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Cell type specificity for circuit output in the midbrain dopaminergic system

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Abstract

Midbrain dopaminergic neurons are a relatively small group of neurons in the mammalian brain controlling a wide range of behaviors. In recent years, increasingly sophisticated tracing, imaging, transcriptomic, and machine learning approaches have provided substantial insights into the anatomical, molecular, and functional heterogeneity of dopaminergic neurons. Despite this wealth of new knowledge, it remains unclear whether and how the diverse features defining dopaminergic subclasses converge to delineate functional ensembles within the dopaminergic system. Here, we review recent studies investigating various aspects of dopaminergic heterogeneity and discuss how development, behavior, and disease influence subtype characteristics. We then outline what further approaches could be pursued to gain a more inclusive picture of dopaminergic diversity, which could be crucial to understanding the functional architecture of this system.

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Introduction

Specific circuits within the brain are formed by distinct classes of neuronal cell types. A formidable challenge in neuroscience is to comprehend how properties of individual neurons contribute to the computational properties of neural circuits and ultimately to behavior. The classification of neurons into cell types is thus one of the prerequisites for the systematic and reproducible analysis of the computational function of neural circuits in health and disease. However, the definition of a cell type is conceptually difficult, as it can refer to highly-specific molecular, anatomical, physiological properties that, moreover, are not necessarily fixed and can change during development, in various metabolic or disease states, and during aging. Nevertheless, new technical approaches in single-cell transcriptomics, circuit tracing, functional imaging and fine-grained behavioral analysis have given us unprecedented insight into molecular, anatomical and functional neuronal diversity across brain areas.

In contrast to fast acting neurotransmitters (e.g. glutamate and GABA), modulatory neurotransmitters such as monoamines affect the properties of downstream neurons in the short to long term by regulating synaptic transmission, excitability, plasticity, protein transport, and even gene transcription. Neuromodulators signal via G-protein coupled receptors (GPCRs). Whether a specific neuromodulator has a stimulatory or inhibiting downstream effect often depends on the GPCR type expressed and the type of downstream second messenger signaling cascade activated in the receiving neuron [1]. Although neuromodulators only indirectly affect neuronal and network activity, they have profound effects on the functional properties of neurons, circuits and entire brain regions. Therefore, understanding the function of neuromodulatory circuits is crucial to understanding information processing in the brain.

An example of a monoaminergic neuromodulatory system that exerts a strong influence on a variety of behaviors by modulating local and long-range circuits in different brain regions is the dopaminergic (DA) system. Within monoaminergic systems, the DA system has unique anatomical features, since the collateralization of DA neurons is limited (see below). This means that a single DA neuron typically projects primarily to a single brain area, making it plausible that the modulatory function in which a DA subpopulation is involved is determined by its target specificity. In the mammalian brain, DA neurons are primarily located in the midbrain (mDA), where they can be anatomically subdivided into the retrorubal field (RRF), substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), with few cells located in the SN pars reticulata and pars lateralis (SNl). Together with further cell groups in the

ventral periaqueductal grey (vPAG), dorsal raphe region, as well as in the olfactory bulb and hypothalamus, these small nuclei supply dopamine to almost the entire brain [2]. Accordingly, dopamine modulates a wide variety of neuronal functions (Table 1) and is involved in a multitude of brain disorders such as Parkinson's disease, schizophrenia, anxiety or addiction [3–6]. To this date, it remains a fundamental question how a single neurotransmitter, released from a comparably small number of neurons, can control these different behaviors and be of importance in various neurological diseases.

Addressing this functional heterogeneity has been challenging. Therefore, researchers have employed diverse approaches to categorize DA neurons into subtypes focusing on disentangling different mDA subclasses. This includes an anatomical classification originally based on mDA soma location in midbrain subnuclei or on mDA axonal projection pathways [2]. In recent years, with the availability of improved tracing tools, imaging techniques and analytical methods, it has become possible to determine the correlation between the location of the soma, axonal projections and even inputs with increasing accuracy [7,8]. Alternatively, molecular markers have been used to differentiate between mDA subtypes, an approach that has been boosted by new techniques such as single-cell RNA sequencing [9,10]. In addition, by using electrophysiological or imaging approaches in behaving animals, ideally in combination with machine learning techniques to define behavioral segments, it is possible to classify mDA neurons based on their activity during specific behaviors and also determine their anatomical position and/or axonal projection targets (see for example [11–14]). In this review, we aim to highlight the importance of these cell type classifications for comprehending the heterogeneous mDA circuit function and discuss how to achieve a more complete understanding of the intersection between different modalities, which will be necessary to gain even deeper insights into functional mDA ensembles and how the mDA system modulates specific behaviors.

