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学 位 論 文

Characterization of nigrostriatal dopaminergic appositions across striatal
cell types and regions using AAV1-mediated trans-neuronal labeling
(AAV1を用いた順行性神経間標識による黒質線条体ドーパミン作
動性軸索の接触様式における細胞型および領域特異性の解析)

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List of Publications and Conference Presentations

Publications

1. Yang Y., Karube F., Kobayashi K., Fujiyama F. (2025). Nigral dopaminergic contact patterns related to striatal regions and cell-types in male mice. *Neurosci. Res.*, 223, 105011. <https://doi.org/10.1016/j.neures.2025.105011>
2. Karube, F., Yang, Y., Kobayashi, K. and Fujiyama, F. (2024). Anterograde trans-neuronal labeling of striatal interneurons in relation to dopamine neurons in the substantia nigra pars compacta. *Front. Neuroanat.*, 18, 1325368.

Conference Presentation

Karube, F., Yang, Y., Fujiyama, F. (2023). Anterograde trans-synaptic visualization of the striatal neurons innervated by dopaminergic neurons in the substantia nigra pars compacta in mice. Poster presentation at the Neuroscience 2023 (Society for Neuroscience Annual Meeting), Washington, D.C., USA, November 2023.

Summary

[Background and Objective]

Dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) are essential for movement, motivation, and learning. Their degeneration causes the characteristic motor and cognitive symptoms of Parkinson's disease, making the nigrostriatal pathway a key model for studying DA regulation. Each SNc neuron forms an extensive axonal arbor that spreads across the dorsal striatum, releasing DA from thousands of varicosities to influence diverse neuronal populations. This widespread axonal organization raises a central question: how can DA achieve selective modulation within such a broad network?

The dorsal striatum consists of two functional territories—the dorsomedial striatum (DMS) supporting goal-directed and the dorsolateral striatum (DLS) supporting habitual behavior. Its neuronal composition includes two major classes of projection neurons, the direct- and indirect-pathway medium spiny neurons (dMSNs and iMSNs), as well as several types of interneurons, which play distinct roles in shaping striatal output. Previous studies have revealed that distinct SNc subpopulations preferentially project to these subregions, suggesting that DA transmission may be spatially and functionally organized. However, the structural basis of this organization—specifically, which types of striatal neurons receive DA input and how these differ between regions—remains largely unknown.

To address this issue, I first tested whether adeno-associated virus serotype 1 (AAV1)–mediated anterograde trans-neuronal labeling, by which pre- and post-synaptic neurons are simultaneously labeled, can be applied to the DA nigrostriatal system. Because AAV1 is known to exhibit limited retrograde transport in some projection systems, I focused on striatal neurons whose axons do not directly project to the SNc. Specifically, one of the principal inhibitory interneurons, parvalbumin-positive interneurons (PV-Ins), the main modulatory interneurons, choline acetyltransferase-positive interneurons (CINs), and iMSNs were analyzed. It is known that iMSNs can be defined by pre-proenkephalin (PPE-iMSNs). This design minimized potential retrograde transfer and allowed us to evaluate AAV1's suitability for mapping neuromodulatory circuits. Based on this methodological validation, I then investigated whether SNc-derived DA axons form organized, cell-type-specific, and region-dependent appositional patterns within the dorsal striatum. Clarifying this organization is critical for understanding how DA regulates striatal microcircuits across distinct regions and neuronal types that underlie goal-directed and habitual behaviors.

[Materials and Methods]

AAV1-Cre was injected into the SNc to induce anterograde trans-neuronal expression of Cre, while Cre-dependent fluorescent reporters were injected into the striatum to label Cre-expressing neurons using wild type C57BL/6 male mice. This approach enabled simultaneous visualization of presynaptic SNc DA neurons and postsynaptic elements in the striatal neurons. The DA nature of labeled axons was confirmed by DA transporter (DAT) immunofluorescence and the absence of cholinergic, glutamatergic, or GABAergic terminal markers. Based on these labeling results, I next identified the striatal neuron types expressing Cre and analyzed their morphological and connectional features in detail.

High-resolution confocal microscopy was used to obtain image stacks of labeled striatal cells and DA axons, followed by three-dimensional reconstruction. Putative appositions (PAPs) were defined as DA varicosities directly contacting the soma or dendritic surface in 3D space. For each neuron, I quantified the number of PAPs, the number of axons forming PAPs, and the soma-to-PAPs distance. Morphometric parameters such as soma volume, total dendritic length, and number of primary dendrites were also compared among neuron types. Statistical analyses were performed using ANOVA with appropriate post hoc tests to evaluate differences across neuron types and regions

[Results]

AAV1-Cre injection into the SNc, combined with Cre-dependent reporters in the striatum, successfully induced Cre expression in postsynaptic striatal neurons, demonstrating that AAV1 can undergo anterograde trans-neuronal transfer in the DA pathway. Immunofluorescence confirmed that nearly all labeled axons were dopaminergic (DAT-positive) and not cholinergic, glutamatergic, or GABAergic.

Among the Cre-expressing neurons, immunostaining identified three major classes: PV-INs, CINs, and PPE-iMSNs. Morphometric data supported these findings: CINs (8 neurons in DMS, 6 in DLS) had the largest somata ($1235.69 \pm 392.55 \mu\text{m}^3$ in DMS) and longest mean dendrites ($186.17 \pm 148.23 \mu\text{m}$ in DMS), PV-INs (7 in DMS, 4 in DLS) had more primary dendrites (6.43 ± 1.90 in DMS) but smaller soma volume ($575.35 \pm 220.44 \mu\text{m}^3$ in DMS), and PPE-iMSNs (5 in DMS, 4 in DLS) exhibited small soma with spiny dendrites, consistent with previous reports. Confocal imaging showed multiple DA appositions on somata and dendrites of these neurons, validating AAV1-based labeling for visualizing pre- and postsynaptic components.

Quantitative analyses showed that DA appositions were broadly distributed but

followed distinct organized patterns across neuron types and regions. Interneurons generally received more proximal appositions to their somata, whereas PPE-iMSNs exhibited more distal dendritic appositions. CINs received the highest number of somatic PAPs per neuron (10.3 ± 4.7 in DMS and 6.2 ± 3.9 in DLS), followed by PV-INs (7.8 ± 3.1 and 5.4 ± 2.8) and PPE-iMSNs (3.2 ± 1.8 and 2.5 ± 1.4). The proportion of axons forming somatic appositions was significantly higher in DMS CINs than in DLS CINs ($p < 0.05$). Regarding spatial distribution, the average soma-to-PAP distance was shortest in CINs ($24.8 \pm 9.3 \mu\text{m}$), intermediate in PV-INs ($31.4 \pm 10.7 \mu\text{m}$), and longest in PPE-iMSNs ($46.8 \pm 12.5 \mu\text{m}$ in the DMS; $72.3 \pm 15.1 \mu\text{m}$ in the DLS).

[Discussion]

This study demonstrates that AAV1 can serve as a reliable anterograde trans-neuronal tracer in the nigrostriatal DA system. By focusing on interneurons and iMSNs that lack reciprocal projections, I minimized the possibility of retrograde artifacts and confirmed that the labeling primarily reflected anterograde transfer. Methodologically, my results extend AAV1's applicability to neuromodulatory systems beyond fast neurotransmitter pathways.

I demonstrated both cell-type- and region-specific heterogeneity in DA innervation within the striatum. Interneurons, particularly CINs and PV-INs, received denser and more proximal DA appositions than PPE-iMSNs. The strong somatic contacts on CINs may explain their stronger DA-dependent inhibition, while PV-INs may integrate DA input to fine-tune inhibitory timing within striatal microcircuits. In contrast, the distal contacts on PPE-iMSNs may influence dendritic processing, and thereby modulate indirect pathway activity.

The regional differences between the DMS and DLS suggest that DA signaling is spatially adapted to distinct functional roles. The DMS, associated with goal-directed learning, showed more proximal appositions, while the DLS, related to habitual behavior, exhibited broader dendritic distributions. These anatomical gradients provide structural support for differential DA-dependent regulation across striatal circuits.

[Conclusion]

I established that AAV1-mediated anterograde trans-neuronal labeling can be effectively applied to the nigrostriatal DA pathway. Using this validated approach, I identified structured, non-random patterns of DA innervation characterized by cell-type- and region-dependent organization. PV-INs and CINs received more proximal and frequent contacts, whereas PPE-iMSNs showed more distal appositions. These

findings demonstrate that DA innervation within the striatum is organized in a structured, cell-type- and region-specific manner rather than by random diffusion. The methodological advancement also provides a powerful framework for future studies investigating circuit-specific DA regulation and its disruption in neurological disorders such as Parkinson's disease. The present study relied solely on light microscopy observation and did not provide direct evidence of ultrastructural or functional connectivity. Future investigations combining AAV1 tracing with electrophysiological, ultrastructural, or behavioral analyses will be essential to validate functional relevance and elucidate how these organized DA connections shape circuit activity in health and diseased states.

List of abbreviations

AAV	adeno-associated virus
AP	anterior-posterior
CPu	caudate putamen
Cx	cortex
ChAT	choline acetyltransferase
CHT1	high-affinity choline transporter 1
CINs	cholinergic interneurons
Ctip2	COUP-TF-interacting protein 2
DA	dopamine/dopaminergic
DAT	dopamine transporter
dMSN	direct pathway medium spiny neuron
DMS	dorsomedial striatum
DLS	dorsolateral striatum
DV	dorsal-ventral
GABA	gamma-amino butyric acid
GFP	green fluorescence protein
GPe	globus pallidus external segment
GPi	globus pallidus internal segment
iMSN	indirect pathway medium spiny neuron
LM	lateral-medial
NOS	nitric oxide synthase
NPY	neuropeptide Y
PAPs	putative appositions
PBP	parabrachial pigmented nucleus
PV	parvalbumin
PV-INs	parvalbumin positive interneurons
PPE	prepro enkephalin
PPE-iMSNs	prepro enkephalin positive iMSNs
ROI	region of interest
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SOM	somatostatin
STN	subthalamic nucleus

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TH	tyrosine hydroxylase
VGAT	vesicular GABA transporter
VGluT2	vesicular glutamate transporter 2
VTA	ventral tegmental area

Introduction

1. Background: Dopamine System as a Foundation for Adaptive Behavior

The dopaminergic (DA) system arising from the mesencephalon constitutes one of the most influential neuromodulatory systems in the mammalian brain. DA neurons play integrative roles in motivation, value-based learning, and decision-making, thereby supporting flexible and adaptive behavior. Among the DA nuclei, the substantia nigra pars compacta (SNc) is a principal source of DA projections to the dorsal striatum. This structure is a key node within the basal ganglia circuitry that regulates motor control, action selection, and reinforcement learning. (François et al., 1999; Gerfen and Surmeier, 2011; Klein et al., 2019; for review, see Bjorklund and Dunnett, 2007). Dysfunction of nigrostriatal projections leads to profound behavioral impairments, exemplified by Parkinson's disease, in which the degeneration of SNc DA neurons results in bradykinesia and loss of voluntary movement.

Each SNc DA neuron extends an exceptionally vast axonal arborization within the striatum, forming tens of thousands of varicosities and en passant boutons (Gauthier et al., 1999; Prensa and Parent, 2001; Matsuda et al., 2009), indicating that individual DA neurons can influence broad striatal territories. Yet, at the same time, behavioral precision requires that DA signals be transmitted in a temporally and spatially regulated manner. The discrepancy between the apparently diffuse anatomical network and the functionally specific modulation by DA complicates research on the basal ganglia.

2. Conceptual Development: From General Modulation to Cellular Specificity

Over the past decades, neuroscience has progressively shifted from describing global neuromodulatory effects to identifying cell-type-specific circuit mechanisms. This transition has been greatly facilitated by advances in transgenic technology, viral tracing, and single-cell molecular profiling. Studies across various brain regions have revealed that even within seemingly diffuse networks, neurons form highly organized and selective connections based on their genetic and functional identity (Luo et al., 2018; Mukamel and Ngai, 2019; Luo, 2021).

In the DA system, recent findings suggest a similar organizational principle. Subpopulations of SNc DA neurons differ in gene expression and input-output connectivity, leading to topographically organized projections to distinct striatal compartments (Watabe-Uchida et al., 2012; Menegas et al., 2015, 2018; Poulin et al., 2018; Luppi et al., 2021). These data raise the possibility that DA modulation is not uniformly distributed but instead tailored to specific circuits or neuronal populations.

In the nigrostriatal pathway, SNc DA axons target striatal neurons, which comprise two major categories: projection neurons and interneurons. Projection neurons include direct- and indirect-pathway medium spiny neurons (dMSNs and iMSNs), while interneurons consist of cholinergic (CINs) and various GABAergic types (Kawaguchi, 1993; Kawaguchi et al., 1995; Tepper et al., 2010). The latter have been increasingly subdivided into molecularly and physiologically distinct subtypes (Ibáñez-Sandoval et al., 2011; Muñoz-Manchado et al., 2016, 2018; Assous et al., 2018; for review, see Tepper et al., 2018). For example, parvalbumin-positive fast-spiking interneurons (PV-INs) and low-threshold spiking neurons expressing somatostatin (SOM), nitric oxide synthase (NOS), or neuropeptide Y (NPY) exert diverse inhibitory influences on the local microcircuit. These interneurons, together with projection neurons, express various DA receptor subtypes (Smith and Villalba, 2008; Kreitzer, 2009). Hence, the DA system can modulate striatal activity through multiple pathways—directly acting on MSNs or indirectly through interneurons in a subtype-specific manner (Straub et al., 2016; Silberberg and Bolam, 2015).

From these findings, I began to consider whether the selectivity of DA modulation might be reflected in the microanatomical organization of DA axons themselves. Specifically, could DA neurons establish preferential spatial relationships with certain striatal cell types? If so, such organization could underlie the cell-type-specific responses observed electrophysiologically (Marcott et al., 2014; Chuhma et al., 2023).

3. Morphological Dichotomy: Synaptic versus Volume Transmission

To approach this question, it is necessary to re-examine how DA is released and transmitted. Unlike classical neurotransmitters such as glutamate or GABA, DA release occurs both at synaptic and non-synaptic sites. Electron microscopy revealed that only about 30–40% of DA varicosities form synaptic contacts, while the majority lack recognizable postsynaptic densities and are assumed to release DA into the extracellular space, mediating volume transmission (Descarries et al., 1996; Rice and Cragg, 2008). This dual mode of transmission—precise versus diffuse—might account for the combination of specificity and broad influence.

However, recent evidence challenges this dichotomy. Molecular components resembling active zones have been identified even in non-synaptic DA varicosities (Daniel et al., 2009; Lipton et al., 2018; Liu et al., 2018; Liu and Kaeser, 2019), and functional imaging indicates that only a subset of varicosities are “active” release sites (Pereira et al., 2016). Moreover, postsynaptic markers such as gephyrin and neuregulin 2 were found at certain DA contact sites (Uchigashima et al., 2016; Kim et al., 2023).

These observations prompted me to consider that DA transmission might be more structured than previously thought—that specific axonal branches or varicosities could preferentially engage distinct postsynaptic targets.

4. Regional Context: The DMS–DLS Functional Gradient

The dorsal striatum itself is not homogeneous but divided into dorsomedial (DMS) and dorsolateral (DLS) subregions, corresponding functionally to associative and sensorimotor domains—analogous to the caudate and putamen in primates. The DMS participates in goal-directed learning, whereas the DLS contributes to habit formation and motor execution (Sharpe et al., 2019; Dhawale et al., 2021; Cataldi et al., 2022; Song and Lee, 2024). DA release dynamics differ between these areas during behavior (van Elzelingen et al., 2022; Jørgensen et al., 2023), and pharmacological blockade of DA signaling produces region-specific effects on learning and movement (Hamid et al., 2021; Bernklau et al., 2024). Anatomically, distinct SNc neuron subtypes preferentially project to either DMS or DLS (Poulin et al., 2018). These lines of evidence suggested that if DA appositions are spatially organized, such patterns may vary not only across cell types but also between striatal regions.

5. Methodological Reflection: Toward Direct Visualization of DA Connectivity

To verify this idea, direct anatomical evidence is required. Yet conventional immunostaining or retrograde tracing cannot unambiguously identify which striatal neurons receive input from a particular subset of DA axons. Around this point, my attention turned to the development of adeno-associated virus serotype 1 (AAV1) as a potential tool. AAV1 vectors are capable of synapse-dependent anterograde trans-neuronal transfer, thereby labeling postsynaptic neurons after injection into a projection source (Zingg et al., 2017, 2020; Li et al., 2021). At first, I was skeptical whether such trans-neuronal labeling would be applicable to the nigrostriatal system, since reciprocal connections between SNc and striatum could complicate the interpretation (Zingg et al., 2020, 2022). However, focusing on interneurons whose axons are confined within the striatum seemed to circumvent this problem. Similarly, indirect-pathway MSNs project to the external globus pallidus instead of directly to the SNc, allowing their analysis without this confound. By combining AAV1-Cre with Cre-dependent fluorescent reporters and cell-type-specific immunolabeling, it is possible to visualize both DA axons and their downstream striatal neurons simultaneously.

6. Research Aim and Framework of the Present Study

Based on the above considerations, this dissertation aims to clarify whether DA innervation in the dorsal striatum is organized in a cell-type- and region-dependent manner. Using AAV1-based anterograde trans-neuronal labeling, I analyzed putative appositions (PAPs) between SNc-derived DA axons and three molecularly defined neuron types: PV-INs, CINs, and indirect-pathway medium spiny neurons expressing preproenkephalin (PPE-iMSNs). Direct-pathway MSNs (dMSNs), which project to the SNc and can be labeled retrogradely by AAVs, were excluded. Quantitative three-dimensional reconstructions were used to assess the frequency and spatial distribution of DA varicosities in contact with the somata and dendrites of these neurons in both the DMS and DLS.

Through this analysis, I sought to provide an anatomical framework to test whether DA neurons form structured, cell-type-specific contacts rather than random, diffuse distributions. Such organization, if confirmed, would help reconcile the apparent discrepancy between diffuse axonal arborization and selective DA modulation. Ultimately, this work aims to contribute to a better understanding of how DA systems achieve specificity within widespread networks—an issue central to both basic neuroscience and the pathophysiology of basal ganglia disorders.

