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Subcellular Connectivity Underlies Pathway-Specific Signaling in the Nucleus Accumbens

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Abstract

We found that medium spiny neurons (MSNs) in both the direct and indirect pathways of the mouse Nucleus Accumbens (NAc) receive inputs from the cortex, thalamus and hippocampus. However, hippocampal inputs are much weaker onto indirect MSNs, where they contact small spines located in the distal dendrites. This selective targeting means these inputs must be gated by subthreshold depolarization in order to trigger action potentials and influence NAc output.

Keywords

Nucleus Accumbens; medium spiny neuron; dendrite; spine; synapse; optogenetics; two-photon microscopy; two-photon uncaging

The Nucleus Accumbens (NAc) is important for reward-related behaviors and strongly impacted in drug addiction¹⁻³. Medium spiny neurons (MSNs) are the principal neurons of the NAc and signal via the direct and indirect pathways⁴. Differential activation of these two pathways has powerful and opposing influences on basal ganglia function⁵⁻⁷. In the dorsomedial NAc core, excitatory inputs arrive from the medial prefrontal cortex (mPFC), midline thalamus (MD) and ventral hippocampus (VH)⁸⁻¹¹. Strong mPFC and MD inputs can trigger action potentials⁸, while weaker VH inputs often evoke subthreshold responses¹¹. One untested hypothesis is that these long-range inputs can selectively activate direct and indirect MSNs in the NAc. However, responses to different inputs have primarily been studied *in vivo*, and the underlying mechanisms and pathway dependence remain unexplored.

We assessed the impact of diverse excitatory inputs on the probability of firing direct and indirect MSNs in acute slices of *D1-tdTomato* and *D2-EGFP* mice (Fig. 1a). We first used AAVs expressing mCherry to show that axons from mPFC, MD and VH target the dorsomedial NAc core (Supplementary Fig. 1). We then used wide-field illumination to trigger glutamate release from axons containing channelrhodopsin-2 (ChR2)¹² (Fig. 1b). Sequential recordings from adjacent direct and indirect MSNs minimized any impact of variable ChR2 expression. We found that VH inputs readily triggered direct MSNs ($p_{\text{spike}} =$

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0.69) but could not activate neighboring indirect MSNs ($p_{\text{spike}} = 0.06$, $n = 8$ pairs, $p = 0.004$) (Fig. 1c). In contrast, mPFC and MD inputs reliably triggered both cell types (mPFC: $n = 7$, $p = 0.57$; MD: $n = 8$, $p = 0.43$). These results indicate that all three inputs contact MSNs, but VH inputs have a weaker influence on neurons in the indirect pathway.

This differential activation suggests that VH inputs evoke distinct synaptic responses at direct and indirect MSNs, which we next measured as light-evoked excitatory postsynaptic currents (EPSCs) (Fig. 1d). In voltage-clamp at -70 mV, VH inputs evoked smaller EPSCs at indirect than direct MSNs (ratio = 0.14, $n = 7$ pairs, $p = 0.02$) (Fig. 1e). In contrast, mPFC and MD inputs consistently evoked similar responses in both cell types (mPFC: $n = 7$, $p = 0.58$; MD: $n = 7$, $p = 0.94$). These EPSCs were monosynaptic, as local inhibition was blocked and there is no recurrent excitation in striatal circuits¹³. In the presence of strontium, VH inputs also generated smaller unitary events at indirect MSNs, while mPFC and MD inputs again evoked similar responses in the two cell types (Supplementary Fig. 2). Together, these results suggest that postsynaptic factors contribute to the contrasting responses to VH inputs at direct and indirect MSNs.

