Supplemental Material: Lammel et al.

Supplemental Figure Legends

Figure S1. Retrogradely Labeled Cells Filled with Neurobiotin Express Tyrosine Hydroxylase.

Sample confocal images of individual retrogradely labeled cells (red beads) that were filled with neurobiotin (NB; green) via the recording pipette and immunostained for tyrosine hydroxylase (TH; blue). Note that all of the recorded neurons in Figure 1D-F were filled with NB and were confirmed to be TH-positive indicating that they were dopaminergic.

Figure S2. Chronic (5 Day) Daily Cocaine Administration Does Not Have a Significant Effect on the AMPAR/NMDAR Ratio in Mesocortical and Nigrostriatal DA Cells.

(A) Diagram of chronic saline and cocaine administration protocol. At the same time each day for 5 consecutive days, mice were administered either cocaine (15 mg/kg dissolved in saline, ip) or saline (volume matched for cocaine injections). Recordings were performed 24 hours after the final cocaine or saline injection.

(B) Sample AMPAR- and NMDAR EPSCs (left panels) and magnitude of AMPAR/NMDAR ratios (right panels) in mesocortical DA neurons following the chronic saline or cocaine administration protocol shown in (A).

(C) Sample AMPAR- and NMDAR EPSCs (left panels) and magnitude of AMPAR/NMDAR ratios (right panels) in nigrostriatal DA neurons following the chronic saline or cocaine administration protocol shown in (A). Number of cells are indicated.

Figure S3. Confocal Analysis of DA and Non-DA Mesocortical Cells in the TH-GFP Mouse Line.

Left panels: Sample confocal images showing a retrogradely labeled mesocortical cell (red beads) expressing GFP (green) and stained for tyrosine hydroxylase (TH; blue) cell in a slice prepared from the TH-GFP mouse line. Note that both green cells in this image stain for TH indicating that they are dopaminergic. All retrogradely labeled cells expressing GFP in the TH-GFP mouse line were found to be immunopositive for TH (n=23/23 cells from 2 TH-GFP mice).

Right panels: Sample confocal images of a retrogradely labeled cell projecting to the mPFC which is GFP-negative and TH-immunonegative indicating it is a non-dopaminergic neuron.

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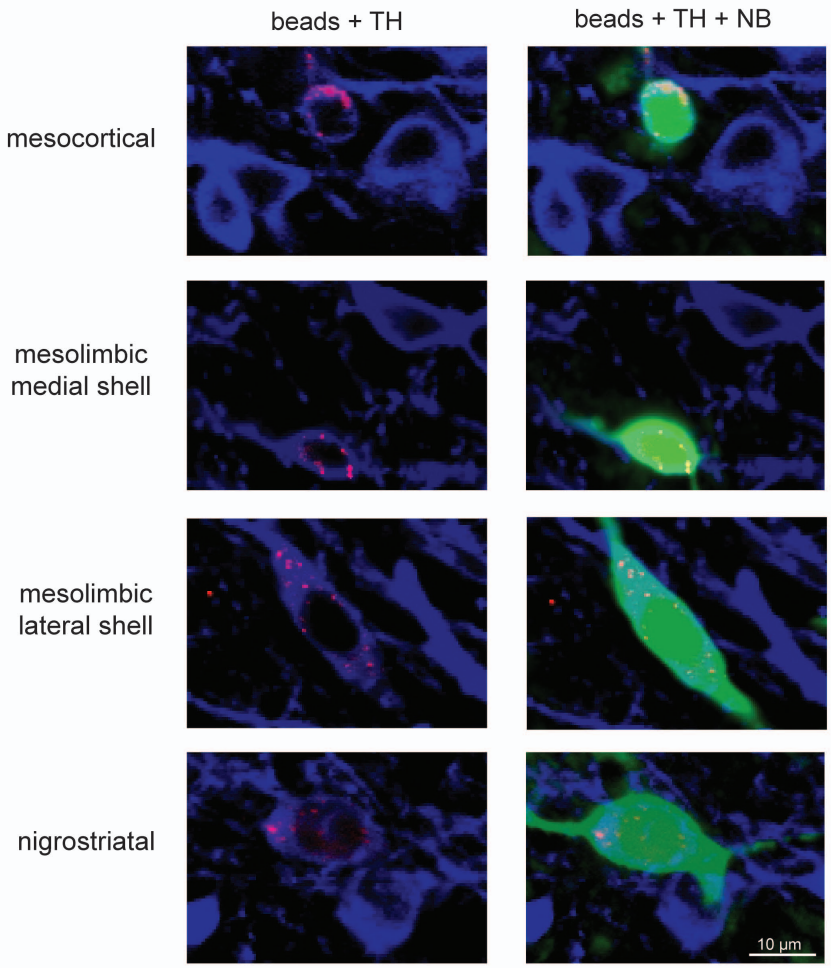


Fig. S2 Lammel et al.

