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Review article

Volume electron microscopy for genetically and molecularly defined neural circuits

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ABSTRACT

The brain networks responsible for adaptive behavioral changes are based on the physical connections between neurons. Light and electron microscopy have long been used to study neural projections and the physical connections between neurons. Volume electron microscopy has recently expanded its scale of analysis due to methodological advances, resulting in complete wiring maps of neurites in a large volume of brain tissues and even entire nervous systems in a growing number of species. However, structural approaches frequently suffer from inherent limitations in which elements in images are identified solely by morphological criteria. Recently, an increasing number of tools and technologies have been developed to characterize cells and cellular components in the context of molecules and gene expression. These advancements include newly developed probes for visualization in electron microscopic images as well as correlative integration methods for the same elements across multiple microscopic modalities. Such approaches advance our understanding of interactions between specific neurons and circuits and may help to elucidate novel aspects of the basal ganglia network involving dopamine neurons. These advancements are expected to reveal mechanisms for processing adaptive changes in specific neural circuits that modulate brain functions.

1. Introduction

Advances in microscopic imaging have improved our understanding of brain structures and functions. Light microscopy (LM) can provide useful information on the general morphology of neurons, including cell bodies, dendrites, and axons, in large observation areas. In contrast, spatial resolution in LM is limited, although several recent advancements such as super-resolution microscopy can help to address this weakness (Sigal et al., 2018). In this regard, electron microscopy (EM) allows us to examine detailed ultrastructure and has revolutionized our understanding of the nervous system, including synaptic connections, the physical connections between neurons, and myelin ensheathment, the glial wrapping that allows for rapid nerve conduction (Palay, 1958; Brightman and Reese, 1969). The brain's information processing is based on a network of neural circuits formed by the neuronal connections. Since observing the details of these connections is still difficult with LM, advancement in three-dimensional (3D) EM imaging, which is achieved by 3D ultrastructural reconstruction from serial EM images and referred to as volume EM (vEM), has significantly improved our ability to acquire the structural basis underlying brain functions (Ohno et al., 2016).

Comprehensive maps of the physical associations between neurons, such as the brain's "connectome", would help to answer critical questions about complex processes of cognition and intellectual abilities as well as processing of sensory and motor information (Lichtman et al., 2008; Seung, 2009). The brain functions are regulated through the interactions of different types of cells. Such cellular diversity can be clarified by single-cell RNA sequencing, which allows for a strategy to characterize cells by analyzing the gene expression patterns at the single-cell level (Zeng and Sanes, 2017). Furthermore, because neuronal interactions involve local cellular networks that typically connect neurons in close proximity, a cellular atlas with spatial information would be required to clarify brain architecture (Piwecka et al., 2023). The structural identification and connections of networks among the cells

Abbreviations: 3D, three-dimensional; CLEM, correlative light and electron microscopy; DAB, diaminobenzidine; EM, electron microscopy; HRP, horseradish peroxidase; LM, light microscopy; PalGFP, palmitoylation site-attached green fluorescent protein; vEM, volume electron microscopy.

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revealed by microscopic approaches, combined with molecular and genetic identification of the cells would lead to a comprehensive understanding of information processing in the brain. However, especially in studies that rely on EM observation, cells and structures are typically classified using morphological criteria, and in those cases of morphological classification, identifying various neural subtypes presents a formidable challenge when based solely on such morphological criteria. Therefore, distinguishing between closely related subtypes of cells in microscopic observation necessitates additional methodology that demonstrates their genetic and molecular identities.

Cellular and molecular labeling techniques are useful for determining the precise identities of structural components because they allow direct visualization of specific molecules along with cellular structures. These techniques frequently provide insights into the molecular and genetic composition related to their functions. At the EM levels, previous studies have developed various methods for visualizing such specific components in specimens during EM analyses. Conventional histochemical methods are used to visualize a limited number of target molecules. Furthermore, immunoelectron microscopy, which uses specific antibodies to target larger numbers of molecules, is a popular method; however, the conditions of fixation and sample preparation have a significant impact on the sensitivity and specificity of the labeling. In this context, recent efforts have resulted in more useful approaches that employ artificial probes, many of which can be seen directly in EM images. In addition, other approaches include correlative microscopy, which combines the expanding tools of LM and EM observation to better understand the consistent spatial distribution of target molecules (Hayashi et al., 2023). Such methodological advancements would result in useful tools for understanding comprehensive interactions between neurons and also glia in the context of unprecedented cellular diversity based on molecular and genetic identities.