Functional anatomy of midbrain dopaminergic outputs

Tracing studies have established that the axonal output pathways of mDA neurons often roughly correlate with soma location (Figure 1). *In vivo* manipulation of dopamine signaling, imaging of mDA axonal activity or dopamine release have provided insight into how dopamine function may be distributed across different brain regions for specific behavioral outputs (Table 1). The correlation between mDA soma location and axonal projections is an important aspect to consider for the functional dissection of the mDA system, since *in vitro* and *in vivo* studies have reported distinct activity patterns in different mDA subnuclei. Many of these patterns can be attributed to the expression of distinct ion channels or neurotransmitter receptors, which often follow a medial-lateral or rostral-caudal expression gradient (see for example [12,14–16]).

Importantly, for both VTA and SNc mDA neurons, a low rate of axon collateralisation between different target areas has been reported [17–21], enabling specific information transmission instead of wide broadcasting of behaviorally-relevant signals. In addition, recent evidence suggests that axons originating from molecularly-defined mDA subtypes innervate only narrowly

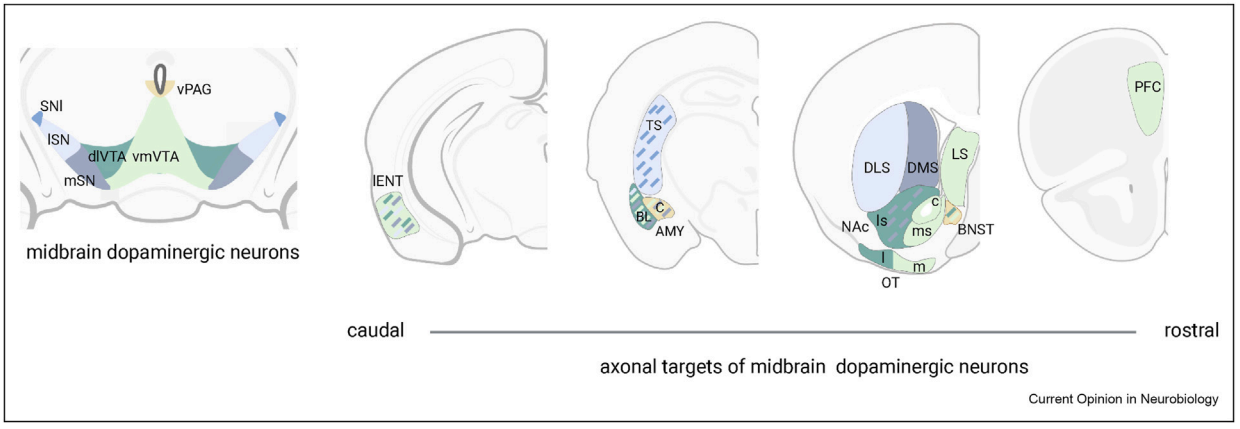
Table 1

Behavioral functions of mDA projections.

Target structure	Dopamine function	References
PFC	Working memory	[102]
NAc core	Pavlovian reward learning	[24]
NAc medial shell	Response in learned instrumental task	[24]
NAc lateral shell	Reward prediction signaling	[103]
Amygdala	Anxiety, appetitive and aversive associative learning and extinction	[69,99,104,105]
BNST	Pain sensitivity ♂, locomotion ♀	[100]
Medial OT	Odor preference	[97]
LS	Promotes aggression	[96]
Lateral ENC	Cue-reward associations	[101]
DMS	Motor control, movement initiation, vigor of movement, goal-directed actions	[26]
DLS	Motor control, skilled movements, habitual actions	[26]
TS	Aversion, threat prediction	[39,106]
Hippocampus	Learning and memory (dopamine partially from locus coeruleus afferents)	[107]

Please note that the functions listed for specific mDA target regions are only examples of the behaviors associated with dopamine signaling in these regions and – especially given the extensive and ever-growing literature on dopamine function – by no means claim to be comprehensive. Abbreviations are defined in the legend of Figure 1.

