amplitude were obtained in mice that had either received saline or cocaine injections 10 to 14 days earlier. In brain slice recordings, transmitter release was desynchronized by substituting calcium with strontium (4 mM) in the superfused ACSF. Asynchronous EPSCs were examined during a 200 ms window beginning 5 ms following optical stimulation. Recordings were analyzed if the frequency of events in this 200 ms window were significantly greater than during the 200 ms window preceding the stimulation. To eliminate the slow exponential decay associated with residual synchronous release, all traces from each cell were averaged and then fit with a single exponential that was subsequently subtracted from each individual trace. In the recordings in which AMPA-NMDA receptor response ratios were determined, the internal solution contained 3 mM QX-314 and cells were held at +40 mV. AMPAR-mediated currents were isolated with the selective NMDAR antagonist AP5. The NMDAR-mediated current was then digitally obtained by taking the difference current before and after AP5 application.

**In Vivo Optogenetic Experiments**
Mice were used for these behavioral experiments starting no less than six weeks after surgery. Optical tethers consisted of a diode-pumped solid state laser (473 nm, 150 mW or 532 nm, 200 mW for ChR2 or NpHR experiments, respectively; OEM Laser Systems) coupled to 62.5 um core, 0.22 NA standard multimode hard cladding optical fiber (Thor Labs) that passed through a single-channel optical rotary
joint (Doric Lenses) prior to being split 50:50 with a fused optical coupler (Precision Fiber Products) (Britt et al., 2012). The intensity of light output was about 15 mW per split fiber for all experiments, except for the NAc shell self-stimulation experiments, where the light intensity was 2 mW. Mice were connected to these optical tethers just before starting each behavioral session.

For cocaine-induced locomotion experiments, mice were either temporarily placed in Med-Associates home cages (20 cm by 25 cm) each day or they resided there for at least two days prior to the start of the experimental sessions. Ten minutes after entering the chamber or, for the home cage group, after being attached to the optical tethers, mice were given intraperitoneal injections of either cocaine (10 mg/kg) or saline (0.9% NaCl). During the 20 minutes immediately following this injection, laser light was pulsed at 4 Hz (5 ms pulse duration) or constantly on for the ChR2 and NpHR experiments, respectively. For open field chamber experiments, the same stimulus settings were used in mice that were placed in activity boxes (40 cm by 40 cm) for 10 minutes. For place preference experiments, modified Med-Associates three-room chambers were used that had the interior walls removed. Mice were left in these chambers for 15 minutes over five consecutive days. On days 2-4, laser light was pulsed at 6 Hz (5 ms pulse duration) whenever mice were physically located in the laser-paired side of the chamber. For self-stimulation experiments, mice were placed in standard Med-Associates operant chambers equipped with active and inactive nose poke operands. Each active nose poke performed by the animal resulted in 30 5-ms pulses of light delivered at 20 Hz, unless otherwise noted. The chamber lights went out and an audible tone was played during the delivery of light. Nose pokes made within 3 seconds of an active nose poke did not activate the laser. Active and inactive nose poke timestamp data were recorded using MED-PC software and analyzed using Microsoft Excel. For all experiments mice were videotaped. Behavior was evaluated in real time and coupled to lasers with Ethovision software.

Data Analysis and Statistics
All data are reported as mean ± SEM. Data was analyzed in Clampex, MiniAnalysis, Ethovision, Excel and Prism. Two-tailed t tests, ANOVAs, and Pearson’s correlation were used for statistical comparisons. Unless otherwise noted, ANOVA post hoc tests were two-tailed t tests using a Bonferroni correction factor for multiple comparisons; * indicates p ≤ 0.05 and was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes additional experimental procedures as well as six figures and can be found with this article online.