Materials and Methods

1. Animals

All experimental procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Hokkaido University (Approval Numbers: 20-0106 and 25-049). Procedures involving adeno-associated virus (AAV) vectors were additionally approved by the Genetic Recombination Experiment Safety Committee of Hokkaido University (Approval Numbers: 2020-019 and 2025-022). Since potential sex differences in the DA system have been reported (Lynch et al., 1999; Eubig et al., 2014; George et al., 2021), I restricted my analysis to male mice in this study. A total of 33 male C57BL/6J mice (6–12 weeks old) were used in this study.

2. Viral vectors

AAVs were obtained from Addgene (Watertown, MA, USA). For trans-neuronal labeling, AAV1-hSyn-Cre (Addgene #10553, titer: 7×10^{12} vg/mL) was used. To achieve Cre-dependent expression of fluorescent proteins, AAVdj-EF1 α -double floxed-hChR2(H134R)-mCherry-WPRE-HGH-pA (Addgene #20297, titer: 1×10^{13} vg/mL), AAVdj-Syn-FLEX-rc[ChrimsonR-tdTomato] (Addgene #62723, titer: 2.2×10^{13} vg/mL) and/or AAVdj-Syn-FLEX-rc[Chronos-GFP] (Addgene #62722, titer: 2.5×10^{13} vg/mL) were employed. These AAVs were used solely for fluorescent labeling, not for optogenetic manipulation of neuronal activity. Accordingly, they are referred to as AAV-Flex-mCherry, AAV-Flex-tdTomato and AAV-Flex-GFP, respectively, throughout the manuscript.

3. Animal surgery

Mice were anesthetized with a combination of three agents: medetomidine (0.3 mg/kg; Domitor, Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), midazolam (4.0 mg/kg; Dormicum, Maruishi Pharmaceutical Co., Ltd., Osaka, Japan), and butorphanol (5.0 mg/kg; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). This anesthetic cocktail, referred to as MMB, was administered subcutaneously at a volume of 0.05 mL per 10 g of body weight. Anesthetic depth was continuously assessed by monitoring heart rate and respiration. To maintain adequate anesthesia, supplemental MMB (0.02 mL) was administered approximately every 40 minutes. During surgery, mice were positioned in a stereotaxic apparatus equipped with ear bars and a nose clamp (Narishige, Tokyo, Japan). Core body temperature was monitored and maintained at approximately 38°C using a BWT 100A animal warming system (Bio Research Center, Nagoya, Japan).

Prior to surgery, the scalp was trimmed with scissors, and locally anesthetized using xylocaine gel (Sandoz K.K., Tokyo, Japan). A midline incision was made to expose the skull. The nose bar was adjusted to align the dorsal surface of the skull such that the bregma and lambda were at the same horizontal level. Small craniotomies were performed using a dental drill to expose the dura mater. Based on the mouse brain atlas (Paxinos and Franklin, 2013), injection coordinates were determined and targeted the following regions: the medial substantia nigra pars compacta (SNc): [3.07 mm posterior to bregma (AP -3.07), 1.1 mm lateral to the midline (LM 1.1), and 4.1 mm deep from the cortical surface (DV 4.1)]; the lateral SNc: [AP -3.07, LM 1.7, DV 3.8]; the dorsomedial striatum (DMS): [AP +0.73, LM 1.3, DV 2.1]; the dorsolateral striatum (DLS): [AP +0.73, LM 2.3, DV 2.5].

For retrograde tracer injection in the striatum, a 5% solution of FluoroGold (FG, Fluorochrome, Denver, CO, United States) was prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The solution of AAV or FG was injected using brief air pressure pulses by a PicoPump (PV820, World Precision Instruments, Sarasota, FL, USA). A glass micropipette with a 20 μ m tip diameter was filled with the solution, positioned at the designated coordinates, and held in place for ~ 5 minutes prior to injection. The solution was delivered using 5 ms air pulses at 20 psi, applied at a frequency of 0.3 Hz. Under these conditions, 200-500 nL of solution was injected over 10 minutes. Following injection, the pipette remained in place for 10 minutes before being slowly withdrawn. Upon completion of all injections, the skull and surrounding tissue were rinsed with sterile saline, and the craniotomies were sealed with bone wax. The skin was sutured, and 10% povidone-iodine solution was applied for disinfection. Mice were then placed on a heating pad until full recovery from anesthesia, and a subcutaneous injection of Meloxicam (5.0 mg/ml, Meloxirin, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) was administered for postoperative analgesia. In the first series of AAV injections (N = 4), AAV1-hSyn-Cre was injected into the SNc of one hemisphere and the mouse was perfused 1 week after injection. In the second series of AAV injections (N = 4), AAV1-hSyn-Cre was injected into the SNc of one hemisphere. After 1 week, AAV-Flex-GFP was injected into the striatum of both hemispheres, and AAV-Flex-mCherry was injected into the SNc in the injected hemisphere using the same coordinates as AAV1-hSyn-Cre. The reverse combination of AAV-Flex-mCherry and AAV-Flex-GFP was applied for one of 4 mice. For trans-neuronal labeling with single neuron analysis, AAV1-hSyn-Cre and AAV-Flex-GFP solutions were mixed at a 1:1 ratio and injected into the lateral SNc of one hemisphere and the medial SNc of the contralateral hemisphere (N = 6). One week later, AAV-Flex-tdTomato was injected into

either of the DLS or DMS per hemisphere, corresponding to the hemisphere previously injected with the mixed viral solution. Two weeks later, the mice were perfused as described below. For FG injection (N = 4 mice), the mice were perfused 4–5 days after injection.

4. Histology

4.1 Fixation

Mice were deeply anesthetized via intraperitoneal injection of pentobarbital (100 mg/kg, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Transcardial perfusion was initiated with 20 mL of a pre-fixative solution containing 50 mM MgCl₂ and 7.5% sucrose in 0.02 M phosphate-buffered (PB). This was followed by perfusion with approximately 50 mL of fixative containing 4% paraformaldehyde (For PPE-labeled samples, use a 2% concentration) and 0.2% picric acid (both from Nacalai Tesque, Tokyo, Japan) in 0.1 M PB. After 2.5 hours of in situ fixation, the brain was carefully removed and rinsed three times (30 minutes each) in 0.1 M PB. The brain was cryoprotected in 15% sucrose in 0.1 M PB until it sank, followed by transfer to 30% sucrose in 0.1 M PB. Once fully equilibrated, the brain was placed in a fresh 30% sucrose solution containing 0.02% sodium azide and kept at 4°C until further processing.

4.2 Tissue sectioning and immunofluorescence

Brains were mounted on a cooling plate using O.T.C. compound (Sakura Finetek, Tokyo, Japan) and frozen with dry ice. Subsequently, brain coronal sections were cut at a thickness of 25 µm using a freezing microtome (Leica SM2000R, Leica Microsystems, Wetzlar, Germany). The sections were washed three times in 0.1 M PB for 10 minutes each. For immunofluorescence staining, sections were incubated in an incubation buffer containing 10% normal goat serum, 2% bovine serum albumin, 0.02% sodium azide, and 0.5% Triton X in 0.05 M Tris-buffered saline (TBS), together with the appropriate primary antibodies (see Table 1). Primary antibody incubation was carried out at room temperature (RT) for 24–48 hours. After three washes in TBS, sections were incubated with a mixture of secondary antibodies (see Table 2) at RT for 3 hours. Following three washes in TBS, sections were mounted on glass slides, air-dried, and coverslipped using ProlongGold (Thermo Fisher Scientific). Slides were stored in the dark at 4°C until imaging.

Table 1 List of the primary antibody

Antigen	Company/catalog ID	Animals	Dilution	RRID
Calbindin	Swant/CB9	Rabbit	4,000	AB_10000348
Cre	Synaptic Systems/257,004	Guinea pig	2,000	AB_2782969
Choline acetyltransferase	Millipore/AB144P	Goat	500	AB_2079751
Choline acetyltransferase	Millipore/MAB305	Mouse	1,000	AB_94647
Dopamine receptor type 2	Frontier Institute/D2R-GP-Af500	Guinea pig	200	AB_2571597
Dopamine transporter	Frontier Institute/MSFR101200	Rabbit	500	AB_2571686
GFP	GeneTex/13,970	Chicken	1,000	AB_371416
GFP	Nacalai Tesque/GF090R	Rat	1,000	AB_2314545
High-affinity choline transporter	Frontier Institute/MSFR100890	Guinea pig	200	AB_2571678
mCherry	Thermo Fisher Scientific/M11217	Rat	1,000	AB_2536611
Nitric oxide synthase	Sigma/N7280	Rabbit	5,000	AB_260796
Neuropeptide Y	Immunostar/22,940	Rabbit	2,000	AB_2307354
Pre pro enkephalin	Neuromics/RA4124	Rabbit	1,000	AB_2532106
Parvalbumin	Swant/PV25	Rabbit	5,000	AB_10000344
Parvalbumin	Synaptic Systems/195,004	Guinea pig	2,000	AB_2156476
RFP	ChromoTek/5f8	Rabbit	500	AB_2336064
Somatostatin	Millipore/MAB354	Rat	250	AB_2255365
Somatostatin	Synaptic Systems/ 366 004	Guinea pig	500	AB_2620126
tdTomato	LSBio (LifeSpan)/ LS-C340696	Goat	2,000	AB_2819022

tdTomato	Frontier Institute/MSFR105900	Guinea pig	1,000	AB_2631185
Tyrosine hydroxylase	Synaptic Systems/ 213 104	Guinea pig	2,000	AB_2619897
Vesicular glutamate transporter 2	Frontier Institute/VGluT2-GP- Af810	Guinea pig	500	AB_2571621
Vesicular GABA transporter	Synaptic Systems/131,003	Rabbit	250	AB_887869

Table 2 List of the second antibody

antigen	company/catalog ID	host species	dilution	RRID
anti-goat Alexa Fluor 546	Thermo Fisher Scientific/ A11056	donkey	500	AB_10584485
anti-chicken Alexa Fluor 488	Thermo Fisher Scientific/ A78948	donkey	500	AB_2921070
anti-guinea pig Brilliant Violet 421	Jackson ImmunoResearch/ 706-675-148	donkey	200	AB_2651104
anti-rabbit Brilliant Violet 421	Jackson ImmunoResearch/ 711-675-152	donkey	200	AB_2651108
anti-rabbit Alexa Fluor 647	Thermo Fisher Scientific/ A-31573	donkey	500	AB_2536183
anti-guinea Pig CF633	Biotium /20171	donkey	500	AB_10854103

5. Microscopic observation and morphological analysis

Two fluorescence imaging systems were employed for specimen observation: a box-type fluorescence microscope (BZ-X710; Keyence, Osaka, Japan) and an epifluorescence microscope (BX51; Olympus, Tokyo, Japan) equipped with a CCD

camera (DP73; Olympus) and a CMOS camera (Orca Spark; Hamamatsu Photonics, Hamamatsu, Japan). Images were acquired using $\times 4$, $\times 10$, and $\times 20$ objective lenses. For higher-resolution co-expression and quantitative analyses, a confocal laser scanning microscope (FV1200; Olympus) was used with $\times 40$ objectives or oil-immersion lenses ($\times 60$ and $\times 100$). All acquired images were adjusted for intensity levels and processed with Fiji (an ImageJ distribution; Schindelin et al., 2012).

For neuronal quantification, serial optical sections were captured at 3- μm z-steps using the FV1200, and neurons were counted stereologically with Fiji. To assess co-localization between AAV-labeled axons and either DA transporter (DAT) or high-affinity choline transporter (CHT1), z-stack images were collected at 0.3- μm intervals using a $\times 60$ objective with $\times 4$ digital zoom. The resulting voxel dimensions were $0.082 \times 0.082 \times 0.3 \mu\text{m}$, corresponding to an image field of $52.48 \times 52.48 \mu\text{m}$ in the XY plane. Because DAT and CHT1 immunofluorescence signal both axonal varicosities and intervaricose segments, co-localization was evaluated along axon segments rather than at terminal boutons. Each AAV-positive axon was manually traced in three dimensions using Neurolucida and NeuroExplorer (MBF Bioscience, VT, USA) and analyzed for the presence of DAT or CHT1 labeling, and the proportion of positive axon segments was calculated.

Co-localization with vesicular glutamate transporter 2 (VGluT2) and vesicular GABA transporter (VGAT) was assessed at the varicosity level, as these transporters are primarily confined to presynaptic boutons. The percentage of VGluT2- or VGAT-positive varicosities was then determined. As presented in Results 3.1, a minor subset of AAV1-labeled varicosities exhibited apparent overlap with VGluT2 or VGAT immunoreactivity. In certain instances, only part of a varicosity showed signal overlap, indicating potential false positives; nonetheless, given the optical resolution limit of the imaging system, such cases were conservatively regarded as co-localization in this study.

To further examine close appositions between AAV-labeled axons and single PV-INs, CINs, or PPE-iMSNs, Z-stack images were acquired using a $60\times$ oil-immersion objective combined with $4\times$ digital zoom, at 0.5 μm intervals. The resulting voxel dimensions were $0.103 \mu\text{m} \times 0.103 \mu\text{m} \times 0.5 \mu\text{m}$ with each image covering an XY field of $52.74 \mu\text{m} \times 52.74 \mu\text{m}$. The morphology of the soma and dendrites was reconstructed in three dimensions from these images using Neurolucida, and analyzed. Morphological parameters such as soma volume, somatic maximum cross-sectional area, number of dendrites, total dendritic length, and average of dendritic length were quantified. Putative appositions (PAPs) were defined as AAV-labeled axonal varicosities in direct

apposition to the surface of the somata or dendrites in three-dimensional space. PAPs were manually identified and annotated. As mentioned above, AAV-labeled axons forming PAPs were also reconstructed, and the spatial distribution of PAPs was quantitatively analyzed, including the number of axons that form PAPs with each neuron, the number of PAPs on the soma, and the number of PAPs on the dendrites.

6. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., Boston, MA, USA), Microsoft Excel, R (R Project for Statistical Computing, Vienna, Austria), and IgorPro (Wave Metrics Inc., Portland, OR, United States). Data are represented as mean $\pm$ standard deviation (SD). Statistical significance for comparing the proportion of Cre-expressing neurons among different neuron types was assessed using Fisher's exact test with multiple comparison correction using Holm-Bonferroni method. For comparisons across cell types and subregions, two-way analyses of variance (ANOVA; factors: cell type $\times$ subregion) were applied. Significant main effects or interactions were followed by simple main-effect tests with Bonferroni-adjusted post hoc comparisons. A p-value < 0.05 was considered statistically significant.

Results

1. Cre expression in the striatum was induced by AAV1 injection into the SNc

To evaluate the applicability of anterograde trans-neuronal AAV1 in DA circuits, I injected AAV1-hSyn-Cre into the SNc of one hemisphere in wild-type mice (Figure 1). One week later, the animals were perfused, and Cre expression in the SNc and striatum was examined using immunofluorescence (N = 4 mice). As expected, Cre-positive nuclei were detected in the injection site (Figure 1A). Because AAV1 injection is difficult to perfectly confine to the SNc, both DA and non-DA neurons expressed Cre, as confirmed by triple immunostaining (Figures 1A–C). In the striatum of the injected hemisphere, scattered Cre-expressing neurons were observed (Figure 1D), and some of them co-expressed parvalbumin (PV) (Figure 1E). Since AAV1-Cre can infect not only DA neurons but also nearby non-DA neurons in the midbrain, it remained unclear whether striatal Cre expression originated specifically from DA neurons.

To address this issue, I first visualized neurons projecting to the striatum by injecting the retrograde tracer FluoroGold (FG) into the DLS (N = 4 mice; Figure 2A). Only a small number of non-DA neurons in the dorsal midbrain were retrogradely labeled (Figure 2A). This indicates that potential tracer leakage along the glass needle track toward the SN was minimal and negligible. In the SN, DA neurons accounted for $95.0 \pm 1.1\%$ of all FG-labeled neurons (N = 733 FG-labeled neurons, N = 4 mice, two sections per mouse; Figures 2B,C). Only four PV-positive neurons were found among these 733 FG-labeled cells. Thus, AAV injection into the SN can reliably label striatum-projecting DA neurons even without transgenic lines.

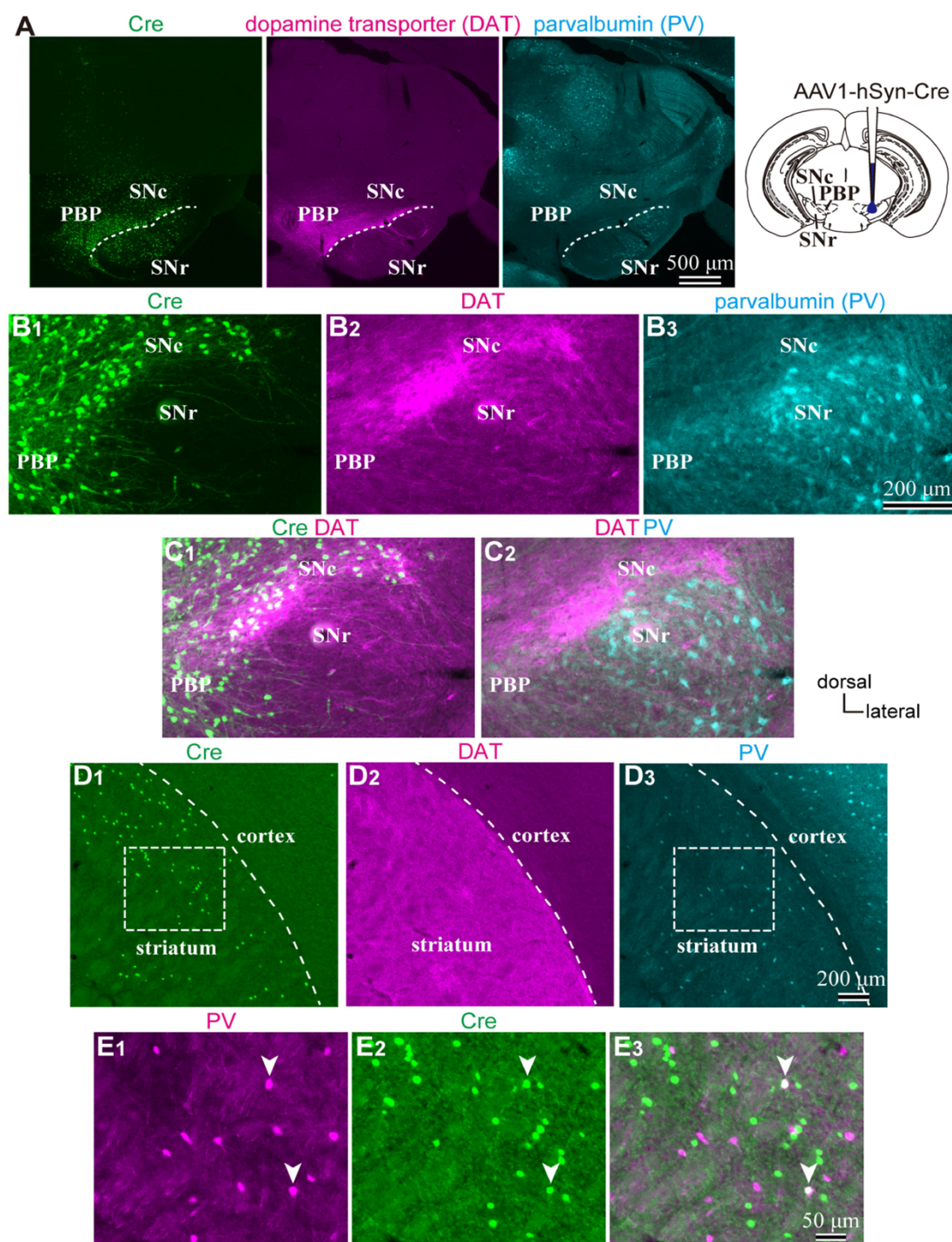


Figure 1. Cre expression in the SNc and striatum following AAV1-hSyn-Cre injection into the SNc. (A) Injection site of AAV1-hSyn-Cre in the SNc. Immunofluorescence for Cre (left), DA transporter (DAT; middle), and parvalbumin (PV; right) is shown. Cre expression was observed throughout the SN and extended medially within the midbrain. (B) Higher-magnification views around the SN region. (C) Merged image corresponding to (B). Cre was expressed in both DAT-positive and DAT-negative neurons, whereas only a few Cre-positive cells were observed in the substantia nigra pars reticulata (SNr). (D) Cre (D1), DAT (D2), and PV (D3) expression patterns in the

striatum from the same mouse shown in (A–C). Cre-positive neurons were scattered within the dorsolateral striatum. (E) Enlarged view of the boxed area in (D). Arrowheads indicate Cre expression in PV-expressing neurons.

