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Classification of midbrain dopamine neurons using single-cell gene expression profiling approaches

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Abstract

Dysfunctional dopamine (DA) signaling has been associated with a broad spectrum of neuropsychiatric disorders, prompting investigations into how midbrain DA neuron heterogeneity may underpin this variety of behavioral symptoms. Emerging literature indeed points to functional heterogeneity even within anatomically defined DA clusters. Recognizing the need for a systematic classification scheme, several groups have used single-cell profiling to catalog DA neurons based on their gene expression profiles. Here we aim to synthesize points of congruence, but also highlight key differences, between the molecular classification schemes derived from these studies. In doing so, we hope to provide a common framework that will facilitate investigations into the functions of dopaminergic neuron subtypes in the healthy and diseased brain.

Keywords

dopaminergic system; molecular diversity; single-cell RNAseq; intersectional; neuroanatomy; cell type

Heterogeneity within the dopaminergic midbrain system

Since their discovery, neurotransmitters and their associated molecular machinery have been used to differentiate one type of neuron from another. This trend has been reinforced by thousands of neuropharmacological studies which have associated diverse behaviors, mental states, or diseases to specific neurotransmitter systems. Too often however, neuronal populations possessing a common neurotransmitter were assumed to be homogeneous, even though they differed significantly based on morphological and physiological properties. Historically, this has been the case for neurons releasing the neurotransmitter dopamine (DA), a small population of less than 500,000 cells in the human brain [1].

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The cellular and molecular properties of DA neurons have attracted great interest, especially since the depletion of DA in the caudate putamen (CP) was shown to underpin the locomotor symptoms of Parkinson's disease (PD) [1]. Since then, dysfunctional DA signaling has been implicated in numerous neuropsychiatric disorders, including depression, chronic pain, drug addiction, schizophrenia, and attention deficit and hyperactivity disorder [2]. Until recently, it remained puzzling how a single neurotransmitter system could be responsible for such disparate symptoms and apparently unrelated diseases. This quandary could in part be resolved by demonstrating the existence of distinct populations of DA neurons, or "subtypes", each possessing unique molecular, physiological, and functional properties. Although this is a somewhat Panglossian point of view since DA-associated neuropsychiatric disorders involve neural impairments that extend beyond DA circuits, identifying subtypes of DA neurons could provide a greater understanding of both the breadth of DA-related symptoms, as well as potentially reveal novel avenues for more precisely targeted pharmaceutical interventions.

The majority of DA neurons are located in three anatomical clusters in the midbrain, namely the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA), and retrorubral area (RR) [2,3]. For decades, neuroscientists have searched for, and identified, markers that were expressed in subpopulations of midbrain DA neurons within these clusters (reviewed in [1,4,5]). Although these studies propelled the field forward in establishing DA diversity, the limited ability to simultaneously look at a multitude of markers made it difficult, if not impossible, to define DA neuron subtypes with certainty since the expression of a single gene is rarely sufficient to identify a molecular subtype. Bulk profiling experiments comparing SNc and VTA neurons did indeed yield some interesting gene expression differences [6–9], but suffered from the caveat that they could not distinguish heterogeneous populations within each anatomical cluster. Indeed, the identification of molecularly distinct neuronal populations requires the measurement of multiple markers in individual cells (reviewed in [10–12]).

Fortunately, in the last decade, advances in the field of single-cell transcriptomics and related bioinformatics made it possible to detect coordinated gene expression profiles within individual cells (Box 1). Such approaches have been used to classify molecularly distinct cell types in an unbiased manner in several brain structures, including midbrain DA neuron clusters. Molecular heterogeneity within the murine DAergic system has been investigated by at least six independent groups, all attempting to classify DA neurons based on gene expression [13–18]. Although these studies differed significantly in their experimental parameters and analysis methods, they identified partially overlapping subtypes, some of which were located in a manner agnostic to traditional anatomical boundaries (SN, VTA, & RR). Unfortunately, the use of different defining markers and naming schemes across studies has made it difficult to investigate and discuss the properties of specific DA subtypes. In this review, we highlight congruencies and incongruencies in the putative DA neuron subtypes identified in these studies. In the two studies that did not validate all the single cell clustering data [17,18], we present our best interpretation of their classification scheme. We then undertake the challenging task of synthesizing a classification scheme for mouse midbrain DA neurons based on all available studies, and when possible, correlate this scheme with

known functional and anatomical properties of DA neurons. Finally, we briefly discuss how this cellular diversity is generated during development.

Defining dopamine neuron subtypes based on their molecular profiles

It is generally agreed upon that groups of neurons that possess a core number of distinctly expressed genes can be classified as the same cell type [10–12]. However, classifying neurons into subtypes is no easy task, particularly when the types are closely related. In the case of midbrain DA neurons, all display a typical neuron molecular signature, in addition to expressing genes necessary for the synthesis, packaging and release of DA (Box 2). Superimposed on this generic midbrain DA neuron signature, which also include mesodiencephalic floor plate transcription factors, is the expression of other genes confined to subpopulations of DA neurons, that can be used to categorize them into distinctive subtypes. Multiple groups have profiled the gene expression of individual midbrain DA neurons in the mouse (Figure 1; [13–18]). These groups observed several differences, both in terms of the number of DA neuron subtypes they identified, as well as the genes used to define them (Table 1). These differences can partly be explained by variations in experimental conditions such as using different cell sources, isolation procedures, developmental stages, technical approaches, and classification criteria (Table 2). Here, we attempt to synthesize the current literature and extract a classification of DA neurons derived from all published single-cell classification studies and other supporting data. We have focused almost exclusively on neurons expressing the dopamine transporter (*Dat*; *Slc6a3*). This is because the majority of DA neurons, as defined by immunolabeling for the rate limiting enzyme involved in DA synthesis tyrosine hydroxylase (TH), are recombined in the adult Dat-IRES-Cre mouse [19]. While *Th* mRNA+ neurons that do not express *Dat* are also observed in the midbrain, it is not clear if these produce functional amounts of DA, and thus they have been omitted from this review [15,19,20] (see Box 2). After assessing the current literature, here we propose seven putative DA neuron subtypes, defined by a unique molecular signature, whose existence is supported by multiple studies. Four of these are more robustly identified by diagnostic markers, whereas the three others are more ambiguous. We next describe each of these subtypes, the data supporting their existence, and when available, their distinctive properties (Figure 1). Here, the list of genes refers to mRNA detected within a subtype and is used for definition purposes (i.e. the functions of the gene product are not necessarily relevant to this discussion).