ACKNOWLEDGEMENTS

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. vHipp Input to the NAc Is Uniquely Concentrated in the Medial NAc Shell**
(A) Representative coronal brain slices showing expression of EYFP (green) after virus injection into the vHipp, basolateral amygdala, or prefrontal cortex. See also Figure S1. The left panel shows images from the sites of virus injection. The right panel shows images of EYFP-expressing afferents to the NAc. To enable comparisons, the NAc images were captured and processed using identical settings. The red lines indicate where patch recordings would be obtained from, as described later in the text.  (B) The average fluorescent signal in each region of the NAc, relative to the brightest signal, shows vHipp input is uniquely concentrated in the medial NAc shell (n = 6, 4, and 6 for the vHipp, amygdala, and prefrontal cortex pathways, respectively; one-way ANOVA for vHipp input, F(2,15) = 13.0, P < 0.01; post hoc tests of medial shell versus core and lateral shell, P < 0.01 for both; one-way ANOVA for other two pathways, P > 0.05). Images are counterstained with the nuclear dye DAPI (blue). All values are mean ± SEM and * indicates p < 0.05. Abbreviations: ac = anterior commissure, AcbC = nucleus accumbens core, AcbSh = nucleus accumbens shell, Amyg = amygdala, vHipp = ventral hippocampus, PFC = prefrontal cortex. Scale bars are 1 mm.

**Figure 2. More Neurons Project to the Medial NAc Shell from the vHipp than from the Basolateral Amygdala or Prefrontal Cortex**
(A) A representative coronal brain slice showing injection site of the retrograde tracer Fluoro-Gold (red) in the medial NAc shell. (B) Representative coronal brain slices showing immunolabeled-Fluoro-Gold (red) in NAc shell-projecting cells in the vHipp, basolateral amygdala and prefrontal cortex. (C) In slices from each region that contained dense populations of NAc-projecting cells, more medial NAc shell-projecting neurons were found the vHipp than in either the basolateral amygdala or prefrontal cortex (n = 3 for each area; one-way analysis of variance (ANOVA), F(2,6) = 29.1, P < 0.01; post hoc tests of vHipp versus amygdala and prefrontal cortex, P < 0.01 for both). (D) Greater immunolabeled-Fluoro-Gold fluorescence was measured throughout the extent of the vHipp than throughout the basolateral amygdala or prefrontal cortex, indicating a larger population of medial NAc-shell projecting neurons in that region (n = 3 for each area; one-way ANOVA, F(2,6) = 29.1, P < 0.01; Fisher’s least significant difference post hoc tests of vHipp versus amygdala and prefrontal cortex, P < 0.05 for both). See also Figure S2. Scale bars are 1 mm.

Figure 3. Pathway-Specific EPSCs Are Largest Following Optical Stimulation of vHipp Input to the NAc Shell
(A) Representative optically-evoked EPSCs recorded in medium spiny neurons from the NAc shell. (B) EPSCs elicited from vHipp fibers are larger than those evoked from amygdala or prefrontal cortex inputs (n = 53, 40 and 50 for the vHipp, amygdala and prefrontal cortex, respectively; one-way ANOVA, F(2,140) = 21.4, P < 0.01; post hoc tests of vHipp versus amygdala and prefrontal cortex, P < 0.01 for both). See also Figure S3. (C) Paired pulse ratios (P2/P1) obtained with 50 ms inter-pulse intervals show that amygdala input exhibits paired pulse facilitation, suggesting a lower presynaptic vesicle release probability in this pathway (n = 52, 39 and 37 for the vHipp, amygdala and prefrontal cortex, respectively; one-way ANOVA, F(2,125) = 9.6, P < 0.01; post hoc tests of amygdala versus vHipp and prefrontal cortex, P < 0.01 for both). (D) Representative asynchronous EPSCs (asEPSCs) obtained from the optical stimulation of selective afferents to the medial NAc shell. Traces are clipped during the initial release event to highlight subsequent asEPSCs. (E) Averaged asEPSCs from representative cells (left). Summary of asEPSC amplitudes in each pathway (right; n = 7, 5, 6 for vHipp, amygdala, prefrontal cortex inputs, respectively; one-way ANOVA, F(2,15) = 1.98, P > 0.05).