Figure 1



Axonal targets of mDA neurons according to soma location in the midbrain. VTA-mDA neurons, particularly in the caudal and ventromedial (vm) VTA, project predominantly to the prefrontal cortex (PFC) and medial part of the ventral striatum (nucleus accumbens (NAc) core (c) and medial shell (ms)) [18,95]. mDA neurons in the vmVTA are also the main source of DA innervation of the lateral septum (LS) [96]. mDA neurons sending projections to the lateral part of the NAc (NAc lateral shell (ls)) are primarily located in the dorsolateral (dl) VTA and medial (m) SNc [18,24,95]. In the olfactory tubercle (OT), medial regions are primarily innervated by mDA neurons in the vmVTA, while the lateral OT (lOT) receives projections from dlVTA-mDA neurons [95,97]. DA innervation of the basolateral (BL) amygdala (AMY) has originally mainly been attributed to the VTA [18], but recent studies have also highlighted pathways from across the SNc [98]. The ventral periaqueductal grey (vPAG) is the primary source of innervation of the central AMY (C-AMY) [25,99] and the bed nucleus of the stria terminalis (BNST) [25,100]. The lateral entorhinal cortex (lENC) appears to receive broad input from both VTA and SNc [101]. mSNc neurons project preferentially to the dorsomedial striatum (DMS), lateral ones (lSNc) to the dorsolateral striatum (DLS), while the tail of the striatum (TS) is innervated by projections from mDA neurons in the lSNc and SN pars lateralis (SNl) [20,37]. Note that there is also a bias of ventral SNc mDA neurons to project to DLS while dorsal ones project primarily to the DMS (not shown) [23,25,51,53]. Comparatively little is known about the projection targets and functions of mDA neurons in the RRF, thus the RRF is not depicted here. Illustration was created with BioRender.

circumscribed anatomical areas within the larger mDA target regions shown in Figure 1. For example, projections from one such subtype densely innervate only the dorsal most part of the dorsolateral striatum (DLS) while axons from another subtype show dense innervation of only the ventral part of the tail of the striatum (TS) [22,23]. In addition, a number of molecularly defined subtypes have been shown to preferentially innervate either the nucleus accumbens (NAc) core or the NAc shell [22,24,25]. Together these data indicate that dopamine signaling can be specific for projection subtypes and thus the postsynaptic target. Such highly specific and compartmentalized target innervation could be of great functional importance, both in the NAc subdivisions and in the dorsal striatum, which is divided into functional subdomains based on cortical and thalamic inputs [26]. Moreover, although it has long been postulated that dopamine is released via volume transmission in target areas, thus lacking spatial accuracy, recent evidence mainly gained by studying dopamine signaling in the dorsal striatum suggests more precise transmission at both the spatial and temporal scale [27,28].

Nevertheless, because of the small number of release sites and the rapid depletion of dopamine in these release sites, the activity of individual mDA neurons may have little predictive value for dopamine release or the effect of dopamine at the target site. In contrast, it has been postulated that the pooled activity of groups of mDA neurons might more closely reflect DA release [27,29]. Whether these mechanisms of release and receptor activation apply to all mDA target areas remains to be investigated.

Finally, when correlating mDA neuronal activity with the effect on target neurons, one needs to consider that subpopulations of mDA neurons have the ability to co-release fast-acting neurotransmitters or slow-acting neuropeptides [30–32]. It should be emphasized, however, that mDA neurons with a specific co-release ability also have some specificity in their projection targets. For example, SNc neurons projecting to the dorsal striatum co-release GABA, while mDA neurons that co-release glutamate are located in the lateral SNc and VTA and send projections to the tail of the striatum and many targets of VTA-mDA neurons [25,33]. VIP-expressing neurons are located in the vPAG and selectively project to the central amygdala and the bed nucleus of the stria terminalis [25]. Overall, this adds another layer of circuit specificity, both at the presynaptic site (e.g. transmitter-specific microdomains or activity patterns that allow for transmitter-specific release) and in the projection area (cell type-specific neurotransmitter receptors and signaling cascades).