This review will provide an overview of recent advancements in the current methodology of labeling technology for molecular and genetic identification of structural components in LM and vEM, as well as perspectives for understanding neural circuits that integrate dopaminergic neurons.

2. Expanding toolbox for molecular and genetic identification in LM and vEM

To uncover cell type-dependent neural connections, knowledge gained from LM is required first. Visualizing neurons with long projection axons is one of the keys to identifying specific neural connections. At the same time, information obtained from vEM provides critical information about detailed connection maps for each process. The advancement of vEM coincided with the development of molecular, cellular, and tissue labeling technologies (Fig. 1). These methods are critical for improving structural identities in EM imaging, as they enable researchers to identify and track specific structures in complex biological systems. There have been several approaches with different strengths and limitations in the labeling for the LM and vEM, that would help us investigate molecularly and genetically defined neural circuits.

2.1. Single neuron tracing

The rules by which choose their synaptic partners in projections have remained elusive and challenging to understand. Historically, the neural circuit has long fascinated many researchers. Golgi, who developed the Golgi staining method, advocated for the reticular theory, while Cajal used the Golgi staining method to promote the neuron theory, both of which received the Nobel Prize. Later, in 1932, EM, as previously mentioned, made significant contributions to our understanding of neural circuits, including the visualization of synapses. Other methods for tracing the pathway, including myelin sheath formation and degeneration (Türck, 1859; Flechsig, 1876), were used. For example, Flechsig showed the early formation of optic radiation in the human neonatal brain by Weigelt's method, which is one of the myelin sheath formation methods (Flechsig, 1876). Labeling for axonal transport came next. Some molecules incorporated into the cell body are transported to the axon terminal via axonal transport, while some molecules incorporated into the axon terminal are transported to the cell body. The first is known as anterograde transport, while the second is known as retrograde transport. Because some molecules are more likely to be transported anterogradely than others retrogradely, it is possible to identify where neurons with cell bodies located at the injection site project their axons, as well as where the cell bodies of neurons with axon terminals are located, and to view images of those axons and cell bodies. In the early days, radioactive amino acids containing tritium were used as such tracers (Grafstein, 1967; Cowan et al., 1972). Since the autoradiographical visualization are far from so-called Golgi-like labeling, other tracers have been employed such as WGA-HRP, PHA-L, BDA, biocytin, cholera toxin subunit, and so on. However, while these methods were useful for qualitative analyses at the regional level of the brain, quantitative verification of specificity at the cellular and subcellular levels proved difficult.

This requires the labeling of individual neurons. The intracellular staining method, which uses a glass microelectrode to stain a single neuron, was then developed. Intracellular labeling, combined with in