Figure 4. NMDARs at vHipp to NAc Synapses Pass Proportionally More Inward Current
(A) Optically-evoked, AMPAR-mediated currents recorded at several holding potentials (+40, +25, +10, -5, -25, -45, -65 mV; top). Summary of normalized current-voltage relationships in pathway-specific AMPAR populations (bottom; n = 10, 5, 8 for vHipp, amygdala, prefrontal cortex inputs, respectively; repeated measures ANOVA, pathway effect, F(2,120) = 0.59, P > 0.05). (B) Optically-evoked, NMDAR-mediated currents recorded at several holding potentials (+35, +15, -5, -25, -45, -65, -85 mV; top). Summary of normalized current-voltage relationships in pathway-specific NMDAR populations shows vHipp to NAc synapses pass proportionally more peak inward current than other synapses (bottom; n = 6, 6, 4 for vHipp, amygdala, prefrontal cortex inputs, respectively; repeated measures ANOVA, pathway effect, F(2,78) = 13.08, P < 0.001; post hoc test of pathway effect between vHipp and both other inputs at -25, -45 and -65 mV, P < 0.05).

Figure 5. vHipp Afferents to the NAc Shell Are Selectively Potentiated Following Cocaine Injections
(A) Experimental timeline showing brain slice recordings were obtained 10 to 14 days after 5 daily injections of either cocaine or saline. (B) The summary of asynchronous EPSC amplitudes obtained in the medial NAc shell show selective cocaine-induced increases in the quantal amplitude of vHipp input (for the saline and cocaine groups, respectively, n = 7 and 7 for the vHipp, n = 5 and 5 for the amygdala, n = 6 and 5 for the prefrontal cortex; two-way ANOVA, cocaine main effect, F(1,29) = 5.4, P < 0.05). (C) Representative AMPA and NMDA receptor-mediated currents recorded at +40 mV in the medial NAc
shell from the optical stimulation of different inputs in saline- and cocaine-treated mice. (D) Summary of AMPA/NMDA receptor response ratios show vHipp input is selectively potentiated following repeated cocaine injections (for the saline and cocaine groups, respectively, \( n = 10 \) and \( n = 9 \) for the vHipp, \( n = 13 \) and \( n = 12 \) for the amygdala, \( n = 8 \) and \( n = 7 \) for the prefrontal cortex; two-way ANOVA, significant interaction, \( F_{(2,53)} = 4.6, P < 0.05 \); post hoc test of cocaine effect on vHipp input, \( p < 0.01 \)). (E) Summary of normalized current-voltage relationships in vHipp to NAc shell synaptic AMPAR populations in naïve and cocaine-treated mice (\( n = 10 \) and \( n = 6 \) for naïve and cocaine-treated mice, respectively). See also Figure S4.

**Figure 6. Activity of vHipp Axons in the NAc Drives Cocaine-Induced Locomotion**

(A) In an unfamiliar environment, optical inhibition of vHipp axons in the NAc reduces cocaine-induced locomotion (\( n = 6 \) for both groups; repeated measures ANOVA, NpHR main effect, \( F_{(1,30)} = 70.6, P < 0.01 \); post hoc test of group effects on days 2-9, \( p < 0.05 \)). This effect strengthens over time (days 1-5) and dissipates in the absence of optical inhibition (days 8-10). During the first session, labeled day 8, mice were only given saline injections. (B) Optical inhibition does not alter locomotor activity of cocaine-naïve mice, measured daily in an open field chamber (\( n = 4 \) for both groups; repeated measures ANOVA, NpHR effect, \( F_{(1,30)} = 0.6, P > 0.05 \)). (C) In animals’ home cages, optical activation of vHipp axons in the NAc enhances cocaine-induced locomotion (\( n = 6 \) and \( n = 7 \) for control and ChR2 groups, respectively; repeated measures ANOVA, ChR2 main effect, \( F_{(1,77)} = 24.9, P < 0.01 \)). This effect does not persist in the absence of optical activation (\( t_{11} = 0.9, p > 0.05 \)). (D) Optical stimulation does not alter locomotor activity of cocaine-naïve mice, measured daily in an open field chamber (\( n = 4 \) for both groups; repeated measures ANOVA, ChR2 effect, \( F_{(1,30)} = 0.6, P > 0.05 \)). See also Figure S5.