In many neurons, selective targeting of inputs onto dendrites and spines has profound effects on synaptic efficacy¹⁴. We hypothesized that subcellular connectivity contributes to the contrasting responses at direct and indirect MSNs. However, long-range inputs to the striatum lack any anatomical organization that can be used to predict this connectivity¹⁵. We used a combination of optogenetics and two-photon microscopy to identify functional inputs by the synaptic calcium signals they evoke at spines^{16,17}. We first isolated a stretch of dendrite that possessed several spines within the same focal plane. While holding neurons at 0 mV, we then triggered local glutamate release with a pulse of focused light and measured fluorescence transients to detect active spines (Fig. 2a). For each recorded neuron, we used high-resolution three-dimensional reconstructions to determine the morphological properties of dendrites and spines (Fig. 2b). Using two-photon uncaging, we confirmed that synaptic calcium signals are present at all spines throughout the dendrites (Supplementary Fig. 3). This approach routinely allowed us to unambiguously identify multiple functional connections onto direct and indirect MSNs.

We first determined if different excitatory inputs map to unique dendritic locations. We found that VH inputs were restricted to the distal dendrites of indirect MSNs ($99 \mu\text{m}$, $n = 51$ spines), but evenly distributed across direct MSNs ($69 \mu\text{m}$, $n = 61$, $p = 0.004$) (Fig. 2c). In contrast, mPFC and MD inputs were evenly distributed across both cell types (mPFC: $n = 55 / 52$, $p = 0.81$; MD: $n = 48 / 91$, $p = 0.14$). Importantly, these results cannot be explained by sampling bias (Supplementary Fig. 4). Instead, they indicate that VH inputs make distinct connections in the dendrites of direct and indirect MSNs.

We next established if these inputs also target spines with unique morphologies. We found that VH inputs contacted smaller spines on indirect MSNs ($0.32 \mu\text{m}^3$) compared to direct MSNs ($0.64 \mu\text{m}^3$, $p = 0.002$) (Fig. 2d). In contrast, mPFC and MD inputs contacted a wide spectrum of spines in both cell types (mPFC: $p = 0.62$; MD: $p = 0.13$). These effects did not reflect dendritic targeting, as spine morphology did not change with distance from the soma

(Supplementary Fig. 5). Together, these results indicate that VH inputs selectively target smaller spines in the distal dendrites of indirect MSNs.

We hypothesized that this selective targeting of VH inputs could explain their weaker influence at indirect MSNs. Subcellular connectivity can strongly influence synaptic responses in other neurons¹⁴, but it was unclear if similar rules applied to MSNs^{18,19}. We next used two-photon uncaging to assess how synaptic responses depend on both spine location (Fig. 3a) and size (Fig. 3b). Average uncaging-evoked EPSPs (uEPSPs) were similar in direct and indirect MSNs and comparable to spontaneous events (Supplementary Fig. 6). However, we found that uEPSPs were reduced in the distal dendrites (direct: $n = 107$ spines, $p = 0.03$; indirect: $n = 96$, $p = 0.0002$) (Fig. 3c). Moreover, we found that uEPSPs were diminished at smaller spines within each dendritic segment (direct: $p = 0.04$; indirect: $p = 0.02$) (Fig. 3d). These results indicate that synaptic efficacy in these neurons depends on both the location and size of targeted spines.

We next combined our mapping and uncaging results to model how subcellular connectivity shapes MSN output. We found that the number of VH inputs needed to trigger action potentials (N) was higher at indirect MSNs ($N = 112$, $n = 7$, $p = 0.0006$) (Fig. 3e). This differential impact of VH inputs was much larger than for either mPFC inputs ($N = -3$) or MD inputs ($N = 12$) ($n = 5$, $p = 0.003$) (Supplementary Fig. 7). In order to assess the importance of spine location and size, we gradually shifted their distributions from those of indirect MSNs to direct MSNs. We found that making both factors similar eliminated the differential impact of VH inputs ($N = -5$), while adjusting either location ($N = 34$) or size ($N = 43$) alone did not ($n = 5$, $p = 0.008$) (Fig. 3f, Supplementary Fig. 7). Together, these results indicate that the subcellular connectivity of VH inputs is sufficient to explain their weaker influence at indirect MSNs.

