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NMDA Receptor-Dependent Cholinergic Modulation of Mesolimbic Dopamine Cell Bodies: Neurochemical and Behavioral Studies

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Abstract

Substance abuse disorders are devastating, costly, and difficult to treat. Identifying the neurochemical mechanisms underlying reinforcement promises to provide critical information in the development of effective treatments. Several lines of evidence suggest that striatal dopamine (DA) release serves as a teaching signal in reinforcement learning, and that shifts in DA release from the primary reward to reward-predicting stimuli play a critical role in the self-administration of both natural and non-natural rewards. However, far less is known about the reinforcing effects of motivationally neutral sensory stimuli, or how these signals can facilitate self-administration behavior. Thus, we trained rats ($n=7$) to perform a visual stimulus-induced instrumental task, which involved lever pressing for activation of a stimulus light. We then microinfused vehicle (phosphate buffered saline), carbachol (acetylcholine receptor agonist), or carbachol in the presence of an N-methyl-D-aspartate (NMDA) receptor-specific drug (NMDA itself, or the antagonist, AP5) into the ventral tegmental area (VTA). This enabled us to directly evaluate how chemical modulation of dopamine cell bodies affects the instrumental behavior, as well as the nature of extracellular dopamine transients recorded in the nucleus accumbens shell (NAc shell) using fast-scan cyclic voltammetry (FSCV). Intra-VTA infusion of carbachol enhanced the magnitude and frequency of dopamine transients in the NAc shell and potentiated active lever responding without altering inactive lever responding, as compared to infusion of vehicle. Co-infusion of carbachol with AP5 abolished dopamine transients recorded in the NAc and attenuated active lever responding without altering inactive lever responding. Finally, co-administration of carbachol and NMDA into the VTA restored both lever pressing and dopaminergic signals recorded in the striatum. Together, these results suggest that acetylcholine and glutamate synergistically act at dopamine cells in the VTA to modulate VTA-NAc shell dopaminergic output, and this underlies motivation to lever press for a motivationally neutral visual stimulus.

Keywords

Glutamate; Acetylcholine; Dopamine; Sensory stimuli; Ventral Tegmental Area

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INTRODUCTION

Sensory stimuli play a critical role in the initiation of food seeking and consumption^{1, 2}, as well as drug use. One example is menthol, a substance that can produce a cool feeling, and is likely to promote cigarette smoking, even for first time cigarette smokers that have not previously experienced the reinforcing effects of nicotine^{3–5}. Similarly, alcoholic beverages are generally flavored prior to consumption, as the flavor contributes to the development of alcohol preference and consumption patterns in both humans and animals⁶. Thus, the role of neutral sensory stimuli in promoting drug use may be partly mediated by the reinforcing effects of the sensory stimulus itself. Multiple studies have reported that sensory stimuli can function as primary reinforcers. For instance, animal studies have shown that the primary reinforcing effects of non-conditioned neutral sensory stimuli (e.g. a stimulus light) play an important role in nicotine^{7–12} and methamphetamine¹³ self-administration experiments. However, the neural mechanisms underlying this phenomenon have not been fully investigated.