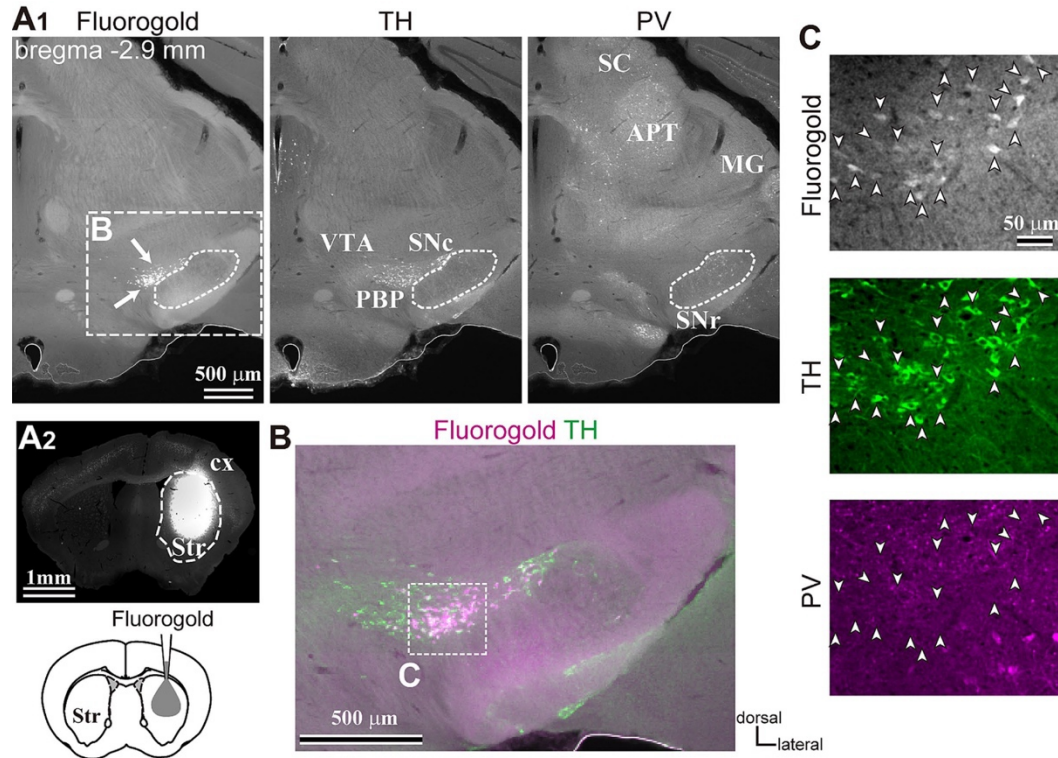


Figure 2. Retrograde labeling of DA projection neurons from the striatum to the SNc. (A) Injection of the retrograde tracer fluorogold (FG) into the striatum (Str, A2) resulted in retrogradely labeled neurons within the substantia nigra (arrows, left panel in A1). The same section was immunostained for tyrosine hydroxylase (TH) (middle) and parvalbumin (PV) (right). Only a few FG-labeled cells were detected outside the substantia nigra within the midbrain. (B) Higher magnification merged image of the boxed area in (A1), showing FG in magenta and TH in green. (C) Confocal images corresponding to the boxed region in (B). All FG-labeled neurons (arrowheads, top) were immunopositive for TH (green, middle) but negative for PV (magenta, bottom), indicating that retrogradely labeled neurons are DA but not PV-expressing. APT, anterior pretectal nucleus; cx, cerebral cortex; MG, medial geniculate nucleus; PBP, parabrachial pigmented nucleus; SC, superior colliculus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area.

Next, I co-injected AAV1-Cre with AAV-Flex-mCherry at the same SNc coordinates. As shown in Figure 3A, no mCherry fluorescence was observed in the contralateral

striatum, whereas strong mCherry expression appeared in the ipsilateral hemisphere, confirming that mCherry expression was Cre-dependent. To identify the neurotransmitter phenotype of mCherry-labeled axons, I performed immunostaining for DAT, high-affinity choline transporter (CHT1), vesicular GABA transporter (VGAT), and vesicular glutamate transporter 2 (VGluT2)—four major transporters expressed by midbrain neurons. All mCherry-labeled axon segments expressed DAT (Figure 3B) but not CHT1 (Figure 3C), confirming their DA nature and excluding contamination from cholinergic projections (DAT in 462/462 segments; CHT1 in 0/462; N = 4 mice). Only a few varicosities exhibited VGAT or VGluT2 immunoreactivity (VGAT 4/138; VGluT2 14/388; N = 4 mice; Figures 3E–G). Importantly, these varicosities were sparsely distributed within individual axons, suggesting that the labeled fibers were not purely GABAergic or glutamatergic.

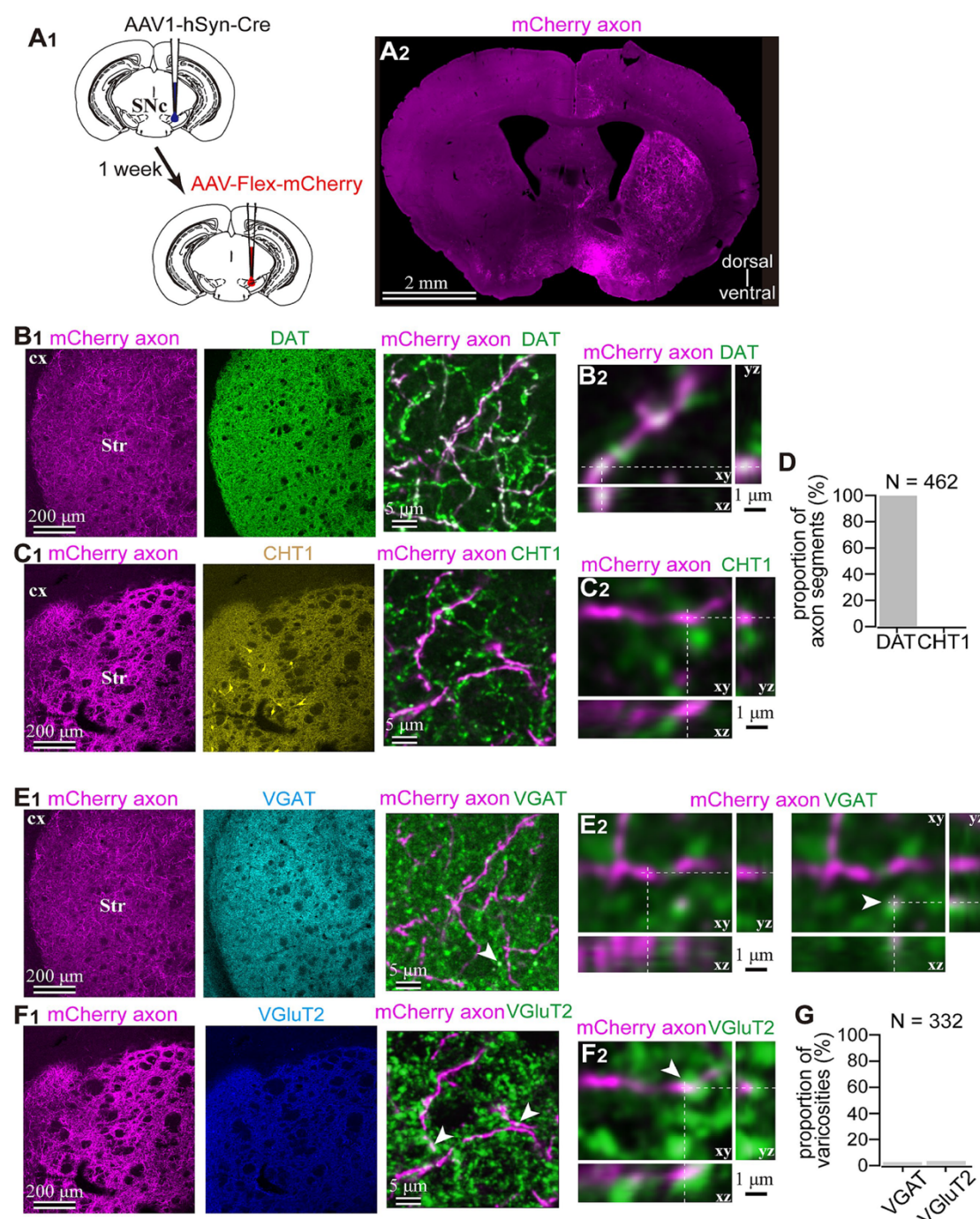


Figure 3. DA identity of striatal axons labeled by AAV1-Cre injection into the SNc. (A) AAV1-dependent labeling of midbrain neurons. (A1) AAV1-hSyn-Cre was injected into the SNc of one hemisphere, followed one week later by an injection of AAV-Flex-mCherry into the same coordinates. (A2) mCherry-labeled axons were observed in the striatum ipsilateral to the injection site. (B–C) Characterization of AAV1-labeled axons in the striatum (Str). (B1, C1) Immunofluorescence for DA transporter (DAT, green in B1) and high-affinity choline transporter (CHT1, yellow in the middle panel of C1; shown in green in the right panel for clarity of co-localization). (B2, C2) High-magnification confocal images in xy, xz, and yz planes. DAT immunofluorescence

overlapped with mCherry-labeled axons (B2), whereas no co-localization with CHT1 was observed (C2). (D) Quantitative summary of DAT and CHT1 co-localization in AAV1-labeled axons. (E–F) Immunostaining for vesicular GABA transporter (VGAT, cyan in the middle panel of E1, green in the right panel) and vesicular glutamate transporter 2 (VGluT2, blue in the middle panel of F1, green in the right panel). (E2, F2) High-resolution confocal images shown in xy, xz, and yz planes. Most mCherry-labeled axon terminals lacked VGAT or VGluT2 immunoreactivity. Occasionally, partial overlap of mCherry with VGAT (arrowhead in E2) or VGluT2 (arrowhead in F2) was observed, although the shapes of the boutons were not identical. (G) Quantification of the proportion of AAV1-labeled varicosities showing VGluT2 or VGAT immunoreactivity.

Given that nearly all AAV1-labeled axons were DA, I next investigated which striatal neuron types expressed Cre. Double immunofluorescence for Cre and neuronal subtype markers revealed that some Cre-positive neurons co-expressed Ctip2, a marker for medium spiny neurons (MSNs) (Figure 4A; Arlotta et al., 2008). Because AAV1 is capable of both retrograde and anterograde trans-synaptic transport (Zingg et al., 2017, 2020), distinguishing between these two labeling modes is difficult. I therefore focused on interneurons, whose axons are restricted to the local striatum, thus, Cre expression in interneurons can be attributed to anterograde trans-neuronal transfer. Indeed, Cre-positive interneurons were detected (Figure 1E). To determine their subtype identity, I performed immunostaining for four canonical interneuron markers: choline acetyltransferase (ChAT), PV, somatostatin (SOM), and nitric oxide synthase (NOS) (Figure 4B).

I then examined whether Cre expression exhibited subtype selectivity among interneurons. As a baseline, the proportions of ChAT-, PV-, SOM-, and NOS-positive neurons were quantified in the DLS of intact mice (N = 20 sections from 3 mice). PV-expressing neurons were slightly more numerous than SOM-expressing ones, but overall, the relative proportions of the four subtypes were comparable (Figure 4C; PV $2.46 \pm 0.35\%$, ChAT $1.94 \pm 0.50\%$, SOM $1.72 \pm 0.07\%$, NOS $2.09 \pm 0.12\%$; $p = 0.045$ for PV vs. SOM, Tukey post-hoc test), consistent with previous data (Muñoz-Manchado et al., 2016). Neuron counts are summarized in Table 3. I then quantified AAV1-driven Cre expression in each interneuron subtype within defined regions of interest (ROIs; $318 \times 318 \mu\text{m}$; N = 4 mice; see Table 4). ROIs lacking Cre signal or a given marker were excluded. As expected from the low abundance of interneurons (Graveland and DiFiglia, 1985), Cre-positive interneurons were fewer than MSNs. Among interneuron

types, ChAT- and PV-positive neurons showed significantly higher Cre expression ratios than SOM- or NOS-positive neurons (Figure 4D1; Table 4). Coefficient of variation (CV) values were also greater for ChAT, PV, and NOS-expressing neurons. When the total Cre-positive neurons were normalized by the number of neurons expressing each marker (Figure 4D2), the ratios were 17.7% for ChAT (N = 283), 15.6% for PV (N = 416), 4.2% for NOS (N = 472), 2.5% for SOM (N = 527), and 8.1% for Ctip2 (N = 14,200). These values were significantly higher in ChAT and PV neurons than in SOM or NOS neurons (Fisher's exact test with Holm correction: $p = 7.12 \times 10^{-4}$ for ChAT vs. PV; $p = 3.37 \times 10^{-21}$ for ChAT vs. SOM; $p = 4.58 \times 10^{-16}$ for ChAT vs. NOS; $p = 5.58 \times 10^{-13}$ for PV vs. SOM; $p = 2.58 \times 10^{-8}$ for PV vs. NOS; $p = 0.155$ for NOS vs. SOM). The overall Cre-positive ratio in Ctip2 neurons (1,058/14,200) was slightly lower than that in the combined interneuron population (148/1,439; $p = 0.00022$, Fisher's exact test), though this difference may reflect sample size disparities. Pooling data from ChAT, PV, NOS, and SOM neurons, the mean Cre-positive proportion was $8.4 \pm 17.8\%$ for interneurons (395 ROIs) and $7.8 \pm 3.9\%$ for Ctip2-positive MSNs (31 ROIs). Although mean values were similar, variance was significantly larger for interneurons ($p = 3.15 \times 10^{-6}$, Wilcoxon signed-rank test).

Table 3 Total counts of the striatal interneurons in intact mice.

Interneuron marker	Number of immunopositive neurons	Number of neurons	Proportion of each interneuron (%)
ChAT	124	6,210	2.00
PV	147	6,474	2.27
NOS	130	6,308	2.06
SOM	105	6,308	1.66

Table 4 Cre-expression in each neuron type

Neuron type marker	Number of ROIs	Proportion of Cre-coexpression in each neuron type in each ROI (%)	CV
ChAT	80	17.4±25.6	1.47
PV	90	14.0±20.3	1.45
NOS	92	4.1±11.6	2.83
SOM	133	2.2±0.8	0.36
Ctip2	31	7.8±3.9	0.5

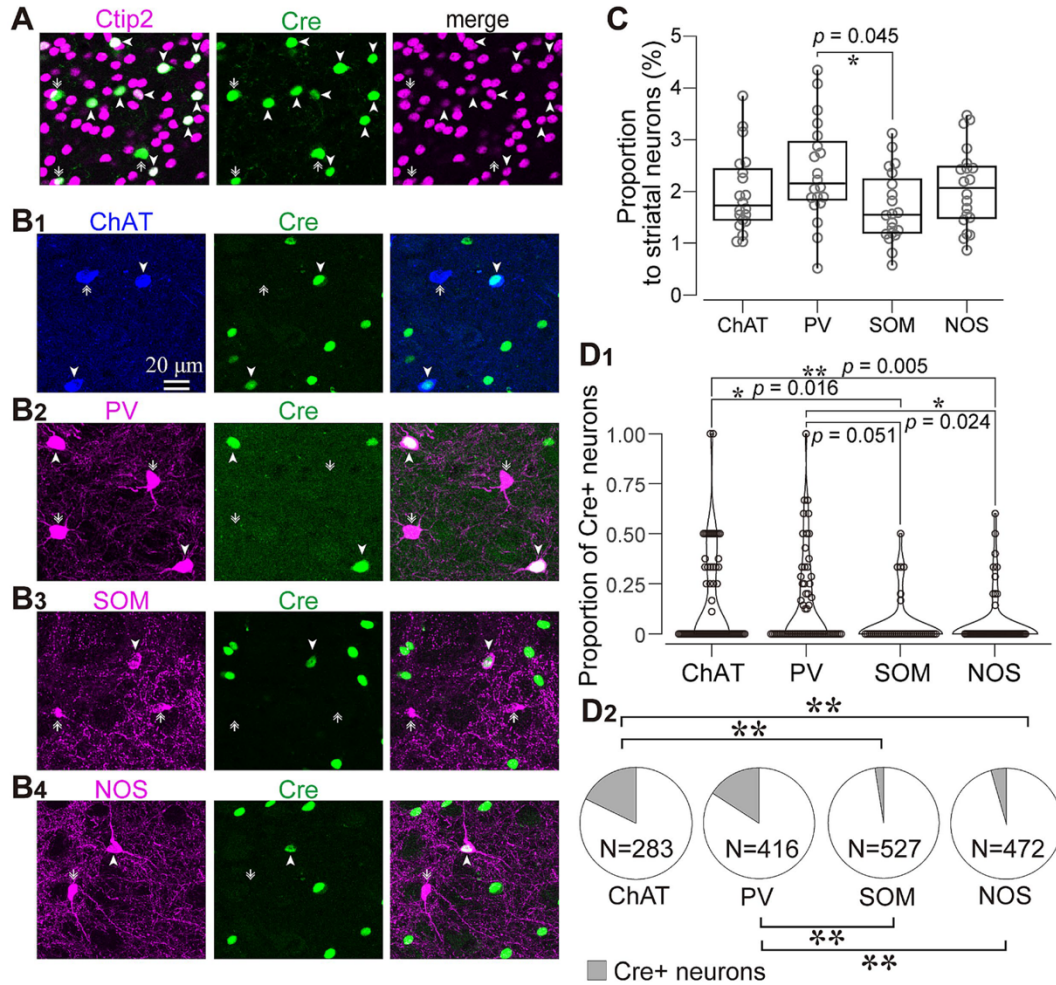


Figure 4. Immunofluorescent identification of striatal cell types labeled by AAV1-Cre. (A) Expression of Ctip2 (magenta) in a subset of Cre-expressing neurons (green) indicates that these cells are medium spiny neurons (MSNs). (B) Expression of striatal interneuron markers in Cre-expressing neurons. Images show immunofluorescence for choline acetyltransferase (ChAT) (blue, B1), parvalbumin (PV) (magenta, B2), somatostatin (SOM) (magenta, B3), and nitric oxide synthase (NOS) (magenta, B4). Arrowheads indicate neurons co-expressing Cre and each interneuron marker, whereas arrows denote neurons expressing the marker without Cre expression. (C) Proportion of four interneuron types relative to the total number of NeuN-positive cells in intact mice. (D) Proportion of Cre-expressing neurons among each interneuron subtype. (D1) Proportion of Cre-positive neurons calculated within each region of interest (ROI). (D2) Cumulative proportion of Cre-expressing neurons across all ROIs. Cre expression was more frequently observed in ChAT- and PV-positive neurons than in other interneuron types. See the text and Table 3 for quantitative details.

