Deep learning-based image-analysis identifies a DAT-negative subpopulation of dopaminergic neurons in the lateral Substantia nigra.

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Abstract

Here we present a deep learning-based image analysis platform (DLAP), tailored to autonomously quantify numbers of neuronal subtypes in defined areas, and of fluorescence signals, derived from RNAscope probes or immunohistochemistry, in defined cellular compartments. As proof-of-principle, we utilized DLAP to analyse subtypes of dopaminergic midbrain neurons in mouse and human brain-sections. These neurons modulate complex behaviour like voluntary movement, and are differentially affected in Parkinson's and other diseases. DLAP allows the analysis of large cell numbers from different species, and facilitates the identification of small cellular subpopulations, based on differential mRNA- or protein-expression, and anatomical location. Using DLAP, we identified a small subpopulation of dopaminergic midbrain neurons (~5%), mainly located in the very lateral Substantia nigra (SN), that was immunofluorescence-negative for the plasmalemma dopamine transporter (DAT), with ~30% smaller cell-bodies, and a ~4-fold higher likelihood of calbindin-$_{d28k}$ co-expression. These results have important implications, as DAT is crucial for dopamine-signalling, and its expression is commonly used as marker for dopaminergic SN neurons.

Introduction

Dopamine releasing (DA) neurons within the midbrain are important for a variety of brain functions and behavioral processes like voluntary movement control, learning, cognition, and motivation (Berke, 2018; Björklund & Dunnett, 2007; Liu & Kaeser, 2019; Wise, 2004). The cell bodies of DA midbrain neurons are predominantly located in two overlapping nuclei, the Substantia nigra pars compacta (SN) and the ventral tegmental area (VTA), with axonal projections to the dorsal striatum, and the ventral striatum and prefrontal cortex, respectively (Gantz et al, 2018; Haber & Fudge, 1997; Halliday et al, 2012; Roeper, 2013). In line with these differential functions and projections, dysfunction of the dopaminergic midbrain system can lead to severe diseases like Parkinson's, Schizophrenia, addiction, or attention deficit hyperactivity disorders (ADHD) (Chen et al, 2021; Klein et al, 2019; Lerner et al, 2015; Montero et al, 2021). Importantly, not all DA midbrain neurons are affected equally in these diseases. More precisely, in Parkinson's disease (PD), the second most common neurodegenerative disorder, a subpopulation of mesostriatal SN DA neurons is progressively degenerating, while neighboring VTA DA neurons remain largely intact (Bloem et al, 2021; Damier et al, 1999; Hirsch et al, 1997; Surmeier et al, 2017). In contrast, in Schizophrenia, mesocorticolimbic VTA DA neurons display complex dysfunctions, while SN DA neurons are mainly unaffected (Jauhar et al, 2022; Kegeles et al, 2010; Owen et al, 2016). The cause for this differential vulnerability of dopaminergic midbrain neurons is still unclear, and up to now, only unspecific, symptomatic therapies are available, with their respective side-effects (Smeland et al, 2020; Vijiaratnam et al, 2021).

One prerequisite for curative and cell-type specific therapies is a better understanding of the distinct subpopulations of dopaminergic neurons and their selective pathophysiology (Gantz et al, 2018; Roeper, 2013). Analysis of mRNA and protein expression with single cell resolution and its correlation with anatomical locations and projections in health and disease states is an essential approach for identifying molecular determinants of differential neuronal vulnerability (Dumrongprechachan et al, 2021; Glock et al, 2021; Hobson et al, 2022a; Hobson et al, 2022b; Paget-Blanc et al, 2022). To address this, labelling of mRNA or proteins in tissue sections, followed by microscopic imaging, are important tools, as spatial distribution and tissue context are preserved (Jonkman et al, 2020; Lichtman & Conchello, 2005; Reilly & Obara, 2021). However, manual neuronal mapping, cell counting, and quantification of mRNA- and protein-derived fluorescence signals is very time consuming and prone to human error (Maier-Hein et al, 2018; Sommer & Gerlich, 2013; Xu et al, 2019).

Here we present a deep learning-based image analysis platform (DLAP) that overcomes these issues. Our single cell DLAP approach and six individual algorithms were tailored to autonomously quantify (i) the number of distinct neuronal types in defined areas, as well as (ii) fluorescence signals, derived from either RNAscope probes (mRNA) or immunohistochemistry

(proteins), in defined cellular compartments - more precisely, in plasma-membranes, cell body/cytoplasm, and nuclei of individual neurons. This is desired, as advanced methodologies are leading to increasingly large biological datasets (Greener et al, 2022), and the general need for their efficient, unbiased, and reproducible analysis (Krenn et al, 2022; Moen et al, 2019). We utilized artificial neuronal networks (ANN) based deep learning approaches for image analysis. ANN are biologically inspired computer programs, designed to simulate the way in which the human brain processes information. ANN gather their knowledge by detecting the patterns and relationships in data (segementation), and learn (or are trained) through experience, not by programming (Agatonovic-Kustrin & Beresford, 2000; Hassabis et al, 2017; LeCun et al, 2015; Yang & Wang, 2020). ANN consist of artificial neurons, arranged in different layers (Angermueller et al, 2016; Greener et al, 2022), where each consecutive layer obtains inputs from its preceding layer. As only the first (input) and the last (output) layer are visible/accessible, all layers in between are referred to as hidden layers. Network architectures containing hundreds of hidden layers are called deep networks (LeCun et al, 2015). After suitable training, deep learning networks can effectively extract relevant cellular features for automated image analysis (Falk et al, 2019).

To facilitate easy adaption to the most common respective tasks in life-science, we have predesigned six algorithms, based on two distinct network structures, DeepLab3 (DL3) and fully convolutional neural networks (FCN) (Chen et al, 2018; Long et al, 2015). We trained them to detect, count, and analyse individual DA midbrain neurons, labelled for tyrosine hydroxylase (TH), the key-enzyme for synthesis of dopamine and other catecholamines (Daubner et al, 2011). However, the flexible design of the algorithms and the workflow allows quick and easy adaptions to other cell types and specimens, and thus is of general interest.

Here we detail the DLAP pipeline, including all six predesigned algorithms for automated image processing, and we demonstrate its reliable identification and count of cells in defined areas, as well as fluorescence-signal quantification in cellular compartments. By using this approach for quantification of the immunofluorescence signal of the plasmalemma dopamine transporter (DAT) in TH-positive SN neurons, we identified a small subpopulation of DAT immuno-negative DA neurons, mainly located in the very lateral parts of the SN (~5 % of TH-positive SN neurons). The DAT is an electrogenic symporter and a member of the Na^+/Cl^- dependent transporter family (SLC6). It is crucial for re-uptake of dopamine at somatodendrites and axons, and also mediates electric conductances (Ryan et al, 2021). The identification of a DAT-negative SN DA neuron population has not only physiological relevance (Bu et al, 2021; Miller et al, 2021; Savchenko et al, 2022), but it has additional implications, as DAT is commonly used as marker for SN DA neurons and for their specific targeting, e.g. to generate DA neuron specific knock out mice, expressing Cre-recombinase under the control of the DAT-promoter (Gong et al, 2007; Lammel et al, 2015; Papathanou et al, 2019; Stuber et al, 2015).

Results

Based on the Wolution web-interfaces (https://wolution.ai/), we developed a deep learning based, automated image analysis platform (DLAP) as well as six tailored algorithms (two based on FCN, four based on DL3) for fast and reliable identification and quantitative analysis of TH-positive midbrain neurons within the SN and VTA of mouse and human *post mortem* brain sections. These six DLAP algorithms were trained by hundreds of manually marked TH- and DAPI-positive cell bodies of midbrain neurons. **Figure 1** illustrates the general workflow. **Table 1, 2 and S1** provide details of algorithms, trainings, and performances (further details in methods and supplement). For all approaches, the region of interest (ROI) for cell type identification and quantification (i.e the SN) was marked manually, according to anatomical landmarks (Paxinos & Keith B. J. Franklin, 2007). All six algorithms allowed reliable identification of target cells, as well as their quantification in a given ROI (DLAP-3 & 4), and/or quantification of mRNA-derived (DLAP-1 &-2) or protein-derived (DLAP-5 & 6) fluorescence signals of distinct genes of interest in these target cells - all common tasks in life science.

Table 1: Quality evaluation parameters for all six algorithms. Performances were determined by comparing DLAP analysis results of the test image sets to the ground truth data (provided by manual labeling). Class name defines the identified molecular or cellular structures. The following quality measures were assessed separately for each class: pixel error rate, rate for true positive (TP), true negative (TN), false positive (FP), false negative (FN) detection, as well as relative specificity (spec.) [defined as TN/(TN+FP)], and sensitivity (sens.) [defined as TP/(TP+FN)].

DLAP algorithm	class name	Error [%]	TP [%]	TN [%]	FP [%]	FN [%]	Spec [%]	Sens [%]
1	cell body	5.08	10.3	84.7	4.1	0.8	95.3	92.7
	mRNA		0.3	98.9	0.7	0.0	99.3	92.5
2 (human)	cell body	4.13	5.2	91.0	2.5	1.4	97.3	80.8
	mRNA		0.2	98.9	0.9	0.0	99.1	94.7
3	nucleus	0.61	0.5	98.9	0.5	0.1	99.5	85.5
4	nucleus	0.26	0.1	99.7	0.2	0.0	99.8	59.8
5	nucleus	3.3	0.9	98.5	0.3	0.3	99.7	74.5
	cytoplasm		4.1	92.9	2.2	0.8	97.7	83.7
6	nucleus	5.49	3.0	93.5	2.5	1.0	97.4	75.6
	cytoplasm		9.7	89.0	0.8	0.5	99.1	95.0
	membrane		11.7	85.2	2.2	0.9	97.5	92.4

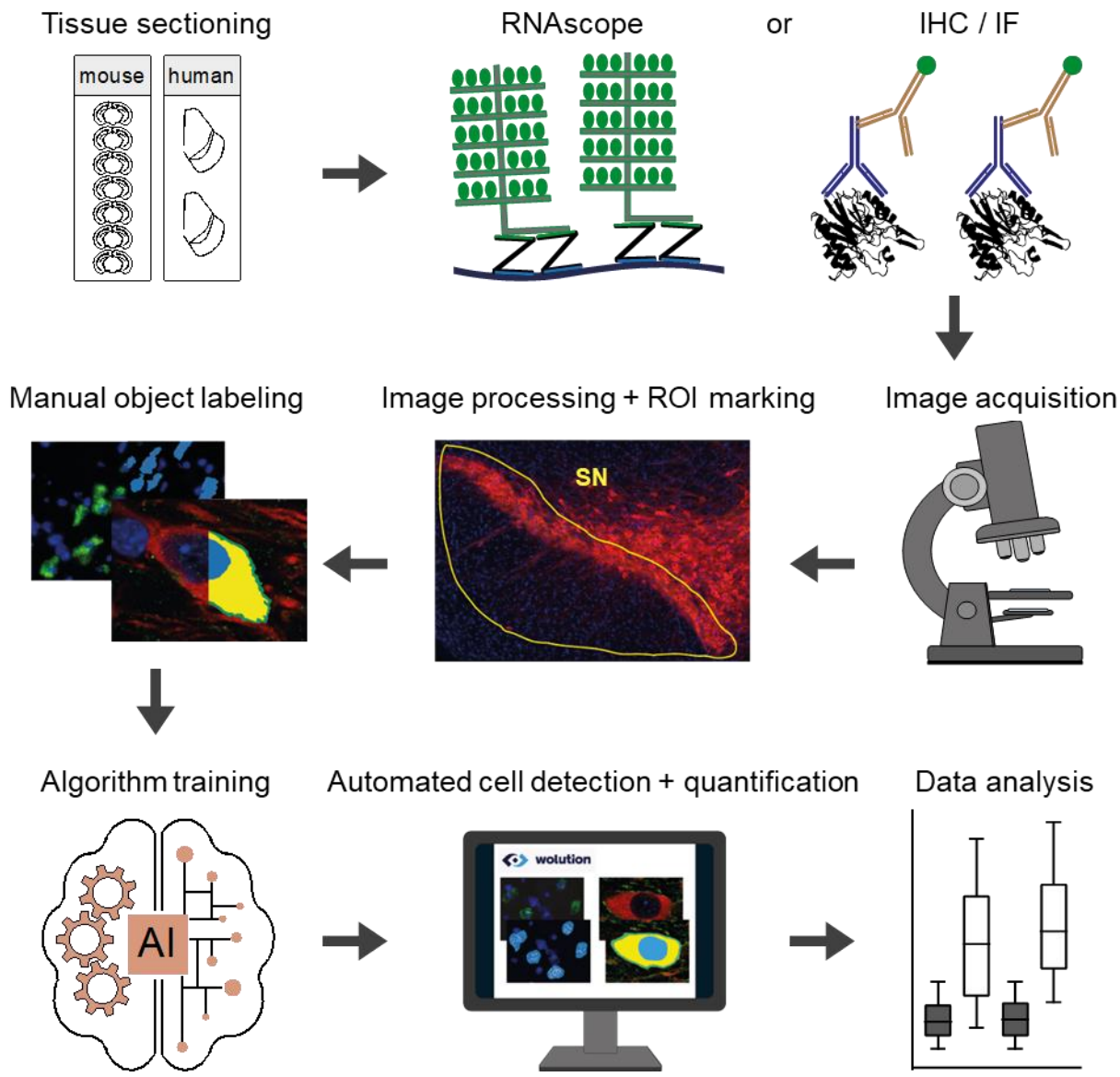

Figure 1: Graphic illustration of deep learning-based image analysis platform (DLAP). After sectioning of murine/human midbrain samples either mRNA- (RNAscope) or protein- (IHC/IF) staining is performed. According to image acquisition, acquired images are preprocessed and the ROIs (e.g. SN) are manually marked. Distinct cell types and compartments (e.g. plasma membrane, nucleus) are manually labeled in a training data set to generate the ground truth data that is then used to train the individual networks. After successful training, images can be automatically analyzed.

Table 2: Overview of six individual DLAP algorithms and workflow, optimized for distinct image sets and analysis tasks. For detailed description, see methods. Abbreviations: BF brightfield, DAB diaminobenzidine, DAPI 4′,6-diamidino-2-phenylindole, FCN fully convolutional neural network, HE hematoxylin, IF immunofluorescence, NM neuromelanin, RGB red-green-blue, TH tyrosine hydroxylase. * For training, only the cellular nuclei were marked, not the full cell-body, similar as in stereology; only clearly visible, full nuclei were counted.

DLAP algorithm	Trained by / optimized for	Algorithm task	Network-Type	Training-set		post training image analysis optimization (a-e)
				images	cells	
1	RNAscope fluorescence: TH-mouse (DAPI)	1) Algorithm identifies cells of interest and delineates the shape of cell bodies by TH-derived fluorescence signals (green channel). 2) Within these marked cell bodies, target gene derived individual fluorescent dots (red channel) are detected, marked, and counted.	FCN	100	~1000	(a) watershed algorithm for improved separation of mRNA molecules. (b) morphological operations (type dilation and erosion) applied, for better separation of neurons.
2 modification of algorithm 1	RNAscope fluorescence: TH-human (DAPI)	3) A fourth BF channel was included, as a measure for NM-content. After Step (1) and (2) from algorithm 1, in an additional step, the BF signal intensity is quantified within the marked cell bodies.	FCN*	200	~500*	(a) watershed algorithm for improved separation of mRNA molecules. (b) morphological operations (type dilation & erosion) applied, for better separation of neurons.
3	DAB-labelling: TH-mouse	Algorithm identifies and counts nuclei of DAB-positive cells (TH-stained).	DeepLab3*	100	~3000*	none
4 modification of algorithm 3	DAB-labelling: TH-mouse & HE-counterstain	Algorithm extends the automated counting of DAB-positive cells (TH-stained) to sections that are counterstained by HE.	DeepLab3*	300	~2500*	(a) watershed algorithm for improved separation of individual nuclei. (c) filling holes within recognized compartments.
5	IF-labelling: TH-mouse, DAPI DAT-mouse	1) Algorithm identifies and counts cells of interest (i.e TH-positive cells). It delineates the shape of the cell body by immunofluorescence signals for TH (red channel) and nucleus by DAPI fluorescence signal (blue channel). 2) Within these marked compartments, the relative signal intensities are quantified for all three fluorescent channels.	DeepLab3	95	~500	(a) watershed algorithm for improved separation of neurons. (c) filling holes within recognized compartments. (d) removing target gene channel during cell identification. (e) RGB intensity threshold for background-signal determination.
6	IF-labelling: TH-mouse, Kv4.3-mouse, DAPI	1) Algorithm identifies cells of interest and delineates the shape of the cell body by immunofluorescence signals for TH (red channel), of the nucleus by DAPI fluorescence signal (blue channel), and of the membrane (originally learned by Kv4.3 staining, after training no membrane marker needed). 2) Within these marked compartments, the relative signal intensities are quantified for all three fluorescent channels.	DeepLab3	94	~110	(c) filling holes within recognized compartments. (d) removing target gene channel during cell identification. (e) RGB intensity threshold for background-signal determination.

Deep learning networks for image segmentation of complicated scenes, such as the Pascal Visual Object Classes (VOC) data set (Everingham et al, 2015), that we used in the DeepLab3 based algorithms, are usually very large, and their filters are particularly trained to learn abstract concepts such as “tree” or “car”. Particularly, for the analysis of simple, RNAscope derived fluorescence signals (i.e. individual small dots, Ø ~ 0.3 µm), we found that such a complicated pre-trained neural network was not of benefit. RNAscope derived fluorescence signals are mainly small dots (Ø ~ 0.3 µm), within the somata, which makes threshold based automatic recognition/segmentation of the cell body difficult. Thus, we custom-designed and optimized two less complex FCN-based algorithms (DLAP-1 and 2), and we demonstrate that they were better suited for the analysis of RNAscope data, compared to DeepLab3 approaches. The latter algorithm failed to recognize the small dots (mRNA molecules) as evident from the sensitivity-value of 0.0% for this class, compared to 92.5% and 94.7% for the FCN-based algorithm (**Table S1**). For protein-derived immunohistochemistry signals (diaminobenzidine, DAB and immunofluorescence IF), tailored DeepLab3 algorithms gave better allover-results as indicated by lower overall error (**Table S1**; DLAP-3 to -6).

DLAP based automated quantification of mRNA molecules in individual target cells via RNAscope approaches.

RNAscope *in situ* hybridization allows absolute quantification of distinct mRNA molecules, without cDNA synthesis, via hybridization-probes, with each fluorescence dot representing one target mRNA-molecule (Jolly et al, 2019; Wang et al, 2012). Due to multiple probes for each target-gene, it is hardly affected by degraded RNA. We have used RNAscope for determining mRNA molecule numbers in individual TH-positive SN DA neurons in fresh-frozen, PFA-postfixed mouse midbrain sections (Benkert et al, 2019). For automated quantification of numbers of RNAscope probe derived fluorescence dots, two custom designed FCN based algorithms were used (DLAP-1 & 2). The general principle of the FCN algorithms is given in **Figure 2**. DLAP analysis of RNAscope signals was performed in two steps: in a first step, the cell body was recognized and marked according to the TH-signals, and in a second step, individual target gene mRNA dots (labelled with a different fluorophore) were identified and counted within the marked TH-positive cell body, including the nucleus. DLAP-1 is optimized for detecting TH-positive DA neurons from mice, DLAP-2 for detecting human TH-positive DA neurons, containing neuromelanin (NM), a dark pigment present in DA neurons from humans and primates (Bazelon et al, 1967; Vila, 2019), visible only in bright field (BF).