Finally, *in vivo* recordings suggest that ongoing activity enhances the ability of VH inputs to trigger action potentials in MSNs²⁰. We hypothesized that subthreshold depolarization might allow VH inputs to activate both direct and indirect MSNs. Using our model, we first demonstrated that depolarization decreased N (-80 mV = 112, -55 mV = 48, $n = 5$, $p = 0.008$) (Fig. 3g). In current-clamp recordings, we then validated this prediction, as depolarization enabled firing of indirect MSNs (p_{spike} : -80 mV = 0.06, -55 mV = 0.95, $n = 6$ pairs, $p = 0.002$) (Fig. 3h). Thus, selective targeting forces VH inputs to be gated by subthreshold depolarization in order to influence the output of the indirect pathway.

Together, our findings reveal a new mechanism for how direct and indirect MSNs in the dorsomedial NAc core process diverse long-range excitatory inputs. VH inputs make weaker connections onto indirect MSNs, where they contact small spines located in the distal dendrites. To our knowledge, this is the first demonstration of selective targeting of functional synapses in the dendrites and spines of MSNs. This subcellular connectivity governs the influence of VH inputs on indirect MSNs and forces them to be gated by subthreshold depolarization. These results reconcile previous *in vivo* findings^{8,10,11,20}, and show how different inputs can uniquely influence the output pathways of the NAc.

ONLINE METHODS

Preparation

Medium spiny neurons (MSNs) were studied in acute coronal slices of the dorsomedial core in the Nucleus Accumbens (NAc) from hemizygous P21 – P28 *BAC D1-tdTomato*²¹ or *BAC D2-EGFP*²² mice of both sexes in a Swiss Webster background. For experiments in Figures 1 and 3, we used hybrid animals containing both reporters for direct comparison of the two cell types. Mice were anaesthetized with a lethal dose of ketamine / xylazine and perfused intra-cardially with ice-cold external solution containing (in mM): 65 sucrose, 75 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 25 glucose, 2.5 KCl, 1 CaCl₂, 5 MgCl₂, 0.4 Na-ascorbate, and 3 Na-pyruvate, bubbled with 95% O₂ / 5% CO₂. Coronal slices (300 µm thick) were cut in ice-cold external solution and transferred to ACSF containing (in mM): 119 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 21 glucose, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 0.4 Na-ascorbate, and 3 Na-pyruvate, bubbled with 95% O₂ / 5% CO₂. After 30 min in ACSF at 35°C, slices were stored for 30 min at 24°C. All experiments were conducted at room temperature (22 – 24°C) except for current-clamp recordings (30 – 32°C). In all experiments, 10 µM D-serine and 10 µM gabazine were present to prevent NMDA-R desensitization and block GABA_A-Rs, respectively. In two-photon mapping experiments, 1 µM TTX, 100 µM 4-AP and 4 mM calcium were present to block action potentials, maximize presynaptic depolarization, and enhance release probability, respectively²³. For experiments in Supplementary Figure 2, extracellular calcium was replaced with 2 mM strontium. All chemicals were from Sigma or Tocris. All procedures followed guidelines established by the NYU animal welfare committee.

Virus injections

Stereotaxic injections were performed on P10 mice anaesthetized with 40 mg ketamine and 5 mg xylazine per kg body weight. The head was leveled on a stereotaxic and injection coordinates determined relative to bregma (all Medial / Lateral, Dorsal / Ventral, Rostral / Caudal; mPFC: +0.3, –2.3, +1.7, MD: +0.4, –3.0, –0.6, VH: +2.8, –4.6, –3.1)²⁴. A small hole was created in the skull, a glass pipette was inserted into the brain, and ten 14 nl measures of virus (AAV2/9-mCherry or AAV2/9-hChR2-mCherry, UPenn Vector Core) were injected over the course of 10 min, for a total volume of approximately 140 nl. The pipette was left in place for an additional 10 min to minimize diffusion and then slowly removed before sealing the wound. Expression occurred in the injected brain region until preparation of acute slices for physiology experiments. The locations of all injection sites in mPFC, MD or VH were verified in slices before each recording session.