2. AAV1-Cre dependent visualization of striatal neurons

To further determine whether the observed trans-neuronal transport was associated with DA axons, I examined Cre-expressing neurons in both the SNc and the striatum. Specifically, one week after injecting AAV1-Cre into the SNc of one hemisphere, I injected AAV-Flex-mCherry into the same coordinates within the SNc. In addition, AAV-Flex-GFP was injected into the striatum of both hemispheres, and the reverse combination of these AAVs was also tested. As shown in Figures 3A and 5A, no fluorescence was observed in the contralateral striatum relative to the AAV1-Cre-injected SNc, whereas bright mCherry and GFP signals were clearly detected in the ipsilateral hemisphere. These findings indicate that Cre-dependent fluorophore expression was strictly confined to the injected side, with negligible leakage. Next, I identified the subtypes of striatal interneurons labeled by Cre-dependent fluorophore expression. Figure 5B shows fluorescently labeled somata and proximal dendrites of striatal neurons, along with immunostaining for specific neuronal markers (N = 4 mice; N = 433 neurons with triple labeling for PV, NOS, and Ctip2; N = 130 neurons with triple labeling for ChAT, SOM, and NPY). All tested markers were detected in neurons labeled in an AAV1-Cre-dependent manner. Among these, 60.89% of labeled neurons expressed Ctip2, identifying them as MSNs (Figures 5B,C). ChAT-positive neurons accounted for 7.69% of the labeled population, similar to the proportions of PV-positive (6.35%) and NOS-positive (5.85%) neurons. In the first triple-labeling combination, no co-expression was observed among Ctip2, PV, and NOS. In the second combination, consistent with previous reports that NPY and SOM are often co-expressed (Kawaguchi et al., 1995; Muñoz-Manchado et al., 2016), SOM-positive neurons constituted a smaller fraction (0.77%) than NPY-positive neurons (3.08%). The remaining ~18% of labeled neurons likely represented other interneuron types, inferred from their aspiny dendritic morphology. When comparing these proportions with those observed in the DLS of intact mice (Figure 4C; Table 3), the AAV1-driven labeling was significantly enriched in ChAT-positive ($p = 5.74 \times 10^{-11}$), PV-positive ($p = 4.48 \times 10^{-6}$), and NOS-positive ($p = 6.44 \times 10^{-6}$) neurons, while the difference for SOM-positive neurons was not significant ($p = 0.249$; Fisher's exact test).

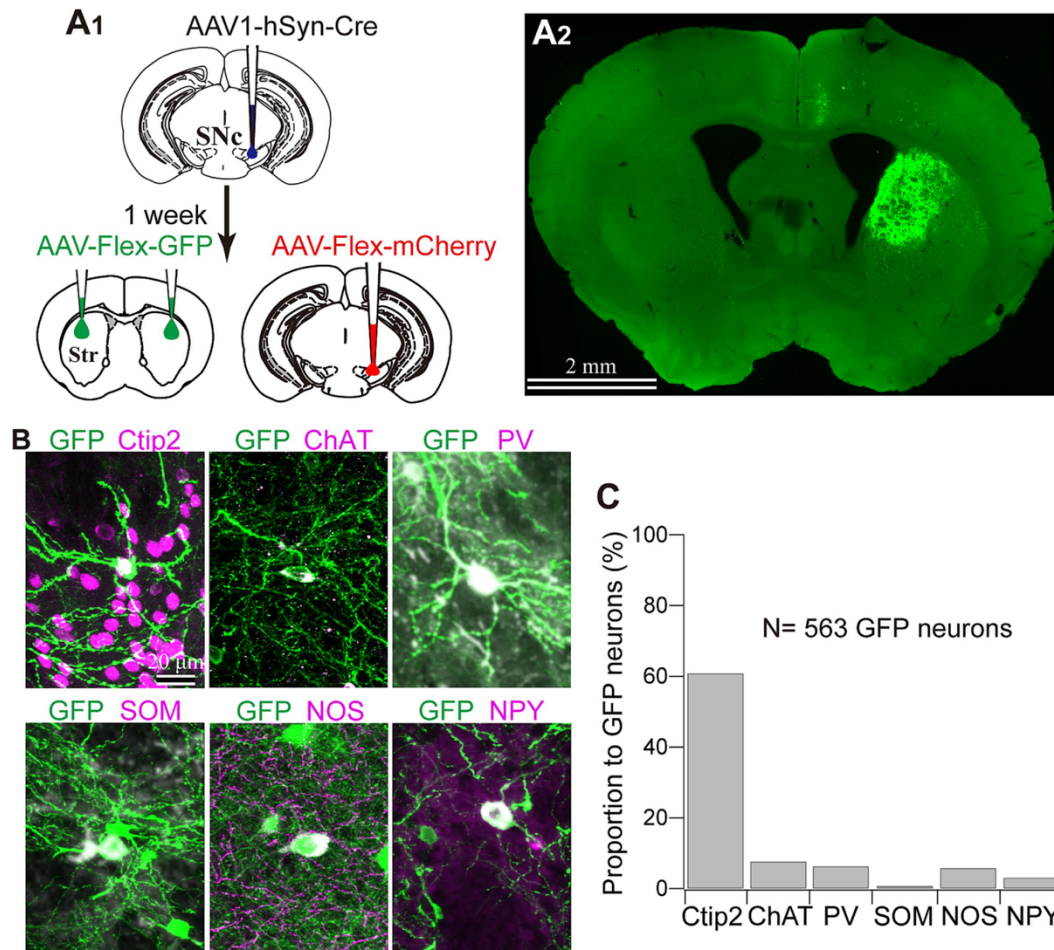


Figure 5. Striatal neuron types labeled by AAV1-mediated trans-neuronal transfer. (A) AAV1-Cre-dependent visualization of striatal neurons. (A1) AAVs encoding mCherry and GFP were injected into the substantia nigra pars compacta (SNc) and striatum (Str), respectively, one week after AAV1-hSyn-Cre injection into the SNc. (A2) GFP expression was detected only in the striatum ipsilateral to the AAV1-Cre-injected SNc, indicating monosynaptic anterograde transfer of Cre recombinase. (B) Representative GFP-positive striatal neurons co-expressing distinct cell-type marker proteins. See Figure 4 and the main text for detailed descriptions of the marker proteins. (C) Quantitative proportion of GFP-expressing neurons positive for each cell-type marker.

3. Relationship between single DA axons and striatal interneurons

Then, in a subset of fluorescently labeled neurons, I directly examined the spatial relationship between individual DA axons and the labeled striatal neurons. Using confocal laser scanning microscopy, I carefully traced and reconstructed the somata and dendritic arbors of these neurons (Figure 6). I then analyzed putative appositions (PAPs) between DA axons and the reconstructed neurons. An example of a non-spiny neuron

is presented in Figure 6A. This neuron lacked ChAT immunoreactivity and possessed a small soma. PAPs were identified on both the soma and the proximal dendrite. In ChAT-positive neurons, PAPs were consistently observed (Figures 6B–D; $N = 8/8$ ChAT neurons). DA axons also established PAPs on PV-positive neurons ($N = 2/2$). In some cases, a single continuous DA axon segment formed multiple PAPs on the same soma and/or proximal dendrites. Furthermore, DA receptor type 2 (D2R) immunoreactivity was occasionally detected adjacent to these PAPs, suggesting the potential presence of functional DA synapses (Figure 6D).

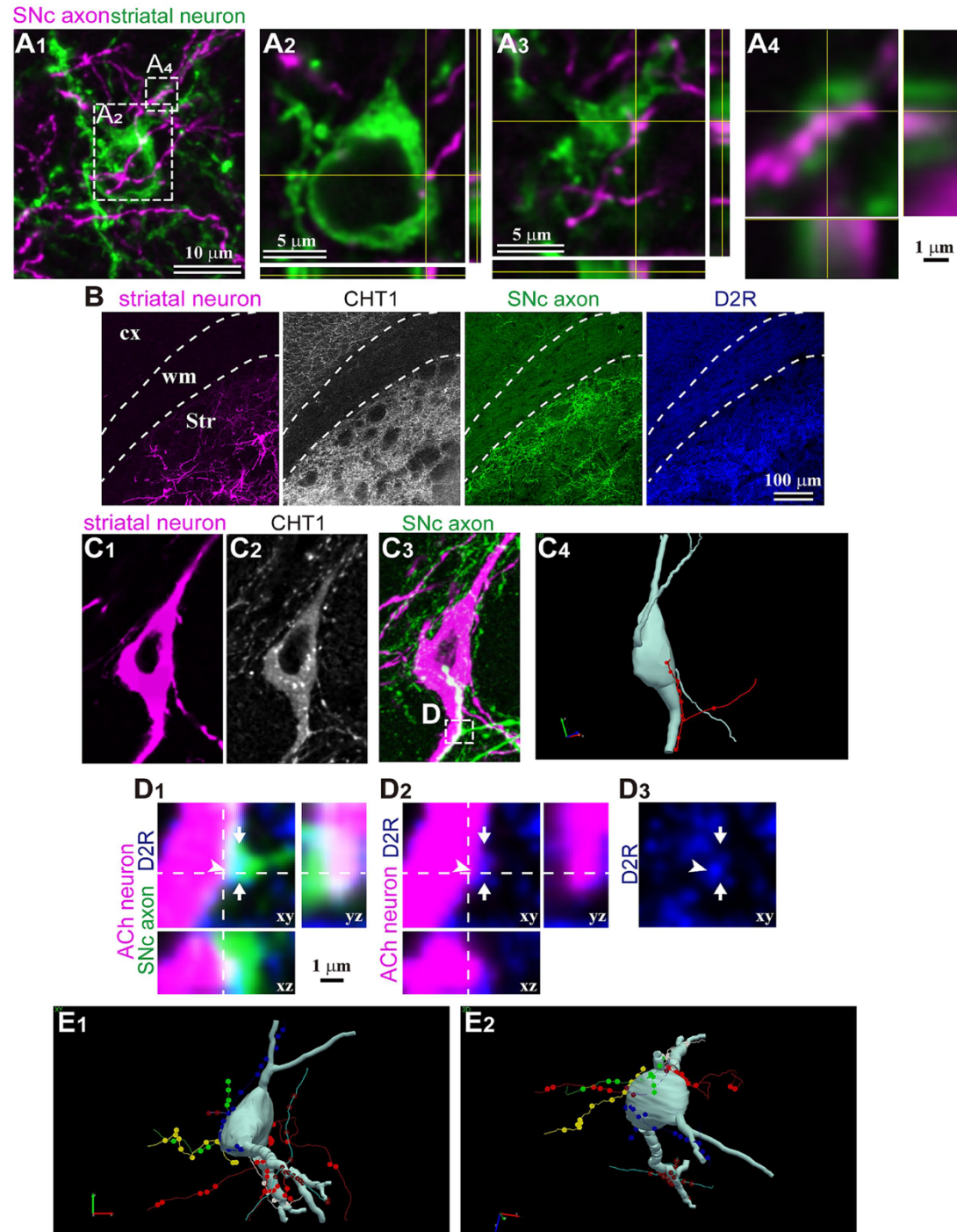


Figure 6. High-magnification confocal views of trans-neuronally labeled striatal neurons and their appositions with SNc axons. (A) Confocal images of an aspiny striatal neuron that did not express any of the tested marker proteins. (A1) The SNc-derived axon (magenta) and the GFP-labeled soma and dendrites of the neuron are shown in xy, xz, and yz planes. (A2–A4) Magnified views of the boxed area in (A1). (A2) One close apposition was identified on the soma. (A3) Two appositions were observed on a proximal dendrite, and (A4) additional appositions were found along the same dendritic branch. (B) Immunofluorescence against high-affinity choline transporter (CHT1) and

DA D2 receptor (D2R) was used to evaluate the relationship between SNc-derived axons and striatal neurons labeled via AAV1-mediated transsynaptic transfer. (C) Example of a cholinergic neuron receiving multiple appositions near its soma. A single SNc axon segment formed a series of ten appositions along the cell surface. (C1) Striatal neurons were visualized using AAV-Flex-mCherry. (C2) The labeled neuron expressed CHT1, confirming its cholinergic (ACh) identity. (C3) SNc axons were labeled with AAV-Flex-GFP. (C4) A rotated three-dimensional reconstruction of the same neuron (gray) showing an axon segment forming close appositions (red). (D) Enlarged confocal view of the boxed region in (C). The section was immunostained for D2R (blue). An axonal varicosity (arrow) formed a close apposition (arrowhead) adjacent to a D2R-positive site. (E) Three-dimensional reconstruction of another cholinergic neuron. Six distinct SNc axon segments (each color-coded) formed close appositions on the soma and proximal dendrites. Two different viewing angles are presented (E1, E2). cx, cerebral cortex; Str, striatum; wm, white matter.

Based on the findings described above, I next performed a detailed analysis of the appositions between SNc DA axons and striatal neurons, including both interneurons and projection neurons. I focused on two major classes of interneurons—PV-INs, CINs—and defined iMSNs by their expression of PPE (PPE-iMSNs). In addition, I examined potential regional differences between the DMS and DLS in those types of neurons.

4. Trans-neuronally labeled striatal neurons show no significant DMS–DLS bias in PV-INs or CINs populations

To identify striatal neurons that receive direct input from SNc DA neurons, I applied a modified AAV1-Cre-based trans-neuronal labeling strategy that was originally established in my laboratory (Fig. 7A). In previous study (Fig. 5; Karube et al., 2024), AAV1-hSyn-Cre was injected one week before AAV-Flex-GFP, a procedure that occasionally resulted in minor mismatches between the two injection sites. To reduce this technical limitation, I injected a premixed solution of AAV1-hSyn-Cre and AAV-Flex-GFP was injected into either the medial or lateral SNc. One week later, I injected AAV-Flex-tdTomato reporter into the corresponding striatal region (DMS or DLS). With this configuration, AAV1-hSyn-Cre was transferred anterogradely through DA axons, inducing tdTomato expression in postsynaptic striatal neurons.

Using this approach, I identified 18 PV-INs co-expressing tdTomato in the DMS and 25 in the DLS, respectively (N = 3 mice). These corresponded to 34.6% (18/52) of PV-

INs and 10.6% (25/235) of all tdTomato-positive neurons in the DMS and DLS, respectively. From the same animals, I also identified 14 CINs in the DMS and 41 in the DLS, respectively (out of 28 CINs and 200 tdTomato-labeled neurons in the DMS, and 140 CINs and 468 tdTomato-positive neurons in the DLS (Fig. 7 B, C, D). Quantitative analysis revealed no significant differences in the proportion of labeled PV-INs or CINs between the DMS and DLS, nor between the two interneuron types within each region (Fig. 7B; $p = 0.277$ in DMS PV-INs vs DLS PV-INs; $p = 0.745$ in DMS CINs vs DLS CINs; $p = 0.422$ in DMS PV-INs vs DMS CINs; $p = 0.99$ in DLS PV-INs vs DLS CINs). This result indicates that the AAV1-Cre system labeled striatal neurons without detectable regional or cell-type differences. . This validation supports the use of this system for subsequent morphological and appositional analyses. A relatively higher proportion of interneuron labeling, compared to their actual proportion among total striatal neurons, was similarly observed in the previous sections (see Discussion).