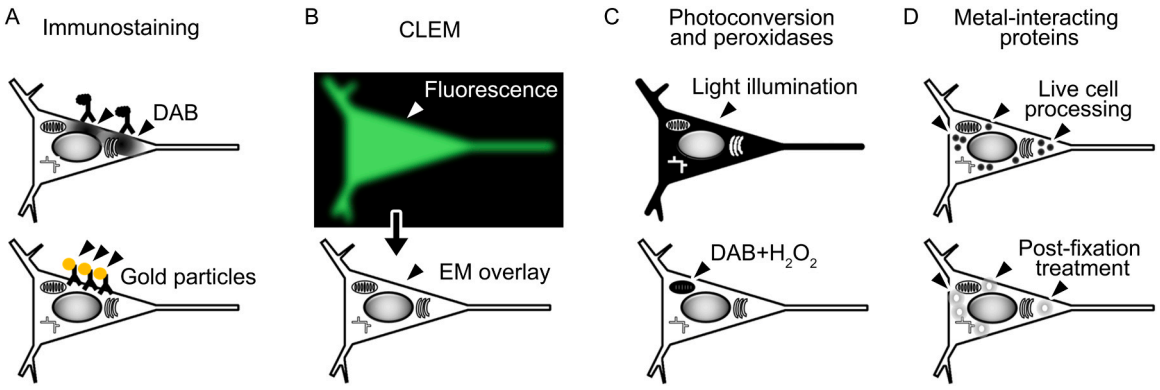

Fig. 1. Illustration of various labeling methods used in volume electron microscopy. Immunostaining involves antibodies binding to specific cells or structures, which are then visualized using osmicated DAB deposition or metal particles like gold (A, arrowheads). CLEM involves light microscopic observation of fluorescently labeled cells and structures, followed by observation with EM and overlaying of the images (B, arrowheads). Cells or structures labeled with fluorescent molecules and proteins can be identified in EM images after photoconversion (C, upper panel, arrowhead). Alternatively, peroxidases expressed in the cytosol or specific organelles can be identified as osmicated DAB deposition after DAB and hydrogen peroxide (H_2O_2) treatments (C, lower panel, arrowhead). Targeted expression of metal-interacting proteins can be detected by treating live cells with metals such as iron or by metal staining after fixing them for EM imaging (D, arrowheads).

vivo-recording, became an important tool for determining the relationship between morphology and function. However, the method is not so technically simple, particularly when labeling long projecting axons with their fine collaterals. A subsequent method was to use genetically modified viruses as tracers to visualize the entire entity of long and fine axons. As a method of gene introduction, viral vectors are also commonly used. If a virus is genetically modified to prevent proliferation, only neurons infected with the virus at the injection site express the reporter molecule. Among neurotropic viruses, adenovirus, adeno-associated virus, herpes simplex virus, lentivirus, and Sindbis virus are commonly used. To visualize the axon of a single neuron, we used a replication-deficient vector based on a Sindbis virus that expresses membrane-targeted palmitoylation site-attached green fluorescent protein (palGFP) under the control of the subgenomic promoter (Tamamaki et al., 2000; Furuta et al., 2001). Because a large amount of palGFP is produced and distributed on both the somatodendritic and axonal membranes, the vector has been used as an anterograde tracer (Nakamura et al., 2004; Tomioka et al., 2005). Furthermore, using a sufficiently diluted viral solution, we can use the vector for single-cell labeling studies (Kuramoto et al., 2009; Matsuda et al., 2009; Fujiyama et al., 2011; Koshimizu et al., 2013; Unzai et al., 2017).

2.2. Immunostaining

Immunostaining is a common and versatile approach for visualizing specific structures within tissues, particularly in vEM (Fig. 1A). In vEM, array tomography and ATUMtome-based section collection on carbon nanotube tapes are permissive approaches in which specific molecules are immunostained and serial images are acquired for 3D ultrastructural information with distribution of immunoreactivity (Collman et al., 2015; Kubota et al., 2018). Furthermore, conventional pre-embedding immunohistochemical methods for antibody visualization that use oxidation and polymerization of diaminobenzidine (DAB) can be used in vEM to label cellular surfaces, even without treatment for antibody penetration that damages tissue ultrastructure (Fig. 2) (Katoh et al., 2017). Alternatively, visualization techniques for immunolabeling make use of gold particles. While large gold particles impede penetration of conjugated antibodies and labeling of deep tissue areas, tiny probes like gold nanoparticles can improve penetration and, when combined with gold/silver enhancement, visualize precise molecular localization in the tissue volume (Mikuni et al., 2016; Liang et al., 2023). In contrast, immunolabeling with extrinsic probes can be hampered by poor cellular ultrastructure as a result of milder chemical fixation and/or probe penetration-enhancement treatments. It has been reported that improvements for larger tissues, such as the preservation of extracellular spaces or the use of smaller probes such as nanobodies, facilitated penetration while maintaining tissue ultrastructure (Pallotto et al., 2015; Fang et al., 2018). While such approaches allow for the flexible and efficient visualization of various structures in electron microscopic images by highlighting the target molecules and structures as electron-dense profiles in EM images, labeling and identifying different types of targets requires additional methodological development. Therefore, developing better visualization techniques is critical for the three-dimensional reconstruction of neural connections, allowing for more precise tracking of neural projections and identification of synaptic components.