Electrophysiology

Whole-cell recordings were obtained from direct and indirect MSNs identified by fluorescently labeled cell bodies and targeted with IR-DIC. For sequential recordings of direct and indirect MSNs, both neurons were identified before patching in a single field of view at the same depth. The first recording alternated between direct and indirect neurons to avoid any complications associated with rundown. Dorsomedial NAc core was defined as the region between the anterior commissure and ventral tip of the lateral ventricle between bregma +1.7 and +0.7. Borosilicate recording pipettes (2 – 3 MΩ) were filled with one of

two internal solutions. Current-clamp recordings used (in mM): 135 K-gluconate, 7 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 Mg₂-ATP, and 0.4 NaGTP (290 – 295 mOsm, pH 7.35 with KOH). Voltage-clamp recordings used (in mM): 135 Cs-gluconate, 10 HEPES, 10 Na-phosphocreatine, 4 Mg₂-ATP, and 0.4 NaGTP (290 – 295 mOsm, pH 7.35 with CsOH). For current-clamp recordings, the internal solutions contained 30 μ M Alexa-594 and 200 μ M Fluo-5F. For voltage-clamp recordings, the internal solution contained 10 mM TEA and 2 mM QX-314. For two-photon mapping experiments, the internal solution contained 30 μ M Alexa-594 and 1 mM Fluo-4FF. The concentration of Fluo-4FF was chosen to maximize the ability to detect calcium signals, which were sometimes outside the linear range²⁵. Neurons were filled via the patch pipette for at least 15 min before imaging. Recordings were made using a Multiclamp 700B amplifier, filtered at 5 kHz, and sampled at 10 kHz.

Optogenetics

Glutamate release was triggered by illuminating ChR2 in the presynaptic terminals of different inputs to the NAc. For whole-cell experiments, wide-field illumination was performed with a 2 ms (current-clamp) or 0.5 ms (voltage-clamp) pulse of blue light from an 473 nm LED (ThorLabs). The power of the LED was measured as 4 – 7 mW at the back of the objective and was constant between cell pairs. For two-photon mapping experiments, focused illumination was performed with a 1 – 3 ms pulse of blue light from a 473 nm laser, as previously described for one-photon uncaging²⁶. The power of the laser was set to 3 mW at the back of the objective, which minimized photo-damage while maximizing ChR2 activation. The spot size of the laser was ~30 μ m, which allowed for local excitation while avoiding stimulating areas that were outside the imaging window.

Two-photon microscopy

Two-photon imaging and glutamate uncaging was achieved with a custom microscope²⁷. For two-photon imaging, 810 nm light from a Ti:Sapphire laser was used to excite Fluo-4FF (green = G) and Alexa-594 (red = R). Reference frame-scans were taken between each acquisition to correct for any spatial drift of the preparation over time. Baseline fluorescence was monitored and recordings were discarded if an increase was detected, which could indicate photo-damage. At the end of each experiment, a high-resolution stack of the entire cell was taken to reconstruct its morphology.

For rapid calcium imaging experiments, green and red fluorescence were collected during line-scans across multiple spine-dendrite pairs. Line-scans were interleaved with and without stimulation to confirm the absence of photo-bleaching. Calcium signals were quantified as change in green fluorescence to red fluorescence ($\Delta G/R$), normalized to the maximal green fluorescence to red fluorescence (G_{sat}/R), giving a final measurement as $\Delta G/G_{\text{sat}}$. After each recording, the value of G_{sat}/R was measured using the internal solution with saturating calcium²⁵ in a pipette positioned directly above the recorded cell.