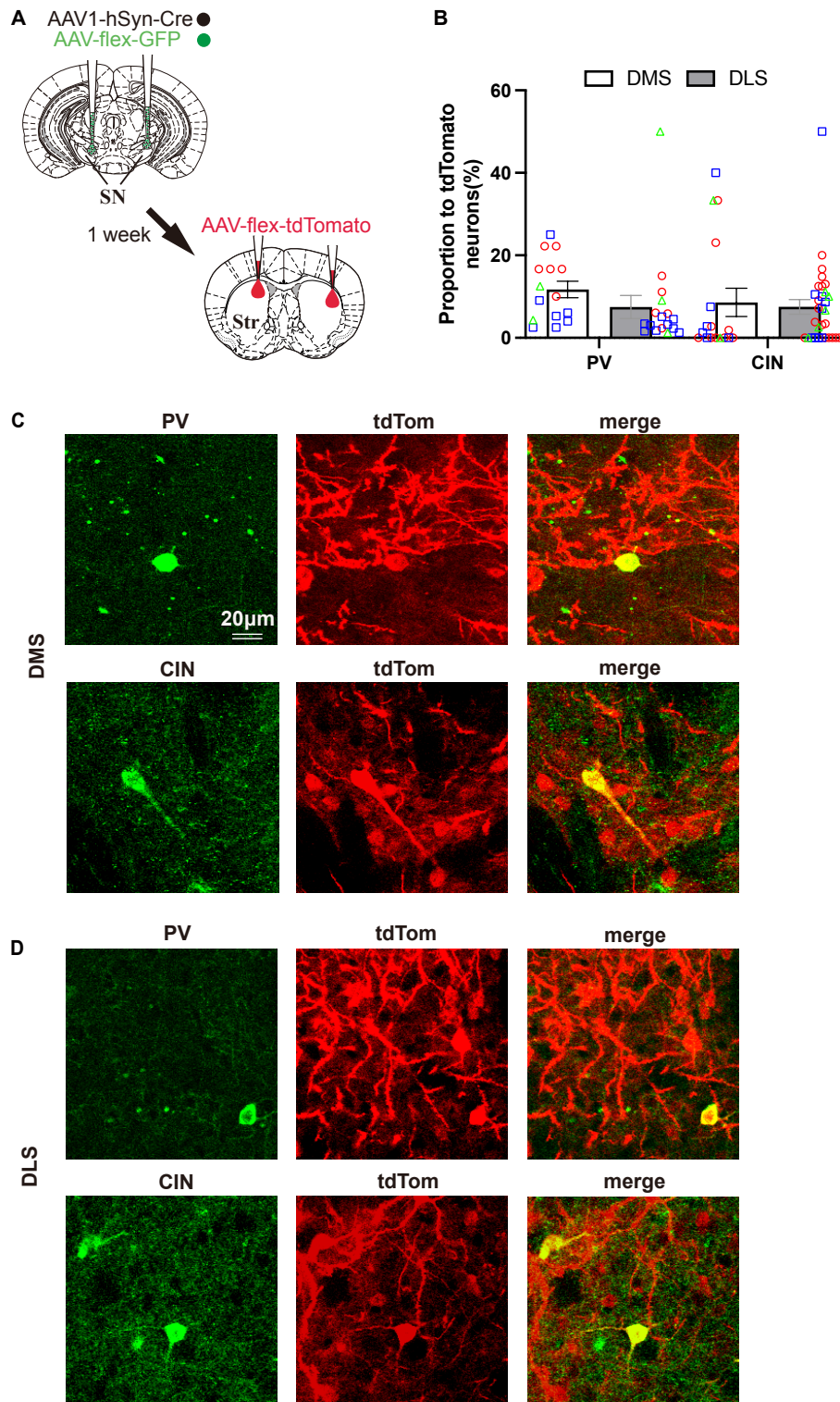


Figure 7. Trans-synaptic labeling of striatal neurons using AAV1-Cre. (A), Schematic illustration of AAV injections. 1:1 mixture of AAV1-hSyn1-Cre and AAV-Flex-GFP was injected into either the medial or lateral SNc. One week later, AAV-Flex-tdTomato was injected into either the dorsomedial striatum (DMS) or dorsolateral striatum (DLS) of the corresponding hemisphere. This approach enabled trans-synaptic labeling of

striatal neurons innervated by SN. (B), Proportion of PV-positive (PV) and cholinergic (ChAT-positive neurons; CIN) interneurons among total tdTomato-labeled striatal neurons in the DMS and DLS. Each dot represents an individual sample from one hemisphere. (C, D), Representative images of tdTomato-labeled striatal PV neurons (top panels) and CIN (bottom panels) in the DMS (C) and DLS (D). Immunofluorescence for PV and ChAT is shown in green (left), and tdTomato expression in red (middle). Merged images are represented in the rightmost panels. All images are shown in the same scale. Data points from individual animals are distinguished by symbol: red circles, green triangles, and blue squares represent the three animals in each group.

5. DA axons form close appositions with striatal PV-INS, CINs, and PPE-iMSNs

To evaluate whether DA axons form close structural appositions with striatal neurons identified by trans-neuronal labeling and immunofluorescence, I analyzed the spatial organization between GFP-labeled axons derived from SNc DA neurons and tdTomato-positive striatal neurons.

For detailed analyses at the level of single neurons, I selected PV-INS, CINs, and PPE-iMSNs sampled that exhibited well-preserved somatic and dendritic morphology among immunohistochemically identified populations (Fig. 8). Confocal Z-projection images showed that GFP-positive axons densely surrounded tdTomato-expressing neurons, and double labeling with DAT immunostaining confirmed the DA identity of these axons (Fig. 8A-C, magenta). Three-dimensional confocal images further revealed numerous GFP-positive axonal varicosities forming PAPs along both the somata (Fig. 8B) and dendrites (Fig. 8C) of labeled neurons. Equivalent analyses demonstrated similar PAPs from DA axons in all three types of striatal neurons, with PAPs distributed throughout both the somatic and dendritic compartments. These findings indicate that DA axons establish close structural associations with diverse striatal cell types in both DMS and DLS, potentially serving as conduits for AAV1-Cre transfer.

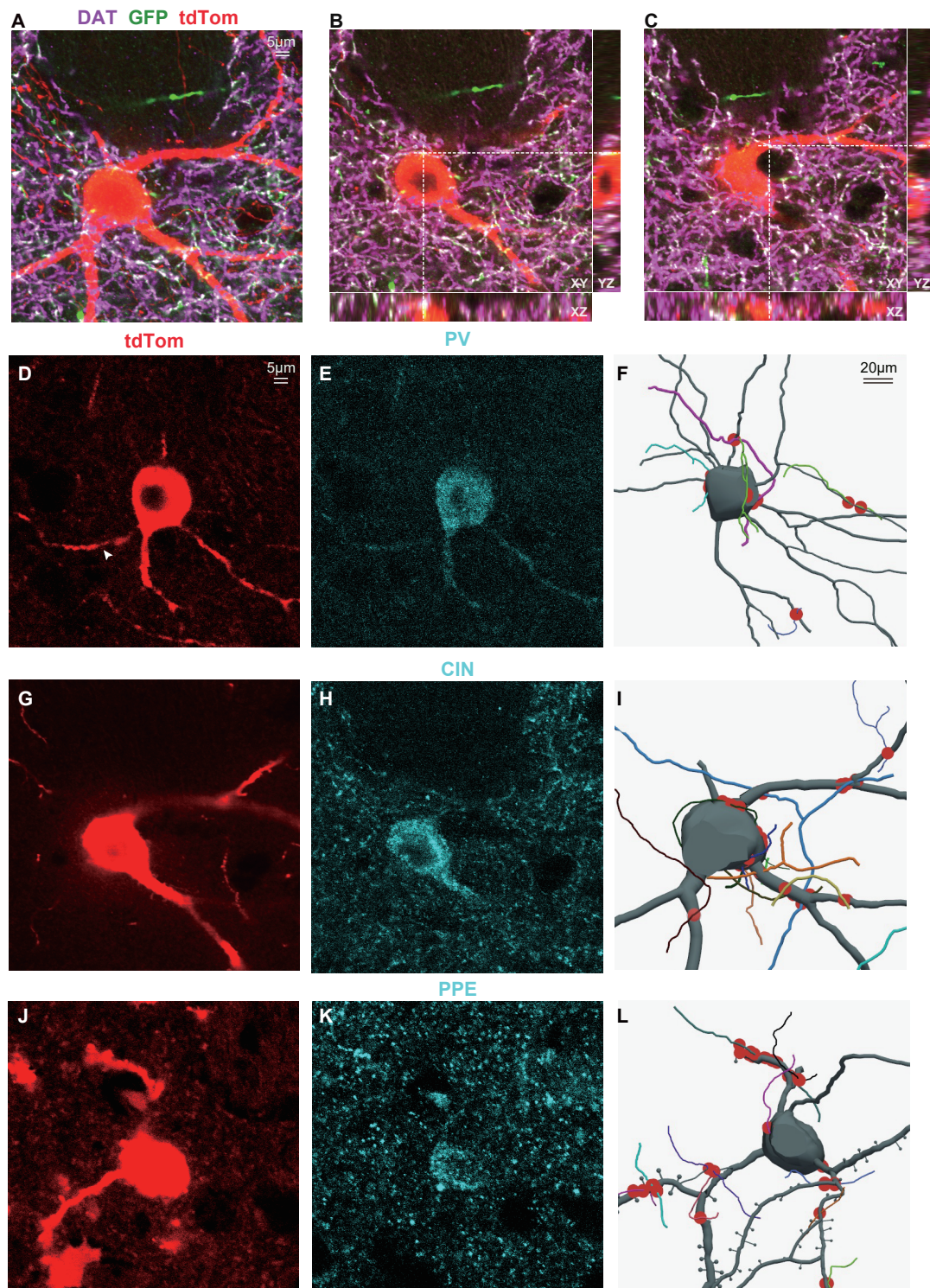


Figure 8. Close appositions between GFP-labeled DA axons and tdTomato-labeled striatal neurons. (A), Representative confocal Z-projection images of a cholinergic (ChAT-positive neurons; CIN) interneuron (red) surrounded by GFP-labeled axons (green), Immunofluorescence of DA transporter (DAT; magenta) confirms the DA identity of GFP-labeled axons. (B, C), Three-dimensional confocal images visualized

multiple GFP-positive axonal varicosities forming PAPs onto the soma (B) and dendrites (C) on the same CIN sample as shown in panel A. (D, G, J), Representative images showing tdTomato-labeled somata of PV, CIN, and PPE neurons, respectively, and arrows indicate dendrites not originating from this neuron. The CIN in panel G corresponds to the same neuron shown in panels A–C. (E, H, K), Corresponding images confirming neuronal identity by immunostaining with antibodies against PV, CHT1/ChAT, and PPE, respectively. (F), Corresponding 3D-reconstruction of the same neuron shown in D, soma and dendrites are shown in dark gray. GFP-labeled axons forming PAPs are reconstructed and shown as colored line drawings. PAPs are indicated as red circles on the soma and dendrite. (I), Equivalent confocal images and 3D-reconstructions of the same CIN shown in panels A–C and G. (L), Equivalent images and 3D-reconstructions for prepro-enkephalin (PPE)-positive neuron, representing putative indirect pathway medium spiny neuron (iMSN). Scale bars shown in the panels A, D, and F apply to the corresponding panels.

6. Morphological and quantitative analysis of DA appositions onto striatal PV-INs, CINs, and PPE-iMSNs

To enable quantitative analysis, I performed three-dimensionally reconstructions of individual selected tdTomato-labeled striatal neurons together with the DA axon segments apposed to them. Reconstructions were generated from confocal Z-stack images (Fig. 8D–E, G–H, J–K) using Neurolucida software (Fig. 8F, I, L). Overall, the reconstructed morphologies of the three neuron types exhibited characteristic structural features consistent with those reported in previous studies (Cutuli et al., 2011; Poppi et al., 2021; Bicanic et al., 2017; Park et al., 2025).

The reconstructed neurons analyzed in this study are shown in Figs 9–11, including 7 PV-INs in the DMS (Fig. 9A) and 4 in the DLS (Fig. 9B); 8 CINs in the DMS (Fig. 10A) and 6 in the DLS (Fig. 10B); as well as 5 PPE-iMSNs in the DMS (Fig. 11A) and 4 in the DLS (Fig. 11B). For each neuron, the 3D reconstruction shown in Figures 9–11 was rotated to display the distribution of PAPs optimally. Enlarged views highlighting PAPs located on the soma are shown in Fig. 13, whereas dendritic PAPs are presented in Fig. 14. Reconstructed PV-INs displayed moderately sized somata, with volumes of $575.35 \pm 220.44 \mu\text{m}^3$ in the DMS and $709.38 \pm 244.58 \mu\text{m}^3$ in the DLS, and possessed an average of 6.43 ± 2.07 primary dendrites in the DMS and 6.75 ± 0.96 in the DLS, matching previous reports (Cutuli et al., 2011). Reconstructed CINs exhibited substantially larger somata in both the DMS ($1235.69 \pm 392.55 \mu\text{m}^3$ in soma volume) and DLS ($695.72 \pm 147.08 \mu\text{m}^3$ in soma volume) (cf. Poppi et al., 2021).

Notably, CINs in the DMS had significantly larger cell bodies than those in the DLS ($p = 0.0064$ in soma volume, $p = 0.0468$ in somatic maximum cross-sectional area, and $p = 0.0043$ in soma surface area, Fig. 15A-C). Within the DMS, CINs also exhibited significantly larger cell bodies than PV-INs ($p = 0.0016$ in soma volume, $p = 0.0013$ in somatic maximum cross-sectional area, and $p = 0.0014$ in soma surface area; Fig. 15A-C). With respect to dendritic architecture, CINs in both the DMS and DLS possessed significantly fewer dendrites than PV-INs ($p = 0.0019$ in the DMS, $p = 0.0387$ in the DLS; Fig. 15D). However, CINs did not show regional differences in the number of dendrites ($p = 0.36$; Fig. 15D) or in total dendritic length ($p = 0.076$; Fig. 15E). In contrast, the average dendritic length was greater in DMS CINs than in DLS CINs ($p = 0.012$, Fig. 15F). Reconstructed PPE-iMSNs were characterized by relatively smaller somata, particularly in the DMS, where the cell bodies of PPE-iMSNs are significantly smaller than those of CINs ($p = 0.0027$ in soma volume, $p = 0.0041$ in somatic maximum cross-sectional area, and $p = 0.0007$ in soma surface area; Fig. 15A-C). Although a similar tendency was observed in the DLS, this difference did not reach statistically significant ($p = 0.6436$ in soma volume, $p = 0.2166$ in somatic maximum cross-sectional area, and $p = 0.69$ in soma surface area). Those results fall within the quantitative morphometric ranges previously reported for striatal medium spiny neurons, including soma size, primary dendrite number, and total dendritic length (Bicanic et al., 2017; Park et al., 2025).

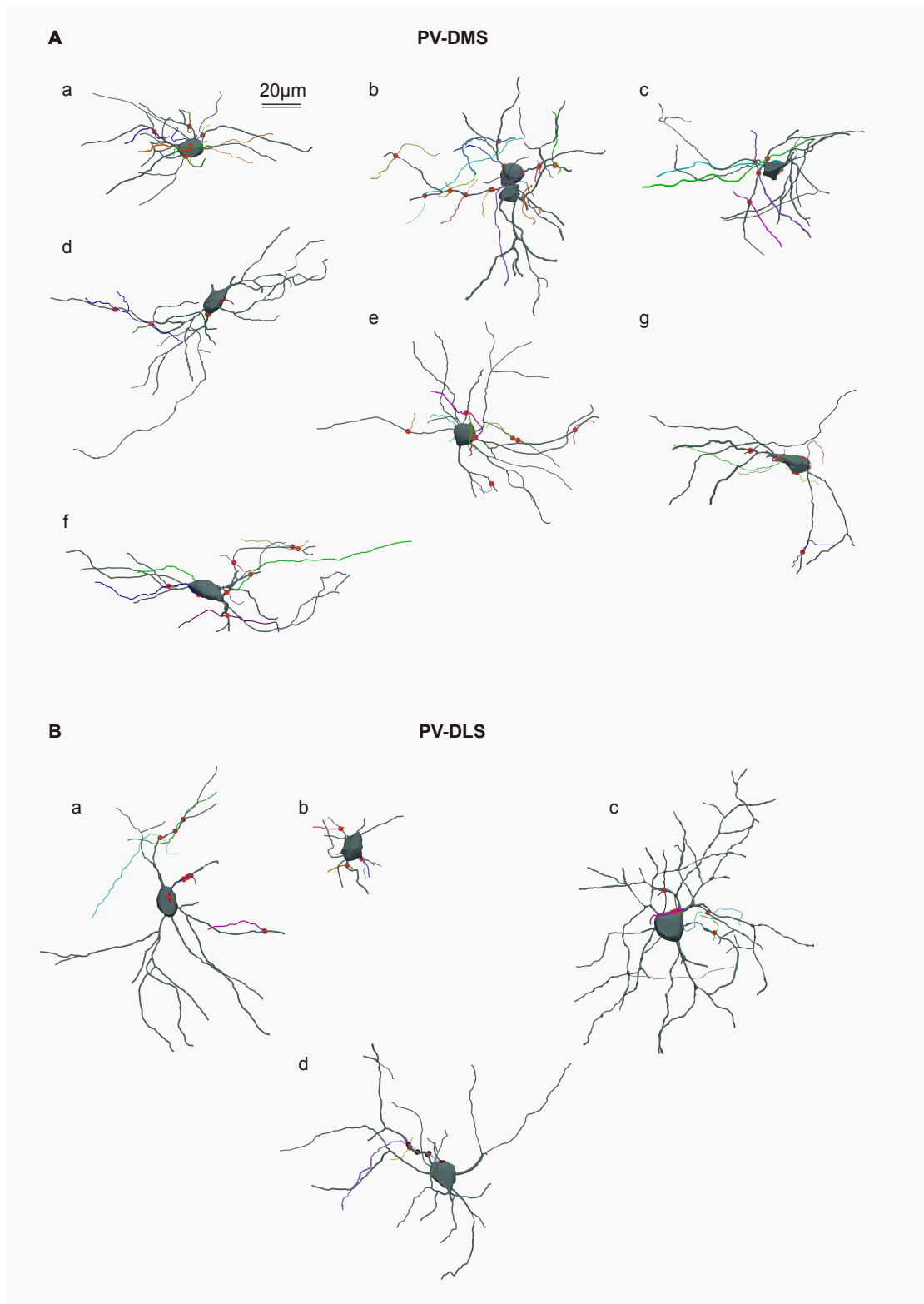


Figure 9. 3D-reconstructions of tdTomato-labeled PV-INs. (A), PV-positive interneurons in the DMS. Reconstructions are presented using the same conventions as in Figure 2, with soma and dendrites rendered in dark gray and GFP-labeled DA axons

represented as colored line drawings. PAPs are shown as red circles. (B), PV-positive interneurons in the DLS. The scale bar shown in A applies to all panels.