2.3. Correlative light and electron microscopy (CLEM)

Given that antibody-based labeling methods may obscure cellular details in vEM due to electron-dense deposits on target molecules, one of the most widely used methods for molecular and genetic identification is a correlative approach, also known as CLEM (Fig. 1B). CLEM is a method that combines LM and EM, frequently involving LM observation of labeled cellular components followed by identification of the same components in EM for ultrastructural analysis (de Boer et al., 2015; Hayashi et al., 2023). Recent multimodal observations include the identification of EM profiles along with data from various modalities of analyses, such as transcriptomic data combined with ultrastructural data. However, this approach is largely limited to reference in related data, such as serial sections and/or in sets of reference data (Vergara et al., 2021; Androvic et al., 2023). In such approaches, it is difficult to observe the same structures across different modalities, as most CLEM approaches do. To facilitate the observation of the same targets in LM and EM, landmarks such as blood vessels or artificial markings are frequently used to align LM and EM images in the tissue architecture; the landmarks, in addition to the targets, are observed in LM and subsequently identified in EM after embedding in resins (Luckner et al., 2018; Maclachlan et al., 2018; Goto et al., 2024). Additionally, other efforts, such as the exploration of osmium-resistant fluorescent proteins or in-resin CLEM, which use fluorescent molecules visible in fluorescent LM even after resin-embedding, can significantly ease the process of

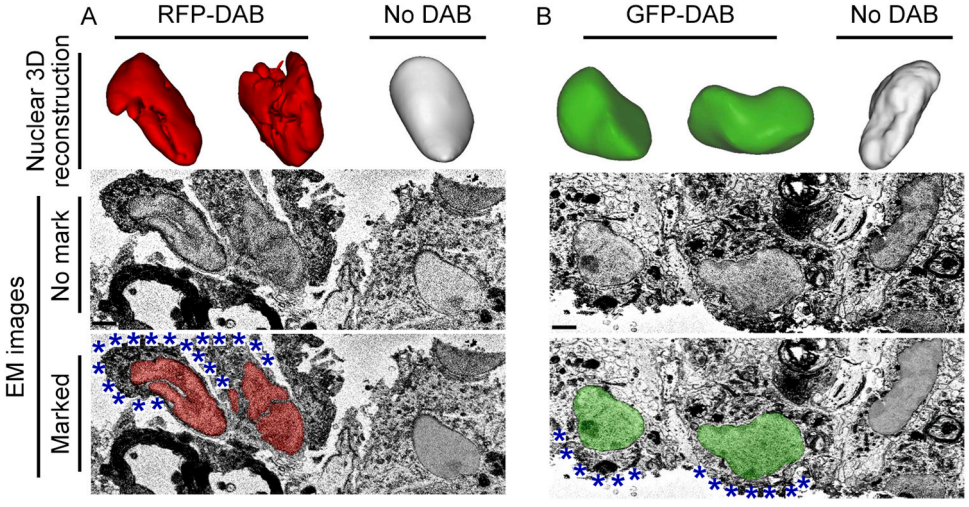

Fig. 2. Immunostaining followed by volume EM, revealed distinct nuclear morphology of macrophages in the spinal cord of a demyelinating disease model. In the experimental autoimmune encephalomyelitis model of double-heterozygous $Ccr2^{rfp}$::$Cx3cr1^{gfp}$ mice expressing RFP in monocyte-derived macrophages and GFP in microglia-derived macrophages, RFP-positive cells labeled with surface DAB by RFP immunostaining (A, blue asterisks) have a complex nuclear morphology (A, red 3D reconstruction) and GFP-positive cells with surface DAB labeling by GFP immunostaining (B, blue asterisks) have a spherical nuclear morphology (B, green 3D reconstruction). Scale bars: 2 μm. The images were reproduced from Katoh et al. (2017) with the necessary permission.

correlating fluorescence and EM images, as the same targets in identical sections can be sequentially observed (Paez-Segala et al., 2015; Fu et al., 2020; Tanida et al., 2020). The combination of two-photon live imaging and subsequent observation with EM is frequently used to observe the structural basis for physiological characteristics; for example, calcium indicators introduced into targeted cells are observed under LM, followed by 3D ultrastructural investigations of structural connections in the functionally relevant wiring network (Bock et al., 2011; Briggman et al., 2011; Lee et al., 2016). Furthermore, in conjunction with immunostaining, fluorescent nanobodies specific to cellular markers and molecules associated with Alzheimer's disease pathology have recently used to clarify ultrastructural features of immunofluorescently identified profiles in the Alzheimer's disease model (Han et al., 2023). While imaging deep within tissues requires further improvement, CLEM is a widely used and useful approach for identifying multiple defined targets in vEM imaging, and it has yielded many successes.