**Figure 7. Photostimulation of vHipp Axons in the NAc Can Reinforce Instrumental Behaviors**

(A) Summary of time spent in different sides of a modified place preference chamber over five consecutive days in which mice had complete freedom of movement (\( n = 6 \); repeated measures ANOVA, significant interaction, \( F_{(4,40)} = 24.6, P < 0.01 \); post hoc test of room effect on day one, \( p < 0.01 \)). During the three test sessions, vHipp axons in the NAc were optically activated whenever mice entered and remained in the ChR2-paired side of the chamber. (B) Summary of instantaneous room exit probabilities each day in the place preference chamber (\( n = 6 \); repeated measures ANOVA, significant interaction, \( F_{(4,40)} = 51.7, P < 0.05 \); post hoc test of room effect on days two and three, \( p < 0.01 \)). See also Figure S6. (C) Cumulative-activity graph of nose pokes made in the first behavioral session to obtain optical stimulation of vHipp axons in the NAc (\( n = 9 \)). Solid lines represent the mean and dashed lines are ± SEM. (D) Summary of active and inactive nose poking behavior in ChR2 and EYFP control mice made during the first behavioral session (\( n = 9 \) and \( n = 6 \) for ChR2 and EYFP groups, respectively).

**Figure 8. Excitatory Input from Different Sources and Direct Stimulation of Medium Spiny Neurons Can Each Reinforce Instrumental Behaviors**

(A) Irrespective of the specific pathway activated, mice spend more time on the side of a chamber that is paired with ChR2-mediated activation of glutamatergic axons in the NAc shell (\( n = 6, 5 \) and \( n = 6 \) for the vHipp, amygdala, prefrontal cortex input, respectively; repeated measures ANOVA, significant effect of session, \( F_{(4,56)} = 19.8, P < 0.01 \)). (B) Summary of active nose pokes made in the third behavioral session to obtain pathway-specific optical activation of ChR2-expressing axons in the NAc (\( n = 6, 5 \), and \( n = 6 \) for vHipp, amygdala, prefrontal cortex pathways, respectively). (C) Representative coronal brain slice showing tracts of implanted optical fibers and expression of ChR2-EYFP (green) in the NAc shell after local virus injection. Image is counterstained with the nuclear dye DAPI (blue). (D) Cumulative-activity graph of nose pokes made in the first behavioral session to obtain optical stimulation of medium spiny neurons in the NAc shell (\( n = 5 \)).
FIGURE 1

A

vHipp

Amyg

PFC

B

medial
lateral
NAc core

NAc shell

Relative fluorescence

Relative fluorescence

Relative fluorescence

* *

*
A

Treatment: cocaine or saline

Day: 1 2 3 4 5

10-14 days → record

B

asEPSC amplitude (% of saline)

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* indicates statistical significance

C

Saline

Cocaine

vHipp

Amyg

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D

AMPA/NMDA

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* indicates statistical significance

E

Relative current vs. $V_m$ (mV)

-60 -40 -20 20 40

-1.0 -0.5 0.5 1.0

Naive

Cocaine

FIGURE 5
**FIGURE 6**

(A) Graph showing distance traveled (m) against day, with data points for light on and light off conditions. The graph includes error bars indicating variability.

(B) Bar chart showing comparison of distance traveled (m) between EYFP and NpHR groups across days 1 to 5. The chart includes error bars.