Aldh1a1+, Sox6+ DA neurons are located in the ventral SNc

Aldh1a1 and *Sox6 mRNAs* are the defining markers for this DA neuron subtype, a population overtly represented in two studies: DA^{1A} in [13] and DA-SNC in [14]. These cells also display *Aldh1a7* [14,17] and *Ndnf* [13,14] mRNA expression. In [15], we believe that this population has been grouped together with *Aldh1a1*+, *Sox6*- cells, possibly because *Sox6 mRNA* was not robustly detected in this study. In contrast, this population was split into two distinct groups in [17] based on the expression of *Anxa1 mRNA*. In [18], the *Sox6*+, *Aldh1a1*+ population seems to have been split into at least three subpopulations named SN_4–5, SN_4–8, and SN_4–9. The defining genes for these populations are *Vcan,*

Anxa1 (also characterized by the highest expression levels for *Aldh1a1*), and *Grin2c* respectively, although these markers have yet to be validated by secondary methods.

Prominent projections of ALDH1A1+ DA neurons of the SNc have been observed in the dorsal caudate putamen (CP; [20–22]), with fibers being more dense in rostral sections and less so in caudal regions. In the dorsal CP, these neurons preferentially innervate some μ-opioid receptor+ (MOR) patches (commonly known as striosomes), although this is not exclusive [20–22]. Interestingly, ALDH1A1 belongs to the aldehyde dehydrogenases family and its presence in DA neurons may alter their function in at least four ways. First, aldehyde dehydrogenases catalyze the conversion of retinaldehyde to retinoic acid (RA), which may be implicated in maintenance of MOR expression in patches, potentially via transsynaptic RA signaling [23]. Second, ALDH1A1 may also mediate the oxidation of the cytotoxic side product of dopamine synthesis, DOPAL, into a less reactive species. Third, ALDH1A1 might enable the synthesis of GABA through a non-conventional enzymatic pathway [24]. Finally, this enzyme could also play a role in modulating DA release, as there is a reduction in DA release by the ALDH1A1+ fibers targeting the striosomes compared to the matrix, an effect not observed in *Aldh1a1* null mice [21]. A recent report supports a function of *Aldh1a1+* DA neurons in the acquisition of skilled movements, as ablation of these neurons results in significant rotarod learning deficits [22]. In addition, *Aldh1a1*+ DA neurons of the SNc are selectively decreased in a mouse model of PD [13], as well as in human brains affected by the disease [25]. Taken together, these studies strongly suggest that these neurons of the ventral SNc have distinctive properties, some of which are conferred by *Aldh1a1* function.

Sox6+, Aldh1a1- DA neurons are located in the SNc, parabrachial VTA, and RR

While this subtype is somewhat more ambiguous, the presence of *Sox6* mRNA and the absence of *Aldh1a1* transcript was considered to be its defining signature. Such neurons have been identified in three studies (DA-VTA1 in [14], DA^{1B} in [13], and SN_4–3 in [18]). These cells also co-express high levels of many of the mRNAs aforementioned in the previous population (*Ndnf*, *Igf1* and *Sncg)*, but in contrast, some of these neurons also express *Calb1* [13,14], *Lypd1* [14], *Tacr3* [13,18], and Cyp26b1 [18] mRNAs. In addition to lacking *Aldh1a1* mRNA, they also do not express *Anxa1* [14,17,18] and *Aldh1a7* [14,17,18]. It is possible that [16] may have grouped this subtype together with *Aldh1a1*+ cells based on high mRNA *Sox6* expression and overall similarity in gene signature. In [15], these cells are likely represented within the population of *Th*+, *Aldh1a1*- neurons displaying high expression of *Dat* (referred to as T-Dat-high). In immunolabeling studies, there is a substantial SOX6+ population that is ALDH1A1- [13,14,26]; these cells are located more dorsal to the ALDH1A1+ neurons in the SNc, the parabrachial pigmented (PBP) region of the VTA and the RR. Because *Aldh1a1* mRNA expression is one of the few diagnostic features separating the two *Sox6+* cell types, further work will be required to define additional markers in order to facilitate their investigation *in vivo*.

The functional roles of the *Sox6*+, *Aldh1a1+* and *Sox6+*, *Aldh1a1*- cohorts might at least be influenced by the expression of ALDH1A1, and its cognate enzymatic functions like retinoic acid synthesis or DA degradation [21,22]. However, it remains to be seen if these subtypes

can be differentiated by other features, such as their physiological properties. With regard to projections, the overall SOX6+ cohort projects densely to the rostral CP and lateral shell and core divisions of the nucleus Accumbens (ACB), whereas the projections to the caudal CP appear less dense [20]. *Sox6*+ projections, which encompasses *Aldh1a1*+ projections, show significant innervation of the medial and ventral CP. Comparing projections of SNc DA neurons labeled using Sox6-Cre and Aldh1a1-CreER^{T2} drivers, it could be inferred that *Sox6*+, *Aldh1a1*- neurons which are located mainly in the PBP region, spanning the junction between the SNc and VTA [20], project to the medial and ventral CP and lateral shell of the ACB. Additional identification of unique molecular markers, as well as the generation of a Cre drivers, will validate the existence and facilitate the investigation of this putative subtype.

Vgat+ DA subtype is located mostly in the VTA

A DA subtype distinguished by the expression of *Vgat* mRNA (*Slc32a1*), was identified in four studies: DA^{2A} in [13], DA-VTA4 in [14], GT-Dat-Low in [15], and based on our interpretation, SN_3–7 in [18]. This group of cells is enriched for *Vgat* [13–15,18], *Calb1* [13,14], *Crhbp* [15,18], *Gad2* [15,18] and *Wnt7b* [15,18] mRNAs. In [18], this subtype was grouped with GABAergic neurons of the SNc rather than with the DA clusters, possibly because this subtype has molecular features of both DA and GABA neurons. Many of these neurons also express *Vglut2* (a.k.a. *Slc17a6*), a glutamate vesicular transporter [13,18], and also have lower *Dat* and *Th* expression [15]. This latter observation may explain why these neurons were not observed in [16], which utilized a *Th*-eGFP transgenic line for isolating DA neurons (Table 2). Anatomically, *Vgat*+ DA neurons have been mainly observed in the VTA [13–15]. While these cells were numerically abundant in single-cell isolation/profiling, the proportion of this population observed by *in situ* hybridization and immunofluorescence is significantly less, with one study suggesting that these comprise less than 5% of all adult midbrain DA neurons [15].