2.4. Photoconversion and peroxidases

Because sequential observation of identical structures in CLEM is often technically demanding and sometimes difficult to achieve complete overlapping, other useful approaches include the expression of genetically encoded probes that can eventually be seen in EM (Fig. 1C). Such probes are frequently visualized using common substrates such as polymerized DAB, which then binds to osmium (Seligman et al., 1968). While DAB polymerization can be achieved by oxidation through light illumination, also known as photoconversion, this oxidation of DAB is inefficient in the case of many fluorescent proteins. Therefore, a genetically encoded probe, miniSOG, has been developed to facilitate both the DAB reaction and fluorescence observation of the same molecule (Shu et al., 2011). miniSOG is a fluorescent molecule that can be seen as osmium black in EM images due to the oxidation of DAB caused by reactive oxygen species generated during light illumination. Therefore, miniSOG would be suitable for CLEM, whereas these molecules' efficient generation of reactive oxygen species may be toxic for live imaging (Ryumina et al., 2013; Xu and Chisholm, 2016). Other widely available molecules that exhibit horseradish peroxidase (HRP)-like properties for visualization of DAB include artificial enzymes such as APEX and its derivatives (Martell et al., 2012; Lam et al., 2014; Joesch et al., 2016). APEX is a plant-derived artificial peroxidase that can deposit DAB in specific cells or organelles via molecular targeting and treatment with hydrogen peroxide in conjunction with DAB. This property makes it useful for cellular and organelle labeling even in destructive vEM methods, like FIB-SEM or SBF-SEM. Importantly, derivatives of APEX are thought to label relatively deep structures, because DAB is a small molecule in comparison to probes like antibodies. Furthermore, organelle-targeted labeling of its derivative, dAPEX2, allows for differential labeling of various types of cells and structures (Zhang et al., 2019). These labeling methods are useful for identifying specific neural circuits in large tissue areas and can be combined with fluorescent LM (Hirabayashi et al., 2018). Using DAB labeling by organelle-targeted peroxidases, multimodal experiments including vEM revealed the recurrent spinal neural circuits of cerebrospinal fluid-contacting neurons in mice, which regulate locomotion via connections with motor neurons (Fig. 3) (Nakamura et al., 2023). Furthermore, multiplexed labeling with differentially targeted APEX derivatives revealed a significant convergence of cortical motor and sensory inputs to proximal dendrites of thalamic neurons, indicating that many individual thalamic neurons integrate signals from various cortical regions (Sampathkumar et al., 2021). These techniques would better connect molecular identities and ultrastructural analyses, making them useful for understanding complex cellular communications in tissue architecture.

2.5. Metal-interacting proteins

Finally recent research has introduced additional labeling methods based on metal interaction that apply to vEM (Fig. 1D). A new artificial ferritin particle, FerriTag, can be attached to specific proteins using rapamycin treatments, allowing for nanometer-level visualization of targets (Clarke and Royle, 2018). Ferritin is known to incorporate iron, so the resulting electron density of ferritin can be identified in EM (Singer, 1959; Sri Ram et al., 1963; Jutz et al., 2015). While a wider application in vivo is awaited, Ferritag's unique properties enabled the combination of fluorescence observation and nanoscale visualization under EM. Another study describes a method for conducting a more comprehensive analysis of defined targets in the tissue ultrastructure. The development of "EMcapsulins", engineered spherically symmetric and concentric barcodes, enabled the classification of multiple types of labeling structures in EM images (Sigmund et al., 2023). EMcapsulin barcodes were created by attaching varying numbers of metallothioneins to the inner surface of different-sized nanospheres (Giessen, 2022). Furthermore, targeting EMcapsulins to different subcellular compartments and generating unique patterns with cross-linkers resulted in approaches for combinatorial multiplex labeling. This genetically encoded probe was used in multiple biological models, including human cells and fruit flies, as well as a computational tool for segmenting EM images. While the applications of these new approaches are still limited and require further evaluation, particularly in *in vivo* models, they would allow for multiple labeling of genetically diverse cells in brains, elucidating the network of specific types of cells.