Behaviorally salient stimuli are thought to initiate burst firing of dopamine (DA) neurons in the ventral tegmental area (VTA) as a key step in reward processing^{14–16}. These DA neurons are regulated by many inputs that contain a variety of chemical modulators ranging from small molecules to larger neuropeptides. For instance, a large excitatory input originates from two glutamatergic/cholinergic groups of mesopontine tegmental area neurons: the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus^{17–19}. Cholinergic and glutamatergic regulation of the VTA is well established. Local application of the N-methyl-D-aspartate (NMDA) antagonist, AP5, into the rat VTA has been shown to robustly attenuate phasic DA release in the naïve rat NAc^{20, 21}, to increase the latency to lever press for electrical self-stimulation of the SN/VTA complex²⁰, and to attenuate cue-induced cocaine seeking²¹ and reward-related learning²². Similarly, the selective genetic inactivation of NMDA receptors in mouse VTA DA neurons attenuates both DA neuron burst firing and subsequent DA release and reward-related learning²³. However, intra-VTA microinfusion of NMDA itself does not reliably initiate an increase in spontaneous DA transients in the NAc shell²⁰ (though it has been shown to increase the magnitude of electrically-evoked DA release in the core²⁴), and when DA neurons are recorded from slices in which afferent input has been severed, these neurons cannot be made to phasically fire in response to glutamate agonist administration alone^{25, 26}. Thus, it is likely that several mechanisms contribute to the generation of DA transients in the ventral striatum. Electrophysiological studies have suggested that acetylcholine plays a role in driving phasic firing in presumed VTA DA neurons^{27–30}. This is likely critical for the reinforcing properties of intra-VTA self-administered carbachol³¹. Acetylcholine receptors in the VTA have been shown to play a key role in eliciting electrically-evoked DA release in the NAc²⁴ and to modulate the reinforcing properties of ethanol-associated cues³². Furthermore, muscarinic (but not nicotinic) acetylcholine receptors in the VTA underlie conditioned reinforcement for a cue previously paired with food reward³³. Collectively, these studies have begun to elucidate the role of glutamatergic and cholinergic modulation of the VTA in conditioned reinforcement; however, far less is known about how these signals regulate the reinforcing effects of motivationally neutral sensory stimuli. Given that DA

transients in the NAc are critically involved in motivation and reinforcement^{34–37}, that DA release in the NAc is both necessary and sufficient for cue-mediated reward-seeking behavior³⁸, and that even neutral sensory stimuli can facilitate drug self-administration³⁹, it is imperative to evaluate how cholinergic and glutamatergic modulation of neuronal firing in the VTA underlies the intrinsic reinforcing effects of salient sensory stimuli that have not been previously paired with reward.

In this work, we utilized fast-scan cyclic voltammetry (FSCV), which can provide selective measurements with high spatial and temporal resolution, to monitor rapidly fluctuating DA concentrations in the NAc. We monitored the effects of intra-VTA microinfusion of carbachol (non-selective cholinergic receptor agonist) by itself, or in combination with AP5 (NMDA glutamatergic receptor antagonist) or NMDA (glutamatergic receptor agonist) on the production of DA transients in the shell subregion of the NAc in freely moving animals. Additionally, we evaluated the effects of these pharmacological manipulations on a visual-stimulus-reinforced instrumental behavior task, which was designed to examine the reinforcing effects of neutral sensory stimuli. Overall, this study sheds light on the synergistic role of cholinergic and glutamatergic signaling in modulation of VTA-NAc shell dopaminergic output, and its correlation with motivation to press for a visual stimulus.

RESULTS AND DISCUSSION

Cholinergic and glutamatergic mechanisms in the VTA modulate DA transients in the shell subregion of the NAc

Over past two decades, FSCV has developed into a powerful tool for studying the role of DA transients in freely moving animals. These rapid DA fluctuations are thought to be the behaviorally relevant mode of DA neurotransmission, as they can become time locked to cues that predict reward availability^{15, 40–44}, and DA release intensifies with increasing proximity to reward^{45–47}. Studies have demonstrated DA's role in motivation and reward-based learning, as well as behavioral conditioning^{48–52}. For instance, phasic stimulation of VTA DA neurons is sufficient to drive behavioral conditioning to environmental context and to elicit dopamine transients in the NAc⁴⁹. However, it remains unclear if there is a causal link between DA signaling and the salience of an environmental cue.