(C) Graph showing distance traveled (m) against day, with data points for light on and light off conditions. The graph includes error bars indicating variability.

(D) Bar chart showing comparison of distance traveled (m) between EYFP and ChR2 groups across days 1 to 5. The chart includes error bars.
FIGURE 7

A

B

C

D

*
**FIGURE 8**

A) Graph showing the fold change in time spent on the laser-paired side across different sessions.

B) Bar graph illustrating active nose pokes/hr for different brain regions: vHipp, Amyg, PFC.

C) Image of a brain with labeled regions.

D) Line graph depicting the number of nose pokes over time for active and inactive conditions.
Inventory of Supplemental Information

Supplemental Information includes additional experimental procedures as well as six figures that directly support main figures presented in the paper.

1) Supplemental Experimental Procedures. This section details the Fluoro-Gold immunohistochemistry procedures as well as the composition of solutions used in electrophysiological experiments.

2) Figure S1, related to Figure 1. This figure presents additional images that complement those in Figure 1, providing readers the ability to judge inter-animal variability.

3) Figure S2, related to Figure 2. This figure shows the correlation between fluorescence signals and manual cell counts, thus justifying the relevance of the data presented in Figure 2D.

4) Figure S3, related to Figure 3. This figure contains additional evidence supporting the claim of Figure 3 that vHipp input to the medial NAc shell is predominant.

5) Figure S4, related to Figure 5. This figure shows that effect presented in Figure 5 is not dependent on the location where cocaine was administered.

6) Figure S5, related to Figure 6. This figure presents representative images of brains from mice that were used in the experiments shown in Figure 6. It also shows that the manipulations did not alter anxiety-related behaviors.

7) Figure S6, related to Figure 7. This figure presents evidence that further supports and describes the claims made in Figure 7. For example, the data shown in panel A justifies the relevance of the data presented in Figure 7B.
Supplemental Information includes additional experimental procedures as well as six figures that directly support main figures presented in the paper.

**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

**Fluoro-Gold Experiments**
Immediately following paraformaldehyde perfusions, brains were removed, post-fixed overnight in 4% paraformaldehyde and sectioned in 100 µm coronal slices on a VT-1200 vibratome (Leica, Nussloch, Germany). Sections were permeabilized for 15 minutes in PBS with 0.2% Triton-X 100 (PBST), washed three times, five minutes each, in PBS, blocked 1 hour in 3% normal goat serum in PBS, washed again three times, five minutes each, in PBS and incubated overnight in rabbit anti-Fluoro-Gold (AB153; Millipore; Billerica, MA) diluted 1 to 10,000 in PBST with 1% normal goat serum. The next day, sections were washed five times, five minutes each, in PBS, incubated 1 hour in Cy3-conjugated anti-rabbit (Jackson Immunolabs) diluted 1 to 500 in PBS, washed three times, five minutes each, in PBS and mounted on glass slides. Following brief air drying, sections were mounted using Vectashield with DAPI (Vector Labs).

Slides were scanned on a confocal microscope (Olympus) with a 10X objective, isolating a single Z-plane. One section was chosen in each brain region from each mouse that represented the maximal labeling for each brain region. Cells in the ipsilateral hemisphere were manually counted in each of these sections by identifying the number of discrete cell bodies visible with signal clearly above background. Background for each brain region was determined by examining tissue from a control mouse that did not receive an injection of Fluoro-Gold, but was prepared and immunostained in parallel with the other mice. Total fluorescence was calculated in each brain slice in the specific areas of the prefrontal cortex, basolateral amygdala, and ventral hippocampus where NAc-projecting cells were located.