The projections and function of this subtype has not been directly determined, although these might correspond to a population of DA neurons GABA co-releasing and projecting to the medial shell of the ACB [27]. Alternatively, they might correspond to the mesohabenular *Gad2*+, *Th*+ neurons described in Stamatakis et al. [28], although these neurons might not have the capacity to release DA. Unlike the non-canonical GABA synthesis and vesicular loading that has been proposed for GABA co-release from SNc neurons [24,29,30], these transcriptomic studies collectively open the possibility that this VTA enriched subtype may synthesize and co-transmit GABA through canonical mechanisms involving GAD and VGAT.

Otx2+, Aldh1a1+ DA neurons in the ventromedial VTA

Cells co-expressing *Aldh1a1* and *Otx2* mRNAs were observed in six studies and named DA^{2B} [13], DA-VTA2 [14], SN_4–1 [18], and MB.1 [16]. Cluster #8 of [17] also appears to match this subtype, which likely correspond to previously VTA neurons observed in immunolabeling-based studies [31,32], as well as those profiled in ACB projection-based transcriptomic analyses [33]. Based on co-expression of other genes in this subtype, we derived the following mRNA expression signature: *Aldh1a1* [13,14,17,18], *Lpl* [13,14,18],

Otx2 [13,14,16,18], *Neurod6* [13,14,16–18], *Gpr83* [14,17,18], *Grp* [13,17,18], and *Cbln4* [17,18]. In [15], it is possible that all *Aldh1a1* expressing DA neurons, from both SNc and VTA, were lumped together (named AT-Dat high). *Otx2+, Aldh1a1+* neurons are located in the ventromedial VTA [13,14,17,31,32,34], and they roughly comprise between 12–36% of VTA DA neurons, depending on the method of quantification. Multiplex *in situ* hybridization suggested that both *NeuroD6* and *Grp* transcripts might not be found in all neurons of this subtype, with *Neurod6* transcript being more restricted [17]. Additionally, one study showed that only 12% of *Neurod6*+ cells co-express *Vglut2* [35], although two other studies suggested that a substantial number of these cells were *Vglut2*+ [13,18], the difference possibly explained by sensitivity of techniques used.

This population has been accessed using *Aldh1a1* and *NeuroD6* based drivers, and it appears to direct major projections to the medial shell of the ACB, and minor projections to the lateral septum [17,20,22,36]. In *Neurod6* KO mice, DA projections to the lateral septum are reduced, suggesting a dependence on NEUROD6 function [36]. Another study, using *Aldh1a1*-CreER^{T2} strain to access this population, postulated some projections to the dorsomedial striatum [22]. Physiologically, DA neurons labeled with Neurod6-Cre have a smaller I_h, more depolarized membrane potential, higher membrane resistance, less pronounced afterhyperpolarization, and shorter action potential height when compared to SNc neurons [17]. These characteristics are consistent with those observed for medial shell projecting DA neurons [37]. When optogenetically stimulated, *Neurod6*+ neurons induce place preference behavior [35]. Furthermore, these neurons appear to co-release glutamate [35], consistent with a larger body of work showing glutamate co-release in the medial shell [38–45]. In studies using *NeuroD6* as a driver, however, it has been dutifully reported that it is expressed in non-DA neurons of the VTA [17,35,36]; thus experiments based on these drivers must be interpreted with caution.

Vip+ DA neurons are the most caudally located population

Four studies identified a subpopulation of DA neurons expressing high levels of the neuropeptide VIP: DA-VTA3 in [14], DA^{2D} in [13], VT-Dat-high in [15], MB.3 in [16]. On a neuroanatomical basis, this subtype is located in the periaqueductal gray (PAG) and dorsal raphe (DR) regions, accounting for about 49% of DA neurons [13] in these regions, but more sparsely in the VTA [13–15,46](Figure 1). Based on the concordance of other genes co-expressed in *Vip*+ neurons [13–16], we developed the following mRNA signature for *Vip* neurons: *Vip*+ [13–16], *Gipr*+ [15], *Calb1*+ [13,14], *Sox6*- [13,14], *Aldh1a1*- [13,14]. These neurons are also *Vglut2*+ at neonatal timepoints [13], consistent with functional data showing glutamate co-transmission from PAG/DR DA neurons in the adult [47,48]. However, since the PAG/DR contains both *Vip+* and *Vip*- DA neurons, definitive proof showing adult *Vip+* neurons co-releasing glutamate requires further experimentation.

The projections of these neurons are peculiar in that they are particularly focal – they have been mapped to the bed nucleus of the stria terminals (BST) and central nucleus of the amygdala [13,20], and appear to innervate a much smaller target volume than other DA neurons (e.g. [49] and [50]). The function of the *Vip*+ DA subtype has never been examined in isolation. However, some studies have investigated the functions of DA neurons located in

the PAG/DR and have found that they were involved in wakefulness and social behaviors [47,48,51]. DA neurons in this region also might be embedded in the circuits that transduce the antinociceptive effects of morphine [52], as well as in fear-induced learning [53]. Since *Vip+* DA neurons constitute a fraction of TH+ neurons of the PAG/DR, direct testing will be necessary to know if Vip+ and Vip- DA neurons of this region have distinct roles in behavior.

