Supplemental Methods

Subjects

Male, Sprague Dawley rats weighing between 280-320 g ($n = 46$, Charles River Laboratories, Wilmington, MA) were group housed and handled prior to training. Rats were maintained on a food-deprived schedule whereby they received 10-12 g of their maintenance diet daily in order to maintain approximately 85% free-feeding body weight. Rats had free access to tap water in the home cage and were fed approximately 3-4 h after each day's training session. All procedures were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the University of California Los Angeles Institutional Animal Care and Use Committee.

Behavioral Training

Apparatus

Training and testing took place in a single Med Associates (East Fairfield, VT) operant chamber housed within a sound- and light-resistant shell. The chamber contained 2 retractable levers that could be inserted to the left and right of a food cup located on the floor at the base of the front wall. A 3-watt, 24-volt house light mounted on the top of the back wall opposite the food cup provided illumination. The chamber was equipped with a pellet dispenser that delivered a single 45 mg sucrose pellet (Bioserv, Frenchtown, NJ) into the food cup.
Action Sequence Training

Each session started with illumination of the house light and insertion of the levers where appropriate and ended with the retraction of the levers and turning off of the house light. Rats received only one training session per day.

Magazine training: All rats received 2 days of magazine training in which they were exposed to non-contingent sucrose pellet deliveries (20 outcomes over 30 min) in the operant chamber with the levers retracted, in order to learn where to collect the sucrose reinforcement.

Single action instrumental training: All rats were then given 2 days of single action training on the lever to the right of the magazine with the sucrose delivered on a continuous reinforcement schedule. Each session lasted until 20 outcomes had been earned, or 30 min elapsed. After this training, the 0-day training group underwent surgery for guide cannula and stimulator implantation. The 5- and 10-day training groups continued with action sequence training as described below.

Action sequence training: Initially, only the distal lever was present (i.e., the lever to the left of the magazine). One response on this lever resulted in the presentation of the right, proximal lever, a response on which resulted in delivery of a single sucrose pellet and retraction of the proximal lever. Both the initial and terminal links of the sequence were continuously reinforced. Sessions continued until 20 outcomes were earned, or 30 min elapsed. Rats received either 5 (5-day group) or 10 sessions (10-day group) of action sequence training, after which they underwent surgery for guide cannula and stimulator implantation.

Surgery

Standard aseptic stereotaxic procedures (see (1, 2)) were used for implantation of a unilateral guide cannula, a reference electrode and a bipolar stimulating electrode. Rats were anesthetized with isoflurane (5% induction, 1-2% maintenance) and implanted with a guide cannula (Bioanalytical Systems, West Lafayette, IN) cut to protrude 2.5 mm into the brain aimed
above the nucleus accumbens core (coordinates from Bregma and the skull surface: AP: +1.3 mm; ML: +1.3 mm; V: -2.5 mm), a bipolar stimulating electrode (Plastics One, Roanoke, VA) into the medial forebrain bundle (AP: -5.2 mm, ML: +1.0 mm, V: -8.2 mm) and an Ag/AgCl reference electrode (0.5 mm silver wire chlorinated in 0.1 M HCl then rinsed with saline immediately prior to implantation) into the contralateral cortex. Following surgery rats were individually housed and allowed to recover for 4 days. After recovery, rats were placed into the operant chamber, tethered to a commutator and retrained for one session on either the proximal action (0-day group) or full sequence of actions (5- and 10-day groups) for sucrose pellets.

**Fast-Scan Cyclic Voltammetry Test**

*Voltammetric Dopamine Measurement at Test*

Prior to test, carbon fiber microelectrodes (6 µm diameter, 75-100 µm length) were prepared as described previously (3). At test, the electrodes were inserted into custom-made micromanipulators (University of Illinois Machine Shop, Chicago, IL) that fit and locked into the implanted guide cannulae. Rats were tethered to the voltammetric recording unit through an electrical swivel (Crist Instrument Co, Hagerstown, MD) and the electrodes were lowered into the ventral striatum (V: -5.7 mm). A custom-made voltammetric potentiostat was used to apply a triangular waveform to the carbon fiber microelectrode through a head-mounted voltammetric amplifier (see previous description (4)). The applied potential was held at -0.4 V (vs. the Ag/AgCl reference) and then ramped to +1.3 V and back to -0.4 V at 400 V/s, repeating every 100 ms for a sample rate of 10 Hz. Application of this waveform results in redox reactions in electroactive brain chemicals at the carbon fiber microelectrode, which can be recorded as current changes. Dopamine is oxidized to dopamine-o-quinone at approximately +0.64 V on this waveform, which is then reduced back to dopamine at -0.2 V. Background-subtraction elucidates these oxidative and reductive peaks providing the dopamine cyclic voltammogram signature for dopamine detection, described previously (3, 4). Waveform generation and
resultant data acquisition were carried out using two PCI multi-function data acquisition cards and custom software written in LabVIEW (National Instruments, Austin, TX).