Thus, this study first examined the VTA mechanisms that are involved in modulation of spontaneous DA transients in the shell subregion of the NAc. Figure 1A shows a representative color plot containing 150 background-subtracted voltammograms that demonstrate DA release. Color plots allow for the discrimination of specific substances in complex brain environments, as they depict the current collected at each potential⁵³. This color plot shows DA release evoked by electrical stimulation of the VTA (asterisk), as well as a naturally occurring DA transient (pound sign). Signature cyclic voltammograms extracted from this color plot (Figure 1 C, D) serve as a means to identify DA at both time points. The current collected at the DA oxidation potential (0.6V – horizontal dashed line) was converted to concentration using a calibration factor, and was plotted in Figure 1B.

To examine the role that cholinergic and NMDA receptors in the VTA play in modulation of DA transients in the NAc shell, a microinfusion of saline, carbachol, a carbachol/AP5

cocktail, or a carbachol/NMDA cocktail was delivered to the posterior VTA. Immediately thereafter, the effects on DA transients recorded in the NAc (frequency and amplitude) were monitored. Figure 2A shows a representative color plot collected 2–4 minutes after microinfusion of saline (left) or carbachol (right). The DA transients are marked by white asterisks. The concentration versus time traces are shown in Figure 2B. A subsequent microinfusion of the carbachol/AP5 cocktail inhibited DA transients (Figure 2C,D **left**). Finally, microinfusion of a cocktail of carbachol/NMDA reinstated a robust DA signal (Figure 2C,D, **right**). This unequivocally demonstrates that the recording site still retained the ability to support DA signaling, and that the lack of signal upon administration of AP5 was not due to a broken sensor or electrode fouling.

A summary of the electrochemical data is presented in Figure 3. Figure 3A demonstrates the effects of intra-VTA pharmacological manipulations on the frequency of DA transients recorded in the NAc, as a percent of baseline transient frequency, ($[F(3, 156) = 63.90]$, $p < 0.0001$, $n = 6$). Tukey's post-hoc comparisons indicate that carbachol microinfusion significantly increased the frequency of DA transients compared to saline, and addition of AP5 abolished the effects of carbachol. In sharp contrast, intra-VTA microinfusion of the carbachol/NMDA cocktail elicited robust DA transients in the NAc. Tukey's multiple comparison test indicated $p < 0.0001$ for all 6 comparisons. Figure 3B demonstrates a significant effect of these manipulations on the amplitude of DA transients recorded in the ventral striatum, ($[F(3, 33) = 10.39]$, $p < 0.0001$, $n = 6$). Tukey's post-hoc comparisons show that administration of carbachol/NMDA elicited a robust increase in the amplitude of the DA response as compared to saline ($p < 0.001$), carbachol ($p < 0.05$), or carbachol/AP5 ($p < 0.0001$). Histological examination of the placement of the working electrode showed that all recordings were made in the NAc shell (Figure 4).

The effects of carbachol reported herein appear to be mediated by stimulation of acetylcholine receptor signaling in the VTA, but the exact acetylcholine receptor subpopulations have yet to be identified. Carbachol is a non-selective cholinergic receptor agonist that can stimulate both muscarinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR). Stimulation of mAChRs in the VTA increases naturally-occurring DA release in the NAc^{54–56}, and stimulation of nAChRs in the VTA persistently activates VTA dopaminergic neurons⁵⁷. While studies have demonstrated that various subtypes of nAChRs and mAChRs are expressed on VTA dopamine neurons^{58–61}, the $\alpha 4$ and $\beta 2$ -containing nAChRs are also expressed on GABAergic interneurons⁶². Furthermore, the $\alpha 7$ nAChRs are most densely localized on pre-synaptic glutamatergic, but not cholinergic, terminals in the VTA^{63, 64}. Thus, we are not able to ascribe the behavioral and neurochemical effects of carbachol described herein to the stimulation of a specific acetylcholine receptor subpopulation, or cell type, within the VTA. Future studies will be necessary to examine the contribution of specific acetylcholine receptor subpopulations in the VTA to modulation of mesolimbic DA neurotransmission.