Vglut2+,Calb1+ DA neurons are ambiguous and likely encompass two or more distinct populations

A *Vglut2*+, *Calb1*+ population is represented in at least three of the studies, although this remains somewhat ambiguous owing to the lack of more specific markers [13,16,18]. In [13], this population, named DA^{2C}, was somewhat indistinct, characterized by *Vglut2* and *Calb1* mRNA expression, but low or sporadic expression of transcription factors like *Otx2* and *Sox6*, and little to no expression of *Aldh1a1* and *Vip.* In [16], this was described as a neuroblast-like population, although we interpret it as an adult neuronal population, since a population expressing these diagnostic markers is observed in the adult brain [18,20]. In [18], this population is likely to be represented within cluster SN_4–2, and is also defined by the marker *Cbln1.* It is unclear whether this represents a single population or multiple DA subtypes. One possibility is that the *Vglut2*+, *Calb1*+ population encompasses at least two subtypes, one located in the SNc and the other in the VTA. The first of these populations may be found in the dorsolateral part of the SNc, including a region known as the *pars lateralis* (SNpl) [20]. Indeed, in recent studies, a population of cells in the dorsal SNc and SNpl could be labeled by viral injections into the SNc of *Vglut2*-Cre, *Th*-Flpo mice [20,54]; only 8% of these were reported to be *Aldh1a1*+[20]. These cells show a predominant projection to the tail of the striatum, but also to more discrete regions in rostral striatum [20,54]. Functionally, these cells are likely to match a recently described class of tail of striatum-projecting SNc neurons, that have distinctive inputs and show responses to aversive stimuli [55–57], but direct and careful testing is required to confirm this prediction. In toxin models of PD, *Vglut2+* DA neurons in the SNc are particularly resistant to degeneration [58,59], and VGLUT2 itself may play a neuroprotective role by inducing a BDNF/TRKB dependent signaling cascade [59].

A second, closely related population of mRNA *Vglut2*+, *Calb1*+ DA neurons is found in the VTA. This is distinct from the *Otx2*+, *Aldh1a*1+ subtype mentioned previously, judged by the absence of many markers including *Aldh1a1* expression. Comparing the projections of *Vglut2*+, *Aldh1a1*- and *Vglut2*+, *Aldh1a1*+ VTA cohorts, we observed significant overlap in the ACB, olfactory tubercle (OT), and lateral septum (LS). In comparison, we observed projections in the BLA, entorhinal and prefrontal cortex, exclusively from *Aldh1a1*-, *Vglut2*+ projections [20]. However, additional analysis will be required to define these neurons on a molecular basis, and thereby unambiguously distinguish this subtype from *Aldh1a1*+ DA neurons of the VTA, and from *Vglut2*+ cells in the SNc. Overall, these data are consistent with the abundant literature on TH+ VTA neurons that can release glutamate in the ACB [38,43,45,60–63], but also in the basolateral amygdala, entorhinal and prefrontal cortex [43,54,64,65]. Interestingly, there is evidence of *Vglut2* and *Vmat2* segregation in

Vglut2+ DA axons in the ACB, suggesting distinct release sites and mechanisms for these two neurotransmitters [41,42,66].

Generating midbrain DA neurons diversity during embryonic development

The embryonic primordia of midbrain DA neurons is well defined. These neurons originate from the mesodiencephalic floorplate (FP), a region at the ventral midline defined by the mRNA expression of *Shh*+ and *Foxa2*+ [67–73]. The boundaries of this primordium have been well defined by transcription factor expression. The caudal extent is defined by the *Otx2*/*Gbx2* boundary coincident with the morphological mid-hindbrain boundary [74]. The rostral extent is defined by the *En1*/*Dbx1* gene expression boundary which is located in the diencephalic region [75–78]. The dorsal extent is defined by the *Lmx1a*/*Nkx6.1* gene expression boundary [71,79–81]. The primordium of the majority of midbrain DA neurons therefore can be described as the ventral mesodiencephalic region progenitors that express *Foxa*1/2, *Lmx1a/b*, *Otx2* and *En1*. Current evidence suggests that most, if not all, midbrain DA neurons observed in the adult mouse brain arise from this primordium. While the DA primordium has been precisely demarcated, how and when DA neuron heterogeneity is generated remains to be clearly elucidated.

A starting point for answering this question is to determine whether the DA primordium can be subdivided further into molecularly discrete regions that foreshadow subtype identity. Consistent with this possibility, several genes are known to be expressed in a non-homogeneous manner in the DA primordium [82]. Some genes like *Shh* are dynamically expressed [67,70]; other genes like *Wnt1* and *Aldh1a1* are expressed specifically in lateral regions of the primordium [83,84], whereas *Corin* is expressed in more medial regions [85]. Some genes, for instance *Wnt1* and *En1*, are expressed in graded fashion along the rostro-caudal axis, being more robustly expressed near the mid-hindbrain boundary. How these non-homogeneous expression patterns in the progenitor zone correlate with diversity remains to be determined. Some recombinase-based lineage studies have been performed to address this question, but differences in Cre drivers, TAM regimens, and mosaicism have provided only a blurry picture of DA subtype specification [67,70,83,86,87]. More recently, *Sox6* has been shown to be expressed in the medial aspect of the progenitor pool, and based on immunolabeling experiments, this progenitor pool has been proposed to be the source of the majority of the SNc but not the VTA [26]. Lineage tracing approaches will be required to test this model. In addition to the aforementioned lineage studies, knockouts of various transcription factors and other developmental molecules have also begun to illuminate the developmental basis of DA neuron diversity, and this has been reviewed recently [82,88].

Single-cell transcriptomics has recently been applied to developing DA neurons and has begun to provide a picture of the emerging diversity within maturing DA neurons. For instance, [14] identified three types of embryonic DA neurons (E11.5-E18.5) in the mouse: 1) an immature type expressing *Th* and other key markers of DA neurons, 2) a more mature neuron type expressing both *Th* and *Dat*, and 3) neurons with a similar signature but also expressing *Aldh1a1* and *Lmo3*. By contrast, [16] identified only two embryonic types (E15.MB.1 and E15.MB.2) representing a neuroblast population and a more mature DA neuron. Finally, [15] suggests that elements of DA neuron diversification might be present as

early as E13, but further diversification continues throughout development. In the authors' elegant pseudotime plot of developmental trajectories, it is apparent that DA neuron subtypes from older mice (P90) are further apart compared to similar cells obtained from younger animals (E18.5 to P7). This indicates that, as DA neurons are maturing and integrating into defined neural circuits, they continue to refine their molecular signature. Supporting the possibility of extrinsically driven refinement of gene expression, a recent study suggested that contact with striatal neurons could modulate the expression of *Vglut2* in DA neurons [42]. Taken together, it appears that DA neuron diversification begins to emerge early and probably occurs in a stepwise fashion. However, because of the limitations of single cell transcriptomics (Box 1; reviewed in [10]), the complete developmental dendrogram of midbrain DA neurons remains to be further refined. Additionally, how much of this diversity is encoded in progenitors, or determined by later intrinsic or extrinsic factors, remains to be resolved.