After stabilization of the baseline current (approx. 20 min), the electrode position was optimized (down to a maximum of V: -7.6 mm) for a location at which dopamine transients occurred spontaneously (~1/min) and were reliably elicited by a range of electrical stimulations (biphasic pulse, 2 ms/phase, 90-200 µA, 12-24 pulses, 30-60 Hz) and unsignaled sucrose pellet deliveries (3 pellets, separated by at least 2 min). The behavioral testing commenced approximately 15-30 min after the stimulation protocol. Voltammetric measurements were made continuously throughout the duration of the behavioral test. Rats in the 0-day training group (n = 11) were first allowed to respond on the single proximal lever for a total of 5 sucrose pellet rewards. As in training the proximal lever was continuously reinforced with the presentation of sucrose pellets. After this initial session, the 0-day group was given their first session of training on the full two-action sequence, using the procedure described above, which ended after 30 sucrose pellets had been earned. Rats in the 5- (n = 11) and 10-day (n = 8) training groups were trained only on the full sequence of actions for a total of 30 sucrose pellet rewards. This session lasted on average 76.9 min (SEM = 12.6), 18.07 min (SEM = 5.32), and 17.09 min (SEM = 4.15) for the 0-, 5- and 10-day action sequence training groups, respectively.

After the test, rats were given another medial forebrain bundle stimulation to elicit dopamine release in the ventral striatum (24 pulses, 60 Hz, 125 µA) in order to confirm that electrode sensitivity had not degraded during the experiment. The electrode was then removed from the brain and calibrated in a custom-made flow-cell system, described previously (5). Dopamine standards of 0.25, 0.5 and 1 µM in phosphate buffered saline (pH = 7.4) were passed by the electrode at a rate of 4 ml/min to determine the calibration factor for each electrode. The average calibration factor was 19.13 nM/nA (SEM = 1.67).
**Voltammetric Analysis**

Electrochemical data were analyzed using software written in LabVIEW (National Instruments). Representative initial output, including color plots with corresponding current v. time traces taken at the dopamine oxidative potential and background-subtracted cyclic voltammograms taken from the peak of the current change, are shown in Figure 3. Chemometric analysis (6, 7) was used to isolate changes in current due to dopamine from the cyclic voltammetric data collected during the 5 s before and after each lever press response (both distal and proximal actions). Each comparative training set was rat-specific and included a minimum of 5 stimulated dopamine and pH cyclic voltammograms from the specific electrode and recording location used for the test. During this analysis all dopamine current data were converted to dopamine concentration via the *in vitro* post-test calibration factor.

**Histological Verification of Recording Sites**

At the conclusion of each experiment rats were anesthetized with Nembutal (100 mg/kg, intraperitoneal [i.p.]) and trans-cardially perfused with 0.9% saline followed by 10% formalin saline. The brains were removed and post-fixed in 30% sucrose-formalin, then cryosectioned into 60 μm slices, mounted onto slides and stained with cresyl violet. Light microscopy was used to examine electrode placement. Histological data are presented in Figure 1.

**Flupenthixol Test**

In a separate experiment, rats (*n* = 16) were food deprived and trained on the two-action sequence task using the procedures described above. After 0, 5 and 10 days of behavioral training, rats were injected i.p. with the D1/D2 antagonist flupenthixol (0.5 mg/ml/kg, Sigma Aldrich, St. Louis, MO) or 0.9% sterile saline vehicle (1 ml/kg) 60 min prior to performance of the sequence of actions. This dose and injection interval was selected to ensure high bioavailability of the drug and is known to have significant suppressive effects on food-motivated performance.
in rats (8). For the 0-day test, rats were assigned to drug groups to balance their initial response rate on the proximal action. For subsequent tests, drug groups were based both on response rate and previous drug exposure. After each on-drug test, rats were given 1 day off from training to ensure the effects of the drug would not affect subsequent performance.

**Statistical Analysis**

During the fast-scan cyclic voltammetry test, we monitored rats’ response rate on both the distal and proximal actions (i.e., the number of times that a lever press was performed divided by the amount of time the session lasted) as well as the amount of time it took to complete each sequence. As the distal lever was continuously available and rats, especially those acquiring the task for the first time, often pressed on it several times (unnecessarily) before transitioning to the proximal lever, the total sequence time was calculated as the time from the initial distal press through the remainder of the sequence up until the next initiating distal press. Additionally, we calculated rats’ efficiency in correctly performing the action sequence task as the ratio of proximal/distal presses. An efficiency ratio of 1 is optimal and would indicate that rats did not perform any unnecessary distal presses. For each behavioral measure, data were analyzed with a 1-way analysis of variance (ANOVA), with number of action sequence training days, either 0, 5 or 10, as the between subjects variable.