Cholinergic and glutamatergic mechanisms in the VTA affect motivation to lever press for a motivationally neutral visual stimulus

Previous studies have demonstrated visual stimulus induced operant responding in rodents⁶⁵. However, few studies have examined the underlying neurobiological mechanisms. Numerous studies have demonstrated a critical role for the limbic dopaminergic system in the motivational and activational effects of other primary reinforcers, including food and drugs of abuse^{66, 67}. Thus, we hypothesized that the limbic dopaminergic system may also contribute to the reinforcing effects of a sensory reinforcer (e.g., visual stimulus). Hence, we established a rat model of a visual stimulus-reinforced instrumental behavior and introduced the pharmacological manipulations described above to examine the corresponding behavioral effects.

In these behavioral experiments, animals chose between a counter-balanced active and inactive lever. A press on the active lever resulted in illumination of the stimulus light above that lever for 1 sec under an FR 1 schedule with a 1-s timeout period. Responding on the inactive lever did not result in a programmed consequence. In contrast to a previous study that reported on a visual stimulus induced operant task⁶⁵, our FR1 schedule of visual stimulus reinforcement produced a robust responding. Notably, our behavioral paradigm was somewhat different (lever pressing v.s. nose poking; 1s light onset v.s. 5s light onset; 1s time out v.s. no time out). At the start of behavioral training, active and inactive lever responding gradually declined in all VTA-cannulated ($n = 7$) rats (all time main effects, $F(9, 54)=4.83-5.00$, $p=0.0001$; Figure 5A). However, rats exhibited stable responding on the active lever during the last three training sessions with a within-subject variability of $<20\%$ in active lever responding (session main and interaction effects, $F(2, 12)=1.11-1.31$, $p=0.30-0.36$). Furthermore, all rats exhibited preference on active lever responding during the last three training sessions (lever main effect, $F(1, 6)=15.40$, $p=0.008$). The mean number of active lever responses was 97 ± 13 , and the mean number of inactive lever responses was 44 ± 9 . Cue light illumination across all training days was relatively stable.

During testing, saline, carbachol, carbachol/AP5 or carbachol/NMDA were microinfused into the posterior VTA and their effects on active and inactive lever responding were evaluated (Figure 5B). Bilateral cannula placement was verified in all rats (Figure 4). Intra-VTA carbachol infusions potentiated active lever responding ($F(3, 18)=27.34$, $p<0.0001$), as compared to vehicle (Tukey test, $p<0.05$). In contrast, intra-VTA infusions of carbachol/AP5 attenuated active lever responding, as compared to vehicle (Tukey test, $p<0.05$). Furthermore, intra-VTA infusions of carbachol/NMDA enhanced active lever responding (Tukey test, $p<0.05$), to a similar level as compared to carbachol. Importantly, intra-VTA infusions of carbachol, carbachol/AP5, or carbachol/NMDA failed to alter the inactive lever responding. The effects of these pharmacological manipulations on visual stimulus-reinforced behavioral responding correspond with their effects on dopaminergic transients in the striatum (Figure 3). Hence, the results provide strong justification to further examine a causal link between the occurrence of dopaminergic events in the striatum and operant responding to visual stimuli. Such experiments will require recording dopaminergic events in the striatum of behaving animals.

Overall, our study evaluated the contribution of cholinergic and glutamatergic signaling in the VTA on the generation of DA transients in the NAc shell, and on visual stimulus-reinforced instrumental behavior. However, DA neuronal activity in the VTA is regulated by numerous afferents^{68–70}, and VTA dopamine output encodes various aspects of reward^{15, 40–44, 66}. Thus, we cannot conclude that the dopamine transients in the NAc carry the reinforcing information of a sensory stimulus *per se*. Future studies will investigate this question in behaving animals during the performance of a visual stimulus reinforced instrumental task using FSCV combined with selective activation of neuronal subtypes.