Cell type-specific afferent inputs for distinct functional output

Another essential component for understanding how information is processed in mDA circuits, is whether mDA neurons with specific projection targets receive inputs from selected brain areas and whether these inputs show activity that correlates with the specific behaviors encoded by these mDA neurons. Complex rabies tracing studies analyzing monosynaptic inputs to mDA subtypes defined by their specific projection targets demonstrate that mDA neurons receive in general broad input from many brain areas with no clearly distinct relationship between projection target and monosynaptic input specificity [20,21,34–37]. Nevertheless, a bias in the distribution of direct inputs to mDA subclasses with specific projection targets is evident [20,21,36,38]. As there is a correlation between mDA soma location and axonal projection targets (Figure 1), this input bias may be attributed in part to the topological organization of mDA cell bodies with specific projection targets rather than to their output specificity *per se*. For example, SNc mDA neurons that project to the DLS are located in the lateral SNc and receive more prominent reciprocal projections from the DLS than from the dorsomedial striatum (DMS), while the DMS-projecting mDA neurons that are located in the medial SNc have biased input from the DMS. One population that appears to have more distinct direct inputs, for example from the globus pallidus or the subthalamic nucleus, are mDA neurons located in the lateral part of the SN that send projections to the TS [37,39]. In the VTA, a topological organization of inputs has been described [21]. Since there is also a rough topological organization of outputs in the VTA (see above), this study suggests that the specificity of connections to and from the VTA depends primarily on the mediolateral location of the neurons within the VTA [21]. In depth analysis of these data additionally revealed some input bias based on projection targets. For example, VTA-mDA neurons that project to the medial prefrontal cortex are located in the medial VTA and receive preferential inputs form the habenula and the dorsal raphe, while VTA-mDA neurons that project to the lateral nucleus accumbens are located in the lateral VTA and are preferentially innervated by neurons of the basal ganglia [40]. In addition to the long range monosynaptic inputs, mDA neurons also receive extensive direct local input, including from other mDA neurons. These local inputs fall into three clusters and a biased contribution of these clusters to mDA neurons along the lateral-medial axis of the VTA as well as to projection-target defined VTA-mDA neurons has been demonstrated [41].

Finally, the question remains how mDA neurons integrate input signals to encode behavior, which likely depends on the concerted activity of larger neuronal populations. This has been examined in detail for reward prediction error (RPE) signaling. A study by the Watabe-Uchida lab has demonstrated that input activity received by lateral VTA-mDA neurons is distributed across monosynaptic inputs from various brain areas, some of which respond positively and others negatively to the RPE, potentially resulting in a range of excitatory and inhibitory signals to the RPE-encoding mDA neurons. Still, this complex input combination results in an apparently homogenous mDA neuronal activity in RPE prediction [42]. More recent work shows, however, that populations of VTA-mDA neurons actually encode RPEs as a distributional code, with slight variability resembling optimistic and pessimistic predictions [43]. In consequence, future rewards are not simply presented as a single mean in the mDA system, but as a probability distribution of possible rewards to account for multiple future outcomes [44]. Moreover, it has been demonstrated that mDA neurons heterogeneously encode a variety of task parameters such as motor, sensory and cognitive variables and multiplex these signals [11,13,45,46]. This is in part achieved by coding distinct variables with tonic and phasic activity patterns [45], which can be selectively modified by specific inputs to mDA neurons [47]. In light of this heterogeneous signaling, it will be an important task to identify whether defined anatomical and/or molecular properties can separate functionally specialized subpopulations within the mDA system.

Molecular composition of the dopaminergic system

Recent advances in molecular techniques such as single-cell transcriptomics promise to solve the ongoing debate about what constitutes an mDA subtype, based on their molecular signatures. A recent review has synthesized the results of single cell gene expression profiling studies of postnatal brain or midbrain in the mouse in an effort to delineate subsets of mDA neurons based on the transcriptomic profiles uncovered in these studies and identified at least seven different molecularly defined mDA subtypes, three of them in the SNc [9]. While these classifications are still valid, further transcriptomic studies with large numbers of neurons and/or across multiple species provide a continuous update of what constitutes a molecularly-defined mDA neuron [10,23,48]. For example, a recent study [10], which performed single-nuclei RNA sequencing on a large number of SNc-mDA neurons from different mammalian species, including mouse and human, identified ten different mDA cell types in the SNc alone, one of which is specific to primates. This study highlights another important aspect to be considered when classifying diversity in the mDA system: biologically relevant differences between mDA subgroups are likely conserved across mammalian species (and some possibly even

across tetrapods). It will be interesting to see if and when a consensus eventually emerges on how many distinct subclasses of DA neurons with different gene expression profiles exist in different species and how many of these are conserved across different mammalian species or even beyond.