To summarize, the advancement of labeling technologies has

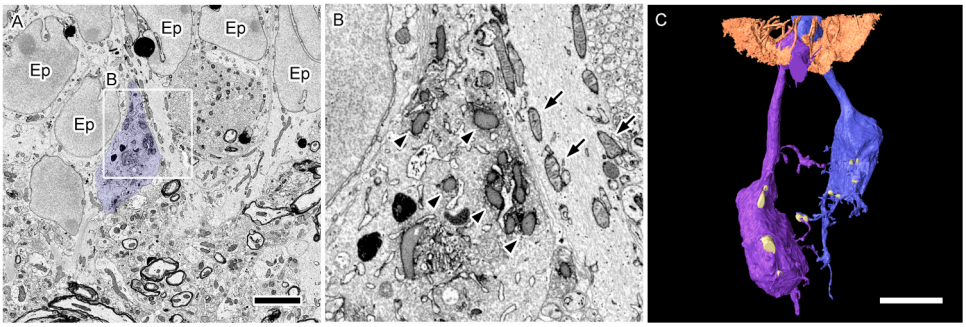

Fig. 3. Neural connections among cerebrospinal fluid contacting neurons (CSF-cN) shown by volume electron microscopy following expression of organelle-targeted dAPEX2 by viral vector injection into Pkd2l1-Cre mice. Electron micrographs show CSF-cN (A, blue, B showing the marked area at a higher magnification) containing mitochondria labeled with diaminobenzidine by expression of mitochondria-targeted dAPEX2 (B, arrowheads), which are distinct from unlabeled mitochondria in adjacent cells (B, arrows). Ep represents ependymal cells. The 3D reconstruction of two labeled CSF-cN (C, blue and purple) reveals thick processes that extend onto the surface of the central canal of the mouse spinal cord (orange) as well as synaptic connections on the surface of their processes and cell bodies (C, yellow). Scale bars: 10 μm. The images were adapted from Nakamura et al. (Nakamura et al., 2023) with the necessary permission.

broadened the capabilities of LM and vEM, potentially allowing for precise and informative identification of cellular and tissue structures using genetic or molecular signatures. Further advancement would result in the identification of a greater number of unique cells or structures in a larger volume of tissues, as well as application to different neural circuits in more diverse animal models. In the following section, we will discuss the potential contribution of such microscopic studies to understanding the basal ganglia network that includes dopaminergic neurons.

3. Perspective to adaptive neural circuits of dopaminergic neurons

3.1. Adaptive neural circuit and dopamine neurons

Animals, including humans, must constantly adapt their behavior to their external environment and internal factors to survive. This adaptability entails choosing behaviors based on expected rewards, which is associated with the cortico-basal ganglia-thalamic loop (Barto et al., 1983; Barto, 1995; Schultz et al., 1997; Doya, 2000; Crittenden and Graybiel, 2011). Dopamine neurons in the substantia nigra pars compacta of the midbrain play an important role in the neural circuit. The projection of these neurons to the striatum has a significant impact by transferring information about reward prediction errors (Schultz et al., 1997, 1998; Reynolds et al., 2001; Bayer and Glimcher, 2005; Cohen et al., 2012; Hart et al., 2014). This information fundamentally shapes the decision-making processes that determine which behaviors to pursue in response to various stimuli. Therefore, efforts have been made to understand the distinct neural projection patterns of dopamine neurons.