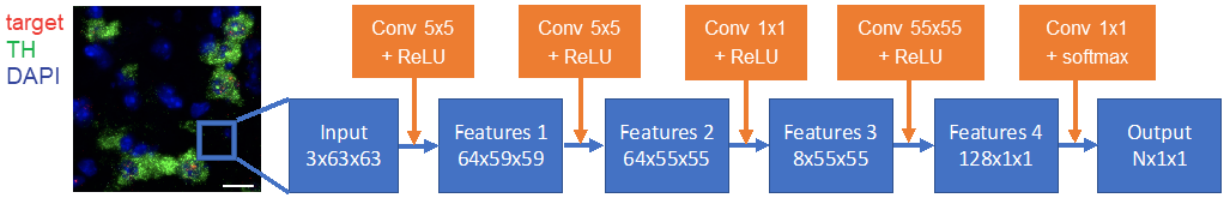

Figure 2: Architecture of the fully convolutional neural network (FCN), used for RNAscope DLAP. The input image on the left shows SN DA neurons RNAscope-labelled for tyrosine hydroxylase (TH) mRNA (green), target mRNA (red) and DAPI (blue). Scale bar: 20 µm. The image is passed through five convolutional layers that are connected by the Rectified Linear Units (ReLU) activation function. The last activation function is the softmax function, which normalized the network output to the probability distribution. The size of the convolutional kernels is specified in the orange boxes. The blue boxes provide the dimensions of the resulting feature maps. The network analyzes image patches of size 63^2 pixels to determine the class of the central pixel in the patch. It is applied convolutionally to every central pixel in the image, so that the final output image has the same dimensions as the input image.

Figure 3a shows a typical image of TH-positive SN neurons in a coronal mouse brain section, after RNAscope *in situ* hybridization, before (input image, left) and after automated identification of TH-positive cell bodies and quantification of individual fluorescence dots, derived from target mRNA molecules. Cell bodies were identified and marked according to the TH-derived fluorescence signal (green), and target mRNA were identified as probe-derived fluorescence-signals (red dots, **Table S2**). Results for two different target genes - with lower (Cav1.3) and higher (Cav2.3) mRNA abundance - are given (**Figure 3b, Table S3, S4**). Both target genes code for a specific voltage gated Ca^{2+} channel α-subunit that has been linked to Parkinson's disease (Benkert et al, 2019; Liss & Surmeier, 2022; Ortner, 2021). The manual and the automated analysis (DLAP-1) resulted in very similar results for both genes, over the full range of mRNA molecule numbers (Cav1.3 manual: 24±1 mRNA molecules, DLAP-1 24±1, N=3, n=499 neurons analysed, p=0.999, R^2=0.92; Cav2.3 manual: 69±5, DLAP-1: 69±5; N=3, n=372; p=0.999, R^2=0.92; **Figure 3b/c, S1, Table S3, S4**). Accordingly, the significant, ~3-fold higher number of Cav2.3 mRNA molecules in mouse SN DA neurons, compared to those of Cav1.3 is robustly detected with manual and with DLAP-1 analysis (p<0.0001 for both), similar as previously described (Benkert et al, 2019).

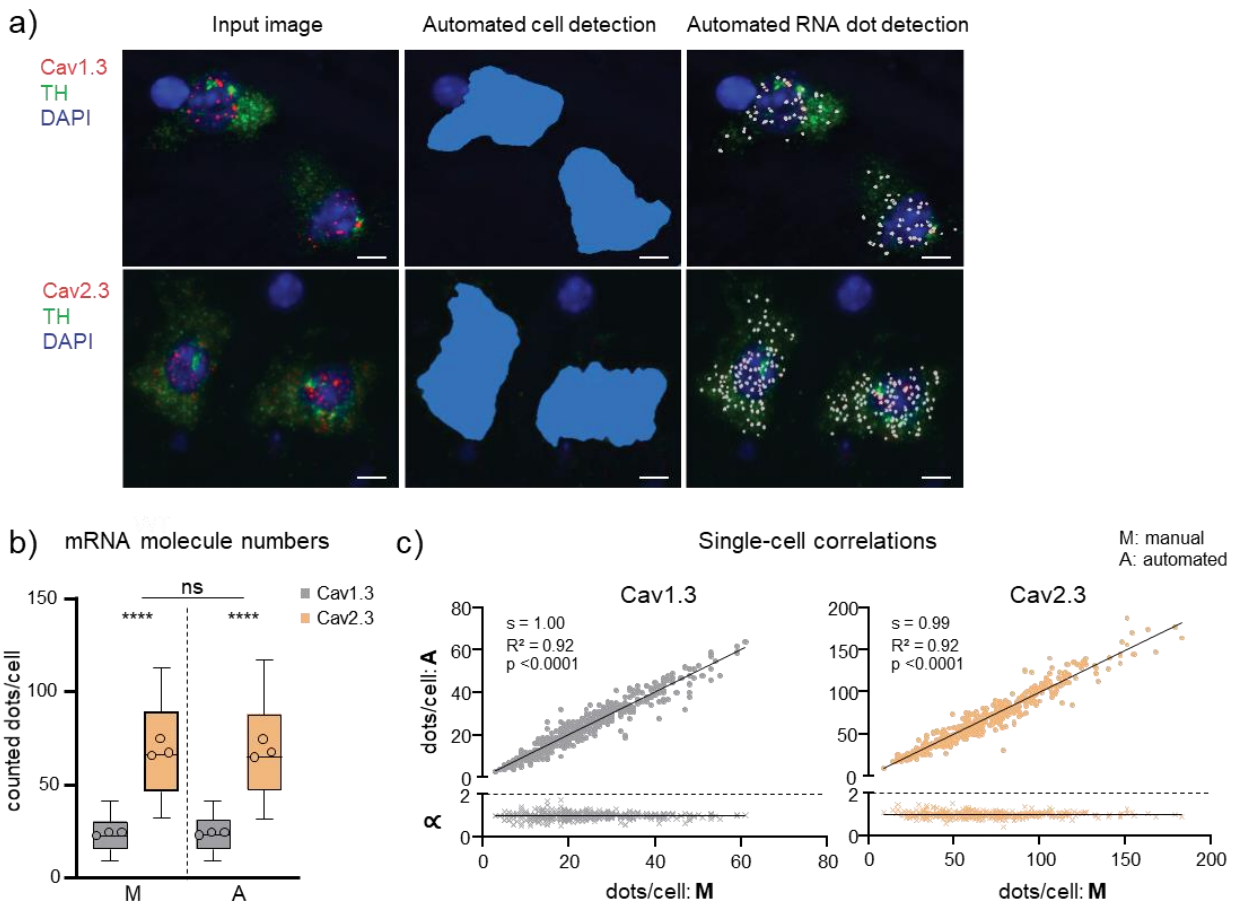

Figure 3: Comparison between manual and automated RNAscope-derived image analysis of mouse midbrain sections. a) SN DA neurons in coronal midbrain section from adult mice after RNAscope for tyrosine hydroxylase (TH, green) and Cav1.3 or Cav2.3 Ca^{2+} channels (red). Nuclei are marked by DAPI-staining (blue). Left: input images, middle and right: images after automated recognition of the TH labelled cell bodies (middle, blue area) and of target mRNA molecules (right, white dots) with DLAP-1. Scale bars: 5 µm. b) mRNA molecule numbers/SN DA neuron, counted manually (M) or automatically (A) via DLAP-1, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analysed neurons (Cav1.3: n = 449, Cav2.3: n = 372). The open circles indicate mean values for each analysed animal (N = 3 each). Significant differences (for analysed neurons) according to two-way ANOVA with Tukey's multiple comparison tests are indicated by asterisks (****: $p < 0.0001$, ns: $p > 0.05$). c) Upper: Single neuron correlations of mRNA-derived dot counts/cell between manual and automated analysis according to Pearson correlation test. Lower: corresponding proportionality constants ∝, calculated from the manual and automated dot count ratios. All data are detailed in Supplementary Table S3/4 and Supplementary Figure S1.

Similar robust results were obtained for RNAscope analysis of human SN DA neurons with the DLAP-2 algorithm. To optimize DLAP for human SN DA neurons, the D-LAP1 algorithm was extended to quantify the brightfield (BF) intensity, as a measure for the variable NM content of human SN DA neurons, to evaluate possible confounding effects of different NM content on RNAscope results (**Figure 4, S2, Table 2**). Cav1.3 mRNA molecules as well as NM content were compared in SN DA neurons from adult and aged individuals (adult: 42±7 years; aged: 78±3, details in **Table S5**). Manual and automated DLAP-2 analysis provided very similar

results, and we detected a strong linear correlation between manually and automatically determined mRNA molecule numbers as well as NM-content (**Figure 4b/d/e, Table S6, S7**). Interestingly, the number of Cav1.3 mRNA molecules was significantly lower (~60%) in SN DA neurons from aged individuals (**Figure 4b left**, manual: adult 38±5, aged 14±3; N=3, n=223; p<0.001; DLAP-2: adult 40±7, aged 17±4; N=3, n=258; p<0.001).

Noteworthy, the BF-value derived NM content was also significantly different between adult and aged samples, with ~30% higher NM content in in aged SN DA neurons (p<0.0001, **Figure 4b right**, **Table S6, S7**). To exclude that the lower number of mRNA molecules determined in aged SN DA neurons were artificially caused by the higher BF values (as measure for NM content), we addressed possible confounding parameters by applying a mixed effects model that we had optimized for these kind of human sample-derived data that are in general more heterogeneous (Duda et al, 2018; Schlaudraff et al, 2014). As the model indicated that the NM content indeed affected the detected Cav1.3 mRNA molecule numbers we corrected the automated data for the NM-content attributed effect. However, as evident in **Figure 4c, Table S6**, we still detected a similar highly significant lower number of Cav1.3 mRNA molecules (~35%) in SN DA neurons from aged donors.

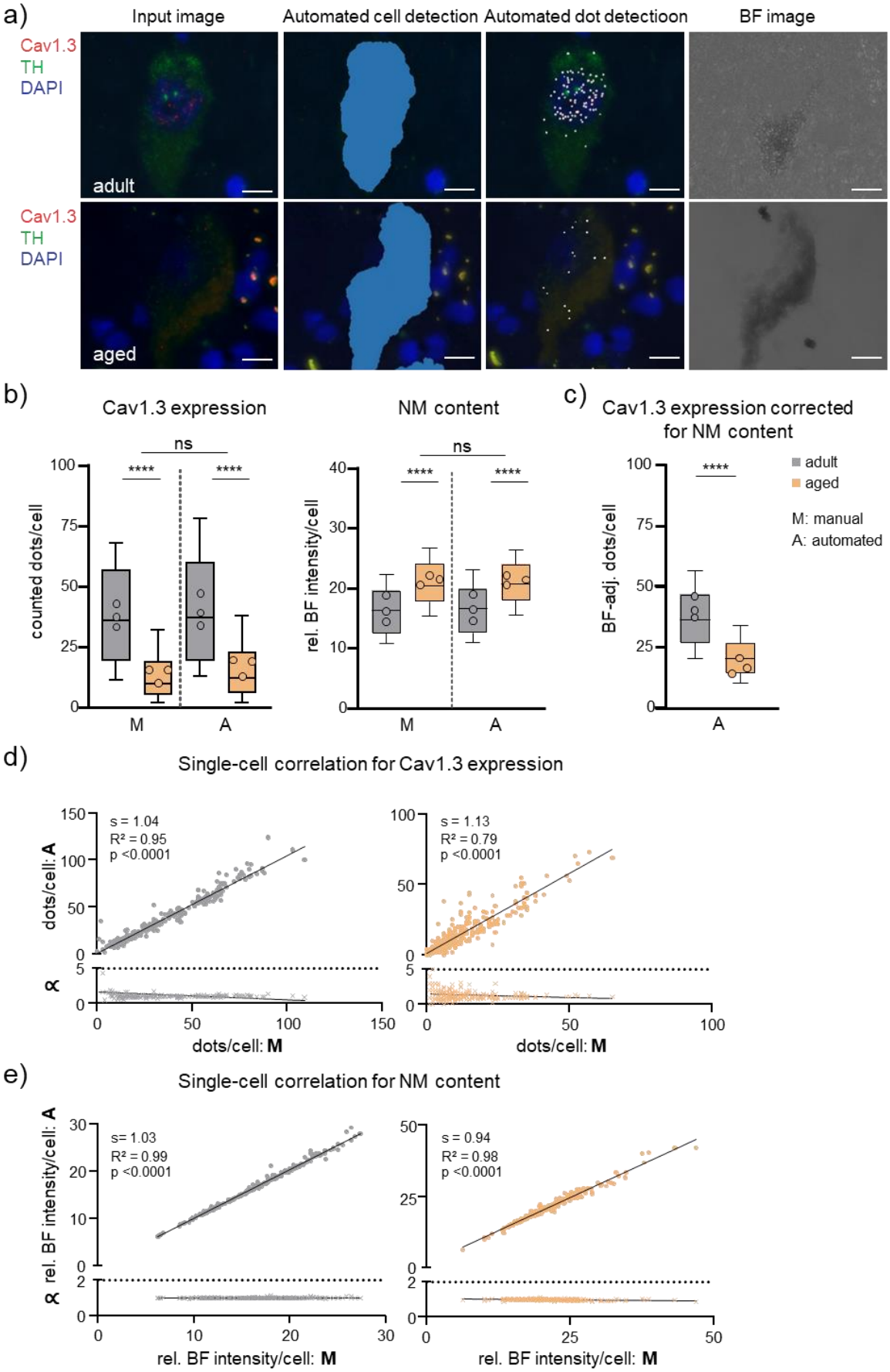
a)
Input image
Automated cell detection
Automated dot detectioon
BF image
Cav1.3
TH
DAPI
adult
aged
b)
Cav1.3 expression
NM content
ns
****
counted dots/cell
rel. BF intensity/cell
M
A
c)
Cav1.3 expression corrected for NM content
BF-adj. dots/cell
adult
aged
M: manual
A: automated
d)
Single-cell correlation for Cav1.3 expression
s = 1.04
R² = 0.95
p <0.0001
s = 1.13
R² = 0.79
p <0.0001
dots/cell: A
dots/cell: M
e)
Single-cell correlation for NM content
s= 1.03
R² = 0.99
p <0.0001
s = 0.94
R² = 0.98
p <0.0001
rel. BF intensity/cell: A
rel. BF intensity/cell: M

Figure 4: Comparison between manual and automated RNAscope-derived image analysis of human midbrain sections. a) SN DA neurons in coronal midbrain section from human adult and aged post mortem brain samples after RNAscope for tyrosine hydroxylase (TH, green) and Cav1.3 Ca^{2+} channels (red). Nuclei are marked by DAPI-staining (blue). Left: input images, middle: images after automated recognition of the TH labelled cell bodies (middle left, blue area) and of target mRNA molecules (middle right, white dots) with DLAP-2. Right: corresponding brightfield (BF) images used to quantify neuromelanin (NM) content. Relative BF intensity is measured within the delineated cell shapes. Scale bars: 10 µm. b) mRNA molecule numbers/SN DA neuron (left) and NM content (rel. BF signal intensity; right), quantified manually (M) or automatically (A) via DLAP-2, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analysed neuros (adult: n = 223, aged: n = 258). The open circles indicate mean values for each analysed brain sample (N = 3 each). Significant differences (for analysed neurons) according to two-way ANOVA with Tukey's multiple comparison tests are indicated by asterisks (****: $p < 0.0001$, ns: $p > 0.05$). c) mRNA molecule numbers/SN DA neuron, determined via DLAP-2, adjusted for NM using the Mixed Effects Model. Data are given as boxplots (median, 10-90 percentile) for all analysed neuros (adult: n = 206, aged: n = 231). The open circles indicate mean values for each analysed brain sample (N = 3 each). Significant differences (for analysed neurons) according to Mann-Whitney test are indicated by asterisks (****: $p < 0.0001$). d/e) Upper: Single neuron correlations of Cav1.3 mRNA-derived dot counts/cell (d) and rel. BF signal intensity/cell (e) between manual and automated analysis according to Pearson correlation test. Lower: corresponding proportionality constants $\propto$, calculated from the manual and automated dot count ratios. All data are detailed in Supplementary Table S5-7 and Supplementary Figure S2.

DLAP based automated quantification of immuno-labeled neuron numbers in tissue sections.

Determination of numbers of cellular populations in defined ROI is a common task e.g. for quantifying selective cell loss in neurodegenerative diseases, and to assess neuroprotective therapeutic effects (Golub et al, 2015). However, commonly used stereological approaches (still the gold-standard), are time consuming, and they provide only a - more or less exact - estimation of cell numbers, depending on the size of the manually counted fraction of cells (Brown, 2017; West, 1999). Approaches have been developed to increase sampling sizes and thus accuracy of estimates (Mouton et al, 2017). Nevertheless, estimates are prone to over- or underestimating total cell numbers, particularly if the target cells are not homogeneously distributed in the ROI, as it is the case e.g. due to differential neuronal vulnerability in degenerative disease (Baddeley, 2001; Miles & Davy, 1976; Noori & Fornal, 2011). However, manual approaches to count all neurons in a given ROI are extremely time consuming and prone to bias/human error. To overcome these issues, we developed three DLAP algorithms, dramatically reducing the time for valid determination of cell numbers, tailored to detect and count target cells, immuno-labelled by either DAB - without (DLAP-3) or in the presence of a hematoxylin counterstain (DLAP-4) - or by IF (DLAP-5). According to our stereological procedures, the DLAP-3 and 4 algorithms were trained by marking the neuronal nuclei only, not the whole cell-bodies, and we also counted only clearly visible nuclei of TH-positive cells.

DAB-labelling is more commonly used for determining cell-numbers in histological samples, as stained sections can be archived and e.g. (re-) analyzed. **Figure 5, S3, Table S8, S11** summarize the results for count of TH-positive SN neurons (unilaterally) from juvenile mice after DAB immunostaining, comparing stereological estimates with automated counting, utilizing DLAP-3. The stereological estimates and automated neuron counts resulted in very similar neuron numbers (Stereology: 4951±1079, DLAP-3: 4317±912 neurons unilaterally; p=0.218, N=10. linear correlation/section: R^2=0.79). These SN DA neuron numbers correspond well to the literature for C57bl/6 mice, ranging from 4000-6000 neurons unilaterally (Brichta & Greengard, 2014; Nelson et al, 1996).