For mapping experiments, initial identification of active spines was achieved with ten trials of 8 Hz frame-scans at 12.8 μ m $\times$ 6.4 μ m. Each trial consisted of seven baseline frames before a brief flash of focused 473 nm light, followed by three additional frames to detect any synaptic calcium signals in spines. Synaptic responses were identified online after each

trial using custom software written in MATLAB (Mathworks). Functional connections were then verified using interleaved 125 Hz line-scans across four spine-dendrite pairs, including the active spine and three adjacent inactive neighbors. For segments containing an active spine, a high-resolution stack ($x = 0.13 \mu\text{m}$, $y = 0.13 \mu\text{m}$, $z = 0.2 \mu\text{m}$ per voxel) of the field of view was acquired for morphological analysis.

For two-photon uncaging, MNI-glutamate was bath applied at 2.5 mM. Glutamate was uncaged using a brief (1 ms) pulse of 725 nm light from a second Ti:Sapphire laser. Uncaging location was at the tip of the spine head in a line directly perpendicular to the axis of the dendrite. Uncaging depth was limited to the top 30 μm of the slice, minimizing any depth effects on the uncaging power. Uncaging power was chosen to mimic synaptic calcium signals evoked by extracellular stimulation (Supplementary Fig. 3).

Image reconstruction and analysis

High-resolution three-dimensional reconstructions of each recorded neuron were performed and analyzed using NeuronStudio²⁸. Sholl analysis (defined here as the number of branch points between increasing three-dimensional spheres emanating from the soma) and total dendrite length were automatically calculated. Active spines were placed on the reconstructed model using the radial coordinates recorded during the experiment, and distance along the dendrite from the soma was automatically calculated. Spine size was quantified as head volume and calculated from reconstructions of magnified (13x) stacks using a rayburst algorithm. Images were deconvolved prior to head volume measurements using custom routines written in MATLAB (Mathworks).

Histology and confocal imaging

Brains were removed from deeply anesthetized mice, transferred to a solution containing 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), and fixed for 24 – 48 hours at 4°C. 50 μm coronal sections were prepared (Leica VT 1000S vibratome), placed on gelatin-coated microscope slides, and sealed under glass coverslips using ProLong Gold antifade reagent with DAPI mounting media (Invitrogen). Images were acquired with an upright confocal microscope (Leica TCX SP5), using a 40x oil-immersion lens with 1.25 numerical aperture. Excitation wavelengths were 561 nm and 405 nm for mCherry and DAPI, respectively. Flat-field correction was performed and montages were stitched using the mosaicJ plugin in ImageJ (NIH).

Computer simulations

The influence of different inputs on the output of direct and indirect MSNs was simulated using a single compartment, leaky integrate-and-fire model²⁹. Intrinsic properties of modeled neurons were adjusted to mimic those measured in current-clamp experiments ($R_{\text{in}} = 200 \text{ M}\Omega$, $C = 50 \text{ pF}$, $V_{\text{rest}} = -80 \text{ mV}$, $V_{\text{threshold}} = -40 \text{ mV}$, $V_{\text{reset}} = -80 \text{ mV}$, refractory period = 50 ms). The properties of different inputs were determined by sampling the distributions of spine morphology and location obtained from mapping experiments. Modeled inputs were initially generated using an alpha function, and subsequently scaled based on the relationships between synaptic responses and both spine morphology and location in the two MSNs. To investigate the impact of a given input on MSN output using

this model, the number of coincident synaptic connections (N) was systematically increased. The probability of spiking (p_{spike}) was calculated over 10 independently resampled datasets for 1 to 350 coincident inputs. The impact on direct and indirect MSNs was quantified as the difference in the number of inputs needed for half-maximal firing ($p_{\text{spike}} = 0.5$) (N). To assess the importance of spine size and location, p_{spike} and N were measured while systematically shifting the distributions of one or both factors from indirect MSNs to those of direct MSNs. This shift was quantified in terms of percent similarity, where 0% indicates no shift and 100% indicates overlapping medians. To assess the impact of ongoing synaptic activity, p_{spike} and N were measured while systematically changing V_{rest} from -80 mV to -45 mV.