Managing multiple cell type modalities in the dopaminergic system

The identification and growing precision of molecularly-defined mDA subclasses leads to the obvious question of how molecular identity relates to the other features of mDA heterogeneity and, in particular, how this identity is mechanistically linked to anatomical and functional modalities of the respective subclasses. While some of the molecular markers that define specific mDA subsets have not (yet) been ascribed any obvious relevance for mDA neuron function, some marker genes encode proteins known to directly influence cellular computations. An example is the calcium binding protein calbindin (CALB1), which is not only associated with somatic regular bursting activity patterns [16], but also regulates dopamine release in a projection target-specific manner [49,50]. In this respect, it will be of interest to investigate in the available single-cell transcriptome datasets whether molecularly defined mDA subtypes show differential expression of genes encoding functionally relevant proteins (e.g., neuropeptide or hormone receptors, or ion channels critically modulating mDA neuron activity patterns). An important consideration in attempting to relate the identity of a neuron as determined by the transcriptome to its functional properties is that gene expression levels do not necessarily correlate with protein expression levels and cannot reveal a great deal about posttranslational modifications affecting protein function or the subcellular location of proteins.

Importantly, it will be key to integrate the molecular classification with the specific anatomical and functional features of mDA neurons. This would raise the questions whether these molecular subclasses have distinct soma location and projection targets, distinct inputs, electrophysiological properties and modulate specific aspects of behavior. Indeed, initial attempts to map the soma location of molecularly-defined mDA subtypes showed preferential location in subnuclei or along the lateral-medial and rostral-caudal axis, for example vGlut2+ mDA neurons are located in the medial VTA, lateral SNc, and SNl, Aldh1a1+ Sox6+ neurons in the ventral tier of the SNc, Aldh1a1- Sox6+ neurons in the dorsal tier of the SNc and the lateral VTA [9,51]. Furthermore, molecular subtypes show distinct susceptibility to neurodegeneration or stress and/or are activated during specific behaviors, e.g. during locomotion, associative learning or to the instructive rewarding or aversive cues [13,22–24,51–53].

Nevertheless, even though anatomically- or molecularly-defined cell types contribute to selective behavioral functions of dopamine signaling, heterogeneity can still be observed within these cell types. One possible explanation could reside in further subtypes within the current classification of mDA cell types, as highlighted above [9]. Indeed, many mDA molecular subtypes are defined by two or several marker genes. Therefore, it will be important to analyze cell type function in terms of combinatorial marker gene expressions. New approaches to target mDA subtypes based on the co-expression of several genes (or lack thereof) include intersectional genetic tools [25,54]. Indeed, the application of intersectional approaches to map projections of molecularly-defined mDA neurons and to investigate them functionally in behaving mice show interesting evidence that the molecularly-defined subclasses innervate highly specific target areas. Examples include preferential yet not exclusive axonal projections of Aldh1a1+, Anx1a+, Sox6+, Bcl11a + or vGlut2+ mDA neurons to distinct striatal areas [22,23,25,51].

Finally, another possible starting point to understand how the molecular profile of an mDA neuron converges with other features of heterogeneity is to consider the molecular mechanisms underlying the development of mDA neurons. For instance, given the relatively high target specificity of mDA neurons described above, mDA neurons that innervate a specific brain area likely express a set of guidance receptors during axonal outgrowth that is distinct from those of other subpopulations, allowing them to find their specific projection target [55]. Similar specificity in the interaction with the environment is likely to be important for the migration of mDA neurons to their correct anatomical position within the midbrain, which in turn may be critical for ensuring that mDA neurons receive the correct local and long-range inputs owing to the correlation between inputs and anatomical position of mDA cell bodies discussed above. Moreover, mDA neurons themselves might contribute to this input selectivity by expressing molecules (secreted factors, cell adhesion molecules etc.) that allow upstream neurons to specifically target them while "ignoring" neighboring mDA populations, a developmental mechanism that has not yet been explored. Indeed, there is growing evidence that diversity in the mDA system arises during embryogenesis, beginning at the progenitor cell level with the spatially and temporally specific generation of distinct types of mDA neurons and the further emergence of features of heterogeneity as mDA neurons differentiate [55,56].