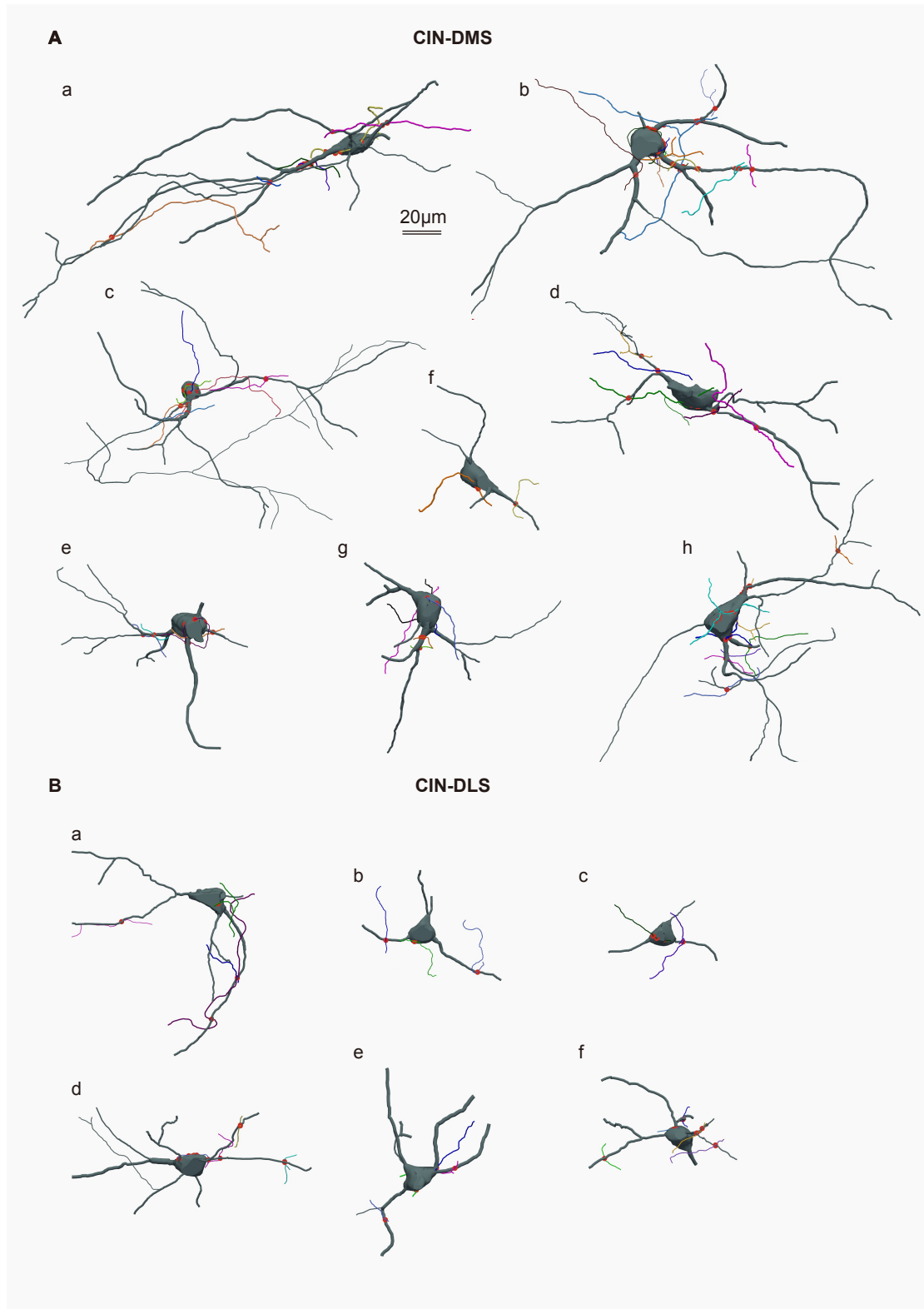


Figure 10. 3D-reconstructions of tdTomato-labeled CINs. (A), CINs in the DMS. Reconstructions are presented using the same conventions as in Figure 9, with soma

and dendrites rendered in dark gray and GFP-labeled DA axons represented as colored line drawings. PAPs are shown as red circles. (B), CINs in the DLS. The scale bar shown in A applies to all panels.

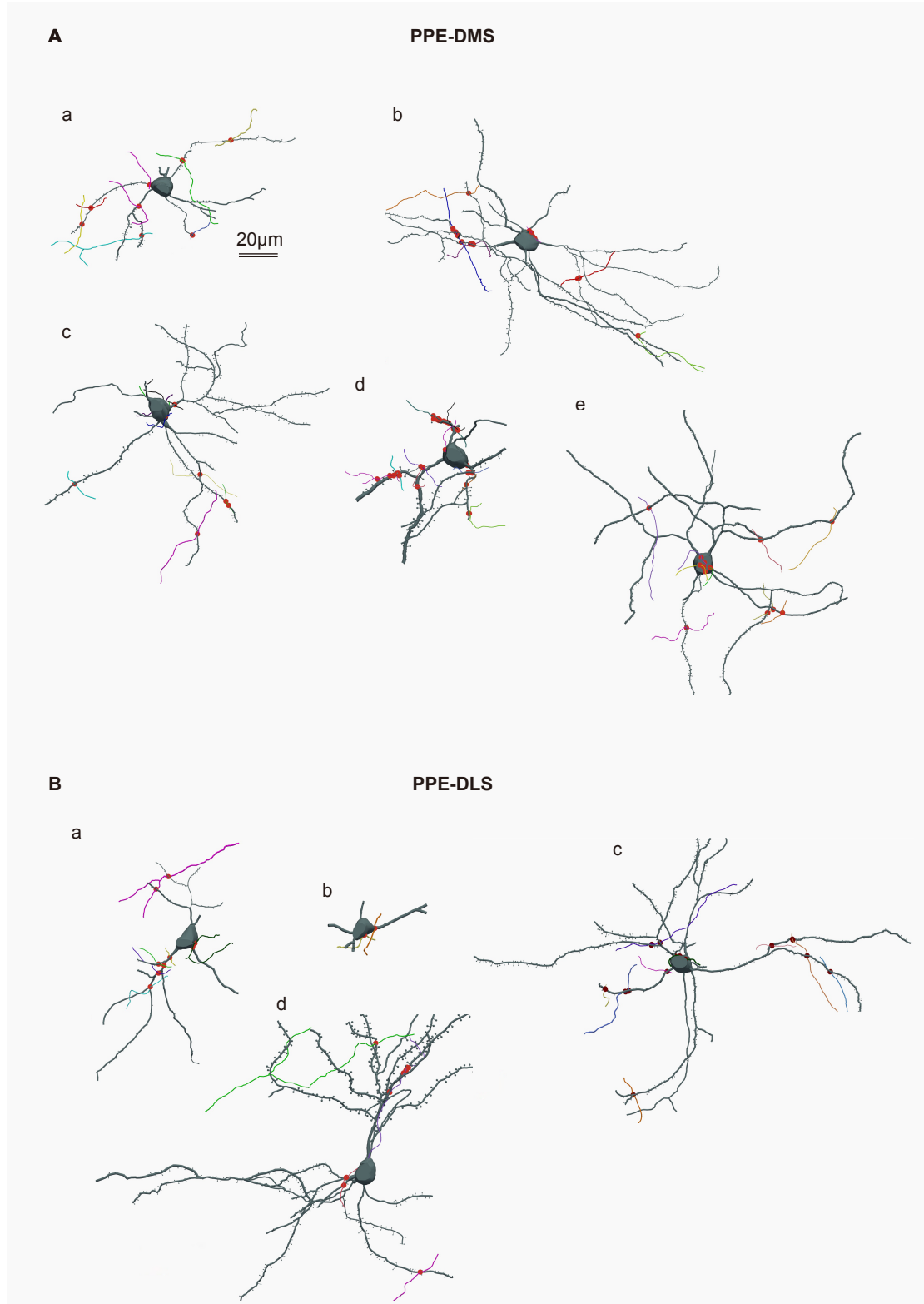


Figure 11. 3D-reconstructions of tdTomato-labeled PPE-iMSNs. (A), PPE-iMSNs in the DMS. Reconstructions are presented using the same conventions as in Figure 9, with soma and dendrites rendered in dark gray and GFP-labeled DA axons represented as colored line drawings. PAPs are shown as red circles. (B), PPE-iMSNs in the DLS. The scale bar shown in A applies to all panels.

To further characterize the spatial interactions between DA axons and different types of striatal neuron, I performed quantitative analyses on high-magnification views of PAPs reconstructed from PV-INs, CINs, and PPE-iMSNs. Such quantitative structural analysis is essential because dendritic architecture of striatal neurons defines the spatial framework for synaptic and nonsynaptic DA signaling (Cazorla et al., 2012; Park et al., 2025). As shown in Figure 12, for somatic PAPs, I quantified the total number of PAPs located on the soma (Fig. 16A); the density of PAPs per unit soma surface area (Fig. 16B); the number of DA axons that form PAPs on soma with each neuron (Fig. 16C); the proportion of axons that form PAPs with the cell body to the total number of axons (Fig. 16D); and the proportion of axons that form multiple (≥ 2) PAPs with the cell body to the total number of axons (Fig. 16E). For dendritic PAPs, I measured the total number of PAPs located on the dendrites (Fig. 17A); the density of PAPs per unit dendritic length (Fig. 17B); the number of DA axons that form PAPs on the dendrites with each neuron (Fig. 17C); the distance from the origin of dendrite to each dendritic PAP (Fig. 17D); the ratio of soma versus dendritic appositions (Fig. 17E); the ratio of DA axons forming soma versus dendritic appositions (Fig. 17F); and the number of PAPs formed by individual DA axons (Table 5). To examine whether the extent of somatic apposition scaled with neuronal size, I conducted Pearson's correlation analyses between the soma surface area and the number of DA varicosities on the soma for the three neuronal types in both the DMS and DLS. No significant correlations were detected for any neuronal type or region (for PV-INs, $p = 0.4887$, $r = 0.3169$ in DMS, $p = 0.1429$, $r = 0.8571$ in DLS; for CINs, $p = 0.7286$, $r = 0.1468$ in DMS, $p = 0.8280$, $r = 0.1152$ in DLS; for PPE-iMSNs, $p = 0.6213$, $r = -0.3021$ in DMS, $p = 0.0754$, $r = -0.9246$ in DLS).

Somatic parameters

Number of somatic DA PAPs

Number of axons forming PAPs on soma

Dendritic parameters

Number of dendritic DA PAPs

Number of axons forming PAPs on dendrites

Distance from dendritic PAPs to the soma

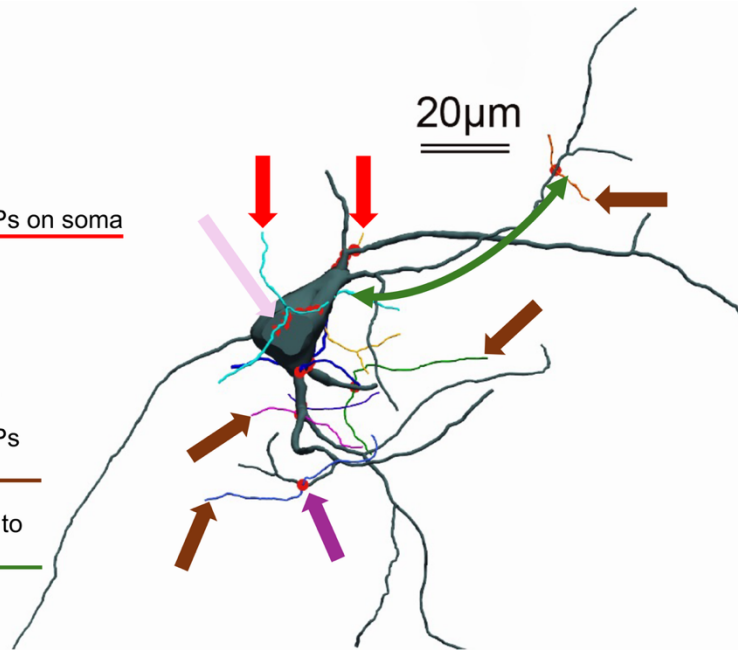


Figure 12. Schematic illustration of quantitative parameters for PAPs analysis. A reconstructed striatal neuron is shown, with the soma, axon, and dendrites depicted in gray. DA axons are represented by colored lines, and PAPs are indicated by red dots. Somatic parameters mainly include the total number of somatic PAPs and the number of DA axons forming PAPs on the soma. Dendritic parameters mainly include the total number of dendritic PAPs, the number of DA axons forming PAPs on dendrites, and the distance from dendritic PAPs to the soma. Identical colored underlines and arrows are used to indicate the corresponding parameters in the schematic. Scale bar, 20 μm .

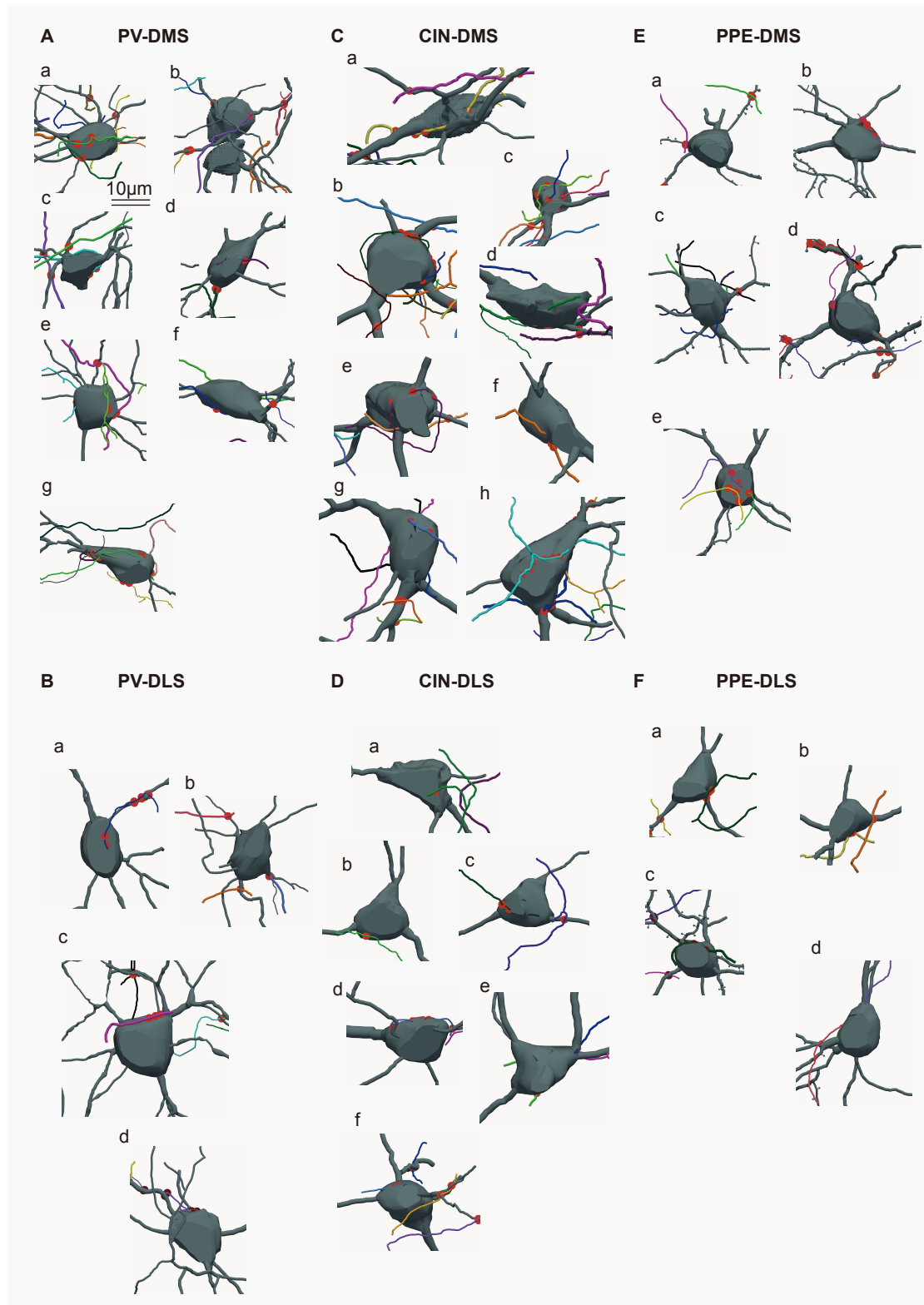


Figure 13. High-magnification views of PAPs proximal to the soma. Detailed reconstructions of striatal neurons shown in Figures 9-11 are presented at higher magnification. Each reconstruction is rotated to emphasize PAPs (red circles). Neurons located in the DMS are in the top panels (A, C, E) and those in the DLS are shown in

the bottom panels (B, D, F). (A, B), PV-INs. (C, D), CINs. (E, F), PPE- iMSNs. The scale bar shown in A applies to all panels.