Concluding remarks and perspectives

Single-cell gene expression profiling has enabled progress in defining DA neuron subtypes, thus revising traditional anatomically based classification schemes. Parsing midbrain DA subtypes is a challenging endeavor since these neurons all share a common developmental origin in the floor plate and share the expression of many terminal selector genes and their downstream cascades. Nonetheless, and although single-cell studies have discrepancies in resulting subtypes, these studies have begun to provide a more granular picture of molecular heterogeneity in the midbrain DAergic system, a significant advance from anatomically based definitions. Additional studies, using larger numbers of cells and ones that are sequenced at greater depth, will further refine or clarify these subpopulations. Spatial transcriptomics provides a methodology to simultaneously locate these subpopulations *in situ* and will be valuable tool towards generating a complete list of midbrain DA neurons. Ultimately, anatomical and functional analyses will be required to corroborate molecularly based classification schemes, to determine if each subtype aligns with a distinct set of physiological and functional features [61,89–94]. Towards this end, selective intersectional genetic approaches have been developed to target DA neuron subtypes that are defined by a combination of genes [20] (Box 3), and initial studies have suggested that indeed DA neuron subtypes expressing given markers have distinctive projection patterns even within a given target region. Such paradigms can be used to target subtypes with intersectional expression of sensors/effectors like opsins [54,95], DREADDs [96,97], or GCaMP [98], in order to study their function in isolation. We envision that a more complete midbrain DA taxonomy can be derived by concerted single-cell profiling studies and functional studies that reinforce, rebut or refine each other.

Establishing a consensus of DA neuron subtypes, is a first step toward a thorough understanding of the DAergic system and its role in diseases. Having an accurate, molecularly defined list of DA subtypes will be crucial for 1) understanding molecular cascades underpinning selective vulnerability in PD, 2) pharmacologically manipulating the DA neurons in a subtype specific fashion towards treatment of DA related disorders like depression and chronic pain, 3) rational and accurate differentiation of iPS cells towards DA subtypes, and 4) evolutionary comparisons across species, including humans. All these goals

would be facilitated by a common nomenclature, which will allow for easier comparison of findings across laboratories, thus providing a shared framework to understand DA neuron diversity. Once a taxonomy of DA subtypes is established, the field will need to attribute specific functional roles to these molecularly defined subtypes. Although it is often assumed that behaviors would neatly segregate between molecularly defined neuronal populations, a recent study of hypothalamic cell types suggests that it is not always that simple [99]. Indeed, in the ventromedial hypothalamus, few transcriptionally defined cell types exhibited a clear correspondence with behavior-specific activation or connectivity. In the DAergic system however, we have observed distinct but partly overlapping projection patterns for molecularly defined types, although these projections often included multiple targets [20]. In the future, mapping behavior-specific activation of genetically defined DA circuits will be an important goal, that might also help understand how disruptions of these circuits can lead to specific symptoms of disease. Although circuit alterations in most DA-associated disorders are by no means restricted to the DAergic system, identifying the specific DA subtypes that are affected may offer insight into molecular or circuit-level mechanisms underlying these diseases.

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Box 1:

Strengths and limitations of single-cell gene profiling technologies

The transcriptional signature of individual cells is currently obtained through single-cell RNA sequencing (scRNA-Seq). As reviewed recently [100,101], single-cell RNAseq experiments initially require the isolation of individual cell and the capture of these cells RNA to generate a library. Depending on the protocol being used, these libraries can be biased toward the 3’ or 5’ end of transcripts, but recent approaches have successfully captured full length RNAs. Analyzing scRNA-Seq data involves reducing the dimensionality by projecting the data into a smaller-dimensionality mathematical space, using methods such as principal component analysis (PCA, linear) and T-distributed stochastic neighbor embedding (t-SNE, non-linear). This facilitates visualization of cell populations by grouping them on the basis of genes expression. This technology is now being used to classify neurons in various brain regions [10–12]. In the future, we envision that a combination of anatomical and sequencing technologies, such as spatial transcriptomics, will allow even greater refinements of cellular identity by simultaneously describing molecular subtypes and their location [102,103].

Single-cell RNA-Seq suffers from limitations, as it has for instance a low capture efficiency compared to “bulk” RNA-Seq, leading to dropout events, that is – a failure to detect the expression of a gene [10,101]. scRNA-Seq data is noisier and more variable than “bulk” RNA-Seq, since cDNA synthesis will only be possible for a fraction of the total transcripts due to Poisson sampling [104], biased towards the most highly expressed genes in a cell. In the context of neural cell-type profiling, this might explain the overabundance of highly expressed genes like neuropeptides, and underwhelming presence of genes with lower expression like transcription factors, in the list of genes defining cell types. This may compromise the ability to segregate closely related subtypes, and could underpin some of the incongruencies in DA neuron classification studies. Another limitation of classifying neurons using single-cell RNAseq results from the imperfect correlation between mRNA and protein levels, such that the transcriptome of a cell provides only a limited insight into protein levels. Strengths and weaknesses of scRNA-Seq have been reviewed in-depth elsewhere [100,101].

Box 2:

Molecular signature of a midbrain dopamine neuron

A DAergic neuron is typically defined as one that releases the neurotransmitter DA. This functional definition is straightforward, and thus well accepted [2,105–108]. However, defining the molecular signature of a DA neuron is more difficult, as many canonical markers of DA neurons are not specific to these neurons. To begin to molecularly define DA neurons, one can postulate that DA neurons are cells that express: 1) a set of genes common to all neurons, and 2) a set of genes that is necessary for the synthesis of DA (or found exclusively in cells that synthesize DA). Both these sets of genes are vital to DA neuron identity, since DA synthesis pathway are present in unrelated cell types of the gut and immune system (e.g. [109,110]). Neuron-specific transcriptional programs have been well characterized and include cohorts of expressed genes that underlie axonal and presynaptic functions. The transcriptional program involved in the synthesis and neurotransmission of DA includes genes encoding tyrosine hydroxylase (*Th*) and Dopa decarboxylase (*Ddc*) that are necessary for the stepwise production of DA from its precursor L-Tyrosine, and vesicular transporter *Vmat2* (Slc18a2) that is necessary for the packaging of DA into vesicles [2]. Since these genes are also expressed in other monoaminergic cell types such as noradrenergic neurons, the absence of dopamine beta hydroxylase (*Dbh*) expression is a requirement to define a DA neuron. Complicating the matter further, a gene such as *Th*, often used to describe DA neurons, shows discrepancies between mRNA and protein in that the mRNA is more broadly detected than the protein in the midbrain as well as other brain regions [19,60]. This raises the question whether all neurons harboring *Th* mRNA have sufficient levels of the protein to synthesize DA, leading some to propose that in the midbrain, *Dat (Slc6a3)* expression, rather than *Th*, represents a more accurate descriptor of bona-fide midbrain DA neurons [19] (see also Box 3).