While our studies have revealed a clear functional interaction between acetylcholine receptors and NMDA receptors in the VTA, the neuronal mechanisms underlying this remain unclear. It is possible that downstream signaling mechanisms inherent to acetylcholine receptor stimulation require NMDA receptor function. Data demonstrating that application of carbachol increases the NMDA receptor-mediated response in neostriatal projection neurons support this hypothesis⁷¹. A functional interaction could also be mediated by mAChRs, which have been shown to facilitate the induction of NMDA receptor-dependent LTP in the hippocampus^{72–74}. Indeed, *in vitro* stimulation of M1 mAChRs potentiates NMDA receptor activation in the hippocampus^{75, 76}. Consistent with this putative mechanism, the present study has demonstrated that the neurochemical and behavioral effects of carbachol are dependent on stimulation of the NMDA receptor. On the other hand, carbachol may also indirectly potentiate NMDA receptor activation by way of nAChRs. For instance, stimulation of presynaptic $\alpha 7$ nAChRs on glutamatergic terminals in the VTA results in glutamate release, which potentiates excitatory inputs to VTA DA neurons, activating NMDA receptors and inducing long-term potentiation (LTP)^{77–79}. The data presented in this work are consistent with this mechanism, in that co-administration of carbachol and NMDA potentiated the frequency and amplitude of DA transients in the NAc (as compared with administration of carbachol alone). Future studies will be important to examine the contributions of specific VTA acetylcholine receptor subtypes in modulation of DA transients in the NAc.

Overall, our findings lend critical insight to the chemical mechanisms that underlie the generation of DA transients in the NAc, and provide putative neuropharmacological mechanisms for the reinforcing effects of a motivationally neutral sensory stimulus. The data unequivocally demonstrate that stimulation of acetylcholine receptors in the VTA increases DA signaling in the NAc and promotes a visual stimulus-reinforced instrumental behavior, and that both of these outcomes are dependent on the stimulation of NMDA receptors in the VTA. However the sources of glutamate and acetylcholine input have yet to be identified. Anatomical studies indicate that the VTA receives excitatory glutamatergic inputs from the prefrontal cortex (PFC), bed nucleus of the stria terminalis (BNST), amygdala, and tegmentum⁸⁰. Unlike the diverse sources of glutamatergic inputs, the VTA receives primary acetylcholine inputs from the tegmentum, which includes the lateral dorsal tegmental nucleus (LDTg) and pedunculopontine tegmental nucleus (PPTg) subregions^{81–86}. Adding to this circuitry, the LDTg also receives significant excitatory input from the medial prefrontal cortex (PFC)⁸⁷. Importantly, cholinergic and glutamatergic afferents projecting from the LDTg to DA cell bodies in the VTA can initiate DA neuron burst firing⁸⁸. Furthermore, intra-VTA administration of muscarinic or nicotinic receptor antagonists

attenuates NAc DA efflux evoked by electrical stimulation of the LTDg⁸⁹. Therefore, the LTDg/PPTg – VTA subcircuitry is well positioned to process the reinforcement information related to various sensory stimuli, as well as other reward-related stimuli in general. Given the diverse and complex nature of this circuitry, future studies using optogenetic or pharmacogenetic manipulation will be important to dissect the role of specific afferents in reward and reinforcement. Such a line of research will advance our knowledge of the neuronal mechanisms that underlie motivated behavior, and will help identify effective therapeutic targets for neurobiological disorders such as drug addiction.

CONCLUSIONS

Our findings demonstrate that intra-VTA infusion of carbachol enhanced the magnitude and frequency of DA transients in the NAc shell. These effects were abolished by co-administration of carbachol and AP5, but restored by administration of carbachol with NMDA. Importantly, intra-VTA infusion of carbachol potentiated active lever responding for a motivationally neutral visual stimulus (as compared to vehicle), without altering inactive lever responding (an index for general motor activity). Furthermore, intra-VTA infusion of carbachol with AP5, but not NMDA, attenuated active lever responding without altering inactive lever responding (as compared to vehicle) during the behavioral test. Taken together, these results suggest that cholinergic and glutamatergic signaling play a synergistic role in modulation of VTA-NAc shell dopaminergic output, and this correlates with the intrinsic reinforcing effects of a salient visual stimulus, even when that stimulus has not been previously paired with reward.