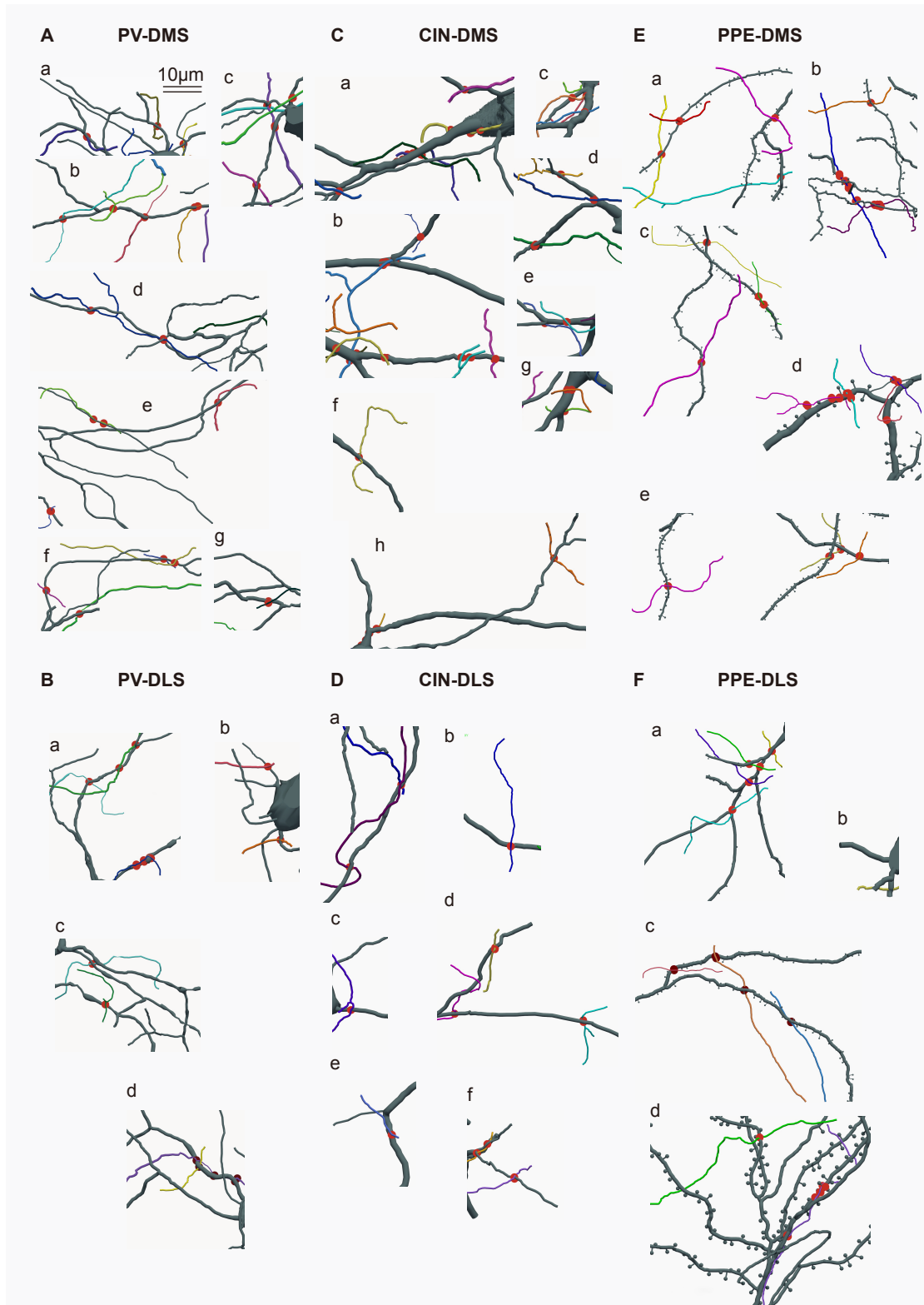


Figure 14. High-magnification views of PAPs distal to the soma. Detailed reconstructions of striatal neurons shown in Figures 9-11 are presented at higher

magnification. Each reconstruction is rotated to emphasize PAPs (red circles). Neurons located in the DMS are in the top panels (A, C, E) and those in the DLS are shown in the bottom panels (B, D, F). (A, B), PV-INs. (C, D), CINs. (E, F), PPE- iMSNs. The scale bar shown in A applies to all panels.

Specifically, on soma, in CINs, the number of somatic PAPs was significantly greater in the DMS than in the DLS ($p = 0.0121$, Fig. 16A). When PAPs counts were normalized to soma surface area, the density of somatic PAPs showed modest regional differences (Fig. 16B): DMS PV-INs tended to exhibit higher values than DLS PV-INs ($p = 0.1604$), and DMS CINs showed slightly higher values than DLS CINs ($p = 0.0729$), yet neither reached statistical significance. Next, for both PV-INs and CINs, the number of DA axons that form PAPs on soma was significantly higher in DMS than in DLS ($p = 0.0443$ in PV-INs, $p = 0.0489$ in CINs, Fig. 16C). To further evaluate the relative abundance of somatic PAPs across neuron type and striatal regions, I quantified the proportion of axons forming somatic PAPs relative to the total number of labeled axons. This analysis revealed a tendency toward a higher proportion in DMS CINs compared with DMS PPE-iMSNs, although the difference did not reach statistical significance ($p = 0.3284$, Fig. 16D). I also assessed the fraction of DA axons formed multiple (≥ 2) somatic PAPs (Fig. 16E). Several non-statistically significant trends were observed, including higher values in DMS CINs compared with DLS CINs ($p = 0.2916$), in DMS PV-INs compared with DMS PPE-iMSNs ($p = 0.3718$), and in DMS CINs compared with DMS PPE-iMSNs ($p = 0.3604$). I then quantified the PAPs on the dendrites. The number of dendritic PAPs did not differ significantly among cell types or striatal regions (Fig. 17A, $p > 0.05$). When dendritic PAPs were normalized to dendritic length, the density of PAPs per unit length showed modest trends (Fig. 17B). In particular, DMS PPE-iMSNs tended to exhibit higher densities than DLS PPE-iMSNs ($p = 0.0623$), whereas DMS PV-INs showed slightly lower densities than DMS PPE-iMSNs ($p = 0.0889$). In addition, I found no significant difference on the number of DA axons forming dendritic PAPs across neuronal types or regions (Fig. 17C). In contrast, the spatial distribution of dendritic PAPs along the dendritic arbor revealed several significant differences (Fig. 17D). Within the DMS, PAPs on PV-INs were located significantly farther from the soma than those on CINs ($p = 0.0356$), but closer than those on PPE-iMSNs ($p = 0.0477$). Within the DLS, PAPs on PV-INs were positioned markedly closer to the soma than those on PPE-iMSNs ($p < 0.0001$). In both the DMS and DLS, CINs exhibited PAPs located significantly closer to the soma than those on PPE-iMSNs (DMS: $p <$

0.0001; DLS: $p = 0.0002$). Furthermore, when comparing the same neuronal subtype across regions, PPE-iMSNs in the DMS displayed significantly shorter distances from PAPs to the soma than those in the DLS ($p = 0.0045$). To assess whether DA appositions preferentially occurred on the soma or dendrites, I calculated two ratio-based measures for each neuronal subtype in DMS and DLS: the ratio of somatic to dendritic appositions (Fig. 17E) and the ratio of DA axons forming somatic versus dendritic appositions (Fig. 17F). Neither ratio revealed significant differences between cell types or between striatal regions. Nevertheless, both ratios displayed consistent trends that mirrored the distribution patterns observed in the absolute counts of apposition. Finally, Table 5 summarizes the number of DA varicosities forming PAPs with individual DA axons for each neuronal subtype. Values shown in parentheses indicate varicosities located on the soma, with the remaining varicosities located on the dendrites. Although part of trends mentioned above did not achieve statistical significance, they nonetheless suggest potential cell-type- and region-dependent variability in the spatial organization and relative density of DA appositions within the dorsal striatum, consistent with the broader structural organization of striatal microcircuits described in recent anatomical and disease-related studies of cholinergic interneurons and medium spiny neurons (Pappas et al., 2015; Paz et al., 2021).

1

2 Table 5. Summary of the number of varicosities on soma and dendrites in each neuron

		number of varicosities in each axon (on soma)											
		axon1	axon2	axon3	axon4	axon5	axon6	axon7	axon8	axon9	axon10	axon11	axon12
PV	DMS	4(4)	2(2)	1(1)	1(0)	1(0)	1(0)	1(0)					
		2(2)	1(1)	1(0)	1(0)	1(0)	1(0)	1(0)	2(0)	1(0)	1(0)	1(0)	
		2(2)	1(0)	2(0)	1(0)								
		3(3)	1(0)	2(0)									
		3(3)	2(2)	3(2)	1(0)	1(0)	2(0)	1(0)					
		5(4)	2(1)	1(0)	1(0)	1(0)	1(0)	1(0)					
		1(0)	1(0)	2(2)	3(3)	2(2)	3(3)	1(0)					
	DLS												
		5(2)	1(0)	2(0)	1(0)								
		1(1)	1(0)	1(0)									
		3(3)	2(0)	1(0)	1(0)								
CIN	DMS												
		6(3)	3(0)	1(0)	2(0)	5(0)	1(0)	1(0)					
		11(11)	6(6)	2(2)	3(1)	1(0)	6(0)	1(0)	1(0)	1(0)	1(0)	2(0)	2(0)
		4(4)	2(2)	2(2)	2(0)	1(0)	1(0)						
		1(0)	1(0)	1(0)	1(0)	7(6)							
		5(3)	2(1)	1(0)	1(0)								
		1(0)	4(4)										
		3(3)	1(1)	1(1)	2(0)	2(0)							
		1(0)	6(3)	6(6)	7(6)	1(0)	1(0)	1(0)	1(0)				
	DLS												
		1(1)	1(0)	1(0)	1(0)								
		1(0)	1(0)	1(1)									
		1(0)	3(3)										
		5(4)	1(0)	1(0)	1(0)								
		4(4)	1(1)	1(0)	1(0)								
PPE	DMS	3(3)	1(0)	2(0)	1(0)	1(0)							
		1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)				
		1(0)	4(0)	3(0)	4(4)	2(0)	1(0)						
		1(0)	1(1)	2(2)	3(3)	1(0)	1(0)	2(0)	1(0)				
		2(2)	2(1)	1(0)	7(0)	2(0)	1(0)	2(0)	1(0)	2(0)	3(0)	1(0)	
	DLS	2(2)	1(1)	3(3)	1(0)	1(0)	1(0)	2(0)	1(0)	1(0)			
		3(3)	2(0)	1(0)	2(0)	1(0)	1(0)						
		3(3)	1(1)										
		3(3)	2(0)	1(0)	2(0)	1(0)	1(0)	1(0)	2(0)	1(0)			
		1(0)	4(0)	2(0)	1(0)								

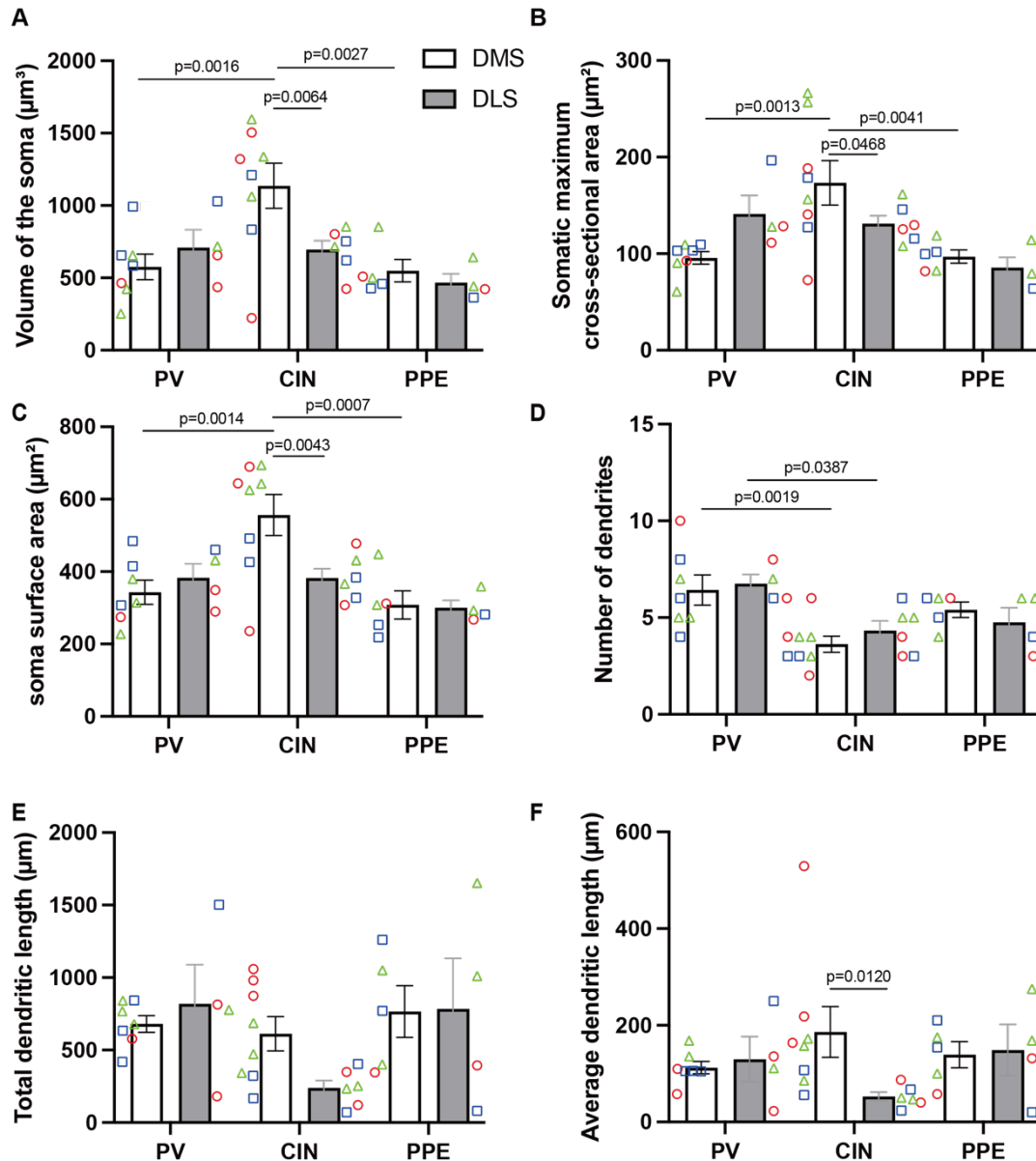


Figure 15. Morphometric parameters of reconstructed striatal neurons. (A), Soma volume of PV-INs, CINs and PPE-iMSNs in the DMS (blank bars) and DLS (gray bars). Each circle represents individual neuron. CINs in the DMS exhibit significantly larger somata than other groups (see Results for details). (B), Maximum cross-sectional area of somata. CINs in the DMS again show the largest somatic profiles among examined groups. (C), Soma surface area. Similarly, CINs in the DMS show the largest somatic profiles among the examined groups. (D), Number of primary dendrites emerging from the soma. PV neurons exhibit a greater number of dendrites than CINs in both the DMS and DLS. (E), Total dendritic length per neuron. (F), Average length of dendrites per neuron. CINs in the DMS exhibit longer dendrites than those in the DLS. Data points from individual animals are distinguished by symbol: red circles, green triangles, and

blue squares represent the three animals in each group.

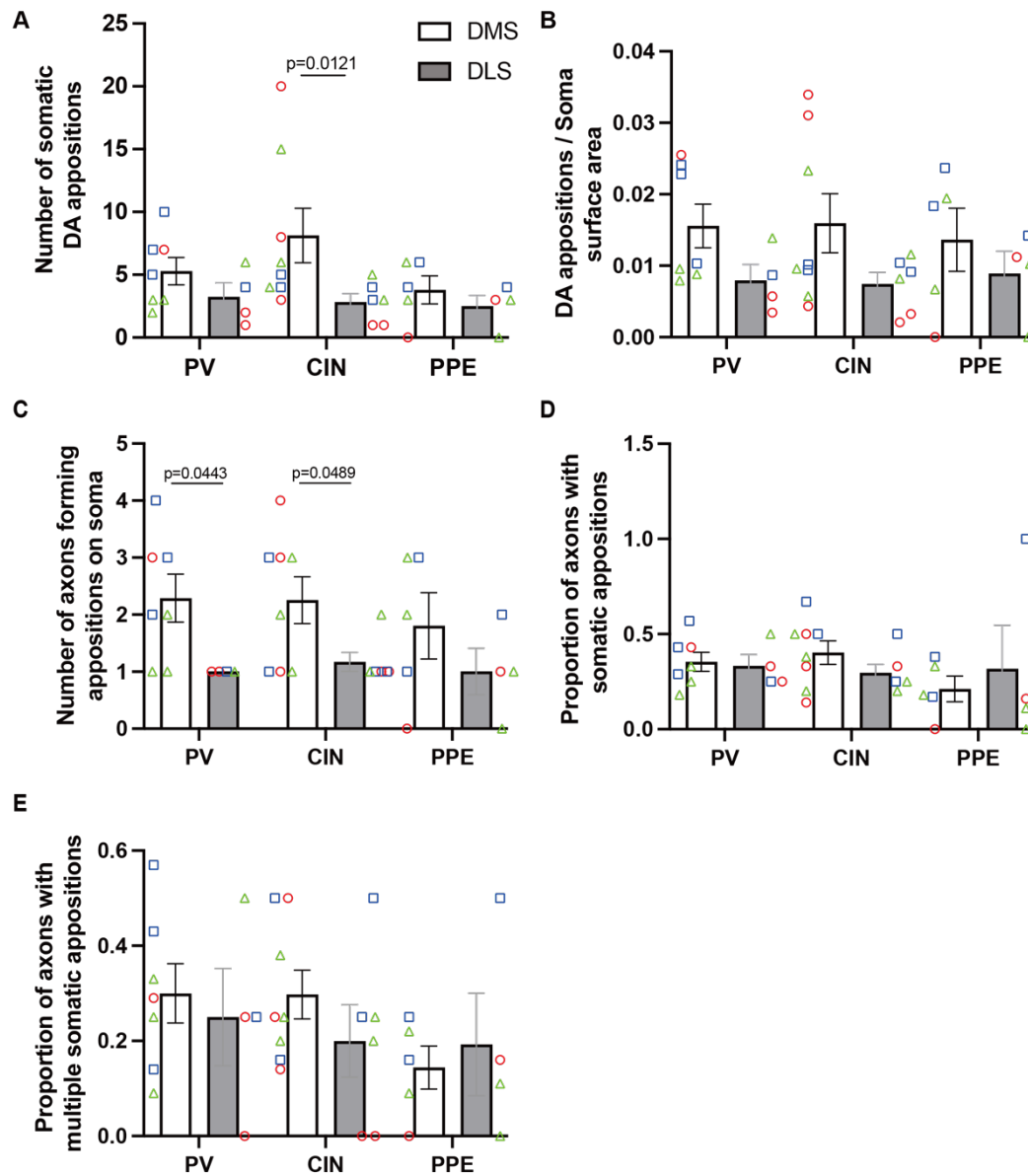


Figure 16. Quantitative analysis of DA somatic appositions in PV-INs, CINs and PPE-iMSNs. (A), Number of DA PAPs per soma. A significant difference was detected between the DMS (blank bars) and DLS (gray bars) for CINs. (B), Density of somatic PAPs per unit somatic surface area. (C), Number of DA axons forming somatic appositions per neuron. CINs and PV-INs in the DMS exhibited a higher number than those in the DLS. (D), Proportion of DA axons forming somatic appositions in PV-INs, CINs, and PPE-iMSNs in the DMS and DLS. (E), Proportion of DA axons forming multiple somatic PAPs on the same neuron. Data points from individual animals are distinguished by symbol: red circles, green triangles, and blue squares represent the three animals in each group.