Data acquisition and analysis

Two-photon imaging and physiology data were acquired using National Instruments boards and custom software written in MATLAB (Mathworks). Offline analysis was performed using custom routines written in Igor Pro (Wavemetrics). The amplitudes of uEPSPs are averages over a 1 ms time window around the peak. The amplitudes of EPSCs are averages over a 10 ms window starting 1 ms after the stimulus. Identification of individual strontium-induced asynchronous events was accomplished using the first derivative of the filtered current trace and subsequent threshold crossings on the raw current trace³⁰. For this analysis, events were identified in a 50 – 500 ms window after the stimulus, and the minimal threshold was set to 5 pA. The amplitudes of NMDA-R calcium signals are averages over a 150 ms time period, starting 50 ms after the stimulus.

Summary data are reported in the text as median. Example electrophysiological and imaging data are shown in figures as the mean $\pm$ SEM from multiple trials of the experiment. Most summary data in figures are in box plot form, showing the median, interquartile range, and 10% – 90% range (whiskers). Spike probability data is summarized as mean $\pm$ SEM. Significance was defined as $p < 0.05$ (*) and determined using the nonparametric Wilcoxon-Mann-Whitney two-sample rank test or the Wilcoxon signed rank test for paired data (when appropriate), both of which make no assumptions about the data distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential activation of direct and indirect MSNs

a, Left, Confocal images of direct (top) and indirect (bottom) MSNs in dorsomedial NAc core. Right, Two-photon images of individual MSNs. Scale bars = 20 μ m. **b**, Responses to VH inputs at neighboring direct (black) and indirect (red) MSNs. Arrow indicates light pulse. **c**, Summary of spike probability for mPFC, MD and VH inputs. **d**, EPSCs evoked by VH inputs, where responses at indirect MSNs are normalized to neighboring direct MSNs. **e**, Summary of normalized EPSC amplitudes. Circles show median values.

Figure 2. VH inputs make unique subcellular connections

a, Top, Two-photon image of an MSN, where box indicates mapped segment. Scale bar = 20 μm . Bottom, Frame-scans of morphology (black) and calcium signals (green) before and after focused light pulse, identifying active spine. Scale bar = 2 μm . **b**, Map of functional inputs, where lines show sampled (black) and unsampled (grey) dendrites, closed circles show active spines, and open circle shows example spine in (a). Scale bar = 20 μm . **c**, Summary of distance of active spines from the soma. Dotted line is median of all spines. **d**, Summary of head volume of active spines. Dotted line is median of all spines.

Figure 3. Subcellular targeting strongly impacts VH efficacy

a, *Top*, Two-photon image of MSN, with red circles indicating three sampled locations (i – iii). Scale bar = 20 μ m. *Bottom*, Average uEPSPs generated at four spines from each location. Arrows indicate uncaging times. **b**, *Left*, Image of a dendritic segment, indicating four sampled spines (i – iv). Orange circles indicate uncaging location. Scale bar = 1 μ m. *Right*, Average uEPSPs generated at each spine. **c**, Summary of uEPSP amplitudes at proximal (<50 μ m) and distal (>100 μ m) locations. **d**, Comparison of uEPSPs generated at the largest (L) and smallest (S) spines in each dendritic segment. **e**, Spike probability of modeled direct (black) and indirect (red) MSNs as a function of number of VH inputs (N). **f**, Summary of N for VH inputs as spine location, size, or their combination become increasingly similar to direct MSNs. **g**, Summary of N for VH inputs as a function of resting membrane potential. **h**, *Left*, Responses to VH inputs at neighboring direct (black) and indirect (red) MSNs held at –80 mV (light) or –55 mV (dark). *Right*, Summary of spike probability at the two membrane potentials.