METHODS

Animals

Male Sprague-Dawley rats (300–400 g) were purchased from Charles River (Wilmington, MA USA) and were individually housed in a temperature/humidity-controlled facility with a 12-h light/dark cycle and food and water provided *ad libitum*. Animal care was approved by the Institutional Animal Care and Use Committee of North Carolina State University and was in accordance with NIH guidelines.

Surgery

Rats were given a minimum of 2 days habituation to the facility post-transport before surgery. The surgical procedure was performed as described previously⁵³, using flat skull stereotaxic coordinates obtained from a brain atlas⁹⁰. Briefly, rats were anesthetized with ketamine/xylazine (80–100mg/kg and 2–12mg/kg; i.p., respectively) and placed in a stereotaxic frame (Kopf Instrumentation; Tujunga, CA, USA). Deltaphase isothermal pads (Braintree Scientific, Braintree, MA, USA) were used to maintain body temperature. Bupivacaine was injected just under the skin and the skull was exposed to reveal bregma and lambda in order to level and drill the holes for electrode placement. For the electrochemical experiments, a combination bipolar stimulating electrode/infusion cannula (Plastics One, Roanoke, VA, USA) was placed above the posterior VTA (–5.9 AP, +0.8 ML, –8.8–9.0 DV). A guide cannula (Bioanalytical Systems, West Lafayette, IN, USA) for recording electrodes

was positioned 2.5 mm deep into the brain over the shell subregion of the NAc (+1.2 AP, +0.8 ML). Reference electrodes were Ag/AgCl, and placed superficially in the contralateral cortex. For behavioral experiments, stainless steel guide cannulae (26 gauge, Plastics One) were bilaterally aimed at the VTA (−5.9 AP, +1.7 ML, −6.6 DV, relative to bregma) with a 10 degree angle.

Microelectrode Fabrication

Carbon-fiber microelectrodes were fabricated in house as described previously⁵³. Briefly, a single 7-μm diameter fiber (Cytec Industries, West Patterson, NJ) was aspirated into a borosilicate glass capillary (1.0 mm x 0.5 mm, A-M Systems, Carlsburg, WA). Using a micropipette puller (Narishige, Tokyo, Japan), the glass was tapered to form sealed microelectrodes. The extending carbon fiber was then cut to ~100 μm beyond the glass seal. An electrical connection between the carbon fiber and a 26-gauge lead wire was established using a small amount of conductive silver paint (GC Electronics, Rockford, IL). Finally, the electrode was loaded into a custom micromanipulator (University of North Carolina, Department of Physics Machine Shop) and slowly lowered into the ventral striatum.

Electrochemical Data Acquisition

The waveform was generated and the current was collected using a multifunction data acquisition board (PCI-6052E, National Instruments, Austin, TX, USA). A PCI-6711E board (National Instruments) was used to synchronize waveform acquisition, data collection, and delivery of electrical stimulation. Electrochemical data were digitized and stored using HDCV Acquisition Software (University of North Carolina, Department of Chemistry Electronics Facility). Signal processing (background subtraction, signal averaging, and digital filtering; 4-pole Bessel filter, 2.5 kHz) was also accomplished with this software.