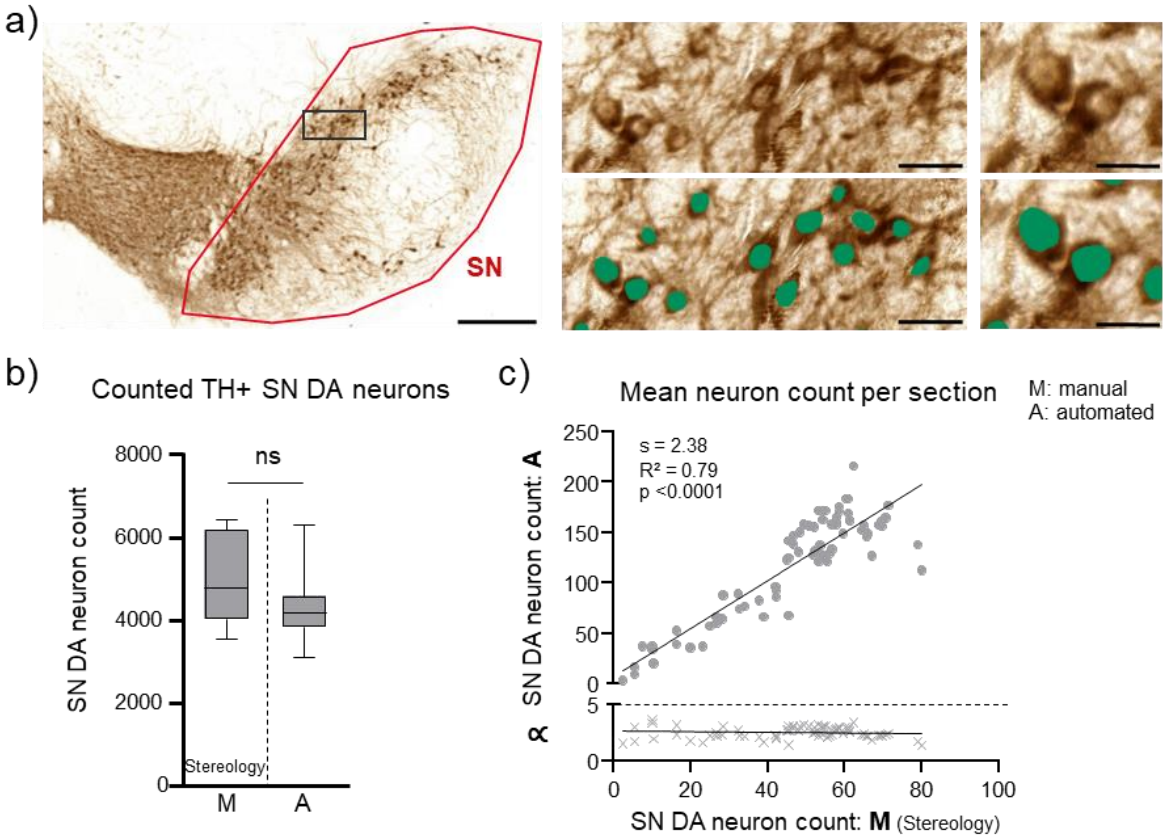

Figure 5: Comparison between unbiased stereology and automated IHC-derived image analysis of juvenile mouse midbrain sections. a) SN DA neurons in coronal midbrain section from juvenile mice after DAB-IHC for tyrosine hydroxylase (TH, brown). Left. input image where total SN is delineated in red. Scale bars: 200 µm. Middle and right: enlarged images before (upper) and after (lower) automated recognition of the nuclei within TH labelled cell bodies (green area) with DLAP-3. Scale bars: 50 µm (middle) and 10 µm (right). b) SN DA neurons/mouse quantified via unbiased stereology (extrapolated via the optical fractionator method, M) or automatically (A) via DLAP-3, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analysed mice (N = 10). Significant differences according to Mann-Whitney test are indicated by asterisks (ns: p > 0.05). c) Upper: correlations of SN DA neuron counts/section between unbiased stereology and automated analysis according to Pearson correlation test. Lower: corresponding proportionality constants ∝, calculated from the manual and automated cell count ratios. All data are detailed in Supplementary Table S8/11 and Supplementary Figure S3.

Immunohistochemistry sections are often counterstained with an unspecific cellular marker (like Nissl, hematoxylin). For analysis of SN DA neurons, such counterstains are often performed to confirm that a loss of TH-immunopositive neurons (e.g. in a PD-model) indeed corresponds to a loss of the respective neurons, and does not reflect a mere downregulation of TH-expression (Alam et al, 2017; Healy-Stoffel et al, 2012; Rausch et al, 2022). As we use hematoxylin-counterstains in such experiments (Benkert et al, 2019; Liss et al, 2005), we optimized DLAP-4 to detect DAB stained TH-positive SN neurons in such counterstained sections. With DLAP-4, we re-analysed a cohort of 21 mice that we had already analysed (Benkert et al, 2019) to quantify the effect of a PD drug (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin, MPTP) that induces preferential loss of SN DA neurons. Adult C57bl/6J mice were either injected with saline (N=9) or with MPTP/probenecide (N=12). We compared DLAP-4 derived data with the respective already published stereology-derived data, and also with another automated neuron count approach, based on the Aiforia platform (**Figure 6, S4, Table S9-11**). We had used the Aiforia platform (https://www.aiforia.com) for this cohort, to confirm relative numbers of remaining SN DA neurons after drug-treatment. Indeed, the relative determined cell loss by the Aiforia approach was similar to that estimated by stereology (~40%, p=0.183; **Figure 6b, Table S10,** and Benkert et al (2019)). However, the absolute number of neurons determined with the Aiforia approach was almost double as high (saline: stereology 3959±643, Aiforia 7048±843, p<0.0001, drug: stereology 2504±933, Aiforia 4108±1596, p=0.004; **Figure 6b, S4, Table S9**). This overcount, and the fact that the convolutional neuronal network (CNN)-based Aiforia system does not allow algorithm training by the users (Penttinen et al, 2018), let us switch to the Wolution-platform in the first instance.

With the DLAP-4 approach, a similar relative % of remaining TH-positive SN neurons in the drug-treated mouse cohort was detected, as with stereology or Aiforia (DLAP-4: 59±21%, stereology: 63±59%, p=0.234; Aiforia: 58±23%, p=0.988). Moreover, also the absolute numbers of SN DA neurons/mouse, counted by DLAP-4 were similar to the respective stereological estimates (Saline: DLAP-4 4614±500, stereology 3959±643, p=0.744; Aiforia 7048±823, p<0.0001. Drug: DLAP-4 2708±975; stereology: 2504±922, p=0.996; Aiforia 4108±1596, p=0.016). The linear correlation coefficients between mean manually and automatically counted neuron numbers were similarly high for both automated approaches. However, the linear regression slope, was higher for the Aiforia algorithm compared to our DLAP-4 algorithm (1.6 vs 1.0), in line with the higher, over-counted neuron-number by the Aiforia approach (**Figure 6, Table S11**).

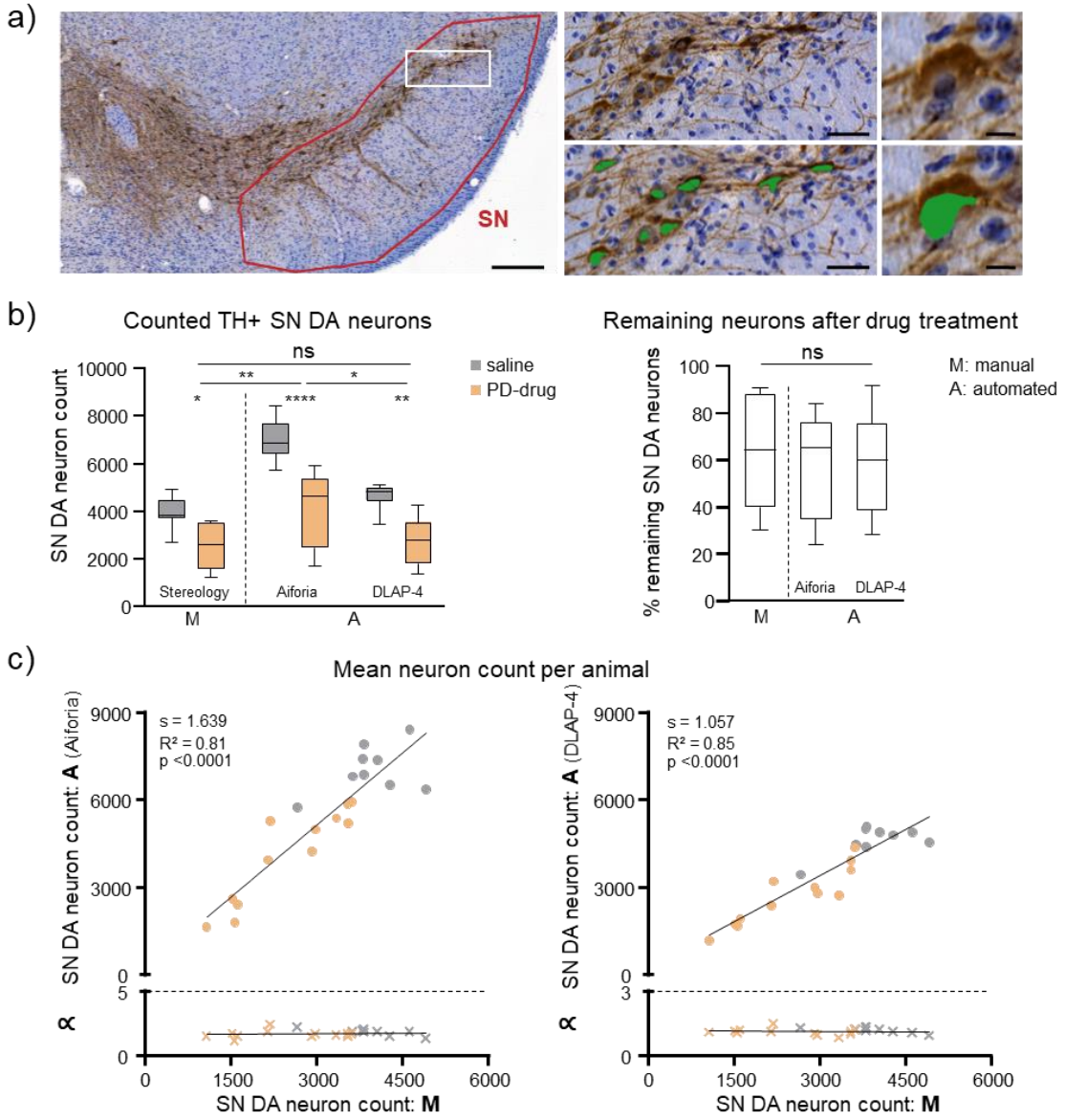

Figure 6: Comparison between unbiased stereology and automated IHC-derived image analysis of adult mouse midbrain sections. a) SN DA neurons in coronal midbrain section from adult mice after DAB-IHC for tyrosine hydroxylase (TH, brown). Nuclei are marked by hematoxylin-staining (blue). Left. input image where total SN is delineated in red. Scale bar: 200 µm. Middle and right: enlarged images before (upper) and after (lower) automated recognition of the nuclei within TH labelled cell bodies (green area) with DLAP-4. Scale bars: 50 µm (middle) and 10 µm (right). b) Left: SN DA neurons/mouse quantified via unbiased stereology (extrapolated via the optical fractionator method, M) or automatically (A) via an algorithm provided by Aiforia or DLAP-4, as indicated. Right: remaining TH-positive neurons [%] in drug-treated mice calculated relative to the mean of the saline-treated group. Data are given as boxplots (median, 10-90 percentile) for all analysed mice (saline: N = 9; PD-drug: N = 12). Significant differences according to two-way ANOVA with Tukey's (absolute counts) or Kruskal-Wallis with Dunn's multiple comparison test(percentage of remaining neurons) are indicated by asterisks (ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$). c) Upper: correlations of SN DA neuron counts/animal between unbiased stereology and automated analysis (left: Aiforia, right: DLAP-4) according to Pearson correlation test. Lower: corresponding proportionality constants ∝, calculated from the manual and automated cell count ratios. Stereology data and Aiforia data modified from Benkert et al (2019). All data are detailed in Supplementary Table S9-11 and Supplementary Figure S4.

DLAP-5 analysis of immunofluorescence labelled cells defines a novel population of DAT-negative neurons in the lateral SN.

We extended our automated cell quantification approach from DAB/BF to cells labelled by immunofluorescence (IF). IF has a greater dynamic signal range (Hofman, 2002; Peck et al, 2016), and allows the parallel quantification of more than one cell-population, defined by distinct markers (Katikireddy & O'Sullivan, 2011). Moreover, it can provide additional information regarding protein localization and relative expression levels.

We trained DLAP-5, based on TH and DAPI signals only, to detect and count immunofluorescence TH-positive SN DA neurons in DAPI co-stained sections, and to mark the neuronal cell-body and the nucleus of TH-positive neurons. After identification of TH-positive cell-bodies, in a next step, DLAP-5 quantifies IF-intensities separately in both identified compartments for up to three distinct fluorescent channels, enabling co-expression analyses. **Figure 7a** illustrates the automated identification of TH IF-positive SN neurons (red) in a coronal mouse midbrain section.

As the expression of the dopamine transporter DAT is commonly used as an additional maker for SN DA neurons besides TH, we used DLAP-5 to quantify the relative DAT-IF signals in the marked TH-positive cell bodies. Our central aim here was to quantify the co-expression of TH and DAT in SN neurons, and their relative expression levels, rather than determining absolute numbers of TH-positive SN neurons (**Figure 7-9, S5, S6**). Thus, we only included those TH-positive SN neurons into further quantitative DAT analysis that were clearly separated/segmented, with marked cell-bodies of proper size and clearly visible full nuclei. Our exclusion criteria are specified in methods and in **Figure S5a,** and resulted in manual exclusion of ~28±4% of the automatically identified TH-positive SN neurons (**Figure S5b, Table S12**). In the remaining number of TH-positive SN neurons (3015±374 neurons/mouse, N=7), we detected only in ~95% a DAT-IF signal above the background signal (2880±340 neurons/mouse, p=0.0156). The remaining ~5% (4.6±1.4%) of TH-positive SN neurons were negative for DAT (**Figure 7b, S5c, Table S12, S13**).

In order to further characterize this small subpopulation of SN neurons, we used the x,y-coordinates of the analyzed TH-positive neurons (automatically provided by the algorithm) to generate 2D anatomical maps for each analyzed mouse and coronal section and merged maps for all mice. These maps revealed that the TH-positive DAT-negative neurons were clustered in the very lateral parts of the SN (**Figure 7c, S6**). This excludes that these DAT-negative TH-positive neuron population was a contamination by VTA DA neurons, overlapping with the medial SN. Interestingly, the DAT-negative TH-positive neurons were significantly enriched in the caudal parts of the lateral SN, as their relative amount of was about 6-fold higher compared to those in the rostral parts of the lateral SN (7.4±2.4% vs 1.2±0.6%, p=0.03; **Figure 7d, S6,**

Table S12). Moreover, the DAT-negative SN neurons were about 35% smaller compared to the DAT-positive neurons (according to x,y-coordinates: DAT-positive: 214±13 µm², DAT-negative: 142±13 µm², p=0.006, **Figure 7e, Table S14**).

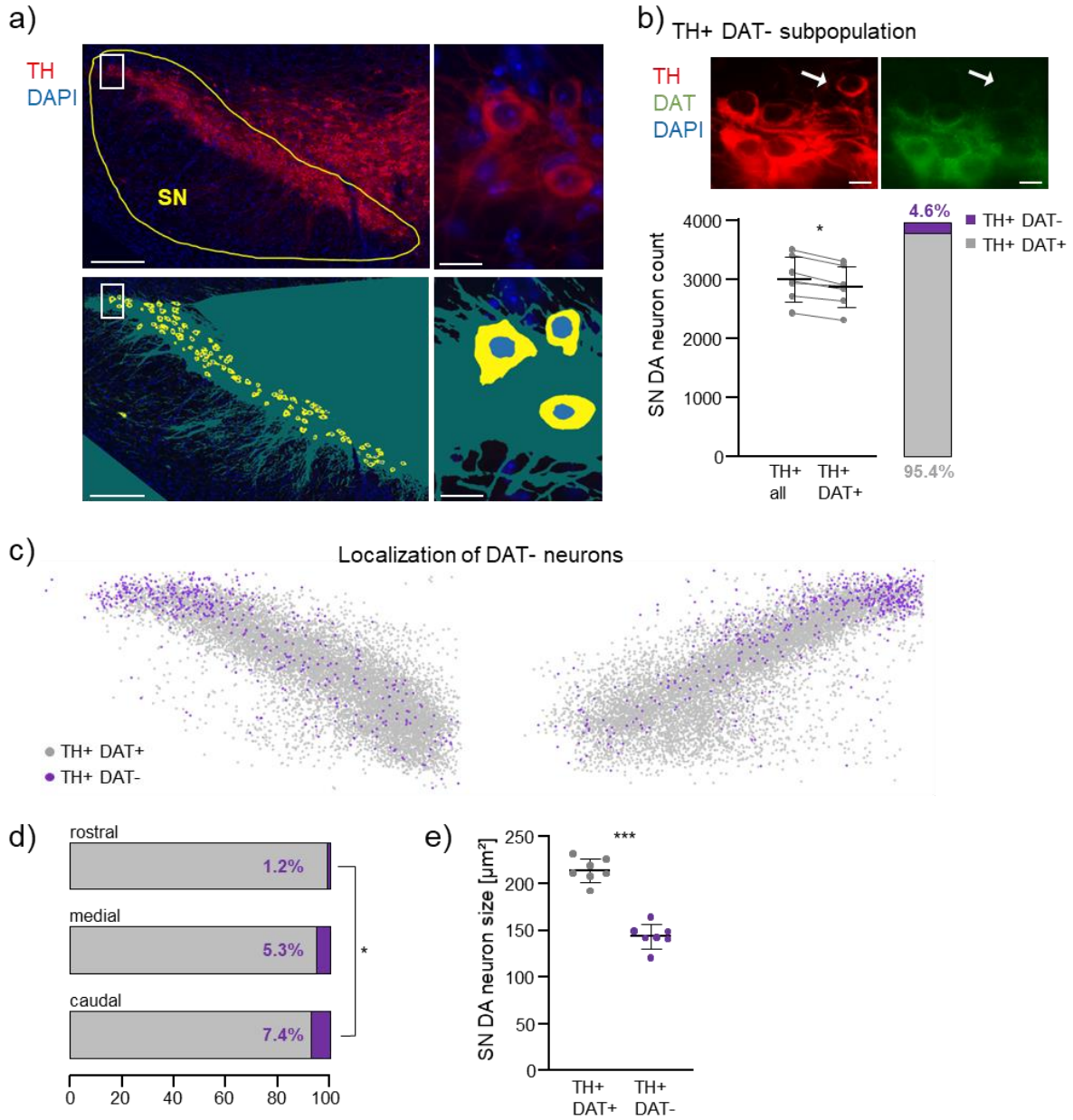

Figure 7: Automated image analysis of IF-derived signals identifies a DAT-negative subpopulation of TH positive SN neurons. a) Top: TH positive neurons in a coronal midbrain section from adult mice after IF for tyrosine hydroxylase (TH, red). Nuclei are marked by DAPI-staining (blue), the SN is delineated in yellow. Bottom: output image after automated recognition of TH labelled cell bodies (yellow area) and corresponding nuclei (blue area) with DLAP-5. Scale bars: 200 µm (left) and 20 µm (right). b) Top: input image, for TH fluorescence (red, left) and DAT fluorescence (green, right). Scale bars: 10 µm. White arrows point to a TH positive (TH+), DAT-negative (DAT-) SN neuron. Bottom left: SN DA neurons/mouse quantified via DLAP-5. Given are the total numbers of analyzed TH positive neurons (TH+), and TH+ DAT+ neurons, as well as the relative percentages of TH+ DAT+ and TH+ DAT- SN neurons. Data are given as scatterplots (mean, SD) for all analysed mice (N = 7). Significant differences according to Wilcoxon test are indicated by asterisks (*: $p < 0.05$).. c) Anatomical map displays the medio-lateral distribution of TH+ DAT+ and TH+ DAT- neurons within the SN. Plotted are

the individual TH+ neurons for all analyzed animals (n = 21106, N = 7), according to their x,y-coordinated, determined via DLAP-5. d) Bars display the relative abundancies of TH+ DAT+ and TH+ DAT- neurons over the caudo-rostral achsis of the SN. Rostral, medial and caudal areas were determined according to the following stereotaxic coordinates: rostral bregma: -2.7 mm to -3.1 mm; medial: -3.1 to -3.5; caudal:-3.5 to -3.9. Significant difference according to Chi square test indicated by asterisks (*: $p < 0.05$). e) Mean neuron sizes of TH+ DAT+ and TH+ DAT- SN neurons, determined via DLAP5 (x,y-area). Data are given as scatterplots (mean, SD) for all analysed animals. All data are detailed in Supplementary Table S12-14 and Supplementary Figure S5/S6.