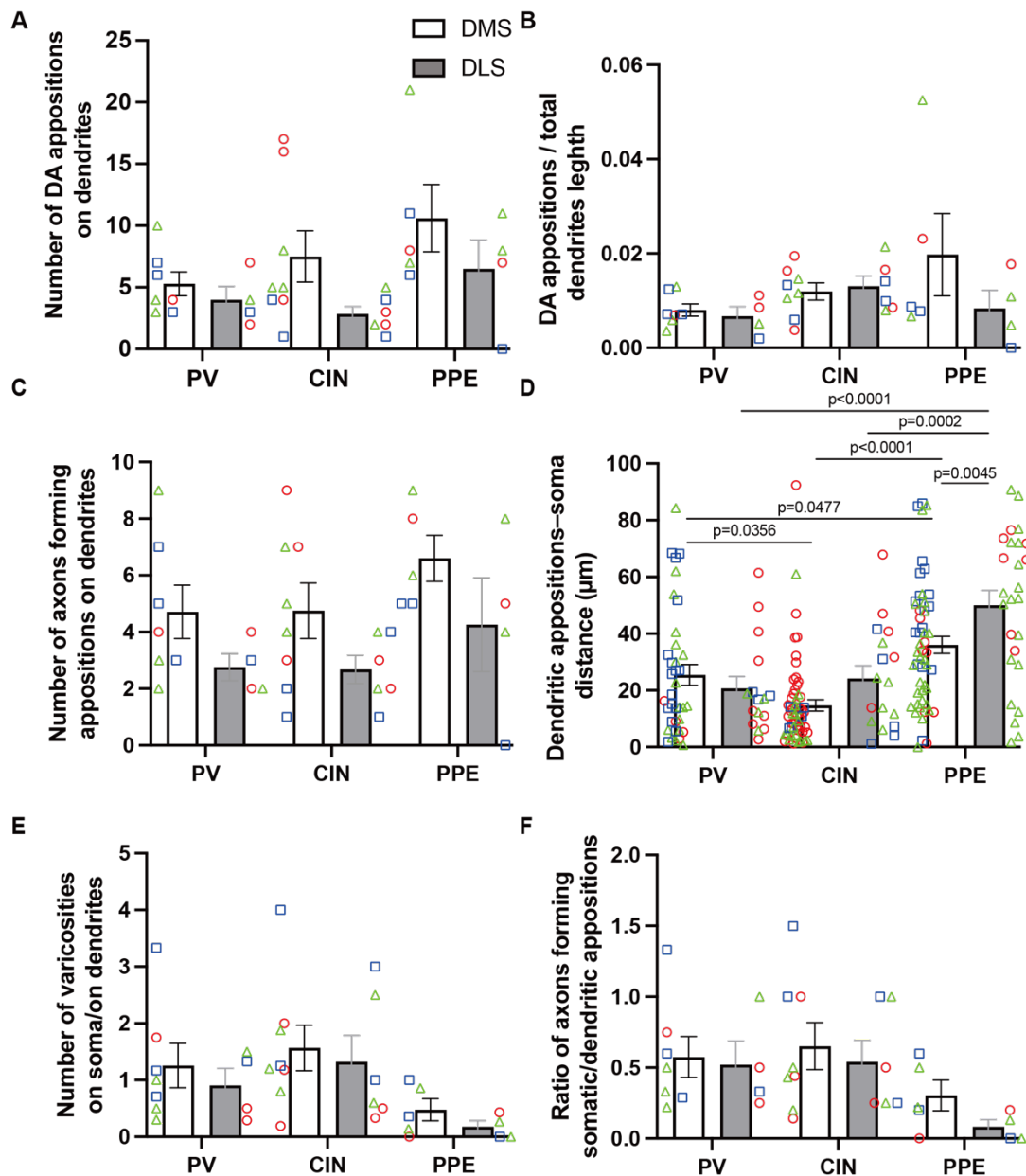


Figure 17. Quantitative analysis of DA dendritic PAPs in PV-INs, CINs and PPE-iMSNs. (A), Number of DA PAPs on dendrite in each neuron on DMS (blank bars) and DLS (gray bars). (B), Density of dendritic PAPs per unit dendritic length. (C), Number of DA axons forming appositions on dendrites. (D), Distance from the soma to each dendritic PAP. PPE-iMSNs exhibited significantly longer distances than PV-INs and CINs in both the DMS and DLS. (E), Ratio of the number of PAPs on the soma to those on dendrites for each neuron type in the DMS and DLS. (F), Ratio of the number of DA axons forming PAPs on the soma to those forming PAPs on dendrites for each neuron

type in the DMS and DLS. Data points from individual animals are distinguished by symbol: red circles, green triangles, and blue squares represent the three animals in each group.

In summary, my three-dimensional reconstruction and quantitative analyses identified clear cell-type-and region-specific differences in the striatal neuronal morphology and DA appositions.

Discussion

In the present study, AAV1 anterograde trans-neuronal labeling was used to investigate how DA axons originating in the SNc structurally relate to identified neuronal populations across the DMS and DLS. The central aim of this work was to determine whether DA connectivity within the dorsal striatum is organized in a manner that reflects both neuronal identity and functional striatal territory. The principal contribution of this thesis is a systematic anatomical comparison of DA inputs across striatal cell types and DMS-DLS subdivisions. These data provide structural insight into previously reported functional heterogeneity of dorsal striatal DA signaling. By focusing specifically on interneurons and iMSNs, the present experimental design minimized interpretational ambiguity arising from potential retrograde viral transport and enabled direct comparison of DA relationships among neuronal classes that subserve different circuit functions. DA axons formed close appositions on both somata and dendrites of PV-INs, CINs, and PPE-iMSNs in both the DMS and DLS, while somatic apposition number and soma-to-PAPs distance exhibited clear cell-type- and region-dependent differences. Recent single-bouton optical imaging further indicates that DA release preferentially occurs at varicosities apposed to post-synaptic DA-receptor-expressing processes, forming synapse-like specializations, thereby supporting the functional relevance of apposition-based structural quantification (Bulumulla et al. 2024). Together, these observations suggest that DA signaling in the dorsal striatum is spatially organized in a manner capable of supporting selective modulation of striatal microcircuits rather than being explained solely by diffuse volume transmission.

Interpretation of AAV1-dependent labeling in DA systems requires careful methodological consideration. Because the mechanism of AAV1 transfer in DA neurons remains incompletely understood, the term “trans-synaptic” was deliberately avoided, as exclusive transfer at bona fide synaptic specializations cannot be definitively demonstrated. Foundational work using AAV1-mediated trans-neuronal labeling emphasized that extensive bidirectional connectivity within basal ganglia circuits can complicate inference of labeling directionality (Zingg et al., 2017; 2020). Accordingly, the present study examined whether the observed labeling pattern is more consistent with anterograde trans-neuronal transfer than with retrograde transport. Several observations support a substantial anterograde contribution. MSNs constitute approximately ninety-five percent of striatal neurons (Graveland and DiFiglia, 1985), yet the fraction of labeled MSNs among all labeled neurons was markedly lower, as

shown in Fig. 5C. If retrograde transport from SNr- or SNc-projecting dMSNs were frequent, MSN labeling would be expected to approximate their anatomical prevalence. In addition, Cre expression was not enriched in Ctip2-positive MSNs relative to interneurons, as summarized in Table 4, further arguing against dominant retrograde transfer. Labeling of iMSNs, which lack direct projections to the substantia nigra, additionally supports an anterograde interpretation, although partial retrograde contribution cannot be completely excluded. Taken together, these findings indicate that AAV1 labeling in the present configuration primarily reflects forward DA connectivity, thereby permitting meaningful comparison of DA relationships across neuronal identity and striatal region, consistent with recent work from our laboratory demonstrating that anterograde trans-neuronal labeling predominates in the striato-pallidal circuitry (Karube et al., 2025).

Differential labeling across interneuron subclasses also required interpretation. ChAT-, PV-, and NOS-positive neurons were labeled more frequently than predicted from their baseline abundance, whereas SOM-positive neurons were relatively underrepresented, as shown in Figs. 4C and 5C and summarized in Tables 3–4, consistent with prior descriptions of interneuron composition (Kawaguchi et al., 1995; Muñoz-Manchado et al., 2016). Injection variability within the SNc has previously been proposed as a potential source of differential labeling across striatal regions (Menegas et al., 2015; Poulin et al., 2018; 2020; Luppi et al., 2021). However, I confirmed the medial and lateral SNc injections did not exhibit significant differences (Fig. 7B). Therefore, this factor is unlikely to be responsible. Alternative explanations therefore warrant consideration. AAV1 transfer may preferentially occur at ionotropic synapses, consistent with glutamate or GABA co-transmission by subsets of DA neurons (González-Hernández et al., 2001; Yamaguchi et al., 2013) and with unconventional VMAT- and GAT1-dependent GABA packaging mechanisms in DA terminals (Tritsch et al., 2012; 2014; 2016; Melani and Tritsch, 2022). Differences in detection sensitivity between Cre immunofluorescence and Cre-dependent reporter expression may further contribute to subtype-specific bias, potentially explaining the higher coefficient of variation observed across regions of interest for ChAT-, PV-, and NOS-positive neurons in Table 4. Consideration of volume transmission provides an additional constraint. Given the extraordinary arbor territory and varicosity density of individual SNc axons (Matsuda et al., 2009), purely diffuse extracellular spread would predict substantially broader neuronal labeling than observed in this study. The restricted labeling pattern therefore favors transfer occurring at spatially limited release sites, potentially

corresponding to synapse-associated specializations, although ultrastructural confirmation remains required.

Beyond methodological interpretation, AAV1-dependent labeling offers a conceptual advantage for investigating DA circuit organization. Electron microscopy remains indispensable for definitive synapse identification, yet the immense spatial extent and dense arborization of nigrostriatal axons make systematic single-cell mapping across multiple neuronal classes technically prohibitive. In contrast, the present approach enables simultaneous visualization of presynaptic DA axons and postsynaptic genetically or immunohistochemically defined neurons, thereby allowing direct comparison across both neuronal identity and DMS–DLS functional domains within a unified anatomical framework. This comparative capability is particularly important because electrophysiological studies have demonstrated region-dependent DA modulation of interneurons and projection neurons (Chuhma et al., 2023), whereas structural correlates of such heterogeneity have remained incompletely characterized. Alignment of AAV1-derived apposition maps with emerging functional approaches, including single-bouton DA imaging and correlative ultrastructural analysis, therefore provides a feasible route toward resolving how synaptic and diffuse DA signaling modes coexist within striatal microcircuits.

The present findings further indicate that DA connectivity is not randomly distributed despite the extensive collateralization of SNc neurons. Only a minority of DA axonal segments formed somatic appositions, and these segments occasionally generated clustered contacts onto individual neurons, as illustrated in Fig. 6C–D, suggesting functional specialization rather than stochastic proximity. Because DA receptors are differentially expressed across interneuron subclasses and MSNs (Kreitzer, 2009), such structural selectivity provides a plausible anatomical substrate for heterogeneous DA physiological responses. Recent electrophysiological analyses demonstrating region-dependent DA signaling onto interneurons (Chuhma et al., 2023) further support the interpretation that DA influence is organized according to both neuronal identity and striatal functional territory. The convergence between structural apposition bias and functional asymmetry therefore supports a model in which DA exerts spatially differentiated modulation across dorsal striatal microcircuits rather than acting as a purely diffuse neuromodulator.

Morphological analysis confirmed that reconstructed neurons fell within previously established quantitative ranges, thereby supporting accurate cell-type classification. Total dendritic length in the present material was shorter, most likely reflecting the use of 25 μm sections rather than true biological reduction, consistent with comparisons to

500 μm volumetric reconstructions of D2R-positive MSNs reported by Park et al. (2025). Importantly, those volumetric reconstructions detected no intrinsic DMS–DLS differences in MSN primary dendrite number or total dendritic length, indicating that regional differences identified here primarily reflect presynaptic DA organization rather than postsynaptic structural variation. This distinction is essential for interpreting DA modulation as a circuit-level specialization.

Direct comparisons across PV-INs, CINs, and PPE-iMSNs revealed clear cell-type- and region-dependent organization of DA appositions. Across all analyzed axons bearing appositions, between approximately twenty-one and forty percent exhibited somatic contacts, and mean somatic apposition number did not correlate with soma surface area, as shown in Fig. 16, arguing against random contact formation.

PV-INs exhibited a higher number of axons forming somatic appositions in the DMS (Fig. 16C) and a tendency toward more frequent somatic varicosities in this region (Fig. 16D–E), consistent with regional differences in inhibitory control and neuronal excitability (Monteiro et al., 2018; Fino et al., 2018). These structural differences suggest that DA inputs may differentially influence PV-IN-mediated inhibitory regulation across striatal subregions, although direct electrophysiological evidence for region-specific DA modulation of PV-INs remains limited.

CINs displayed the most pronounced DMS-biased DA organization, paralleling stronger DA synaptic currents and longer pause responses reported electrophysiologically (Chuhma et al., 2018; 2023). In particular, DA neurons evoke larger D1 receptor–mediated excitatory currents together with stronger D2 receptor–dependent inhibitory responses in CINs within DMS compared with DLS (Chuhma et al., 2023), accompanied by prolonged pause durations following DA activation (Chuhma et al., 2018). The increased number of somatic DA appositions and DA axons contacting CIN somata in DMS observed here therefore provides direct anatomical support for these regionally enhanced DA synaptic effects and their influence on striatal output timing.

In contrast, PPE-iMSNs exhibited few significant regional structural differences, although soma-to-varicosity distance was greater in the DLS (Fig. 17D), which may influence local dendritic processing. Consistent with this structural pattern, direct DA synaptic currents in iMSNs are typically weak or absent under baseline conditions, with measurable inhibition emerging only when specific conductances such as GIRK channels are experimentally enhanced (Marcott et al., 2014). These findings support a model in which DA signaling in iMSNs operates predominantly through modulatory or receptor-dependent mechanisms rather than strong fast synaptic transmission.

An important unresolved component concerns DA organization onto direct-pathway MSNs, which were not anatomically evaluated in the present thesis. Within classical basal ganglia models, dMSNs form the principal striatonigral output facilitating movement, whereas iMSNs exert opposing inhibitory influence (Albin et al., 1989; DeLong, 1990). DA differentially modulates these pathways via D1-receptor-mediated excitation of dMSNs and D2-receptor-mediated inhibition of iMSNs, as demonstrated by receptor-specific gene expression and projection mapping (Gerfen et al., 1990). Selective activation of dMSNs alleviates parkinsonian motor deficits in circuit-manipulation experiments, underscoring their causal importance (Kravitz et al., 2010). Ultrastructural analyses further reveal defined synaptic and perisynaptic relationships between DA terminals and striatal projection neurons (Hersch et al., 1995; Hanley and Bolam, 1997). Determining whether SNc DA axons exhibit cell-type- and region-specific structural bias toward dMSNs, comparable to patterns identified here for interneurons and iMSNs, is therefore essential for completing a comprehensive anatomical model of dorsal striatal DA organization. Future work integrating D1-specific genetic access, anterograde viral tracing, projection-defined labeling, and cell-type-resolved physiological analysis will enable systematic comparison of DA connectivity across the full complement of striatal targets, thereby extending the comparative framework established in this thesis.

Several methodological limitations should be acknowledged. Appositions were used as structural proxies for synapses without ultrastructural verification. Thin-section reconstruction likely underestimated distal dendritic contacts. Substantial cellular heterogeneity within DA and striatal populations may obscure subtype-specific connectivity. Only male animals were examined, precluding evaluation of sex-dependent differences. The precise mechanism of AAV1 trans-neuronal transfer also remains incompletely understood. Nevertheless, these considerations do not alter the principal conclusion that DA connectivity in the dorsal striatum exhibits systematic organization across neuronal identity and functional region.

Such structured DA organization has direct implications for basal ganglia disorders. Differential vulnerability of SNc DA subpopulations and region-specific alterations in interneuron physiology (Lozovaya et al., 2018; Poulin et al., 2020; Yaghmaeian et al., 2024) suggest that degeneration of selectively organized DA inputs may destabilize goal-directed and habitual control through distinct mechanisms (Devan et al., 2011; Sharpe et al., 2019; Vandaele et al., 2019). Clarifying how DA differentially engages interneurons, iMSNs, and dMSNs across DMS and DLS therefore provides an essential anatomical foundation for interpreting clinical heterogeneity and for guiding

development of circuit-targeted therapeutic strategies aimed at restoring balanced DA modulation within basal ganglia networks.

Conclusions

In this research, I established and validated a viral tracing strategy using AAV1-mediated anterograde trans-neuronal labeling to map the postsynaptic targets of SNc DA neurons within the dorsal striatum. Although the nigrostriatal pathway has long been recognized as a major neuromodulatory system, its bidirectional connectivity has raised concerns about potential retrograde viral transfer, limiting the applicability of AAV1-based methods. By focusing specifically on striatal interneurons and iMSNs—cell types whose axons remain confined within the striatum and do not project back to the SNc—I overcame this limitation and provided the first direct experimental evidence that AAV1 can achieve anterograde trans-neuronal transfer in the DA system.

Through high-resolution three-dimensional reconstructions of AAV1-labeled neurons and their apposed DA axons, I revealed that DA appositions are not randomly distributed but are instead organized according to cell identity and regional context. PV-INs and CINs exhibited frequent and proximal contacts, whereas PPE-iMSNs received sparser and more distal appositions, mainly along dendritic compartments. Moreover, the distribution of DA appositions differed between the DMS and DLS, indicating regionally specialized DA regulation that parallels the distinct behavioral functions of these territories—goal-directed control in the DMS and habitual processing in the DLS. These results collectively demonstrate that the nigrostriatal DA projection forms a structurally organized network, capable of target-selective modulation rather than diffuse volume transmission. The validated use of AAV1 in this system provides a powerful tool for future investigations into synapse-specific organization and plasticity of DA circuits. This methodological advancement bridges the gap between anatomical structure and functional modulation, offering new perspectives for understanding how DA orchestrates adaptive behavior and how its selective circuit dysfunction contributes to neurological disorders such as PD.

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At this point in writing, I am filled with emotion.

From the age of seventeen when I first stepped into the world of medicine, more than a decade has passed. Three universities across two countries have shaped my journey, leaving indelible traces on my youth. Over these years, I have grown from naivety, broadened my horizons, gained knowledge, made cherished friendships, and accumulated valuable experiences. I feel deeply grateful and content.

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May the next chapter of life be vibrant and full of possibilities.

Conflict of Interest

The author declares no conflict of interest.

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