Classification of dopaminergic cell types vs. cell states

While the anatomical properties of mDA neurons are likely hardwired from an early developmental stage [55],

some of the molecular markers identified in single-cell transcriptomic analysis of the postnatal brain to define certain subsets of mDA neurons are expressed in changing patterns across the course of development. Of note, vGlut2 (see previous section) is initially expressed in all mDA neurons [57,58], and Sox6-expressing mDA progenitors in the developing brain give rise to both Sox6-positive and Sox6-negative adult mDA populations [51]. It should also be considered that the molecular and thus the functional identity of mDA neurons might further change in adulthood, e.g. in circadian rhythm [59], estrus cycle [60], during healthy aging [61] or in disease [10,62–64]. Taking into account that metabolic states [65], sleep [66] or simple behaviors such as increased motor activity [67] can change gene expression programs in other regions of the brain, it is likely that similar alterations can also be induced in mDA neurons. Expression of molecular markers is thus not necessarily static, but can vary over time and experimental conditions, and thus may represent a cell state rather than a cell type.

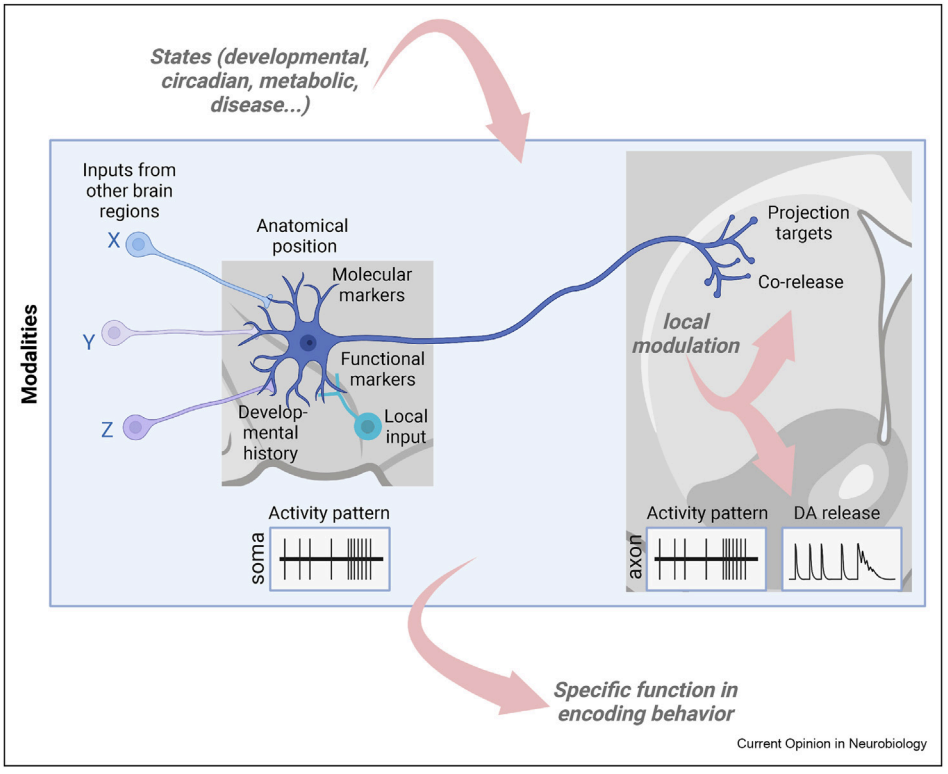
Similarly, the activity of mDA neurons can be modulated by external parameters such as context [68] or behavioral state [69–72]. This includes metabolic needs such as hunger or thirst, which are crucial drivers for goal-directed behavior. For example, cues signaling food rewards are potentiated in the mDA VTA→amygdala pathway during hunger, but are attenuated in satiety. In contrast, responses to aversive cues increase with the transition to satiety, indicating opposing changes in cue salience across behavioral states for this mDA projection [69]. Furthermore, different motivational signals such as social and food stimuli are encoded by overlapping populations of mDA-VTA neurons. Hunger states or opposite-sex encounters can dynamically change this representation, which is accompanied by state-dependent changes in gene expression related to cellular excitability [73]. At this point, it is largely unknown if state-dependent modulation affects selected molecular and/or anatomical mDA subpopulations. On a single cell level, it is likely correlated with expression of distinct genes, for example receptors for distinct neuropeptides or hormones modulating homeostatic needs.