Combined electrochemical data from the fast-scan cyclic voltammetry acquisition test of the 0-day training group was presented and analyzed for the first, middle and last 5 trials (Figure 4). Across groups, electrochemical data were averaged across all 30 trials for each rat, then averaged across rats and plotted separately for each group (Figure 5). Dopamine traces for the 5 s before and after each distal (left) and proximal (right) lever press were averaged across sequences and plotted separately for each phase of the session for the acquisition group (Figure 4 A and B) or training condition (Figure 5 A and B). The peak dopamine response to each task element (preceding the distal action, to the lever insertion cue, preceding the proximal
action and to the reward delivery) was used as the primary output measure. Peak dopamine values preceding the distal action were obtained by taking the largest value in the 2 s period prior to the distal press. After a distal lever press the proximal lever was inserted into the operant chamber, which likely served as a cue to press that lever. This lever cue occurred approximately 0.4 s after the distal press. The peak dopamine value to this lever cue was therefore determined as the largest value in the 2 s period after the distal press. After a press on the proximal action, a sucrose pellet reward was delivered and arrived in the magazine approximately 0.95 s after the proximal press. The reward delivery-related dopamine peak was therefore determined as the largest dopamine signal in the 2 s period after the proximal press. Importantly, peak signals were always at least 3 times the root mean square of the noise. As a result of even slight variations in peak times the average change in dopamine peak quantification (Figures 4C and 5C) will be higher than it appears in the traces (Figures 4 A and B and 5 A and B). This peak dopamine measure is particularly useful in evaluating differences in dopamine signaling across conditions, but does however preclude a report of a no signal change.

Peak dopamine data for the 0-day acquisition group were analyzed with a 2-way repeated measures ANOVA with event (pre-distal action, lever insertion cue, pre-proximal action, reward delivery) and session phase (the beginning, middle or end of the session) as within subjects variables. Additional one-way repeated measures ANOVAs were run to determine if there was an effect of event within each session phase condition and separately to determine if there was an effect of session phase on the dopamine peak for each event. Peak dopamine data, separated for each training condition, were analyzed with a 2-way repeated measures ANOVA to compare the effects of the within subjects variable event (pre-distal press, lever cue, pre-proximal event and reward delivery) and the between subjects variable training group (either 0, 5 or 10 days of action sequence training prior to test) on the dopamine signal. Additionally, we analyzed the peak dopamine concentration to both the earned reward and to unexpected
reward deliveries given prior to the lever-pressing session. These data were analyzed with a two-way ANOVA with within subjects variable, expectancy, and between subjects variable, training group.

Peak dopamine concentration prior to the distal action for each of the 30 total events was compared to the amount of time it took to complete each sequence, both averaged for each trial across rats acquiring the sequence task for the first time at test only (0-day group). These data were analyzed with a Pearson correlation. The peak dopamine concentration change preceding the distal action was also averaged across trials for each rat, including rats in all three training groups and compared to the average amount of time it took each rat to complete the action sequence, as well as the rate of lever pressing on the distal and proximal lever. These data were analyzed with a Pearson partial correlation controlling for the effects of training group. In both cases the peak dopamine concentration was taken as the maximal dopamine peak amplitude in the 5 s prior to the distal press in order to account for the peak time moving further back in time in the 10-day training group (see Results).

The effects of flupenthixol dopamine receptor antagonism on action sequence time were analyzed with a 2-way ANOVA with between subjects variables drug treatment (flupenthixol or vehicle) and training group (0, 5 or 10 days of action sequence training prior to test). Where appropriate, Bonferroni post hoc analyses were used to assess the effects of drug within each training group.
Table S1. Press rate and peri-press dopamine peak correlation analysis. The peak dopamine concentration to each task element was correlated against the average distal and proximal response rate and analyzed with a Pearson correlation, controlling for training group.

<table>
<thead>
<tr>
<th>Control Variable: Training Group</th>
<th>Distal Press Rate</th>
<th>Proximal Press Rate</th>
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<tbody>
<tr>
<td>Pre-Distal DA</td>
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<td>Correlation</td>
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<td>Significance (2-tailed)</td>
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<td>df</td>
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<tr>
<td>Lever Cue DA</td>
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<tr>
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DA, dopamine; df, degrees of freedom.
Supplemental References


2. Wassum KM, Ostlund SB, Maidment NT, Balleine BW (2009): Distinct opioid circuits determine the palatability and the desirability of rewarding events. Proc Natl Acad Sci U S A.