For subpopulations of VTA DA neurons, DAT-expression gradients have been described (Fu et al, 2012; Lammel et al, 2008; Li et al, 2013; Morales & Margolis, 2017), and a lower DAT/TH ratio for lateral SN DA neurons has been briefly discussed (Fu et al, 2012). To systematically address whether a gradient in DAT expression is present within the SN, we used the relative, non-saturated fluorescence signal intensities (RF) of each DAT-analyzed TH IF-positive neuron, determined by DLAP-5, and the respective x,y-coordinates to generate heat-maps, color-coding relative IF-signal intensities (**Figure 8a**). Those maps indicated a medio-lateral DAT-protein gradient, with lowest DAT-immunosignals, within those of the background in the very lateral SN. Corresponding heat-maps for TH suggested a similar, but less prominent gradient (**Figure 8b**), similar as in (Blanchard et al, 1994; Fu et al, 2012). To our knowledge, such DAT-gradient and neurons in the very lateral SN that are immune-positive for TH but immuno-negative for DAT have not yet been systematically described. We enabled this by analysing DAT-expression in more than 20,000 SN neurons - hardly possible without the automated DLAP-5 approach. However, this algorithm does not allow conclusions regarding the sub-cellular location of the IF-signal.

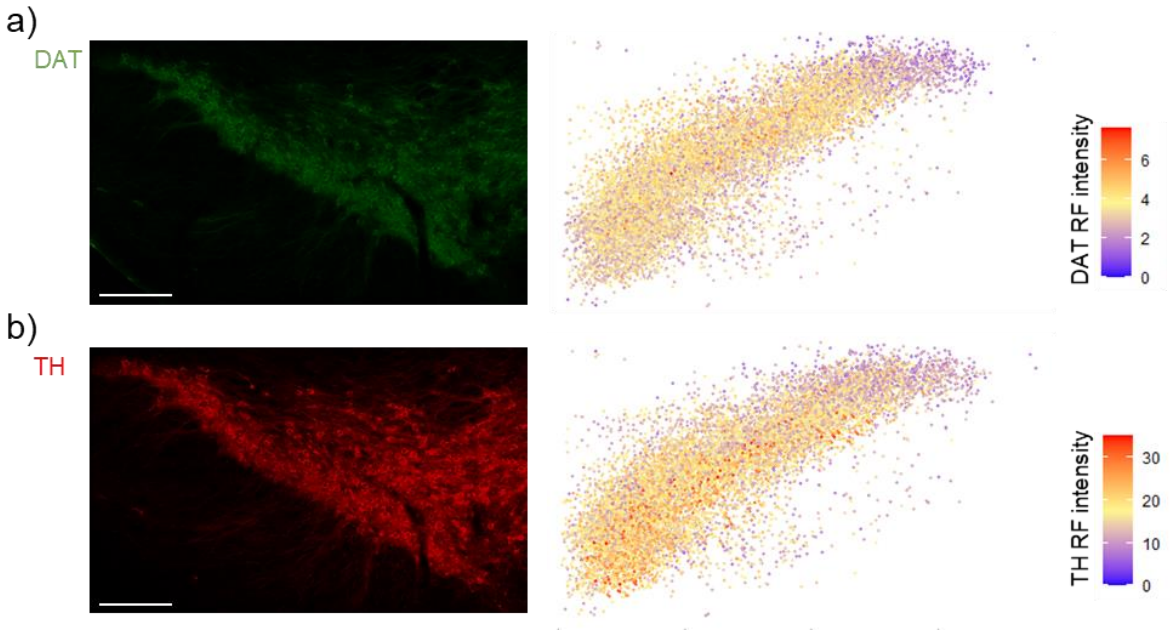

Figure 8: IF-derived relative TH and DAT protein levels in mouse SN neurons. Left: SN neurons in coronal midbrain section from adult mice after IF for the dopamine transporter (DAT, green, a) and

tyrosine hydroxylase (TH, red, b). Scale bars: 200 µm. Right: Anatomical maps display the medio-lateral distribution of all analyzed TH+ SN neurons, and the relative TH and DAT IF signal intensities (RF) for all analyzed animals (n = 21106, N = 7), according to their x,y-coordinated, determined via DLAP-5. RF-values are color-coded as indicated.

We also analysed the co-expression of the cytosolic Ca^{2+} binding protein calbindin-$_{d28k}$ (CB) in DAT-negative and DAT-positive SN DA neurons (**Figure 9, Table S14**), a presumed marker for less vulnerable SN DA neurons (Brichta & Greengard, 2014; German et al, 1992; González-Hernández et al, 2010; Liang et al, 1996). As expected, only a small percentage of TH- & DAT-positive SN neurons co-expressed CB (8.7±0.8%, n= 681 from 8051 neurons), and these neurons were ~20% smaller compared to the CB-negative SN DA neurons (DAT+ CB+: 181.9±8.5 µm^2, DAT+ CB-: 205.4±10.1 µm^2, p<0.0001, **Table S14**). Moreover, in TH-positive but DAT-negative SN neurons, the CB co-expression rate was ~4-fold higher (37.4±7.3% n= 142 from 395 neurons; p<0.0001), and those neurons had the smallest cell bodies from all four TH-positive groups; they were only about half the size of that of classical DAT-positive and CB-negative SN DA neurons (DAT- CB+: 109.8±9.9 µm^2; p<0.0001).

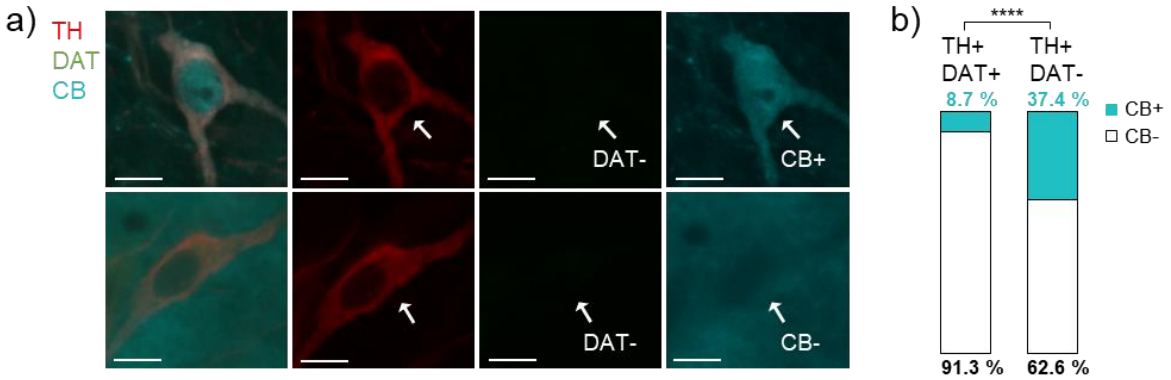

Figure 9: Calbindin co-expression in DAT-negative SN DA neurons. a) SN DA neurons in coronal midbrain section from adult mice after IF for tyrosine hydroxylase (TH, red), dopamine transporter (DAT, blue) and calbindin-$_{d28k}$ (CB, cyan). Nuclei are marked by DAPI-staining (blue). Scale bars: 10 µm. White arrows indicate a TH positive, DAT negative neuron each, positive (top) or negative (bottom) for CB. b) Co-expression rate of CB in DAT-positive and DAT-negative SN DA neurons for all analyzed animals (n = 8466, N = 3). Significant difference according to Chi square test indicated by asterisks (****: p < 0.0001). Data are detailed in Supplementary Table S14.

DLAP-6 based relative quantification of immuno-fluorescence derived signals in cellular compartments.

As proteins can mediate different functions in dependence of their cellular localization, analysis with higher resolution is desired. To enable relative quantification of immunofluorescence signals in plasma-membranes, cytoplasm and nucleus, we developed the DLAP-6 algorithm, optimized to detect and analyse neurons in high-resolution confocal fluorescent images.

DLAP-6 was specifically trained by using TH as marker for the cell body/cytoplasm, DAPI as marker for the nucleus, and the ion channel subunit Kv4.3 as marker for the plasma-membrane of SN DA neurons. Kv4.3 is highly expressed in plasma-membranes of SN DA neurons, and it is the pore-forming subunit of voltage and Ca^{2+} sensitive A-type K^+ channels that modulate the activity and the vulnerability of SN DA neurons (Aidi-Knani et al, 2015; Dragicevic et al, 2015; Haddjeri-Hopkins et al, 2021; Liss et al, 2001; Serôdio & Rudy, 1998; Subramaniam et al, 2014). The Ca^{2+}-sensitivity of Kv4.3 channel complexes is mediated by plasma-membrane associated KChip3. However, in the cytoplasm, KChip3 is also known as the enzyme calsenilin, regulating presenilins, and in the nucleus, KChip3 acts as gene-transcription-repressor DREAM (downstream regulatory element antagonist modulator), illustrating the importance of cellular localization-analysis (Burgoyne et al, 2019; Naranjo & Mellström, 2012). A prerequisite for DLAP-6 training was the availability of a suitable Kv4.3 antibody that we and others had already used for immunoelectron-microscopy (Haddjeri-Hopkins et al, 2021; Liss et al, 2001; Subramaniam et al, 2014).

Figure 10a shows a typical confocal image of a TH-positive SN neuron in a coronal mouse brain section, after IF-staining, before automated cell-body detection (input image), and after manual as well as algorithm-based identification of the cell cytoplasm (TH, red), the nucleus (DAPI, blue), and the plasma-membrane (Kv4.3, green). As expected, the highest relative fluorescence (RF) signals each were detected in these respective compartments, with manual as well as with DLAP-6 (RF for TH in cytoplasmic compartment: manual 55±3% vs. DLAP-6 57±4%, p=0.5862; DAPI/nucleus: 76±7% vs. 76±8%, p=0.9270; Kv4.3/membrane: 75±8% vs. 68±9%, p<0.0001; **Figure 10b, Table S15, S16**).

Particularly for detecting the RF-signal in the plasma-membrane compartment, the algorithm appeared superior or more consistent/precise than the manual analysis. Accordingly, the single cell correlation manual vs DLAP-6 is less good - but still very high - (R^2=0.93 for membrane compared to 0.99 for cytoplasm and nucleus, **Figure 10c, Table S16**). Most importantly, the DLAP-6 algorithm has successfully learned to identify SN DA neuron plasma-membranes, irrespective of a Kv4.3 derived fluorescence signal, and thus enables a reliable identification of the membrane-compartment in the absence of a membrane-derived fluorescence-signal.

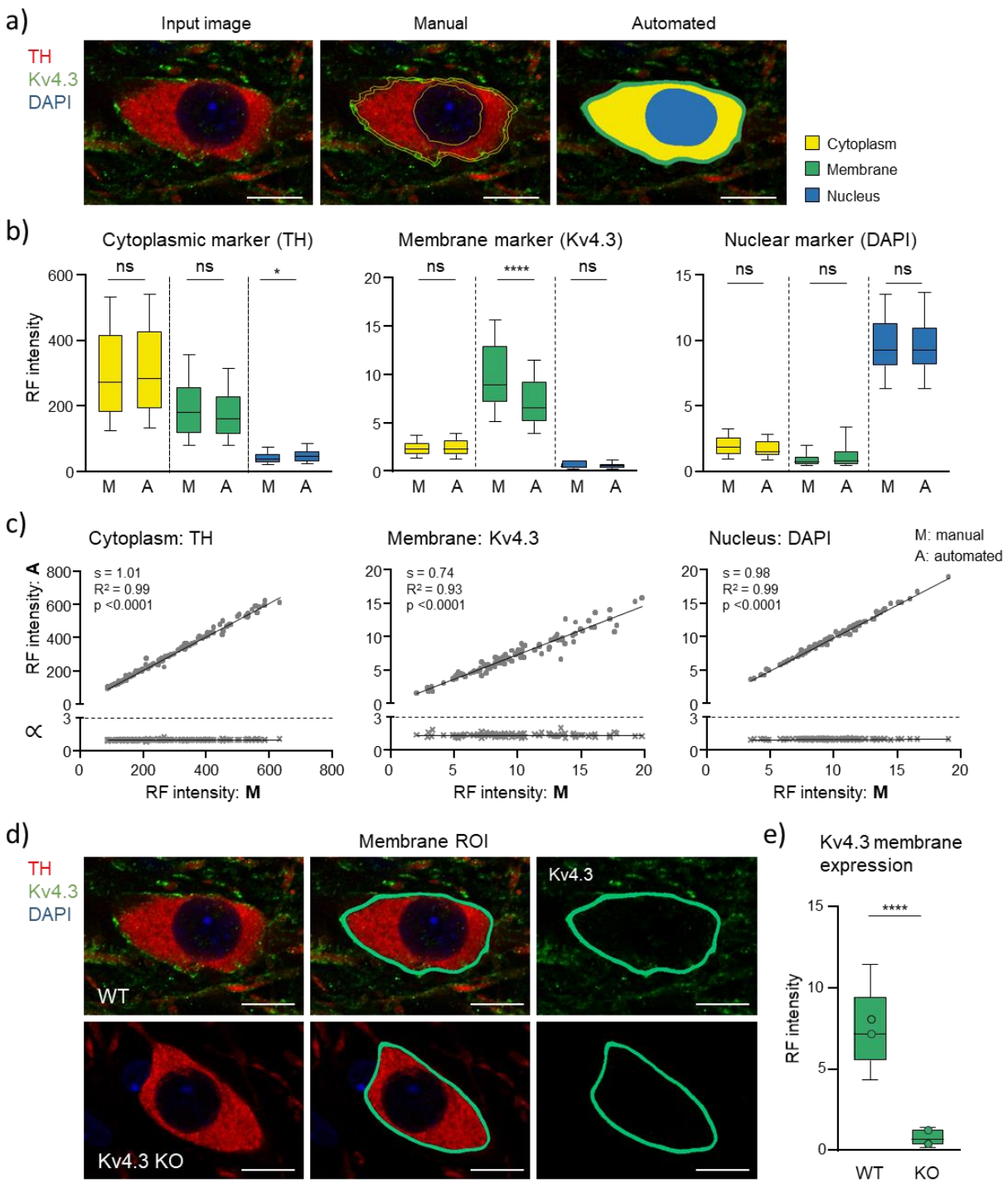

Figure 10: Comparison between manual and automated IF-derived image analysis of mouse midbrain sections. a) SN DA neurons in coronal midbrain section from adult mice after IF for tyrosine hydroxylase (TH, red) and Kv4.3 K^+ channels (green). Nuclei are marked by DAPI-staining (blue). Left: input image, middle: image after manual labeling of cell body, nucleus and membrane, right: image after automated recognition of the TH labelled cell body (yellow area), nucleus (blue area) and membrane (green area) with DLAP-6. Scale bars: 10 µm. b) Relative fluorescence (RF) intensity/SN DA neuron in different sub-cellular compartments, quantified manually (M) or automatically (A) via DLAP-6, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analysed neurons (n = 94). Significant differences according Mann-Whitney test are indicated by asterisks (ns: $p > 0.05$, ****: $p < 0.0001$). c) Upper: Single neuron correlations of RF intensities/cell for manual and automated analysis for three marker genes in their respective compartment according to Pearson correlation test.

Lower: corresponding proportionality constants ∝, calculated from the manual and automated dot count ratios. d) SN DA neurons in coronal midbrain section from adult WT (top) and Kv4.3 KO (bottom) mice after IF for tyrosine hydroxylase (TH, red) and Kv4.3 (green). Nuclei are marked by DAPI-staining (blue). Left: input images, middle and right: images after automated recognition of Kv4.3 labelled plasma membrane with DLAP-6. Scale bars: 10 µm. e) Kv4.3 relative fluorescence (RF) intensity/SN DA neuron membrane in WT and Kv4.3 KO mice, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analysed neuros (WT: n = 179, Kv4.3 KO: n = 200). The open circles indicate mean values for each analysed animal (N = 2 each). Significant differences according to Mann-Whitney test are indicated by asterisks (****: $p < 0.0001$). All data are detailed in Supplementary Table S15-17 and Supplementary Figure S7.

For a direct proof, we tested the DLAP-6 approach on SN DA neurons from Kv4.3 knock-out (KO) mice (**Figure 10d, S7, Table S17**). As expected, SN DA neurons from Kv4.3 KO mice did not show any Kv4.3 derived membrane-signal higher than the background, while in wildtype (WT), mean RF-values were ~10-fold higher (DLAP-6: RF KO: 0.8±0.5, RF WT: 7.7±2.9, $p<0.0001$). Nevertheless, the plasma-membrane compartment was marked by the DLAP-6 approach in TH-positive neurons from KO mice with similar robustness as for neurons from wildtype mice - a nearly impossible task by manual analysis.

Discussion

Here, we detail a deep learning-based image analysis platform (DLAP), including six pre-designed algorithms for automated image analysis, that allows rapid and unbiased cell count, as well as quantification of fluorescence signals (e.g. derived from mRNA or proteins) in a given ROI. These are common tasks in cell biology, but manual analysis is time consuming and prone to human error (Sommer & Gerlich, 2013; Xu et al, 2019). However, automated AI based and related approaches like ImageJ/Fiji plugins such as the Trainable Weka Segmentation (Arganda-Carreras et al, 2017), CellProfiler (Carpenter et al, 2006; McQuin et al, 2018), QuPath (Bankhead et al, 2017), U-Net (Falk et al, 2019), and DeepImageJ (Gómez-de-Mariscal et al, 2021), or the HALO® image analysis platform by Indica Labs (Brodie, 2020), require significant computational skills and/or high-end hardware, a significant barrier for their routine use (Kraus et al, 2017; Levet et al, 2021; von Chamier et al, 2019). Hence, manual approaches are still widely used for image-analysis in life sciences (Brown, 2017; Helmstadter et al, 2021; Holland & Davies, 2020).

Our DLAP approach overcomes these issues by providing already pre-trained neural network algorithms that can learn to detect new labels and extract desired features, with only a small amount of additional training-images. The six DLAP algorithms are pre-designed to suit most common life science tasks, and are easily adapted to distinct scientific needs.