Besides neuronal identity genes and DA synthesis genes, a third axis is required to define midbrain DA neurons: genes that are characteristic of a developmental origin in the mesodiencephalic floor plate. These would include developmental genes like *Foxa1/2*, *Lmx1a/b*, *Nurr1* that are expressed in all midbrain DA neurons, although possibly at varying levels [72,79,111–116]. These genes are not coordinately expressed in hypothalamic or olfactory DA neurons (A11–16), which are not floor plate derived [67,111], and appear to be quite distinct from midbrain DA neurons [6,16,117]. Conversely, these genes are also expressed in non-DA neurons, like those located in the rostral linear nucleus, as well as in neighboring floor plate derived populations like the subthalamic nucleus (STN) and ventral premammillary nucleus (PMv), and thus by themselves are not sufficient to describe midbrain DA neurons [75,76]. Expression of Engrailed 1 (*En1*) and its downstream target *Pitx3* [75–77,118,119], when added to the analysis, can be used to exclude diencephalic floor plate derived neurons (e.g. STN and PMv neurons), although not neurons of the rostral linear nucleus [75]. In summary, all midbrain DA neurons, located in three clusters RR, SNc, and VTA (A8–10) have a

characteristic signature defined by an overlap of neuron-specific genes, DA pathway genes, and midbrain floor plate derived transcriptional cascades.

Box 3:

Genetic targeting of midbrain dopamine neurons

Investigating DA neuron function is best accomplished by genetic targeting using site-specific recombinases. For instance, Cre recombinase can be driven by transcriptional regulatory elements active in midbrain DA neurons and can be used to activate a genetically encoded effector within a viral vector or a mouse reporter. A thorough characterization of the driver used is critical for a proper interpretation of experimental results. For instance, two distinct Th-Cre drivers have been generated and used to drive expression of opsins. In one of these lines [120], Cre recombinase has been knocked into the 3'UTR of the tyrosine hydroxylase (*Th*) locus, and by virtue of the internal ribosome entry site (IRES) sequence, has the advantage of preserving two functional alleles of the gene. For most genes, mRNA and protein levels are highly correlated, however in the case of *Th*, many midbrain cells show mRNA expression in the absence of protein (e.g. [20]). Accordingly, Th-IRES-Cre mice have displayed recombination in cells where TH protein could not be detected [19]. For this reason, Dat-IRES-Cre is now the driver of choice as it does not appear to drive recombination in TH immunonegative neurons in the midbrain. However, some TH- cells are labeled in the nearby premammillary ventral nucleus [75,121] and Dat-IRES-Cre might not completely recombine neurons that express low levels of *Dat*, depending on the reporter used. Further, DAT levels are decreased in the available strains [122,123], possibly interfering with the interpretation of behavioral experiments.

Genetic targeting of DA neuron subpopulations can be achieved by two methods: 1) projection specific retrograde viruses or 2) intersectional genetic approaches. Projection specific approaches typically involve injection of viruses that show retrograde capabilities, into a target region of choice, allowing the efficient segregation of DA neuron subtypes based on their projections; indeed, these approaches have been used to segregate functionally distinct subpopulations within the VTA and SNc with extraordinary success [61,91,92,94]. Projection-specific approaches typically are performed in wild type mice, or in mice harboring only a single allele (e.g. Dat-IRES-Cre) and therefore require minimal husbandry. However, a caveat is that these approaches require accurate and reproducible placement of viruses, often at multiple sites. Additionally, these approaches are predicated on the idea that each target region receives innervation from a single DA subtype, which does not always appear to be the case [20]. Intersectional genetic approaches require more than one recombinase to achieve the required specificity. One way to accomplish this is by using a combination of Cre and Flp recombinases, thus activating a genetically encoded effector that is defined by two drivers [124]. Genetic targeting using Boolean logic is highly flexible and exploits the numerous Cre and Flp drivers available in mouse repositories. To facilitate prospective targeting of DA neuron subtypes, we previously generated a Th-2A-Flpo driver [20]. Combining this Flp driver with a subtype specific Cre line and an intersectional reporter [125] or virus [95] allows genetic targeting of subsets of DA neurons. Another intersectional strategy to target DA neuron subtypes makes use of the tetracycline expression system, in conjunction with Cre recombinase. To this effect, a Dat-tTA mouse was generated in

which the tetracycline-controlled transactivator protein (tTA) is driven by the *Dat* gene and can be turned off by doxycycline injection [126]. Combining this driver with a subtype specific Cre line and a TRE reporter that is Cre-dependent allows genetic targeting of subsets of DA neurons [20]. While intersectional approaches require mice with multiple targeted alleles, their main strength is that they are predicated on molecularly defined DA subpopulations, rather than solely by projection targets. In the future, we envision even greater selectivity of targeting by combining projection specific approaches with intersectional approaches using the plethora of new recombinases available.