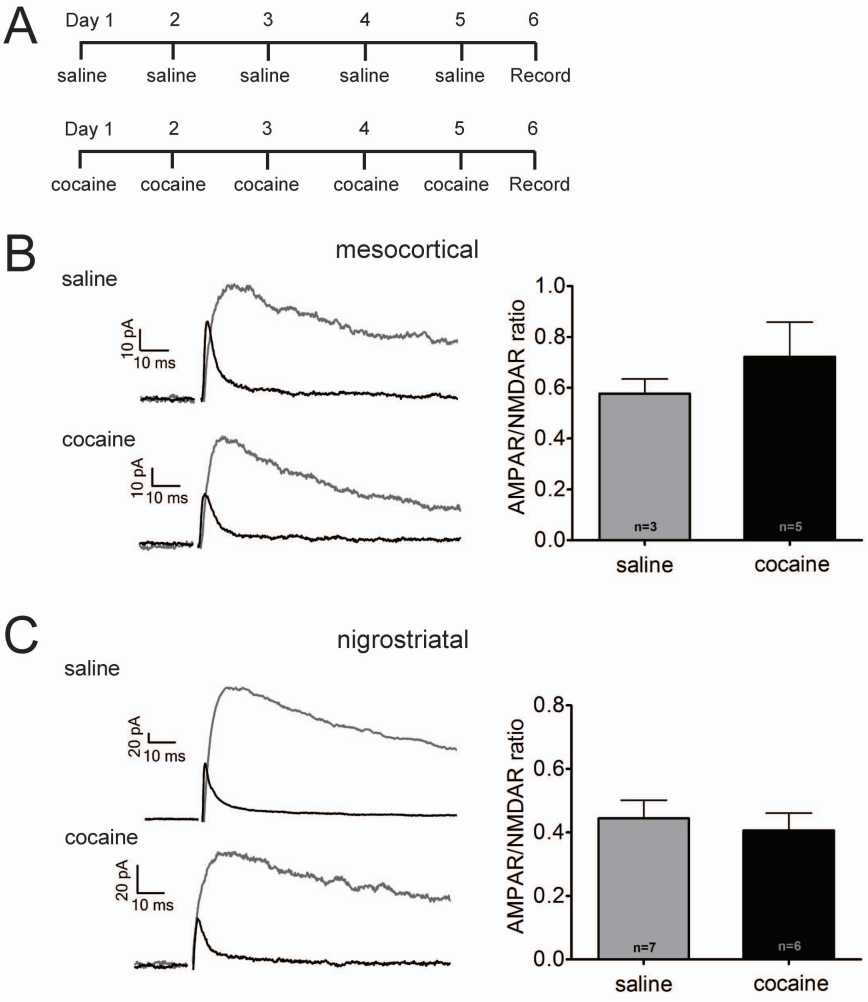
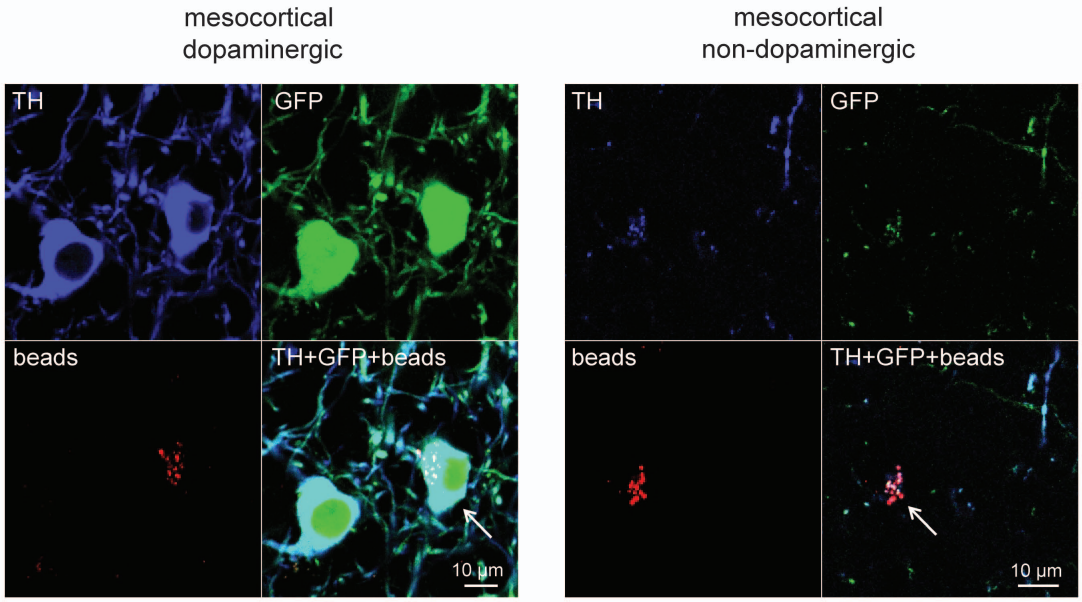