The electrodes were connected to a head-mounted amplifier (UNC Electronics Shop) attached to a commutator (Crist Instrument Company, Hagerstown, MD, USA). Electrodes were conditioned at 60 Hz for 15 min with a triangular waveform (−0.4 V to 1.3 V vs Ag/AgCl, 400 V/s), followed by 15 min of cycling at 10 Hz. The microelectrode position was optimized by monitoring naturally occurring and electrically evoked (biphasic, 2 ms/phase, 24 pulses, 60 Hz, 125 μA) DA release. Electrical stimulation was computer generated and passed through an optical isolator and constant-current generator (Digitimer Ltd, Hertfordshire, England). Stimulated dopamine release was evoked at the end of each session to ensure neuronal viability, and electrodes were calibrated *in vitro*. Drugs (Sigma Aldrich, St. Louis, MO, USA) were unilaterally administered with a syringe pump (0.5 μL for 60 s, Kent Scientific Corporation, Torrington, CT, USA) via an infusion cannulae (33 gauge) inserted into the implanted guide.

Experiments consisted of 10 min of baseline collection, 20 min of recording over a period that encompassed microinfusion of saline or drug, and an electrical stimulation that evoked dopamine release (n = 6). The first microinfusion into the VTA consisted of saline (0.9%), and the process was repeated ~20 minutes later with microinfusion of a drug (n = 5). Subsequent microinfusions were counterbalanced across rats and they occurred at a minimum of 1-hour intervals. These were carbachol (acetylcholine receptor agonist, 0.5

nmol/0.5 μ L, dissolved in sterile saline), a cocktail of carbachol (0.5 nmol) and ($\pm$)2-amino, 5-phosphopentanoic acid (AP5; 5 nmol dissolved in 0.5 μ L of sterile saline), or a cocktail of carbachol (0.5 nmol) and NMDA (0.15 nmol) dissolved in 0.5 μ L of sterile saline. The doses of these pharmacological agents were chosen based on previous studies on the role of cholinergic and glutamatergic neurotransmission on reinforcement^{20, 31}. Responses were expressed as a ratio of post- to pre-microinfusion measurements.

Visual Stimulus-Reinforced Instrumental Behavior

Visual stimuli-reinforced instrumental behavior training was conducted during daily 1-h sessions on a minimum of ten consecutive days during the rats' light cycle in a 43 $\times$ 43 $\times$ 53 cm Plexiglas chamber housed in a sound-attenuated cubicle (Med Associates, St. Albans, VT, USA). One side of the chamber had two retractable levers (Coulbourn Instruments, Allentown, PA, USA) 17 cm apart, with a stimulus light located 6 cm above each lever. A house light (100 mA), which was mounted 18 cm above the floor on the opposite wall, was illuminated continuously during each session.

Rats ($n = 7$) were trained to press a lever (counterbalanced across animals) to illuminate the stimulus light above the lever for 1 s under an FR 1 schedule with a 1-s timeout period. Responses on this active lever during the 1-s timeout period did not result in a programmed consequence. During the sessions, responses on a second, inactive lever had no programmed consequence but were recorded. Daily behavioral training sessions were continued until a rat reached the stability criterion (i.e., less than or equal to 20% variability in active lever responding across two consecutive sessions on a minimum of ten training days). Rats were adapted to the intracranial infusion procedure on training day 5. To this end, injection cannulae were inserted bilaterally into the rat's guide cannulae to a depth 2 mm below the tip of the guide cannulae. The injectors were left in place for 4 min, but no fluid was infused.

Once rats reached the stability criterion, they received four test sessions for visual stimuli-reinforced instrumental behavior, as described above. Five minutes prior to each test session, rats received bilateral microinfusion of vehicle, carbachol, carbachol/NMDA, or carbachol/AP5 into the VTA at the doses described above. Infusions were administered at a rate of 0.25 μ L/min, and injectors were left in the cannulae 1 min before and after the infusions. The order of testing was counterbalanced based on mean active lever responding during the last three behavioral training days. Between test sessions, rats received additional training sessions until they re-obtained the stability criterion (described above). During the period of experiment, rats did not any receive food restriction.