DLAP dramatically reduces the respective analysis-time, and it is straightforward to use (utilized by under-graduate students in our lab). Moreover, as training and image-analyses are carried out via an easy-to-use web-based interface (https://wolution.ai/), and Wolution provides in case in-depth support for specific adjustments, our approach does not require any sophisticated hardware, software, or programming skills. Importantly, DLAP details are not proprietary, but all steps and strategies are provided here for free and in full detail, to facilitate distribution and use within the scientific community. These features represent important advantages in comparison to other cloud-based analysis pipelines, for example Aiforia (Benkert et al, 2019; Penttinen et al, 2018), CDeep3M (Haberl et al, 2018), or Visiopharm (Maffeis et al, 2019) that do not disclose insights into the underlying algorithms, do not allow user-based modifications, or require advanced programming experience (e.g. DeepCell Kiosk (Bannon et al, 2021)).

We have trained our DLAP procedures to detect, count, and analyse individual dopaminergic neurons from fixed mice and human brain samples, identified by tyrosine-hydroxylase (TH) expression, but the flexible design allows their quick and easy adaption to other cell types, specimens, and scientific questions. The here detailed DLAP approach allows the systematic quantitative analysis and anatomical mapping of several thousands of neurons in reasonable time, and thus facilitates the identification and characterization of small cellular subpopulations. Accordingly, by DLAP-analysis of more than 20.000 TH-immuno-positive SN neurons in PFA-fixed brain-sections from adult mice, and by generation of expression-maps according to anatomical coordinates, we defined a small subpopulation of neurons (~5% of all TH-positive SN neurons). These neurons were immuno-negative for the dopamine transporter DAT, were mainly located in the caudo-lateral SN, and had ~30% smaller cell bodies. DAT is a major determinant of activity and excitability of DA neurons, and of dopamine homeostasis and transmission (Bu et al, 2021; Condon et al, 2019; Miller et al, 2021). DAT is inhibited e.g. by methylphenidate and amphetamines as treatment for ADHD and depression (Torres et al, 2003; Vaughan & Foster, 2013), and changes in DAT expression have been reported in schizophrenia, ADHD, and Parkinson's (Palermo & Ceravolo, 2019; Salatino-Oliveira et al, 2018).

However beyond disease states, up to now, only for VTA DA neurons lower DAT expression has been systematically analyzed (Fu et al, 2012; Lammel et al, 2008; Li et al, 2013). On the contrary, for SN DA neurons, DAT is commonly used as marker besides TH (Ciliax et al, 1995; Palermo & Ceravolo, 2019; Poulin et al, 2020), and for their targeting, for instance, via DAT-Cre transgenic mouse lines, expressing the Cre-recombinase under the DAT-promotor (Gong et al, 2007; Lammel et al, 2015; Papathanou et al, 2019; Stuber et al, 2015). Our results imply that with such approaches, target-gene expression might not be affected in those lateral SN DA neurons that are immuno-negative for DAT protein. However, it must be noted that we did

not perform an absolute DAT-protein quantification. Hence, we explicitly do not exclude very low expression of DAT protein in the plasma-membranes of these lateral SN neurons, within the range of the respective detected DAT-background immuno-signals (similar applies for CB). Such a low DAT expression would be sufficient for successful DAT-Cre recombination. As using the TH-promotor instead of DAT cause issues due to its non-specificity to dopamine neurons (Lammel et al, 2015; Stuber et al, 2015), an alternative marker for all SN DA neurons might be DCC (deleted in colon cancer), the receptor for the axon guidance molecule netrin. In the midbrain, DCC is largely restricted to TH-positive neurons (Brichta & Greengard, 2014; Osborne et al, 2005), but we have not yet systematically analysed DCC expression in DAT-negative SN DA neurons.

We did however analyzed the co-expression of the Ca^{2+} binding protein calbindin, a marker for less vulnerable SN DA neurons in the dorsal tier (Brichta & Greengard, 2014; González-Hernández et al, 2010). In terms of differential vulnerability, it is important to note that the (caudo-) lateral SN DA neurons project into the (rostro-) dorsolateral striatum (Ambrosi & Lerner, 2022), and correspond to the ventro-lateral SN DA neurons in humans (Düzel et al, 2009; McCutcheon et al, 2019). These neurons display a particularly high vulnerability in Parkinson's compared to rostro-medial SN DA neurons (Damier et al, 1999; German et al, 1989; Hassler; Jellinger, 2012). In fact, the ventro-lateral SN DA neurons are almost completely lost in early PD, at a time point were other SN DA neurons are still largely intact (Kordower et al, 2013).

With DLAP-5, we identified that only ~10% of all TH-positive SN neurons from adult mice co-express CB, in line with previous reports (Fu et al, 2012; Gerfen et al, 1985; Liang et al, 1996; Neuhoff et al, 2002; Ricke et al, 2020). However, TH and CB co-expression was ~4-fold more frequently detected in DAT-negative as in DAT-positive SN neurons; the latter were almost double in size, compared to the CB-positive DAT-negative SN neurons. Although in classic DAT-positive SN DA neurons, CB expression is seen as a marker for less vulnerable neurons (Brichta & Greengard, 2014; Fairless et al, 2019; German et al, 1992; Lammel et al, 2008), we do not conclude a lower vulnerability of the here identified DAT-negative neurons in the lateral SN, as for very lateral SN DA neurons a higher sensitivity towards PD-stressors is described, compared to other SN DA neurons in general (German et al, 1992; Rodríguez et al, 2001), and particularly to the resistant CB-positive dorsal tier SN DA neurons (Brichta & Greengard, 2014; Yamada et al, 1990). Similarly, there is no clear positive correlation between DAT-expression and vulnerability described (González-Hernández et al, 2010), as suggested in earlier studies (Hurd et al, 1994; Uhl et al, 1994). Hence, we would rather speculate that the here described DAT-negative population of very lateral SN DA neurons is particularly affected by PD-stressors.

Very lateral SN DA neurons have also been reported to be negative for aldehyde dehydrogenase 1 (Aldh1a1) and the transcription factor SOX6, and positive for the vesicular glutamtate transporter Vglut2 (Carmichael et al, 2021; Poulin et al, 2020). Whether they are also negative for DAT, and a possible co-expression pattern with CB has not yet been systematically addressed. However our preliminary single nuclei sequencing data suggest that in the here described DAT-negative population of SN DA neurons, SOX6 expression is at least significantly reduced. Indeed, with a similar approach, it recently has been shown for different species that one type of SN DA neuron clusters preferentially expressed SOX6 while other clusters preferentially expressed CB (Kamath et al, 2022). Sox6-negative SN DA neurons have been shown to project to the medial, ventral, and caudal striatum and respond to rewards (Pereira Luppi et al, 2021).

What is known about the function of lateral SN DA neurons? Vglut2- and CB-positive but Aldh1a1-negative lateral SN DA neurons are important for responses to novel cues and salience (Kim et al, 2015; Menegas et al, 2017; Todd et al, 2022). Some lateral SN DA neurons are activated by aversive stimuli or cues that predict aversive stimuli (Matsumoto & Hikosaka, 2009). Lateral SN DA neurons *in vivo* display a higher burst activity (that is particularly metabolically demanding) compared to medial SN DA neurons (Farassat et al, 2019). *In vitro*, the pacemaker frequency of lateral but not medial SN DA neurons is positively coupled to the activity of Cav1.3 L-type voltage gated Ca^{2+} channels (Shin et al, 2022). These channels have been linked to defining the distinct vulnerabilities of DA neurons in PD (Duda et al, 2016; Liss & Surmeier, 2022; Ortner, 2021; Sandoval et al, 2022). However, it should be noted that the definition of the “lateral SN” in most of these studies is much broader, and likely includes the here defined very lateral SN DA neurons, but also more medial, DAT-positive neurons in the lateral SN.

One very recent study has indeed addressed the electrophysiological properties exclusively of only very lateral SN DA neurons, expressing either CB or Vglut2, most likely similar to the very lateral DAT immuno-negative neuronal population that we describe here. These neurons had a higher input resistance and excitability, and they displayed a less precise pacemaker, with lower frequencies and less prominent after-hyperpolarisations, compared to classical SN DA neurons (Sansalone et al, 2022). However, future studies are necessary to fully characterize the here defined and quantified subpopulation of lateral SN DA neurons, in terms of their distinct molecular identity, their axonal projections, their physiological function, and their fate in disease.

Author contributions/Acknowledgment

NB carried out the RNAscope analysis. SR carried out the protein quantification in cellular compartments, and assisted MH with neuron count and protein quantification in IF images. DW and MM implemented and customized all algorithms for automated analysis. SM performed IHC and IF stainings, JW performed stereology and automated cell count analysis for juvenile mice, HH and JB for adult mice. JRF and JMG provided Kv4.3 KO mice, and high resolution Kv4.3 immunofluorescence images. MF performed model-based data adjustments. CP generated 2D maps, JD and RP supervised experiments and helped with analysis. BL designed and supervised the study, and wrote the manuscript together with NB and SR. All authors revised the manuscript.

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Methods

DLAP algorithms 1-6; neural networks, training, post processing parameters

Convolutional neuronal networks - DeepLab3 and FCN.

We have used two very different neuronal network architectures, based on either the DeepLab3 network architecture or on a fully convolutional neural network (FCN). DeepLab3 based networks are more powerful for detecting complicated larger objects, like complex neuronal structures, and beyond, while FCN perform in general better for fine structures (like RNAscope probe derived, small individual dots), rather than large global concepts (Chen et al, 2017; Long et al, 2015). Both types of neural networks belong to the class of convolutional neuronal networks (CNN), commonly used for image analysis (Greener et al, 2022). Convolutional layers do not receive their inputs from the whole previous layer but only from a certain area (called 'filter' or 'kernel') which increases their computational efficiency (Wang et al, 2019). Moreover, they assign to each pixel in the image a class, such as cellular nucleus or background. This type of analysis is called semantic segmentation. The performance of deep learning based semantic segmentation depends strongly on the neural network architecture, training parameters and in particular the quality of the training data set. By specific training and further optimizing these two network-types for distinct tasks, we generated six distinct algorithms, as specified in **Table1 and 2**.

For cell counting and immunofluorescence signal quantification (DLAP-3 to 6), the large pre-trained neural network DeepLab3 (Chen et al, 2018) was further trained and optimized past training for the respective specific tasks. DeepLab3 is one of the best performing image segmentation algorithms on the Pascal Visual Object Classes (VOC) dataset (Everingham et al, 2006). This is a standardized image data set for building and evaluating algorithms, often used as a benchmark for segmentation quality. In general, the key innovation of DeepLab algorithms is the use of so called dilated or atrous convolutions. This type of convolutions has a dilated filter size, increasing the area covered by each filter, and the context that can be incorporated. Therefore, they have a higher resolution and can integrate information over larger areas, while keeping the number of parameters of the convolutions the same (Chen et al, 2017). Specifically, for our algorithms 3 to 6, we used xception65 as the CNN backend, provided by Google [https://github.com/tensorflow/models/tree/master/research/deeplab] (Chollet, 2017), which was pre-trained on the Pascal VOC 2012 segmentation dataset (Everingham et al, 2015).

For analysis of RNAscope data, we developed (DLAP-1 and 2), belonging to the class of FCN, as the trained respective algorithms based on DeepLab3 were not optimal for reliable detection of RNAscope-derived dot-numbers (**Table S1**). The most important modification of the FCN architecture is that it uses only convolutions, rather than including fully connected linear layers

(Long et al, 2015). Replacing the linear layers with fixed dimensions by convolutions allows the network to accept arbitrarily large images for processing and making predictions (called interference), and to process them very efficiently - limited mainly by the graphics processing unit (GPU) memory size. The FCN architecture, illustrated in **Figure 2**, is our own development, using the Tensorflow library [https://www.tensorflow.org/]. Our FCN network uses five convolutional layers, connected by the Rectified Linear Units (ReLU) activation function. This function only transfers the direct output of the previous layer to the next layer, if it is positive, while it returns zero for negative outputs (Nair & Hinton, 2010). Our network does not include any downsampling/downsizing to reduce image resolution through dropout or strided (kernel step size > 1) convolutions. Therefore, the output image has the same resolution as the input image, and fine structures are not smoothed out. We used another approach of neural network architectures, the so-called 1x1 convolutions, to reduce the number of feature maps and keep the parameter number tractable (Lin et al, 2013). Such a convolution is used between feature map 2 and feature map 3 to reduce the number of kernels by a factor of 4, thus reducing the parameter size of the following convolution by a factor of 16. To conserve image dimension, we extend the outer frame at the boundary of each image, which increases the space for the filter/kernel to scan the image, a process also called mirror padding. This architecture provided a good compromise between simplicity (and thus computational speed) and segmentation quality.

CNN training parameters for six individual tasks.

Training dataset need to have sufficient quality and size, depending on data variability (Moen et al, 2019; von Chamier et al, 2019). However, using too large training datasets can lead to overfitting, which leads to decreased generalization, and the algorithm fails to optimally apply learned concepts on a sample that it has not already seen during training. We ensured the quality (e.g. resolution, frame size) of our training datasets, and performed a quality evaluation during training (see below), in additional to data-validation by comparison with manual analyses. Images showing different neuronal shapes/forms in different brightness intensities were used for training to ensure the identification and recognition over a vast array of brightness and cellular structure.

As our FCN is not based on a pre-trained neural network (no "transfer learning"), it was trained by us from scratch, by only using our own training data. The FCN-based algorithms 1 and 2 analyse image patches of size 63^2 pixels. To train the network, we randomly cropped the images from our manually labelled training data set (ground truth data) into such patches. Training was performed using the stochastic first-order gradient-based Adam optimizer with default settings (learning rate α=0.001, estimates decay rates β_1=0.9, β_2=0.999, to prevent division by zero $\varepsilon=10^{-8}$) (Kingma & Ba, 2014). For DeepLab3 based algorithms DLAP-3 to 6,

we adjusted the neural network weights by further training with our own data sets using the training script provided by Google Research (Chollet, 2017). This was performed on randomly sampled patches of size 512^2 pixels much larger than the 63^2 pixels we use in the FCN algorithm, as Deeplab3 is designed to detect large, complex objects within an image.

For both network-types, the training time on a modern GPU was several hours for each of the data sets. The number of images and neurons each that were used to train each of the individual algorithms is given in **Table 2**. We used 80% of our manually labelled training data set for training and 20% (referred to as 'test data') to evaluate the segmentation quality. For this performance-evaluation of the individual algorithms, the 'test data' was analyzed and the results were compared to the ground truth provided by the labels. For all algorithms, we assessed typical quality measures (**Table 1**, **S1**): The pixel error rate, i.e. the global percentage of pixels that are wrongly classified. This global error rate was calculated by defining for each segmentation class the rates of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN), each given in % of the total pixels of the whole training data set. The specificity [defined as TN/(TN+FP)] and the sensitivity [defined as TP/(TP+FN)], for each segmentation class, were also calculated, as these values are more suitable measures in cases of large class imbalance.

We trained each data set until the training error/loss function (difference between output and ground truth) converges minimum. We also verified that we did not overtrain, by tracking the pixel error in the 'test data'. If overtraining is happening, only the training error is still getting lower, while the test error increases. Therefore, training was stopped as soon as the pixel error starts to get worse not better.

Post training image analysis optimization.

After CNN training was completed, the quality of the CNN outputs for each task (summarized in **Table 1**) was further improved by, implementing additional classic image analysis procedures (a) to (e), specified below, into the individual CNN procedures. If an analysis step was further increasing the correlation between algorithm- and manually-obtained results for a distinct task, it was implemented into the respective algorithm. The specific steps (a-e) that were implemented post-training into each of the final algorithms are summarized in **Table 2**.

a) Watershed algorithm. This algorithm requires a set of markers, which are associated with the centers of respective target structures (Kornilov & Safonov, 2018). These markers are obtained by calculating the Euclidean distance transform of the binary mask of target structures cells (detected by the neural network), and then finding peaks in the distance image. Starting from these markers, the watershed algorithm "floods" the image until all pixels are assigned

with a watershed region. We used this algorithm in both FCN based algorithms, for a better separation of mRNA molecule derived dots. The FCN output tend to connect single fluorescent dots of individual mRNA molecules, which biases their number count. We found that by applying a classical watershed algorithm, we can separate these points and get a number count much closer to the truth (manual count). For the DeepLab3 based algorithms 4 and 5, watershed algorithm were used to improve segmentation of neighboring nuclei/cells. This was not necessary for DLAP-3.

b) Morphological operations. A series of morphological operations of type dilation and erosion (Fisher, 1996) were applied to algorithms 1 and 2. These operations add to or remove pixels from the object boundaries. This improves separation of individual neurons and smoothes out uneven boundaries provided by the CNN. This approach was used in both FCN-based algorithms for a better separation of individual neurons.

c) Binary fill-holes-function. This function was applied to the DeepLab3-based CNN outputs for DLAP-4 to 6, were pixel-wise semantic segmentation resulted in holes within the detected compartments, as such holes are of no biological meaning. Therefore, all holes in the segmentation masks of each region were filled using the "*binary_fill_holes*" function (González-Hernández et al, 2010) of the SciPy library http://scipy.github.io/devdocs/reference/generated/scipy.ndimage.binary_fill_holes.html.

d) Remove target gene color channel procedure. This procedure was applied to the DeepLab3 based algorithms 5 and 6 to facilitate identification of the target areas within the ROI (i.e neuronal cell bodies or neuronal compartments), in which the relative target-gene derived fluorescence-signal was quantified. This procedure is removing the target gene channel for the algorithm-based detection of the target areas, to ensure its recognition, independently of the target gene fluorescence-signal. For excluding the target-gene channel, the respective color layer of the image was set to zero before passing the image through the CNN (both for training and analysis), and it was added back, for the target-signal quantification step of the algorithms.

e*) RGB intensity threshold*. Such threshold was included to the DeepLab3 based algorithms 5 and 6, to optimize background intensity quantification for calculation of relative target-gene signal-intensities in identified target areas (i.e. neuronal cell bodies or neuronal compartments) to correct for non-uniform intensity conditions in different images/experiments. It was not always straight- forward to determine the individual background-intensities, because some images contained large regions with no detected target areas, but also with no apparent blank background. We included RGB intensity thresholds (for widefield and for confocal images) of pixel values ranging from 10-40 for each channel, depending on its raw intensities, to exclude pixels higher than this threshold from determining background signal intensities.

Mice

Juvenile (~PN13, Figure 5 data), and adult (~PN90) male mice were bred at Ulm University at a 12-hour light/12-hour dark cycle and were fed ad libitum. Kv4.3 KO mice were obtained from Jean-Marc Goaillard (Haddjeri-Hopkins et al, 2021). All animal procedures were approved by the German Regierungspräsidium Tübingen (Ref: 35/9185.81-3; TV-No. 1043, Reg. Nr. o.147) and carried out in accordance with the approved guidelines.

Drug and saline treated animals (Figure 4 data), were the same, cohort as already published (Cav2.3 +/+ mice from Benkert et al (2019)). Drug treated mice were treated in vivo with MPTP/probenecid to introduce SN DA neuron degeneration. Briefly, MPTP hydrochloride was injected 10 times (every 3.5 days for 5 weeks) subcutaneously at a concentration of 20 mg/kg saline (sigma) together with probenecid intraperitoneally at a concentration of 250 mg/kg 1x PBS (Thermo Fisher). Control mice were injected with saline and probenecid. For all details, including, TH-DAB/hematoxillin staining, stereology, and Aforia-based automated analysis, see (Benkert et al (2019).