3.2. Visualization of dopaminergic neurons

As mentioned in previous sections, various methods for visualizing dopaminergic neurons have been used to uncover their characteristic neural circuitry. Tepper et al. successfully identified dopamine neurons using electrophysiological criteria and visualized them using microiontophoretically injected HRP in vivo (Tepper et al., 1987). Because of the large molecular size of HRP and fine dopaminergic axons, visualization of dopamine neurons was limited to the somata, proximal dendrites, and local thick axons (Tepper et al., 1987). Prensa and Parent performed the microiontophoretic extracellular labeling of single axons using biotin dextran amine in living rats and were successful in tracing individual neurons (Prensa and Parent, 2001). Although these neurons were not 100 % guaranteed to be dopaminergic, the study provided detailed information on nigrostriatal axons, including fine collaterals. However, as discussed in the following paragraph, their axon labeling may not be complete. While intracellular labeling with brain slices has been widely used to provide detailed morphology of the cell body, dendrites, and local axons, it cannot reconstruct the entire length of the axon. As for the dopaminergic neurons, Yung et al. investigated the membrane properties and morphological reconstruction of dopamine neurons in slice preparations of guinea-pig midbrain and discovered heterogeneity in dopaminergic neurons' membrane properties, though visualization was limited to the somata and proximal dendrites and axons (Yung et al., 1991). Matsuda et al. used a modified Sindbis virus to trace single axons of dopamine neurons and discovered unexpected results (Matsuda et al., 2009). The eight dopamine neurons had an average total axonal length of ~467,000 μm, whereas the previous study reported that the seven representative neurons in the substantia nigra had that of 36,000 μm (estimated from the published data, available at https://www.jneurosci.org/content/jneuro/suppl/2009/01/14/29.2.444.DC1/Supplemental_Table.pdf as supplemental material) (Prensa and Parent, 2001), which is <1/10 of the present data. It suggests that the palGFP-expressing Sindbis virus vector was a highly sensitive and efficient method for visualizing the arborization of single axons. As discussed in our previous report (Matsuda et al., 2009), the long and complex distribution of axons of dopamine neurons implies that multiple dopamine neurons redundantly innervate the striatum. This may explain the lack of symptoms in the early stages of Parkinson's disease. It may also mean the complexity of reinforcement learning with the various reward system. Now, the complicated and dense axons of dopaminergic neurons can be efficiently labeled with many genetic tools, as introduced in the next section.

3.3. Unveiling dopamine projections and their synaptic connections

Dopamine neurons in the substantia nigra pars compacta have topographies that project indistinguishably to adjacent striatal regions in each region (Gerfen et al., 1987; Jimenez-Castellanos and Graybiel, 1987; Langer and Graybiel, 1989). The striatum has biochemical compartment structures: the striosome (patch) and the matrix compartments, and we have demonstrated differences in inputs and outputs between these structures (Fujiyama et al., 2006, 2011; Yamada et al., 2016; Unzai et al., 2017; Karube et al., 2019). Dopamine neurons were thought to be divided into two types based on which compartments they projected to (Gerfen et al., 1987). However, a single neuron tracing study using a Sindbis virus with a membrane-targeted palmitoylation site revealed that a single dopamine neuron projects to a broad region that includes both compartments, albeit with a slight preference for one (Matsuda et al., 2009).

Also in this study, theoretical calculations based on experimental data revealed 75,000 striatal neurons in the projection area of a single dopamine neuron (Matsuda et al., 2009). Because there are several types of striatal neurons, it is unclear which ones are more likely to receive dopamine and how the physical synaptic connections are formed on each neuron. Furthermore, unlike glutamate and GABA, dopamine has been shown to transmit volume via diffusion as well as through synaptic sites. Are there any rules governing the interaction of dopamine axons and striatal neurons? The vEM approach may provide useful information about the distribution of synaptic connections, as well as the axonal projections of dopamine neurons and target striatal neurons. Furthermore, cellular labeling techniques for vEM would be required to understand the neural circuits formed between the various types of striatal neurons. To investigate these types of connections between specific types of neurons, we recently succeeded in anterograde trans-synaptic tracing of dopaminergic axons using AAV1 with human synapsin promoter and Cre, an anterograde trans-synaptic tracer (Karube et al., 2024). As a result, it appears that dopaminergic axons from the substantia nigra pars compacta preferentially innervate specific striatal interneurons (Karube et al., 2024). In this study, we only demonstrated the innervation pattern of a specific group of dopamine neurons. We visualized putative pre-synaptic dopaminergic axons and putative post-synaptic striatal interneurons simultaneously. Considering dense and wide axon collaterals of single dopaminergic neurons, how synaptic plasticity, which must require precise control of neuromodulation in time and space, is accomplished? Using AAV1 labeling, single dopaminergic axons formed multiple appositions onto dendrites and cell bodies of single striatal interneurons. Although more detailed observation is requested, the result can offer the possibility of the specific relationship between a single dopaminergic neuron and a single striatal interneuron. In addition, it has been considered that dopaminergic axons possess two forms of dopamine release: synaptic and volume transmission, and only 30–40 % of dopaminergic terminals possess synaptic structures (Descarries et al., 1996). Pereira et al. estimated that ~80 % of varicosities in dopaminergic axons may be silent (Pereira et al., 2016). The rule that governs the formation of synaptic or non-synaptic varicosities in dopaminergic axons related to the single striatal neurons will become an important key to recognize dopaminergic control of plasticity. Therefore, vEM can be a useful tool to uncover these questions. Recent mouse studies have revealed the topographical projections of dopamine neurons in the substantia nigra pars compacta are correlated with variations in gene expression and/or external inputs (Watabe-Uchida et al., 2012;