Proposed classification	*Aldh1a1+, Sox6+*	*Sox6+, Aldh1a1-*	*Vglut2+ (SNc)*	*Vglut2+ (VTA)*	*Vgat+*	*Aldh1a1+, Otx2+*	*Vip+*
Poulin et al. 2014 [13]	DA ^{1A}	DA ^{1B}	DA ^{2C}	DA ^{2C}	DA ^{2A}	DA ^{2B}	DA ^{2D}
La Manno et al. 2016 [14]	DA-SNC	DA-VTA1	N/A	NA	DA-VTA4	DA-VTA2	DA-VTA3
Hook et al. 2018 [16]	MB.4 (?)	MB.4 (?)	MB.2 (?)	MB.2 (?)	N/A	MB.1	MB.3
Saunders et al. 2018 [18]	SN_4-5, 4-8, & 4-9	SN_4-3	SN_4-2	SN_4-2	SN_3-7	SN_4-1	N/A
Tiklova et al. 2019 [15]	AT-Dat ^{HIGH}	T-Dat ^{HIGH}	N/A	N/A	GT-Dat ^{LOW}	AT-Dat ^{HIGH}	VT-Dat ^{HIGH}

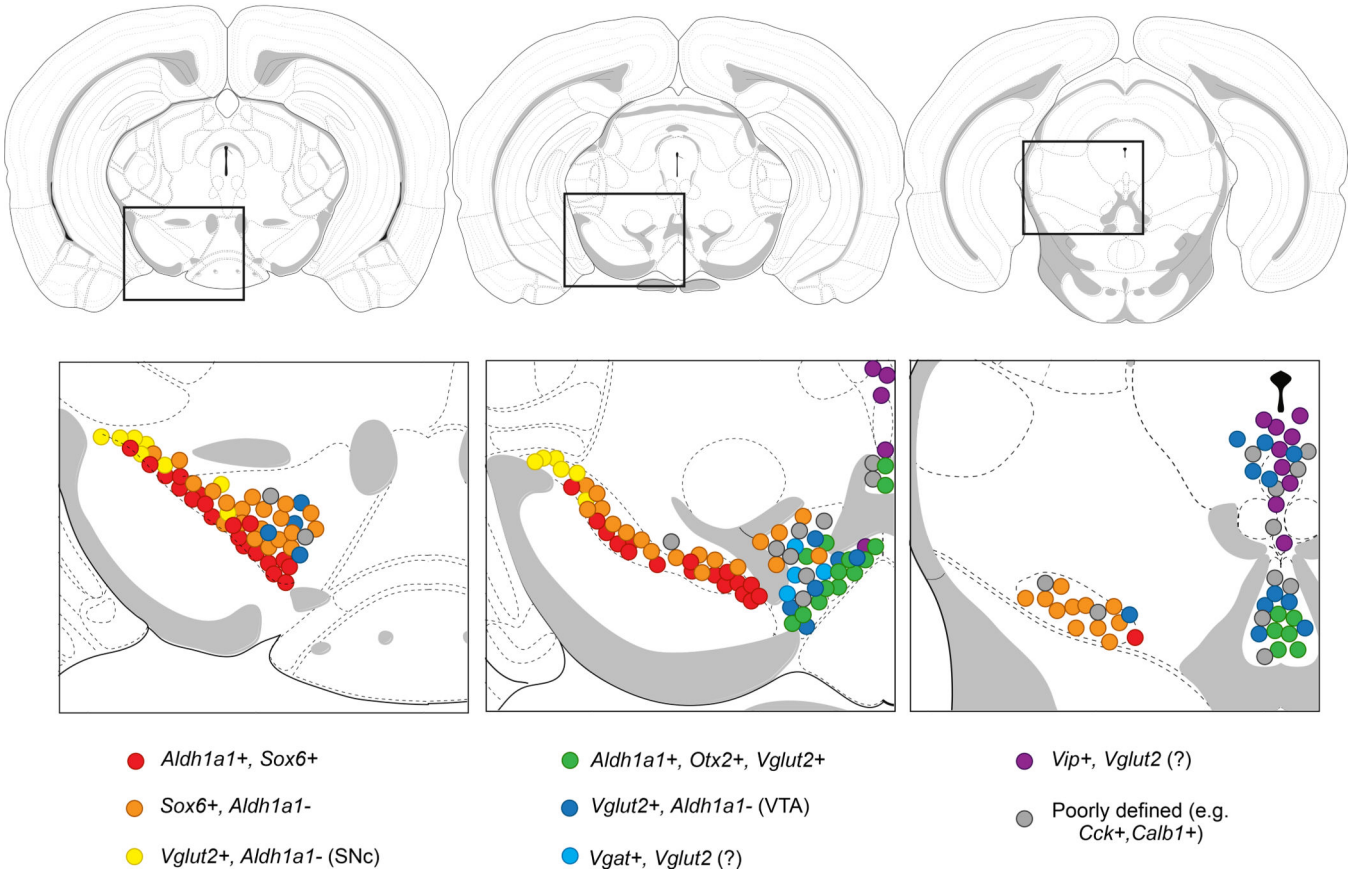

Figure 1.

Molecularly defined DA neuron subtypes.

Our interpretation of putative DA neuron subtypes across several studies with their potential neuroanatomical locations in the adult mouse midbrain. Of note, the subclusters SN_4–4 (*Th, C1ql3*), SN_4–6 (*Th, Cadm2*), and SN_4–7 (*Th, Nefl*) from [18] are not mentioned, as we were unable to unambiguously locate these groups. In relation to [15], we have omitted neurons that have low *Th* and *Dat* expression (N-Dat^{LOW}, G-Dat^{LOW}). NT-Dat^{LOW}, defined by *Npxh4*, likely represents neurons in the rostral linear region, as depicted in that study. For discussion of results from a sixth related study, [17], please see main text; findings from this study have not been listed in this figure due to the limited information on clusters.

Table 1.

List of dopamine neuron subtypes and markers with highest variance identified by single-cell gene profiling studies.