Human brain samples

Human midbrain tissue including the substantia nigra (SN) was obtained from the German Brain Bank (www.brain-net.net) Grant-No. GA76 and GA82), as native cryo-preserved tissue blocks (-80 °C/dry ice). Analysis of the human material was approved by the ethic commission of the German Brain Bank as well as of Ulm University (277/07-UBB/se). Detailed information on human midbrain samples is summarized in **Table S5.** Human tissue was processed, stained, and the RNA integrity was assessed via determining the RNA integrity number (RIN), using the Agilent 2100 Bioanalyzer as described (Duda et al, 2018).

RNAscope® *in situ* hybridization, image acquisition, and data analysis

RNAscope experiments were performed essentially as described (Benkert et al, 2019; Siller et al, 2022). *The* RNAscope® technology (Advanced Cell Diagnostics, ACD) was according to the provided protocol for fresh frozen tissue sections [https://acdbio.com]. Details of the used RNAscope probes are given in **Table S2.** All used chemicals were RNAse free grade.

12 µm coronal cryosections of mouse or human midbrains were prepared, using a Leica CM3050S cryotome as previously described (Gründemann et al. 2008; Duda et al. 2018), mounted on SuperFrost® Plus glass slides (VWR), and allowed to dry in a drying chamber containing silica gel (Merck) at -20°C for one hour. After fixation with 4% PFA (Thermo Fisher Scientific) in 1x PBS (pH 7.4) for 15 min at 4°C and dehydration via an increasing ethanol

series (50%, 75%, 100%, 100%, Sigma), sections were permeabilized for 30 minutes by digestion with protease IV (ACD) at room temperature (RT). Following digestion, respective RNAscope probes (TH probes and target gene probes were processed in parallel) were hybridized for 2 h at 40°C in a HybEZ II hybridization oven (ACD). Signal was amplified using the RNAscope Fluorescent Multiplex Detection Kit (ACD) containing four amplification probes (AMP1-4). In between each amplification step (incubation at 40 °C for 30 min with AMP1/3) or 15 min with AMP2/4), sections were washed twice for 2 min with wash buffer (ACD). After RNAscope hybridization, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) ready-to-use solution (ACD, included in Kit) for 30s at RT. Sides were coverslipped with HardSet mounting medium (VectaShield), and allowed to dry overnight at 4 °C. RNAscope probes for TH were labelled with AlexaFluor488, and target gene probes with Atto550.

Image acquisition: Fluorescent images containing the ROI (i.e. the SN), of murine and human midbrain sections were acquired at 63x magnification using a Leica DM6 B epifluorescence microscope. All images were acquired as z-stacks, covering the full depth of cells, and reduced to maximum intensity Z-projections, utilizing Fiji [http://imagej.net/Fiji]. TH-derived RNAscope fluorescence signals were visualized at 480/40 nm excitation, 527/30 nm emission and 505 nm dichroic mirror, target gene-derived fluorescence signals were visualized at 546/10 nm excitation, 585/40 nm emission and 560 nm dichroic mirror, DAPI-signals were visualized at 350/50 nm excitation, 460/50 nm emission and 400 nm dichroic mirror. Files were aquired using the LASX software (Leica), and stored as PNG files.

Image analysis: For manual analysis, the images were further processed using the Fiji software [http://imagej.net/Fiji]. First, cell bodies of individual TH- and DAPI-positive neurons were encircled, using the “Polygon selection” tool to define the area for RNAscope signal quantification. In the next step, a classifier was trained on images only showing target gene signal, by manually labelling and annotating background and dot regions (around 30 labels for each class) in the “Trainable Weka Segmentation”. This classifier was then used to classify all target gene images into the two groups “dots” and “background”. Afterwards, the threshold for particle recognition was set on classified images, and dots were counted for each cell-specific ROI using the “analyze particle” function. Target probe hybridization results in a small fluorescent dot for each mRNA molecule, allowing absolute quantification of mRNA molecules (via dot counting) independent from fluorescent signal intensity.

For automated quantification of RNAscope signals, custom-designed FCN based algorithms DLAP-1 (for mouse-brain sections) and 2 (for human brain sections) and the Wolution-platform were used [https://console.wolution.ai/] (Wolution GmbH & Co. KG, Planegg, Germany). Processed images were uploaded on the Wolution platform, and the algorithms automatically marked cell bodies of TH-positive cells and quantified the number (and the dot area) of target

gene derived fluorescence dots. After algorithm processing, correct identification of cells was controlled by hand and resulted in inclusion of ~50 % correctly identified cells for analysis. If a quantification of all TH-positive neurons is required, neuronal nuclei must be counted, before excluding neurons with not optimally separated cell-bodies for subsequent RNAscope based mRNA quantification in the target cells.

Statistical modelling for brightfield light adjustment:

To asses and correct for a possible influence of neuromelanin (NM) human-derived RNAscope data for, a linear mixed effect model was applied, similar as we had previously described (Duda et al, 2018; Schlaudraff et al, 2014). Briefly, we applied a bayesian probabilistic model approach conducted in RStan (Stan Development, 2018) with the R package version 2.21.7, https://mc-stan.org/) using R (version 4.2) under RStudio (version 2022.07). We applied normal distributions for priors of all parameters. To achieve a stable non-degenerate solution priors needed to be properly informed by restricting the effective parameter space. . The chosen model, detailed below, is well-informed by the data on all its parameters and shows an acceptable fit on the age level (see posterior-predictive plots in **Figure S2**).

The number of detected dots/mRNA-molecules for each component/gene (defined as Y_G) is assumed to follow a binomial distribution in which the trial size is derived from cell area s:

$$Y_G \sim Binom(s, p_G)$$

The probability pG of detecting a target molecule is expressed according to a logit-link function (linear combination) as:

$$p_G = \frac{p_{max}}{1 + e^{-g(x)}}$$

where $g(x)$ is an equation, indexed by the mixture-component and a binary discretization of a brains donor (adult vs. aged) that considers a cell's NM concentration (as given by brightfield light transmission, T) according to the Lambert-Beer law which states that concentration is proportional to the negative logarithm of light transmission

$$-log_{10}T = l\varepsilon \cdot c$$

Light transmission

T is the fraction of transmitted to incident light intensity

$$T = \frac{\mathrm{I}_t}{\mathrm{I}_i}$$

Since $\mathrm{I}_t < \mathrm{I}_i$ and light intensities are strictly positive, it follows that $0 < T < 1$. For the probabilistic model a distribution for T has to be defined. A continuous distribution bounded on [0, 1] is the Beta-distribution.

$$T \sim Beta(\mu_T, \kappa),$$

The Beta distribution is parameterized by its mean μ_T and the precision κ.

For μ_T we apply a logit-link function

$$\mu_T = \frac{1}{1 + e^{-h(x)}}$$

$h(x)$ is a linear equation, indexed by the mixture component and binarized age. In addition, the intercept considers a random effect on individual brains. The mixture proportion is modeled to depend on binarized age and to consider a random effect on the brain level, given in Wilcoxon notation (Wilkinson & Rogers, 1973)

$$\theta = logit^{-1}(\theta_0 + \Delta_{\theta,age} + (1|brain))$$

To adjust experimental results for a mutual NM influence, data were fitted and then reproduced from the model, with each cell's T set to a constant value according to the mean of all brains' μ_T.The influence of BF light transmission (associated to NM content by –log T) on gene expression / dot detection probability can be considered significant with 95%-credible intervals from -5.1 to -1.5 or from -12.2 to -7.7 for low and high expressing cells, respectively (corresponding intervals from the prior distribution vary from -7.8 to +8.0).

Immunohistochemistry, image acquisition, and data analysis

DAB Immunohistochemistry (IHC) experimental procedures were performed essentially as previously described (Benkert et al, 2019). Briefly, mice were transcardially perfused with and post-fixed (overnight at 4°C) in 4% PFA (Thermo Fisher Scientific) in 1x PBS (pH 7.4). Brains were stored in 0.05% NaN_3 (Sigma) in PBS at 4°C until vibratome (VT 1000S, Leica) cutting (30 µm coronal midbrain sections). All washing and incubation steps during the staining procedure were performed while shaking (300 rpm, microplate shaker, VWR). Free-floating sections were washed (three times in 1xPBS for 10 min) and blocked for 2 hours with 10% normal goat serum (NGS, Vector Laboratories), 0.2% BSA (Carl Roth) and 0.5% Triton X-100 (Merck) in 1x PBS to prevent non-specific antibody binding. After further washing (one time in 1xPBS for 10 min), sections were incubated with rabbit anti-TH primary antibody (1:5000, Merck) in 1% NGS, 0.2% BSA and 0.5% Triton X-100 (in 1x PBS) overnight at room temperature. Sections were then washed three times in 0.2% Triton X-100 in 1xPBS for 10 min and incubated with biotinylated goat anti-rabbit (1:1000, Vector Laboratories) for 2 hours

at room temperature. Immunostaining was visualized via VECTASTAIN® ABC system based on Horseradish peroxidase (HRP) detection (Vector Laboratories) using 3,3'-Diaminobenzidine (DAB, Vector Laboratories) as substrate. The slices were mounted on SuperFrost® Plus glass slides (VWR), dehydrated in ascending ethanol series (50%, 70%, 90%, 100%, 100%, Sigma) for 10 min each, and cleared with xylene (Sigma) two times for 10 min each. Slides were mounted with VectaMount Permanent Mounting Medium (Vector Laboratories).

Hematoxylin-counterstaining: For counterstaining of already DAB-stained and permanently mounted adult mouse brain sections, slides were incubated in xylene (two times for 5 min) to remove coverslips and rehydrated by an ethanol series (100%, 100%, 90%, 70%, 50%) and H_2O (5 min each). After drying for 5 min, slides were incubated with Vector hematoxylin QS (2 min) to counterstain nuclei (Vector Hematoxylin QS Kit, Vector Laboratories). After dehydration in ethanol series and clearing in xylene they were again mounted using VectaMount (all steps as described for DAB-staining).

Stereology: Stereological estimates of TH-positive neuron numbers were determined using a Leica CTR5500 microscope and the unbiased optical fractionator method (StereoInvestigator software; MBF Bioscience), similar as previously described (Benkert et al, 2019; Liss et al, 2005). The SN region throughout the whole caudo-rostral SN axis (Bregma -3.8 to -2.7, according to (Paxinos & Keith B. J. Franklin, 2007) was identified, using well established landmarks (Fu et al, 2012)), and was marked as ROI for TH-positive neuron count. The ROI was marked ab analysed unilaterally on each of the serial DAB-stained sections (37 for juvenile, 40 for adult mice). Sampling grid dimensions were 75 x 75 µm (x,y-axes), counting frame size were 50 x 50 µm (x,y-axes), and counting frame height was 9 or 11 µm for juvenile and adult mice, respectively. Estimated total number of TH-positive neurons (N) was calculated for each animal according to equation (1):

$$1)\ N = \sum Q^{-} \cdot \frac{t}{h \cdot asf \cdot ssf}$$

with ΣQ^{-} = number of counted neurons, t = mean mounted section thickness (i.e. ~10-11 µm), h = counting frame height (i.e. 80 % of the mounted section thickness), asf = area sampling fraction (i.e. 0.44), and ssf = section sampling fraction (i.e. 1 for SN).

Reliability of the estimation was evaluated by the Gundersen coefficient (CE, m = 1) according to equation (2). CE values were all ≤0.05 for all analyzed animals.

$$2)\ CE = \frac{\sqrt{s^2 + VAR_{SRS}}}{s^2}$$

with $A = \sum_{i=1}^{n} (Q_i^{-})^2$; $B = \sum_{i=1}^{n-1} Q_i^{-} Q_{i+1}^{-}$; $C = \sum_{i=1}^{n-2} Q_i^{-} Q_{i+2}^{-}$

s^2 = variance due to noise, and VAR_{SRS} = variance due to systematic random sampling, according to equation (3) for m = 1.

$$3)\ VAR_{SRS} = \frac{3(A-s^2)-4B+C}{240} \text{ for m=1}$$

Automated neuron counting:

Image acquisition: Digital images of DAB-stained sections were acquired using a whole slide scanner (3D-Histech Pannoramic 250 Flash III, Sysmex Deutschland GmbH, Norderstedt, Germany or Aperio Versa 8, Leica Biosystems Nussloch GmbH, Nußloch, Germany) and processed using the Fiji software [http://imagej.net/Fiji] were used to combine these layers.

Image analysis: The digital slides were processed using QuPath [https://qupath.github.io/] to label and cut out the sections of interest (37/40 consecutive SN sections covering the entire caudo-rostral axis). As for stereology, the SN ROI was identified according to the typical landmarks (Fu et al, 2012; Paxinos & Keith B. J. Franklin, 2007) and analyzed unilaterally. Processed sections (showing the ROIs) were afterwards uploaded on the Aiforia® Cloud platform [https://www.aiforia.com/] (Aiforia Technologies Oy, Helsinki, Finland) or the the Wolution platform [https://console.wolution.ai/] (Wolution GmbH & Co. KG, Planegg, Germany). For both automated platforms, in case of flawed sections that could not be automatically analyzed, the counts from the section before and after were averaged to prevent methodological bias (for stereology, these sections were omitted, and this information was taken account for the calculation of the stereological estimates).

The Aiforia-platform was only used for analysis of Hematoxylin-counterstained DAB-stained brain sections. It uses a supervised training to establish a non-disclosed deep CNN algorithm that recognized TH-positive neurons, based on nuclear/cell morphology and TH signal. The Aiforia algorithm was trained with 4064 TH-positive neurons (Benkert et al, 2019) and comprised two layers. The individual TH-positive cells were segmented in the first one and counted in the second one (see Penttinen et al (2018)). For generating the ground truth data used for algorithm training, one circle is placed on top of the nucleus within each TH-positive neuron (instead of marking the whole nucleus), similar as for our respective Wolution-based algorithms DLAP-3 and 4. Only TH-positive cells with a clearly visible/focused nucleus were considered for neuron counting, to avoid counting the same cell on more than one section. Processed images were uploaded on the Wolution platform. For counting of only DAB-stained TH-positive neurons (juvenile mice), DLAP-3 was used, for counting of hematoxylin counterstained TH-positive neurons (adult mice), DLAP-4 was utilized.

Immunofluorescence, image acquisition, and analysis

Immunofluorescence (IF) experimental procedures were performed essentially as previously described (Benkert et al, 2019). Mouse brains were perfused, stored and cut as already described for IHC. All washing and incubation steps were performed while shaking (300 rpm, microplate shaker, VWR).

For automated counting and relative signal-intensity quantification analysis with the DLAP-5 algorithm, immunostaining was performed in two separate cohorts using sequential antibody incubation. 30 µm coronal midbrain free-floating sections were washed (three times in 1xPBS for 10 min) and blocked for 2 hours with 10% normal goat serum (NGS, Vector Laboratories) and 0.5% Triton X-100 in 1x PBS. After blocking, sections were incubated with mouse anti-DAT (1:500, Thermo Fisher Scientific) primary antibody in carrier solution (1% NGS, 0.5% Triton X-100 in 1x PBS) overnight at 4°C. Sections were then washed (three times in 1xPBS for 10 min) and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000, Thermo Fisher Scientific) in carrier solution for 3 hours at room temperature in darkness. All subsequent staining steps were carried out in darkness. For additional primary antibody incubation, sections were washed (three times in 1xPBS for 10 min) and incubated overnight at 4°C with rabbit anti-TH primary antibody (1:1000, Merck) or with chicken anti-calbindin d28K (1:1000, Novus bio) in carrier solution. After washing (three times in 1xPBS for 10 min), sections were incubated for 3 hours at room temperature with Alexa Fluor 546 goat anti-rabbit secondary antibody (1:1000, Thermo Fisher Scientific) or with Alexa Fluor 647 goat anti-chicken secondary antibody (1:1000, Thermo Fisher Scientific), in carrier solution. After washing (three times in 1xPBS for 10 min), the sections -were mounted on SuperFrost® Plus glass slides (VWR) with VectaMount Permanent Mounting Medium with DAPI (Vector Laboratories).

For relative quantification of fluorescence-signal intensities in cellular compartments (plasma-membrane, cytoplasm, nucleus) with DLAP-6, two similar Kv4.3 staining protocols were utilized, adapted from (Haddjeri-Hopkins et al, 2021), leading to very similar results. More precisely, one WT and one Kv4.3 KO brain (dataset 1) was processed, and images were acquired in the Goillard lab essentially as described (Haddjeri-Hopkins et al, 2021). The other brains (dataset 2) were perfused in the Goillard lab, and further processed in the Liss lab, according to the following protocol. 50 µm free-floating coronal midbrain sections were blocked for 1 hour 30 minutes with 5% NGS and 0.3% Triton X-100 in 1x PBS, followed by incubation with a rabbit anti-Kv4.3 primary antibody (1:10000, Alomone Labs) together with chicken (G)/mouse (L) anti-TH primary antibody (1:1000, Abcam / Merck) in a carrier solution (1% NGS, 0.3% Triton X-100 in 1x PBS) overnight at 4°C. Sections were then washed (three times in 0.3% Triton X-100 in 1x PBS for 15 min) and incubated with Alexa Fluor 488 goat anti-rabbit

secondary antibody (1:1000, Thermo Fisher Scientific) and Alexa Fluor 546 goat anti-mouse secondary antibody (1:1000, Thermo Fisher Scientific) in carrier solution for 2 hours at room temperature. After washing (three times in 0.3% Triton X-100 in 1x PBS for 15 min), sections were incubated with DAPI (1.5 µg/mL; Sigma-Aldrich) for 10 min, washed (two times in 1x PBS for 10 min), and mounted on glass slides using Vectashield mounting medium.

Image acquisition: For automated counting of TH-IF positive neurons und and relative signal intensity quantification (DAT-IF), fluorescent images of all sections were acquired with Leica DM6 B epifluorescence microscope (Leica Microsystems) using a 63X oil objective. All image parameters were set using the LAS X software (Leica microsystems) to avoid saturated pixels, and identical acquisition settings were maintained (a prerequisite for relative signal-intensity quantification). TH-IF was visualized at 546 nm (exposure = 300-500 ms, gain = 2), DAT-IF at 488nm (exposure = 600 ms, gain = 2), CB-IF at 660nm (exposure = 500 ms, gain = 2) and DAPI at 405nm (exposure = 300 ms, gain = 1). Fluorescence lamp illumination was kept at 30%. Images were acquired as tile scans from a single plane of focus. Merged images were exported as Leica Image File (LIF).

For relative quantification of fluorescence-signal intensities in cellular compartments, 63X confocal quality images were acquired using a Zeiss LSM780 with the settings defined in (Haddjeri-Hopkins et al, 2021) for dataset 1. For dataset 2, confocal images using 100X/1.4 NA oil objective were acquired with STEDYCON (Abberior Instruments) mounted on an Olympus BX53 upright microscope (Olympus LS), and image parameters were adjusted using the STEDYCON web-based software (Abberior Instruments). To avoid saturated pixels (a prerequisite for relative signal-intensity quantification), photon counts from all channels were monitored in the photon count range indicator using the “fire blue” colour-map. This map indicates saturated image parts in blue over a yellow-red gradient for each individual channel. Fluorochromes were excited using an Argon laser, and the following wavelengths were used for excitation: TH-IF at 546 nm, Kv4.3-IF at 488nm, and DAPI at 405nm. The following acquisition parameters were adjusted at the stedycon user interface: Pixel size 78 nm; pinhole diameter 0.71 AU; one-directional line accumulation acquisition; laser power 10% for TH and Kv4.3, 12% for DAPI. Images were acquired from a single plane of focus and were exported as OsmAnd Binary Maps (OBF) files.