**Electrophysiology**
Brains were sectioned around both the injection site and the NAc in 200 µm thick coronal sections on a VT-1200 vibratome (Leica). The artificial cerebrospinal fluid (ACSF) used to both perfuse the animals and slice the brains was modified to contain (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 26 NaHCO₃, 11 glucose and 3 sodium ascorbate. Slices were placed in a holding chamber filled with 32°C ASCF saturated with 95% O₂ and 5% CO₂ containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2.4 CaCl₂, 26 NaHCO₃, 11 glucose and 1 sodium ascorbate. The ACSF used in the recording chamber was the same except for the exclusion of sodium ascorbate and, to block inhibitory transmission, the addition of picrotoxin (100 µM). Electrodes (3-5 MΩ) were backfilled with an internal solution containing (in mM): 117 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 4 Mg-ATP and 0.4 Na-GTP (280-285 mOsm). Cells were visualized on an upright microscope using infrared differential interference contrast video microscopy. Whole-cell voltage-clamp recordings were made using a MultiClamp 700B amplifier (2 kHz low-pass Bessel filter and 10 kHz digitization) with pClamp 10.3 software (Molecular Devices). Medium spiny neurons in the medial NAc shell were identified by morphology, membrane resistance, and hyperpolarized resting membrane potential. Series resistance (10-25 MΩ) was monitored with a 5 mV hyperpolarizing pulse (50 ms) given every 20 s, and only recordings that remained stable over the period of data collection were used. The current-voltage relationship of excitatory currents was obtained with optical stimulation using an internal solution that contained 3 mM QX-314. AMPAR current-voltage relationships were examined with 50 uM AP5 in the superfused ACSF and 100 uM spermine in the internal solution. NMDAR current-voltage relationships were collected with 10 uM CNQX in the superfused ACSF. All drugs were obtained from either Sigma or Tocris Bioscience.
Figure S1, related to Figure 1. vHipp Input to the NAc is Uniquely Concentrated in the Medial Shell
Representative coronal brain slices showing expression of EYFP (green) after virus injection into the vHipp, basolateral amygdala, or prefrontal cortex. The top row shows images from the sites of virus injection. The bottom rows show images of EYFP-expressing afferents to the NAc. Notice the relatively selective innervation of the medial NAc shell from the vHipp axons.
Immunolabeled-Fluoro-Gold fluorescence significantly correlated with manual cell counts of Fluoro-Gold labeled cells \( (n = 10; R^2 = 0.86, P < 0.01) \). The x-axis represents manual counts of the number of Fluoro-Gold-positive cell bodies within different brain regions (vHipp, amygdala, prefrontal cortex) following Fluoro-Gold injections into the NAc shell. The y-axis represents total fluorescence signal in each brain region. Total fluorescence was calculated within regions of interest (ROI) by multiplying mean fluorescence by the surface area of the ROI. The ROI for each slice was defined by the subregion of tissue containing Fluoro-gold positive cell bodies, and was manually traced. Mean fluorescence for each slice was determined in Adobe Photoshop by measuring the average pixel intensity in the red channel, with background subtracted – defined by average red channel pixel intensity of a region of grey matter without anti-Fluoro-Gold fluorescence.
Figure S3, related to Figure 3. **vHipp Input to the Medial NAc Shell is Uniquely Effective in Driving Postsynaptic Spiking**

(A) Representative current- and voltage-clamp recordings from medium spiny neurons in the medial NAc shell during optical stimulation (20 Hz) of ChR2-positive axons coming from the vHipp (top) and basolateral amygdala (bottom). Physiological recording solutions were used.  
(B) The intensity of the fluorescent signal at the different injection sites, relatively to the brightest average signal, show viral injections and ChR2 expression is similar between injection sites ($n = 5, 4,$ and $5$ for the vHipp, amygdala, and prefrontal cortex injections, respectively; one-way ANOVA, $F_{(2,11)} = 0.5, P > 0.05$).  
(C) The average fluorescent intensity of each region, calculated across brain slices throughout areas where NAc-shell projection neurons were located, suggests a relatively larger proportion of NAc-projecting neurons were virally-infected in the prefrontal cortex ($n = 5, 4,$ and
5 for the vHipp, basolateral amygdala, and prefrontal cortex injections, respectively; one-way ANOVA, $F_{(2,11)} = 10.7$, $P < 0.01$; post hoc test of prefrontal cortex versus vHipp and amygdala, $p < 0.05$). 