Studies	Developmental stages	DA Subcluster (Markers)
La Manno et al., 2016 [14]	E11.5–E18.5	mDA0 (**Th+**, **Cck+**, **Prkca+**)
		mDA1 (**Th+**, **Dat+**, **Calb1+**)
		mDA2 (**Sox6+**, **Th+**, **Dat+**, **Calb1+**, **Aldh1a1+**, **Lmo3+**, **Bnc2+**)
		mNbM (**Sox6+**, **Neurod1+**, **Neurog2+**, **Ebf2+**, **Rnd3+**, **Cnfn2+**, **Nhlh1+**, **Igfbpl1+**, **Pitx3-**)
		mNbMDA (**Pbx1+**, **Ebf2+**, **Cdk14+**)
	P28–P56	mDA-SNC (**Sox6+**, **Calb1−**, **Aldh1a1+**, **Aldh1a7+, Anxa1+, Ndnf+**)
		mDA-VTA1 (**Sox6+**, **Calb1+**, **Ndnf+**)
		mDA-VTA2 (**Sox6−**, **Calb1+**, **Aldh1a1+**, **Aldh1a7+**, **Neurod6+**, **Grp+**, **Gpr83+**, **Otx2+**, **Lpl+**, **Anxa1+, Cbln4+**)
		mDA-VTA3 (**Sox6−**, **Calb1+**, **Vip+**, **Cck+**, **Cbln1+**, **Cbln4+**)
		mDA-VTA4 (**Sox6−**, **Calb1+**, **Vgat+**, **Cbln1+**)
Hook et al., 2018 [16]	P7	P7.MB.1 (**Gpr83+**, **Otx2+**, **Cck+**, **Neurod6+**, **Lpl+,** Tacr3+)
		P7.MB.2 (**Lhx9+**, **Ldb2+**, **Dat−**, Vglut2+, Wnt7b+)
		P7.MB.3 (**Vip+**, **Calb1+**)
		P7.MB.4 (**Sox6+**, **Ndnf+**, Lmo3+, Aldh1a1+, Aldh1a7+, Anxa1+, Sncg+)
	E15	E15.MB.1 (**Lhx9+**, **Ldb2+**, Vglut2+)
		E15.MB.2 (**High Th+**, **high Dat+**, Vmat2+, Pitx3+, Ddc+, En1+)
Poulin et al., 2014 [13]	P4	DA1A (**Aldh1a1+**, **Sox6+**, **Ndnf+**)
		DA1B (**Aldh1a1−**, **Sox6+**, **Ndnf+**, **Tacr3+**)
		DA2A (**Vgat+**, **Calb1+**, **Cck+**, **Vglut2+**)
		DA2B (**Lpl+**, **Adcyap1+**, **Otx2+**, **Aldh1a1+**, **Vglut2+**, **Calb1**, **Cck+**, **Grp+, Tacr3+**)
		DA2C (**Aldh1a1−**, **Calb1+**, **Cck+**, **Vglut2+, Tacr3+**)
		DA2D (**Vip+**, **Calb1+**, **Cck+**, **Vglut2+**, **Sox6−**, **Aldh1a1−**)
Tiklova et al., 2019 [15]	P1-P90	N-Datlow (**Nxph4+**, **Th−**, **C1ql1+**, **Fam19a2+**, **Car10+**)
		NT-Datlow (**Nxph4+**, **Th+ high**, **C1ql1+**, **Fam19a2+**, **Car10+**)
		G- Datlow (**Gad2+**, **Th−**, Vgat+, **Wnt7b+**, **Crhbp+**)
		GT-Datlow (**Gad2+**, **Th+ high**, Crhbp+, Vgat+, **Wnt7b+**)
		T-Dathigh (**Th+ high, Aldh1a1−**, **Sncg+**)
		AT-Dathigh (**Aldh1a1+**, **Th+ high**, Otx2+, Grp+, Cck+, Lpl+)
		VT-Dathigh (**Vip+**, **Th+**, Dlk1+, Cck+, Gipr+, Pou2f2+)
Kramer DJ et al., 2018 [17]	P26-P34	Cluster 1 SNC (**Aldh1a7+**, **Igf1+**, **Bsn+**, **Ntsr1+**, **Kcns3+**)
		Cluster 4 SNC (**Aldh1a7+**, **Igf1+**, **Bsn+**, **Ntsr1+**, **Kcns3+**, **Anxa1+**)
		Cluster 2 VTA (**Ubqln2+**, **Ids+**, **Slc12a5+**)
		Cluster 5 VTA (**Necab1+**, **Cthrc1+**, **Cbln1+**)
		Cluster 6 VTA (**Necab1+**, **Cthrc1+**, **Cbln1+**, **Gucy1a3+**, **Ryr2+**, **Uri1+**)

Studies	Developmental stages	DA Subcluster (Markers)
		Cluster 8 VTA (**Neurod6+**, **Grp+**, **Gpr83+**, **Cbln4+**, **Igfbp4+**)
Saunders et al., 2018 [18]	P60-P70	SN_3–7 (**Vglut2+**, **Gad2+**, **Crhbp+**, Vgat+, Wnt7b+)
		SN_4–1 (**Lpl+**, **Vglut2+**, Cbln4+, Aldh1a1+, Neurod6+, Otx2+, Calb1+, Tacr3+)
		SN_4–2 (**Cbln1+**, Vglut2+, Calb1+, Wnt7b+)
		SN_4–3 (**Cyp26b1+**, Sox6+, Tacr3+)
		SN_4–4 (**C1ql3+**, Calb1+)*
		SN_4–5 (**Vcan+**, Aldh1a1+, Sox6+)
		SN_4–6 (**Cadm2+**)*
		SN_4–7 (**Nefl+**, **Nefm+**)*
		SN_4–8 (**Aldh1a1+**, **Anxa1+**, Sox6+, Igfbp2+)
		SN_4–9 (**Grin2c+**, Aldh1a1+, Sox6+)

Notes:

1) Genes in bold represents those explicitly discussed in the papers, whereas those not in bold represent our own mining of underlying data

2) Asterisk (*) indicates populations of neurons in [18] which we were unable to definitively determine the correspondence to subtypes in other papers, or the localization in the mouse brain.

Table 2.

Key methodological aspects of single-cell dopamine neuron classification studies.

Studies	Mouseline	Age	Isolation	Technique	# of cells
Poulin et al., 2014 [13]	Dat-IRES-Cre, Ai9	P4	FACS for tdTomato	sc-qPCR	159
La Manno et al., 2016 [14]	Dat-IRES-Cre, Ai9	P28-P56	FACS for tdTomato	scRNA-Seq	245
Hook et al., 2018 [16]	Th-eGFP	P7	FACS for eGFP	scRNA-Seq	80
Saunders et al., 2018 [18]	wildtype	P60-P70	No sorting	Drop-Seq	919
Kramer et al., 2018 [17]	Dat-IRES-Cre, Ai9	P26–34	FACS for tdTomato	scRNA-Seq	232
Tiklova et al., 2019 [15]	Pitx3-eGFP	P1-P90	FACS for eGFP	scRNA-Seq	1106