Image analysis: For automated counting of TH-IF positive neurons und and relative signal intensity quantification, images were converted to 8 bit RGB color TIFF images. The Fiji software was used to label and cut out the sections of interest (~28 consecutive SN sections within the caudo-rostral axis, bilaterally), and the ROI was identified according to the typical landmarks for each section, similar as described for IHC/DAB-stained neuron counting (Fu et al (2012), https://mouse.brain-map.org). Processed images were uploaded on the Wolution

platform and DLAP-5 (Wolution GmBH & Co. HG) was used for analysis. The algorithm automatically identified cell bodies and nucleus of both TH- (red channel) and DAPI-positive (blue channel) cells within the ROI and directly quantified the mean IF signal intensity per area for TH, DAPI and the target gene (green channel, DAT) in the cell body and nucleus. Background (BG) regions (for normalization of signal intensities) were taken from regions outside the ROI by application of individual IF intensity thresholds for each RGB channel (as described in post-training procedures). All identified SN cells were manually checked for the correct and clear identification of full cell bodies and nuclei. If cell bodies were not completely separated from each other, or if they were cropped, fragmented, very small, or did not show a full nucleus, these cells were excluded from further semi-quantitative analysis (~30% of identified TH-positive SN neurons, **Figure 5a**, **Table S12**). This strategy was used, as our aim was analysis of TH and DAT co-expression and signal-quantification. For absolute quantification of all TH-positive (or DAT-positive) neurons, a count of neuronal nuclei is required (and possible), as described for DLAP-3/4.

Raw signal intensities of each cell were normalized to the determined BG signals from the respective section. The BG signals determined from each analysed section were then normalized to the mean BG-value for each animal, and outliers were removed. The threshold for defining DAT-negative and CB-positive SN DA neurons was manually determined, and set as the mean normalized BG value for each mouse, plus 2 times SD or 1.5 times SD for DAT (due to different raw signal intensities of the two different mouse cohorts; **Figure S5c**), and plus 4 times SD for CB.

For the generation of anatomical 2D maps, the x and y coordinates (provided by DLAP-5), of all analysed TH-positive neurons were used, to plot the TH and DAT RF signals, using R computational environment (Gantz et al, 2018) version 4.2.1 with Tidyverse package (Carmichael et al, 2021). To merge / compare results from distinct experiments, data were adjusted for average RF-signals for DAT (or TH). More precisely, as cohort 2 (3 mice) had ~1.2 fold lower DAT signals, a corresponding correcting factor, CF = mean DAT cohort 1/ mean DAT cohort 2, was applied.

For relative quantification of fluorescence-signal intensities in cellular compartments with DLAP-6 algorithm, OBF images were converted to 8 bit RGB color TIFF images using the Fiji software. For manual analysis, images were further processed using Fiji. First, the “polygon selection” tool was used to manually delineate a continuous membrane ROI around the cell based on the Kv4.3 IF signal (green channel). The cytoplasm was marked according to the immunofluorescence signal for the cytoplasmic TH (red channel), and nucleus was marked according to the DAPI signal (blue channel). In the next step, the “measure” function (under the “analyze” toolbar) was used to calculate the mean, RF signal-intensity for each

compartment, already normalized to the respective area. For automated analysis, the same high resolution RGB color TIFF images were uploaded on the Wolution platform and analysed with DLAP-6. Each identified TH-positive neuron was segmented into “cytoplasm”, “membrane” and “nucleus”, based on the cytoplasmic TH and nuclear DAPI fluorescence signal (after training, the plasma-membrane was reliably identified without a third membrane-marker). BG signal-intensities were determined from images acquired outside the region of analysis, using individual RGB channel thresholds, and were used to normalize signal intensities for both, manual and automated analyses. All images were manually checked for the correct identification of membrane, cytoplasm and nuclear compartments similar as performed for analysis with DLAP-5 and ~90 % of cells were used for further quantitative analysis. In case of non-continuous membrane compartment identification, the weighted mean (area) of RF signal-intensity in all identified membrane ROIs from each cell was used for analysis.

Statistics and software

Data analysis and graphical illustrations were performed using GraphPad Prism 9 (GraphPad Software, Inc.), Adobe Illustrator CC2015.3 (Adobe Systems Software), Wolution (https://wolution.com/), and Fiji (https://imagej.net/Fiji) software. Statistical tests were performed with GraphPad Prism 9. Anatomical maps were plotted using R computational environment (Gantz et al, 2018) version 4.2.1 with Tidyverse package (Carmichael et al, 2021).

Normal distribution was tested with D`Agostino-Pearson omnibus normality test. Correlations were performed using Pearson correlation test, proportionality constant was calculated from the ratio between two directly corresponding values. In graphs, data are given as boxplots showing median and whiskers representing 10th -90th percentile. For analyzing one parameter non-parametric Mann-Whitney U test and Kruskal Walis test, with post hoc Dunn’s was used. Testing for two independent parameters was performed via two-way ANOVA with Tukey’s multiple comparison test. ROUT outlier test (Q=1) was used to remove outliers. Statistical significances are indicated as ns > 0.05, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Supplementary material

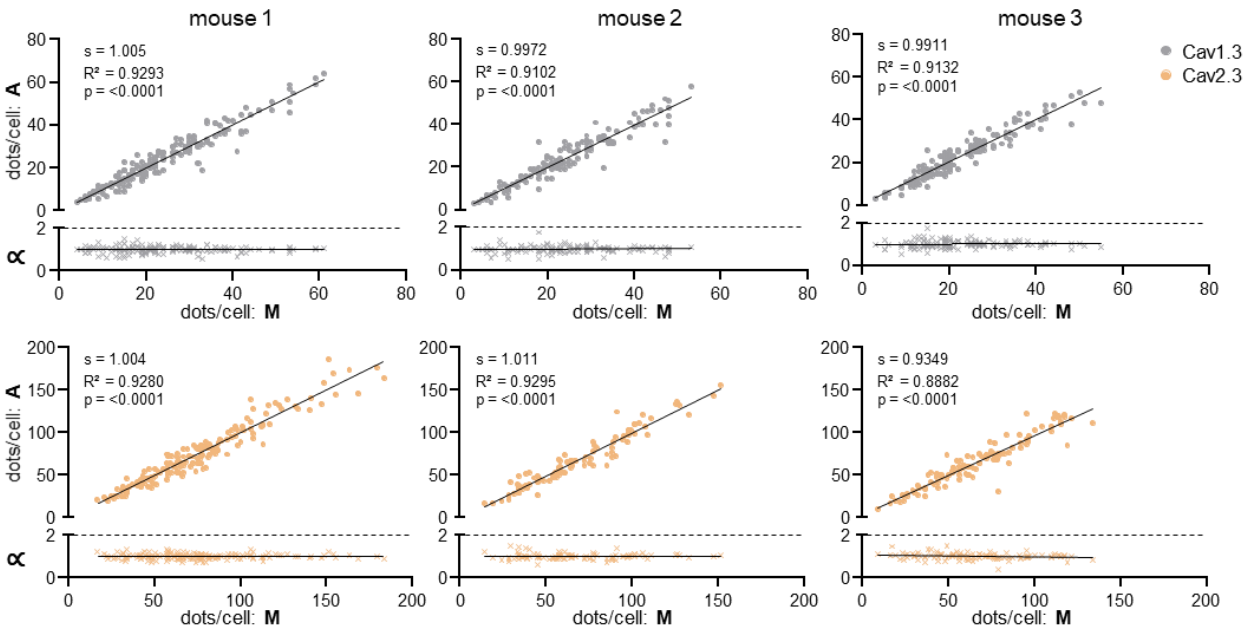

Figure S1: Comparison between manual and automated RNAscope-derived image analysis. Upper: Single neuron correlations of Cav1.3 (grey) and Cav2.3 (light brown) mRNA-derived dot counts/SN DA neuron between manual (M) and automated (A) analysis according to Pearson correlation test separately for each analyzed mouse. Lower: corresponding proportionality constants ∝, calculated from the manual and automated dot count ratios.

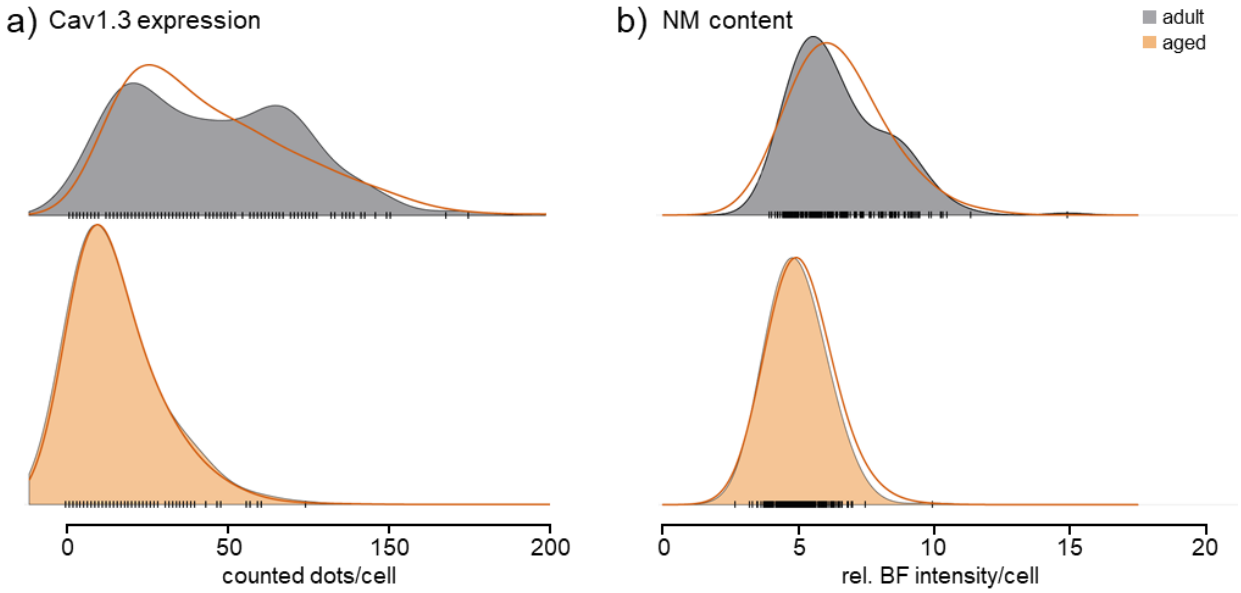

Figure S2: Posterior-predictive control of human RNAscope data model-fits. Cav1.3 expression (a) and NM content (BF light transmission, b) data is represented by vertical black lines and the colour-filled kernel densities for adult and aged brains, as indicated. Red continuous lines display the kernel distribution of data reproduced from the fitted model. Note the bimodal distribution in the group of adult brains in Cav1.3 expression and BF light transmission. The model does attribute for the wider base of the distribution of the number of detected Cav1.3 mRNA molecules (two components mixture of binomials) and BF light transmission (two components of beta), but does not represent the mixture proportion.

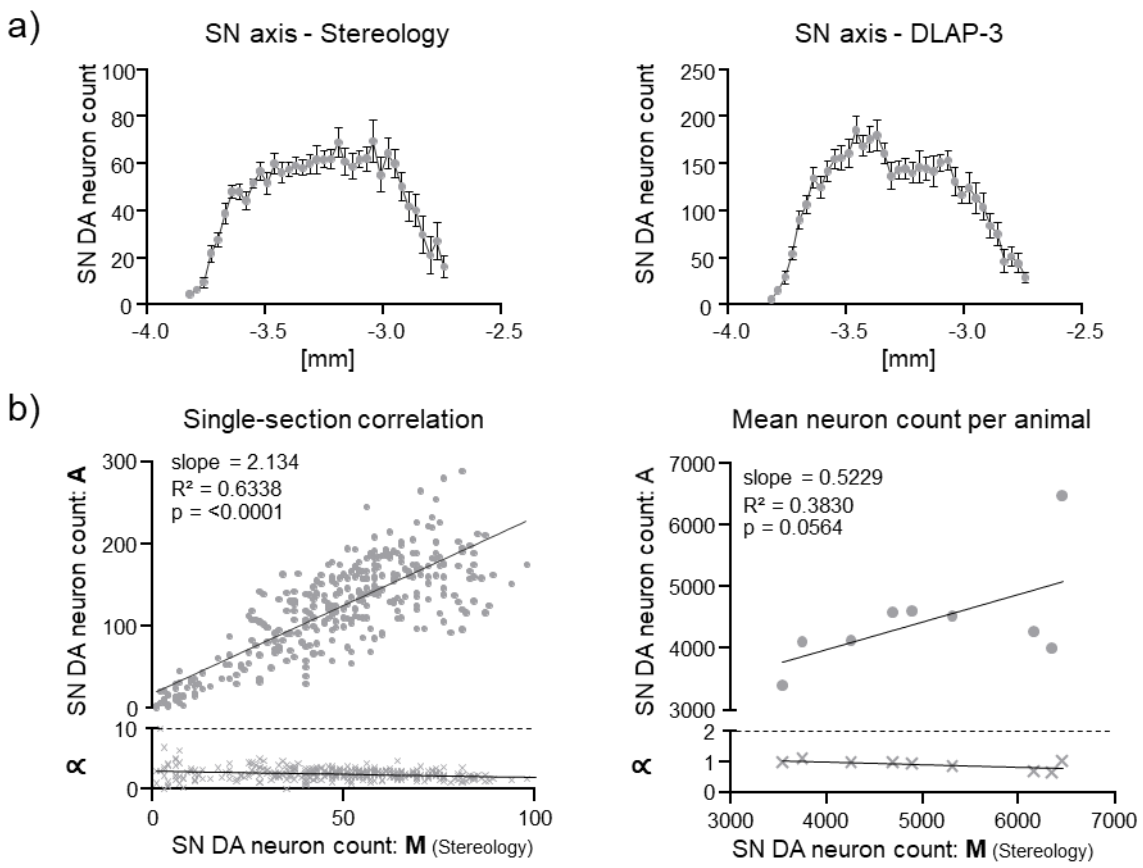

Figure S3: Comparison between unbiased stereology and automated IHC-derived image analysis of juvenile mouse midbrain sections. a) Mean SN DA neuron counts for all animals (N = 10) in all analyzed sections along the caudal-rostral axis quantified via unbiased stereology (extrapolated via the optical fractionator method, M) or automatically (A) via DLAP-3. Data are given as mean ± SEM. b) Upper: correlations of individual SN DA neuron counts/section and animal (left) and mean SN DA neuron counts/animal (right) between unbiased stereology and automated analysis according to Pearson correlation test. Lower: corresponding proportionality constants ∝, calculated from the manual and automated cell count ratios.

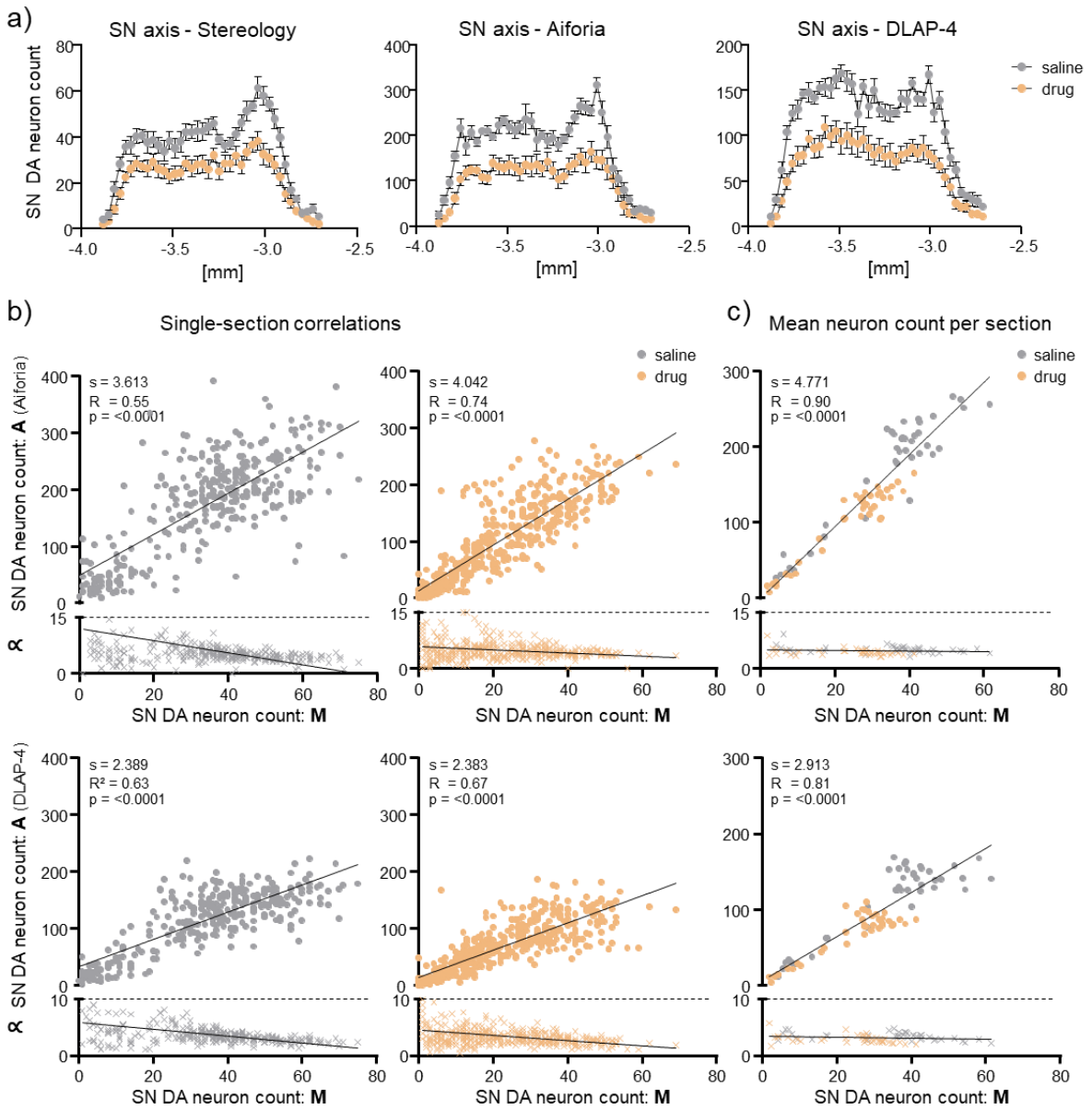

Figure S4: Comparison between unbiased stereology and automated IHC-derived image analysis of adult mouse midbrain sections. a) Mean SN DA neurons for all animals (saline: N = 9; PD-drug: N = 12) in all analyzed sections along the caudal-rostral axis quantified via unbiased stereology (extrapolated via the optical fractionator method, M) or automatically (A) via an algorithm provided by Aiforia or DLAP-4, as indicated. Data are given as mean ± SEM. b/c) Upper: correlations of individual SN DA neuron counts/section and animal (b) and mean animal counts/section along caudo-rostral axis (calculated from the individual values for each animal, c) between unbiased stereology and automated analysis (Aiforia, or DLAP-4, as indicated) according to Pearson correlation test. Lower: corresponding proportionality constants ∝, calculated from the manual and automated cell count ratios. Stereology data and Aiforia data modified from Benkert et al (2019).