(D) Summary of optically-evoked EPSC amplitudes measured at different light intensities in different afferent pathways to NAc shell medium spiny neurons ($n = 7, 6$ and $7$ for the vHipp, amygdala and prefrontal cortex inputs, respectively; repeated measures ANOVA, significant interaction, $F_{(16,136)} = 7.9$, $P < 0.01$). 

(E) Normalized input-output curves from the data presented in (D) suggest each input pathway has comparable amounts of functional ChR2 protein (repeated measures ANOVA, pathway effect, $F_{(2,136)} = 0.1$, $P > 0.05$). 

(F) Paired pulse ratios ($P_2/P_1$) show that only amygdala afferents exhibit paired pulse facilitation, suggesting a lower presynaptic vesicle release probability in this pathway ($n = 52, 39$ and $37$ for both of the vHipp, amygdala and prefrontal cortex measurements, respectively; two-way ANOVA, location main effect, $F_{(2,230)} = 10.8$, $P < 0.05$).

Figure S4, related to Figure 5. vHipp Afferents to the NAc Shell are Potentiated Following Cocaine Injections

Summary of AMPA/NMDA receptor response ratios show vHipp input is selectively potentiated following repeated cocaine injections, regardless of where the cocaine was administered ($n = 10, 9$, and $9$ for the saline, novel cocaine, and home cocaine, respectively; one-way ANOVA, $F_{(2,25)} = 5.5$, $P < 0.05$; post hoc test of cocaine versus saline treatments, $p < 0.01$).
Figure S5, related to Figure 6. Activity of vHipp Axons in the NAc Does Not Alter Anxiety-Related Behavior

(A) Representative coronal brain slices showing tracts of implanted optical fibers and expression of NpHR-EYFP (green; top) or ChR2-EYFP (bottom) in the NAc after virus injection into the vHipp. Images are counterstained with the nuclear dye DAPI (blue).

(B) Summary of the proportion of time mice spent exploring the center of an open field chamber, a measure of anxiety-related behavior (n = 4 for each group; EYFP vs NpHR, t₆ = 0.2, P > 0.05; EYFP vs ChR2, t₆ = 0.2, P > 0.05).
Figure S6, related to Figure 7. Optical Activation of vHipp Axons in the NAc Induces a Place Preference by Reducing the Probability Mice Will Exit from the ChR2-Paired Side of the Chamber

(A) Plotting the distributions of visit durations for each side of the chamber on pre- and probe-test days shows an exponential distribution similar to that observed in classical conditioned place preference experiments. On the probe test day versus the pre-test day, mice made fewer quick visits to the ChR2-paired side (n = 6; repeated measures ANOVA on ChR2-paired side, significant interaction, F(8,45) = 2.7, P < 0.05; post hoc test for visit durations lasting either 3 or 6 seconds or less, p < 0.05).

(B) Summary of locomotor behavior in a place preference chamber where one side is paired with the optical stimulation of vhipp axons in the NAc shell.

(C) Summary of time spent in different sides of a real-time place preference chamber over five consecutive days (n = 12; repeated measures ANOVA, P < 0.05). During the three test sessions, vHipp axons in the NAc were optically inhibited whenever mice entered and remained in the NpHR-paired side of the chamber.

(D) Average number of active nose pokes made by mice to obtain optical activation of vHipp axons in the NAc in sessions spanning 70 days.
Britt et al. characterizes hippocampal, amygdala, and prefrontal cortex input to the nucleus accumbens. Hippocampal input is shown to be predominant in the medial accumbens shell, yet photostimulation of axons from each pathway is sufficient to reinforce instrumental behaviors.
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