Fig. S3 Lammel et al.



DETAILED EXPERIMENTAL PROCEDURES

Retrograde Labeling of DA Neurons

Male adult (3-month-old) C57Bl6 (Charles River) mice were used for all experiments except for those illustrated in Figures 4E-G and Figure S3 for which TH-GFP mice (Sawamoto et al., 2001) were used. All procedures complied with the animal care standards set forth by the National Institutes of Health and were approved by Stanford University's Administrative Panel on Laboratory Animal Care. As previously described (Lammel et al., 2008), under general ketamine-medetomidine anesthesia and using a stereotaxic instrument (David Kopf), Retrobeads (100 nl; LumaFluor Inc., Naples, FL) were injected unilaterally in dorsolateral striatum (DS) (bregma 0.98 mm; lateral 1.9 mm; ventral 2.2 mm), nucleus accumbens (NAc) lateral shell (bregma 1.45 mm; lateral 1.75 mm; ventral 4.0 mm), NAc medial shell (bregma 1.78 mm; lateral 0.4 mm; ventral 4.1 mm), or medial prefrontal cortex (mPFC) (quadruple injection at four different sites: bregma 1.95 mm, 2.05 mm, 2.15 mm, and 2.25 mm; lateral 0.27 mm; ventral 2.1 mm and 1.6 mm). These coordinates resulted in mPFC injections into the prelimibic and infralimbic cortex; little labeling was normally observed in the cingulate and motor cortices (i.e. in the pipette tract). Importantly, a significant proportion (more than 50%) of the animals in which Retrobeads were injected into mPFC did not yield midbrain slices with cells containing Retrobeads, presumably because the prelimbic and infralimbic cortices were not sufficiently labeled with beads. To allow adequate time for retrograde transport of the Retrobeads into the somas of midbrain DA neurons, survival periods prior to sacrifice depended on the respective injection areas: DS and NAc lateral shell, 7 days; NAc medial shell, 14 days; and mPFC, 21 days. Injections sites were confirmed in all animals by preparing coronal sections (100 μm) of injection sites and staining with green Nissl (NeuroTrace 500/525, Molecular Probes, Eugene, OR) (Figure 1A). We routinely carried out complete serial analyses of the injection sites and animals with Retrobead contaminations outside target areas were discarded (see Lammel et al., 2008 for serial analysis of injection-sites and definition of target areas).

In vivo Manipulations

After allowing sufficient time for the retrograde transport of the Retrobeads and at the same time each day, mice were administered either cocaine (15 mg/kg dissolved in 0.9% saline ip) or 0.9% saline (volume matched for cocaine injections). To provide an aversive stimulus mice received a subcutaneous injection of 5% formalin in saline (50 μl) in the plantar surface of a single hind paw (Ma et al., 1993). Following formalin injection, as previously described (Dubuisson and Dennis, 1977), mice displayed spontaneous pain behavior characterized by increased paw flinching, licking, and intermittent paw

elevation. If not otherwise mentioned, acute midbrain slices were prepared 24 hours after the *in vivo* manipulation.