Conclusion and future directions

The rapid development of methodologies that allow unprecedented insights into the anatomical, molecular and functional features have resulted in a tremendous increase in our knowledge on how diverse the mDA system is. However, to fully distinguish mDA cell types, new approaches will be needed to integrate information on the single cell level across distinct behavioral and disease states with a more comprehensive definition of individual anatomical and molecular identity. Taking into account all modalities of mDA cell type heterogeneity, an ideal experiment would need to cover anatomical information (morphology, soma location in the midbrain, inputs and outputs), gene expression analysis (molecular markers, specific ion channels and receptors) and functional properties across behavioral states on a single cell level (Figure 2). While this cannot be achieved (yet) in its entirety with existing technical approaches — and would likely exceed the capabilities of any single research group — we have highlighted recent studies addressing selected combinations of cell type modalities that have substantially improved our understanding of the mDA system. Many new exciting techniques allow to gain a more complete overview of mDA cell type classification, and we are outlining major questions that can now be addressed in the following.

1. *How do unique molecular features overlap with anatomical properties?* More and more platforms and data resources for large-scale spatial transcriptomics are now becoming available [74–76]. These allow linking gene expression with soma location and even projection targets at the single cell level ('EASI-FISH' [77]). Furthermore, new viral connectomic approaches such as 'BARseq' or 'Connect-seq' allow for combinations of circuit tracing with transcriptional analysis, or trans-synaptic labeling from molecularly-defined neuronal subpopulations [78]. This can additionally be combined with assays measuring synaptic strength (e.g. *ex vivo* slice electrophysiology) or *in vivo* monitoring of input activity to elucidate the functional connectivity of mDA subtypes [42,78]. How well these new tools will work for mDA neurons remains to be determined, as viral tropism is still an issue in the mDA system, with popular approaches such as AAV2-retro failing to efficiently label mDA neurons [79]. Labor-intensive screening, e.g. for suitable serotypes of AAVs [80], systematic approaches such as engineered capsid structures [81] or receptor complementation strategies [82] can help to overcome these problems. Alternatively, non-viral tracers such as retrobeads [18] or cholera toxin B [77] are efficient strategies for retrograde labeling of mDA projection pathways and allow incorporating anatomical information with other modalities such as gene expression using spatial transcriptomics, functional recordings with electrophysiology or calcium imaging, or even combinations of these approaches. Finally, to gain a better insight into how developmental, behavioral and disease states influence gene expression and function of mDA subpopulations, it will be critical to integrate the anatomical and molecular profiles of these subclasses with information about their epigenetic features [83].
2. *How are behavioral functions encoded on the single cell level?* For functional assessments, one important consideration is the technical approach of recording neuronal activity. While fiber-based imaging typically allows for reliable recording from a large population of somata or axonal projections, it lacks cellular resolution and

Figure 2



Distinct modalities used to categorize mDA neurons into subpopulations. The modalities of a given subclass are not static, but can be modulated by behavioral states or local modulation. The interplay of these intrinsic determinants and extrinsic factors ultimately results in the functional output of the mDA system. Illustration was created with BioRender.

thus is at risk of missing potential heterogeneity of individual neurons within the recorded population. Both brief and prolonged pauses in spontaneously active mDA neurons are meaningful for circuit computations [12,45,84], but likely to be overlooked with this technique if only displayed in a subgroup and masked by a majority of activated cells or axons. Therefore, any interpretation that the response of a projection- or molecularly-defined population is homogeneous based on fiber-photometry recordings should be made with caution.