Histology

Animals were anesthetized with urethane (2.0 mg/kg). Electrochemical recording locations were marked via an electrical lesion. Animals were transcardially perfused with saline followed by 10% formalin post drop fix. Brains were removed intact, refrigerated, and coronally sectioned at 40 μ m on a vibratome. Sections were mounted onto gelatin-coated slides and stained using cresyl violet (Kodak, Rochester, NY, USA). Placements were then determined using light microscopy. The most ventral portion of each cannula track was mapped onto schematics of appropriate plates from the rat brain atlas⁹⁰.

Statistical and Data Analysis

Substances were resolved with principal component regression using MATLAB (The MathWorks, Natick, MA, USA) ⁹¹. Dopamine concentration transients were defined as events with a signal-to noise ratio greater than five, and were characterized with an in house MATLAB program. Frequency and amplitude were analyzed using a one-way ANOVA with Tukey's *post hoc* test. Behavioral data were analyzed using mixed-factorial or repeated measures analyses of variance (ANOVAs) and Tukey *post-hoc* tests, when appropriate. Statistical significance was designated at $p < 0.05$, and error bars are $\pm$ SEM. All statistical analyses were performed using GraphPad Prism 5 Software Version 5.04 for Windows (Graphpad Software, La Jolla, CA, USA).

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Figure 1.

Background-subtracted voltammograms were collected every 100 msec for quantitative analysis of DA dynamics. (A) A representative color plot, containing 150 background-subtracted voltammograms that demonstrate DA release. *Asterisk* represents release evoked by electrical stimulation, *pound* represents a naturally-occurring DA transient. (B) DA concentration trace and (C, D) cyclic voltammograms extracted from the raw data presented in (A).

Figure 2.

Intra-VTA microinfusion of carbachol, a carbachol/AP5 cocktail, or a carbachol/NMDA cocktail modulates the frequency and amplitude of DA transients in the NAc. (A) A representative color plot collected ~3 min after microinfusion of saline (*left*) or carbachol (*right*). (B) Concentration versus time traces extracted from the data. (C) A representative color plot collected ~3 min after microinfusion of a carbachol/AP5 cocktail (*left*) or a carbachol/NMDA cocktail (*right*). (D) Concentration versus time traces extracted from the data. Asterisks represent the DA transients.

Figure 3.

A summary of the electrochemical data. Effects of intra-VTA pharmacological manipulations on the frequency (**A**) and amplitude (**B**) of DA transients recorded in the NAc, as a percent of baseline. * p<0.05, ***p<0.001, and ****p<0.0001.

Figure 4.

Schematics illustrating the placement of stimulating electrodes, working electrodes, and microinjection cannulae. All electrochemical recordings were made in the NAc shell. The most ventral point of the infusion cannula tracks was located within the ventral tegmental area (VTA). Numbers are relative to bregma in mm. The *black circles* represent placement of stimulating electrodes in the VTA (Experiment 1, $n = 5-6$). The *black diamonds* represent placement of working electrodes in the NAc shell (Experiment 1, $n = 5-6$). The *filled triangles* represent bilateral cannula placement for intra-VTA microinfusion (Experiment 2, $n = 7$). Rats with cannula placements outside of the targeted region were excluded from the data analysis.

Figure 5.

Effects of intra-VTA microinfusion of saline, carbachol, a carbachol/AP5 cocktail, or a carbachol/NMDA cocktail on a visual stimulus-induced instrumental behavior. **(A)** During the behavioral training, a press on the active lever resulted in illumination of the stimulus light above that lever for 1 sec under an FR 1 schedule with a 1-s timeout period. Responding on the inactive lever did not result in a programmed consequence. **(B)** During testing, drugs were microinfused into the posterior VTA and the effects on active and inactive lever responding were evaluated. *Asterisks* represent significant effects as compared with VEH control. *Plus* represents significant effects as compared with carbachol. * $p < 0.05$