Electrophysiological Recordings from Adult Mouse Midbrain Slices

Mice were deeply anaesthetized with pentobarbital (200 mg/kg ip; Ovation Pharmaceuticals, Deerfield, IL). Coronal midbrain slices (200 μm) were prepared after intracardial perfusion with ice-cold artificial cerebrospinal fluid (ACSF) containing elevated sucrose (in mM): 50 sucrose, 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 2.5 glucose (oxygenated with 95% O_2/5% CO_2). After 90 min of recovery, slices were transferred to a recording chamber and perfused continuously at 2-4 ml/min with oxygenated ACSF (22.5 mM sucrose) at 30 ºC. Picrotoxin (50 μM, Sigma) was added to block inhibitory currents mediated by $GABA_A$ receptors. Patch pipettes (3.8-4.4 MΩ) were pulled from borosilicate glass (G150TF-4; Warner Instruments) and filled with internal solution containing (in mM): 117 $CsCH_3SO_3$, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA, 4 MgATP, 0.3 NaGTP, 5 QX314, and 0.1 Spermine (pH 7.3, 270-285 mOsm). Labeled DA neurons were visualized by infrared-differential interference contrast (IR-DIC) video microscopy and epifluorescence (Olympus) for detection of retrobeads.

Excitatory postsynaptic currents (EPSCs) were recorded in whole-cell voltage clamp (Axopatch 1D amplifier, Axon Instruments), filtered at 2 KHz, digitized at 10 KHz (ITC-18 interface, HEKA) and collected on-line using custom IgorPro software (Wavemetrics, Lake Oswego, OR, USA). Series resistance and input resistance were monitored on-line with a 4-mV hyperpolarizing step (50 ms) given with each afferent stimulus. A bipolar stimulating electrode was placed 100–300 μm lateral (in the area of the lemniscus medialis) to the recording electrode, and was used to stimulate excitatory afferents at 0.1 Hz. Neurons were voltage-clamped at -70 mV to record AMPAR EPSCs and at +40 mV to record dual component EPSCs containing NMDAR EPSCs that could be pharmacologically isolated. To calculate the AMPAR/NMDAR ratio at +40 mV, an average of 15 consecutive EPSCs at +40 mV was computed before and after application of the NMDAR antagonist D-AP5 (50 μm for 5 min). NMDAR EPSCs were generated by subtracting the average EPSC in the presence of AP5 from that recorded in its absence. The peak of the AMPAR EPSC (2 ms window compared to a 2 ms window on the baseline) was divided by the peak of the NMDAR EPSC to yield an AMPAR/NMDAR ratio (Saal et al., 2003). AMPAR/NMDAR ratios were also calculated by dividing the peak of the AMPAR EPSC at -70 mV by the peak of the NMDAR EPSC at +40 mV (Figure 2B). The decay time constant (tau τ) of the NMDAR EPSC at +40 mV was calculated by fitting a double exponential function to each average EPSC and using the following formula $\tau_W = [(A_1 x \tau_1)+(A_2 x \tau_2)]/(A_1+A_2)$; where A_1 and A_2 are the amplitudes and τ_1 and τ_2 are the decay time constants of the fast and slow components respectively.

To measure the magnitude of Ih and the time-independent inward leak currents, neurons were held at -40 mV, and a voltage step (500 ms) to -120 mV was applied. Leak current was measured as the change in current between the baseline before the voltage step was applied and the current at ~40 ms after the voltage step was applied (Figure 1D). Ih was determined as the change in current between ~40 and 498 ms after the voltage step was applied (Figure 1D). For these experiments, external and internal solutions were as previously described (Lammel et al., 2008) and recordings were performed in voltage-clamp mode using an EPC-10 patch-clamp amplifier (Heka Elektronik, Lambrecht, Germany).

Immunohistochemistry

Immunohistochemistry and confocal microscopy were performed as described previously (Lammel et al., 2008). Briefly, after intracardial perfusion with 4% paraformaldehyde in PBS, pH 7.4, the brains were post-fixed overnight and coronal midbrain slices (50 μm) were prepared. The primary antibody used was rabbit anti-tyrosine hydroxylase (TH) (1:1000; Chemicon, Temecula, CA). The secondary antibodies used were AlexaFluor488 goat anti-rabbit (1:750), Alexa Fluor647 goat anti-rabbit (1:750), AlexaFluor488 streptavidin (1:1000) (all Molecular Probes, Eugene, OR). Image acquisition was performed with a confocal system (Zeiss LSM510) using a 10x/0.30 Plan Neofluar and a 63x/1.40 Oil DIC Plan Apochromat objective. Confocal images were analyzed using the Zeiss LSM Image Browser software.

Statistics

Student's t tests or one-way ANOVA tests were used to determine statistical differences using GraphPad prism 5 (Graphpad Software, San Diego, CA). Bonferroni post hoc analysis was applied, when necessary, to compare means. Statistical significance was set at $p < 0.05$. All data values are presented as means ± SEM.

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