3. *How do functional properties correlate with other cell type modalities?* Deep-brain imaging techniques now allow for recordings of large mDA neuronal populations at single-cell resolution in freely-behaving or head-fixed mice [11,73]. Refined surgical approaches no longer require tissue removal for the implantation of thin lenses and thus allow for measurements of physiological activity patterns in the almost intact brain. Despite lacking temporal resolution, spike-inferring algorithms promise to pry out tonic and phasic mDA neuron activity patterns that are critical to understand coding in mDA neurons from calcium imaging data [85]. Importantly, since these recordings are typically stable for many days, they allow to follow individual neurons across distinct behavioral states, and can even be combined with post-hoc spatial transcriptomics to identify the molecular profile of the imaged cell types ('CaRMA' platform [86]) — making it the almost perfect experimental approach to address all modalities of mDA cell type diversity.
4. *How do somatic and axonal activity translate to dopamine release at the target sites?* Genetically-encoded fluorescent sensors detecting extracellular dopamine in projection areas cannot differentiate for release from molecularly-defined subtypes, but can be combined with optogenetic stimulation or recordings of axonal activity in defined subpopulations [87]. Of note, focusing on mDA target areas instead of their somata would also allow to take local mechanisms of modulation such as action potential initiation in distal axons into account [88—90]. However, which of these local modulatory mechanisms are actually important for DA release in behaving animals requires further research [91,92].
5. *What are the downstream effects of mDA projections on recipient neurons in target areas?* As highlighted above, co-release of other neurotransmitters and peptides has to be considered for many mDA neurons, yet if distinct

activity patterns are necessary for transmitter-specific release is so far unclear. Binding of dopamine itself to its receptors activates or inhibits — depending on the type of receptor expressed — second messenger signaling cascades that ultimately modulate cAMP levels and PKA activity. Thus, examining these downstream readouts may provide additional insight into how dopamine signaling is integrated in the receiving neuron to modulate its functional output [93,94].

In conclusion, a concerted effort to investigate mDA neurons in a multimodal manner by applying new technologies as outlined above will be critical to understand the functional architecture of the DA system. Such a comprehensive insight could provide the foundation for developing therapeutic strategies to specifically modulate abnormal DA functions in disease states in a targeted, subcircuit specific manner.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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In this review article, Poulin et al. compile and integrate data from several single-cell transcriptomic studies to categorize mDA neurons according to their molecular profile. In doing so, they not only emphasize the convergent points but also the distinctions among the mDA subtype classifications that arise from these analyses. Their review establishes an initial framework for molecularly-defined mDA neurons that will serve as a basis for integrating current and future studies to further refine gene expression profile-based categorizations of mDA neurons.

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Kamath, Abdulraouf et al. used single nuclear RNA sequencing, spatial transcriptomics, and computational analysis to investigate the transcriptome and regulons (transcription factors and their targets) of SNc-mDA neurons in humans, macaques, and three rodent species. They identified ten transcriptionally distinct subpopulations of mDA neurons, one of which is specific to primates. They also examined SNc-mDA neurons from patients with Parkinson's disease and dementia with Lewy bodies to determine which of the identified subpopulations are particularly vulnerable in these neurodegenerative diseases.

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In this preprint, Avvisati, Kaufmann et al. utilize in vivo electrophysiological recordings with juxtacellular labeling to investigate the organizational logic of mDA neuron activity patterns during associative learning. This study provides evidence that certain activity patterns are not associated with soma location of mDA neurons in midbrain subregions, but correlated with distinct projection pathways, highlighting that multiple task variables are encoded in dopamine outputs to distinct striatal subregions.

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This study establishes a link between developmental gene expression and specific anatomical and functional features of mDA neurons by demonstrating that SNc- and VTA-mDA subpopulations defined by the expression of the transcription factor Bcl11a during development and in the adult brain have a very specific innervation pattern of forebrain areas and, in the SNc, are particularly susceptible to neurodegenerative processes. Inactivation of Bcl11a in mDA neurons leads to altered mouse behavior and increased vulnerability to neurodegeneration.

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This study demonstrates that distinct genetic subtypes of SNc-mDA neurons exhibit unique responses to rewards, aversive stimuli, accelerations, and decelerations. Importantly, activity patterns within these subtypes (but not between subtypes) are highly correlated between somata and axons when monitored with fiber photometry. These subtype-segregated responses could explain the divergence between dopamine release/axonal activity and activity in mDA somata observed in studies monitoring these parameters independent of the molecular profile of mDA-SNc neurons (but see Liu et al. for an alternative mechanism). These results establish a link between functional and genetic mDA subtypes, and highlight that defining mDA subclasses based on their molecular profile can provide a useful context for studying mDA neuronal functions.

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