Menegas et al., 2015, 2018; Poulin et al., 2018; Pereira Luppi et al., 2021). Furthermore, the relationship between dopamine neuron groups defined by specific gene profiles and vulnerability to Parkinson's disease is being clarified not only in mice (Hook et al., 2018; Kilfeather et al., 2024), but also in humans (Monzon-Sandoval et al., 2020; Aguila et al., 2021; Kamath et al., 2022). In the future, it will be necessary to clarify the innervation pattern of each class of dopamine neurons defined by gene profiles. Furthermore, the reports mentioned above confirmed that the gene profiles of dopamine neurons dynamically change in response to aging and disease. Not only drastic changes in states, but also mild plastic changes in daily learning can affect gene expression, which, in turn, can affect synaptic functions and/or connections themselves. Our successful demonstration of the dopamine neuron output destination using the anterograde trans-synaptic viral vector is significant step toward a better understanding of the relationship between cell types of pre-and post-synaptic neurons, projection, and synaptic functions. The use of a trans-synaptic tracer expressing Cre with cellular labeling in ultrastructural analyses may provide important clues to a better functional understanding of neural circuits formed by connections between specific types of dopamine and striatal neurons.

4. Conclusion

Recently, advances in LM and vEM have contributed significantly to our understanding of complex brain structures and functions. LM tracing of axonal projection has long been used and continually improved to be powerful tools for visualizing neural circuits in relatively large brain volumes, which can be more easily combined with molecular and genetic cellular identities. Advances in vEM have also improved our ability to clarify neural circuits and cellular associations, resulting in comprehensive wiring maps of the brain across multiple organisms. To identify various structural components more precisely in addition to the morphological criteria used in common ultrastructural analyses, particularly in vEM observation, a combination of molecular and genetic labeling is required. There were significant advancements in the use of conventional labeling techniques, such as immunoelectron microscopy. Recent advances in correlative microscopy and genetically encoded probes look promising. At the same time, these approaches need to be refined further to address issues of specificity/sensitivity and structural preservation, as well as to optimize resolution and reliability in large areas. The novel labeling technologies have only recently begun to provide multi-target visualization in vEM imaging, but they will help us better understand cellular interactions and neuronal networks. Although various types of LM tools have been developed and revealed features of neural circuits involving projections of dopamine neurons that are critical to shaping decision-making processes in response to different inputs, questions such as connections between specific types of dopamine and striatal neurons must be addressed to clarify the mechanisms underlying such adaptive behaviors. The ongoing refinement and application of microscopic technologies for molecularly and genetically defined neural circuits is expected to lead us to a better understanding of the structural basis in neural networks involving dopamine neurons, which regulate adaptive behaviors in both physiological and diseased brain conditions.

CRediT authorship contribution statement

Fumino Fujiyama: Conceptualization, Writing – original draft, Writing – review & editing. **Fuyuki Karube:** Conceptualization, Writing – original draft, Writing – review & editing. **Nobuhiko Ohno:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest associated with this manuscript.

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Author contributions

N.O., F.K., F.F.: conceptualization, writing original draft, review and editing.

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