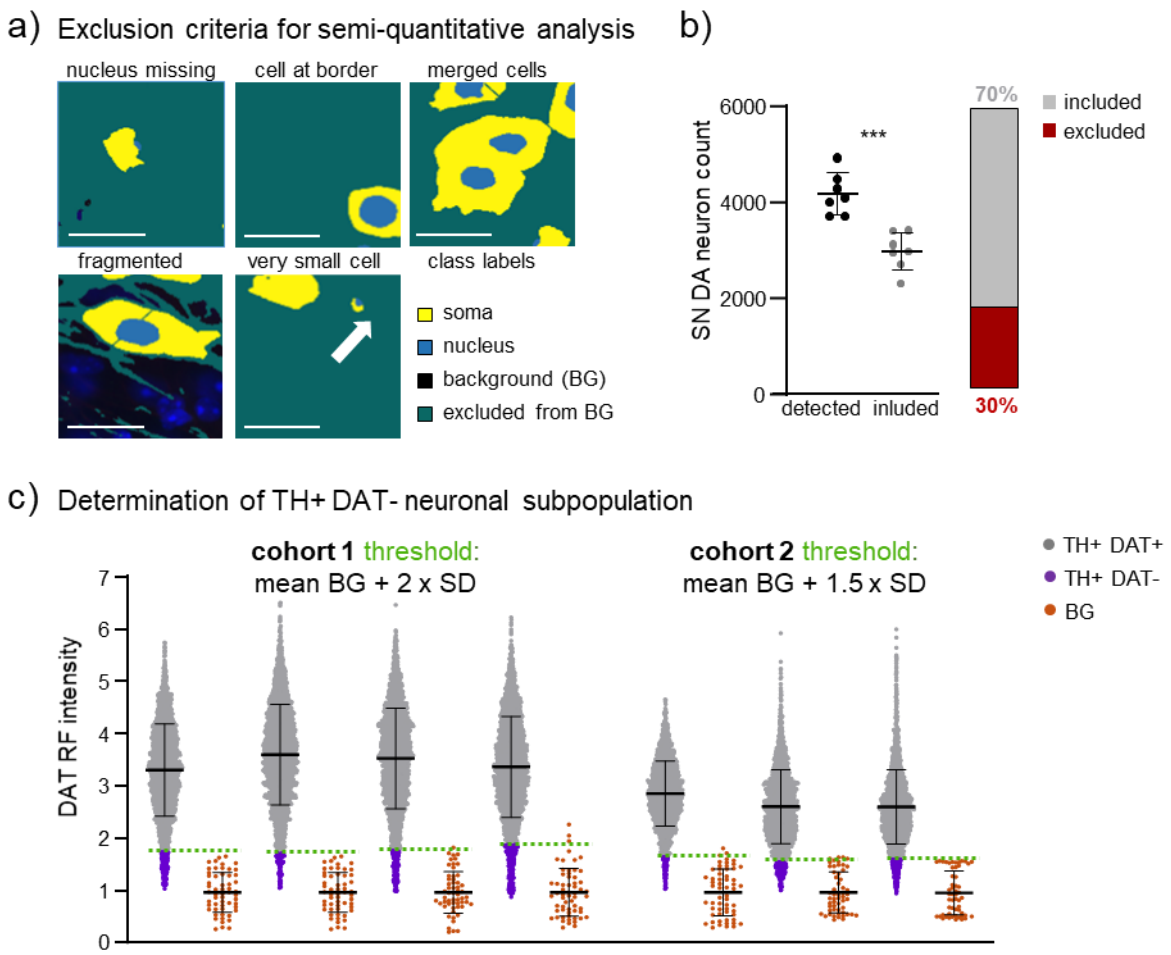

Figure S5: Semi-quantitative analysis with DLAP-5. a) Detected neurons, where cell bodies were not properly marked, cropped, not clearly separated from each other, fragmented, very small, or did not show a full nucleus, were excluded from further semi-quantitative analysis. Scale bars: 20 µm. b) Left: SN DA neurons/mouse quantified via DLAP-5. Shown are the total TH+ neuron count of all detected neurons and the number of neurons included for semi-quantitative analysis. Data are given as scatterplots (mean, SD) for all analyzed mice (N = 7). Significant differences according to Mann-Whitney test are indicated by asterisks (***: $p < 0.001$). Right: Vertical bar giving the percentage of included and excluded neurons. c) DAT relative fluorescence (RF) intensity per SN DA neuron (TH+) or per BG image, as indicated, quantified via DLAP-5. Data are given as scatterplots (mean, SD) for all analysed neurons separately for each animal.

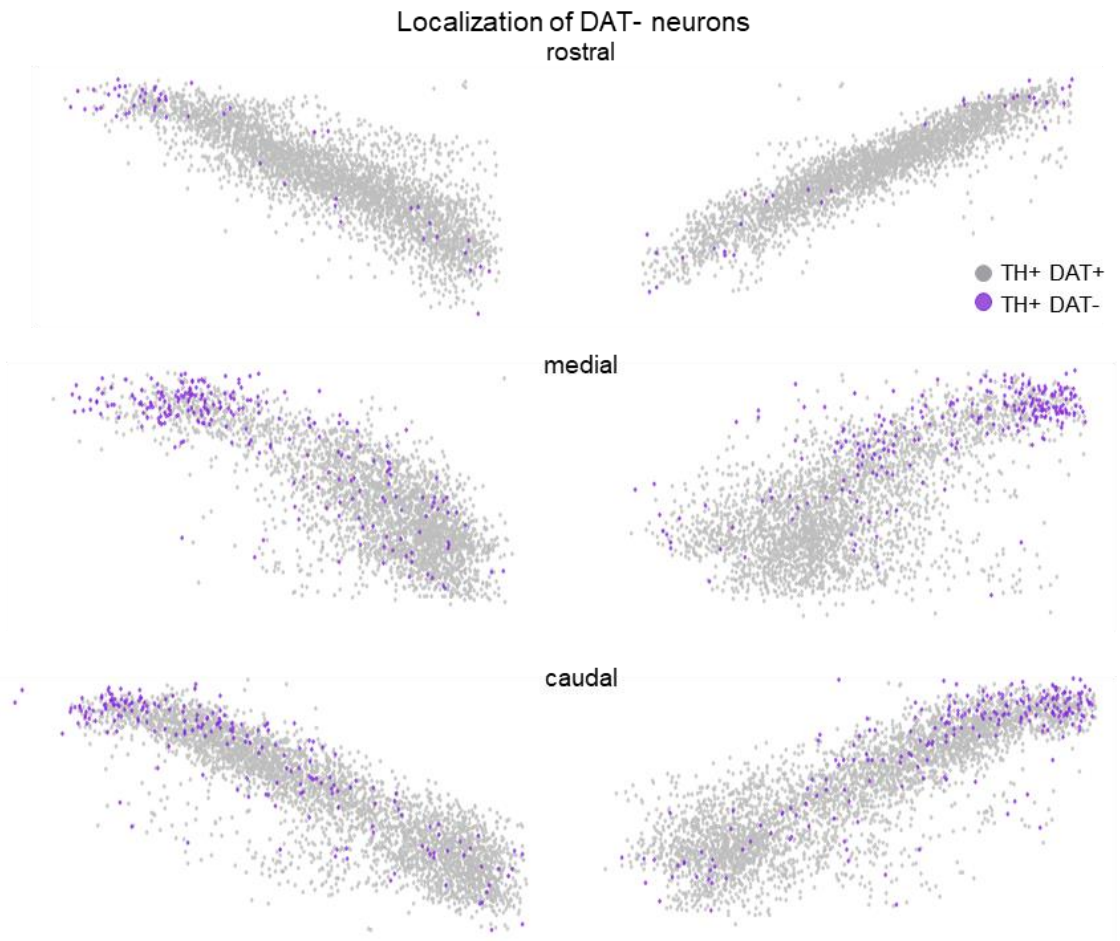

Figure S6: SN region maps after automated IF-derived image analysis of mouse midbrain sections. Localization maps showing the distribution of TH+ DAT+ and TH+ DAT- neurons. Given are the individual neurons separately for rostral, medial and caudal sections (N = 7; rostral: TH+ DAT- n = 85, TH+ DAT+ n = 6584; medial: TH+ DAT- n = 423, TH+ DAT+ n = 7466; caudal: TH+ DAT- n = 469, TH+ DAT+ n = 5869). Rostral, medial and caudal locations were determined according to the respective stereotaxic coordinates (bregma: -2.7 mm to -3.1 mm (rostral); -3.1 to -3.5 (medial); -3.5 to -3.9 (caudal)).

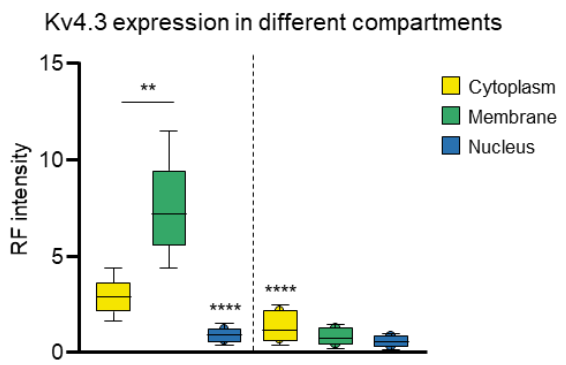

Figure S7: Automated IF-derived image analysis of WT and Kv4.3 KO mice. Kv4.3 relative fluorescence (RF) intensity/SN DA neuron in different sub-cellular compartments, quantified in WT and Kv4.3 KO mice, as indicated. Data are given as boxplots (median, 10-90 percentile) for all analyzed neurons (WT: n = 179, Kv4.3 KO: n = 200). Significant differences according to Kruskal-Wallis with Dunn's multiple comparison tests are indicated by asterisks (**: p<0.01, ****: p < 0.0001).

Table S1: Comparison of FCN and Deeplab3 network performances in distinct tasks. Performances were determined by comparing analysis results the test data to the ground truth data (provided by manual labeling). The following quality measures were assessed separately for each class: pixel error rate, rates for true positive (TP), true negative (TN), false positive (FP), false negative (FN) detection as well as specificity (spec.) [defined as TN/(TN+FP)] and sensitivity (sens.) [defined as TP/(TP+FN)].

D-LAP algorithm		Error [%]	class name	TP [%]	TN [%]	FP [%]	FN [%]	Spec [%]	Sens [%]
1	FCN	5.08	mRNA	0.3	98.9	0.7	0.0	99.3	92.5
			neuron	10.3	84.7	4.1	0.8	95.3	92.7
	DeepLab3	4.39	mRNA	0.0	99.7	0.0	0.3	100.0	0.0
			neuron	11.0	84.6	4.2	0.2	95.2	98.3
2 (human)	FCN	4.13	mRNA	0.2	98.9	0.9	0.0	99.1	94.7
			neuron	5.2	91.0	2.5	1.4	97.3	80.8
	DeepLab3	2.49	mRNA	0.0	99.8	0.0	0.2	100.0	0.0
			neuron	6.1	91.4	2.1	0.4	97.8	95.6
3	FCN	0.64	nucleus	0.0	99.4	0.0	0.6	100.0	1.7
	DeepLab3	0.61	nucleus	0.5	98.9	0.5	0.1	99.5	85.5
4	FCN	0.31	nucleus	0.2	99.5	0.3	0.0	99.7	46.7
	DeepLab3	0.26	nucleus	0.1	99.7	0.2	0.0	99.8	59.8
5	FCN	7.08	nucleus	1.0	97.3	1.5	0.2	98.4	80.9
			cytoplasm	3.7	90.8	4.9	0.7	94.8	83.8
	DeepLab3	3.3	nucleus	0.9	98.5	0.3	0.3	99.7	74.5
			cytoplasm	4.1	92.9	2.2	0.8	97.7	83.7
6	FCN	8.34	nucleus	9.4	87.9	1.2	1.5	98.7	87.2
			cytoplasm	12.4	82.8	4.1	0.8	95.3	93.4
			membrane	2.3	94.2	1.7	1.7	98.2	58.9
	DeepLab3	5.49	nucleus	3.0	93.5	2.5	1.0	97.4	75.6
			cytoplasm	9.7	89.0	0.8	0.5	99.1	95.0
			membrane	11.7	85.2	2.2	0.9	97.5	92.4

Table S2: Details of RNAscope probes. RNAscope target probes were obtained from the Advanced Cell Diagnostics (ACD) library. All probes detected but did not discriminate between known splice variants of the respective target genes. Abbreviations: NCBI: National Center for Biotechnology Information.

Species	Gene	ACD Cat No.	Assay target region according to NCBI Genbank gene accession no. (NM)
mouse	Tyrosine hydroxylase (*Th*)	317621	483 – 1603 of NM_009377.1 (exon 3-13)
	Cav1.3 (*Cacna1d*)	502591	522 – 1994 of NM_001302637.1 (exon 1-10)
	Cav2.3 (*Cacna1e*)	449211	2319 – 3317 of NM_009782.3 (exon 20-22)
human	Tyrosine hydroxylase (*TH*)	441651	596 – 1681 of NM_199292.2 (exon 4-14)
	Cav1.3 (*CACNA1D*)	502591	522 – 1994 of NM_001302637.1 (exon 1-10)

Table S3: Mean mRNA molecule numbers in individual mouse SN DA neurons, determined manually and via DLAP algorithm 1. Data and statistics for graphs in Fig. 3b. Number of total analyzed neurons is given by n, N represents the number of analyzed mice. P-values according to Two-way ANOVA followed by Tukey's multiple comparison (significant values in bold red).

		manual				automated				p-value
	n N	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
Cav1.3	449	22	9-41	24	12	23	9-41	24	12	0.9988
	3	24	22-24	24	1	24	23-24	24	1	
Cav2.3	372	66	32-113	70	31	65	31-117	70	32/	0.9995
	3	67	66-75	69	5	67	65-75	69	5	
	Cav1.3 M vs Cav2.3 M, **<0.0001**; Cav1.3 A vs Cav2.3 A, **<0.0001**									

Table S4: Single cell correlation analyses between manually and DLAP algorithm 1 determined results. Data and statistics for graphs in Fig. 3c and S1a, according to Pearson correlation (slope of the linear regression with its own 95% confidence interval).

	Cav1.3	Cav2.3
all mice (N = 3)		
Pearson r (95% confidence interval)	0.959 (0.950-0.966)	0.959 (0.950-0.967)
R squared	0.919	0.920
p-value	<0.0001	<0.0001
No. of XY pairs	444	372
Slope (95% confidence interval)	0.998 (0.971-1.026)	0.989 (0.960-1.020)
Proportionality constant ∝	1.01 ± 0.16	1.00 ± 0.14
mouse 1		
Pearson r (95% confidence interval)	0.964 (0.952-0.973)	0.963 (0.951-0.973)
R squared	0.929	0.928
p-value	<0.0001	<0.0001
No. of XY pairs	168	167
Slope (95% confidence interval)	1.005 (0.962-1.047)	1.004 (0.961-1.047)
Proportionality constant ∝	1.01 ± 0.15	1.00 ± 0.13
mouse 2		
Pearson r (95% confidence interval)	0.954 (0.936-0.967)	0.964 (0.947-0.976)
R squared	0.910	0.930
p-value	<0.0001	<0.0001
No. of XY pairs	131	96
Slope (95% confidence interval)	0.997 (0.943-1.052)	1.011 (0.954-1.068)
Proportionality constant ∝	1.00 ± 0.17	1.00 ± 0.14
mouse 3		
Pearson r (95% confidence interval)	0.956 (0.939-0.968)	0.943 (0.917-0.960)
R squared	0.913	0.888
p-value	<0.0001	<0.0001
No. of XY pairs	145	109
Slope (95% confidence interval)	0.991 (0.941-1.042)	0.935 (0.871-0.998)
Proportionality constant ∝	1.02 ± 0.16	1.01 ± 0.15

Table S5: Details of human *postmortem* midbrain samples. Samples were provided by the German BrainBank. The mean age of the aged cohort was significantly higher compared to that of the adult cohort (** = 0.0012; t-test), the mean RIN was significantly lower (*0.038; t-test), PMI was not significantly different. Abbreviations: AD: Alzheimer's disease; CERAD: Consortium to establish a registry for Alzheimer's disease; f: female; LB: Lewy Body; m: male; NFT: neurofibrillary tangles; PD: Parkinson's disease; PMI: post mortem interval, RIN: RNA integrity number.

Cohort	Age, gender	PMI [h]	RIN	Braak AD/NFT, PD	CERA-stage	LB-pathology
adult	46, f	27	8.5	- , 0	0	no
	34, m	10	8.3	- , 0	0	no
	46, m	19	8.4	- , 0	0	no
mean±SD	42 ± 7**	19 ± 9	8.4 ± 0.1*			
aged	76, m	24	6.9	I, -	A	no
	76, f	11	6.4	0, -	0	no
	81, f	18	4.0	II, -	0	no
mean±SD	78 ± 3	18 ± 7	5.8 ± 1.6			

Table S6: mRNA molecule numbers per human SN DA neuron, determined manually and via DLAP algorithm 2. Data and statistics for graphs in Fig. 4b and c. Number of total analyzed neurons is given by n, N represents the number of analyzed brain samples. P-values according to Two-way ANOVA followed by Tukey's multiple comparison (significant values in bold red).

Cav1.3 mRNA-derived dots/cell										
		manual				automated				p-value
	n N	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
adult	223	36	11-68	39	23	37	13-78	42	25	0.6376
	3	38	34-43	38	5	39	34-47	40	7	
aged	258	10	2-32	13	12	12	2-38	17	15	0.2982
	3	16	10-16	14	3	19	13-20	17	4	
	adult M vs aged M, **<0.0001**; adult A vs aged A, **<0.0001**									
rel. BF signal intensity/cell										
		manual				automated				p-value
	n N	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
adult	223	16.4	10.8-22.3	16.4	4.5	16.7	11.0-23.1	16.7	4.7	0.9964
	3	16.2	14.5-18.9	16.5	2.2	16.6	14.7-19.0	16.7	2.2	
aged	258	20.4	15.4-26.8	21.2	5.4	20.7	15.6-26.5	21.1	5.1	>0.9999
	3	21.6	20.7-22.2	21.5	0.8	21.5	20.6-22.2	21.4	0.8	
	adult M vs aged M, **<0.0001**; adult A vs aged A, **<0.0001**									
BF-adjusted Cav1.3 mRNA-derived dots/cell										
		manual				automated				p-value
	n N	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
adult	180	31	18-50	32	12	31	17-49	32	12	>0.9999
	3	33	30-34	32	2	33	29-34	32	3	
aged	224	17	9-30	19	9	20	11-35	21	10	**0.0436**
	3	18	14-21	17	3	21	16-22	20	3	
	adult M vs aged M, **<0.0001**; adult A vs aged A, **<0.0001**									

Table S7: Single cell correlation analyses between manually and DLAP algorithm 2 determined results. Data and statistics for graphs in Fig. 4d and S1b and c, according to Pearson correlation (slope of the linear regression with its own 95% confidence interval).

	Cav1.3 mRNA dot counts / cell	rel. BF intensity / cell	Cav1.3 molecules, NM/BF-adjusted according to model
all human samples (N = 6)			
Pearson r (95% confidence interval)	0.964 (0.957-0.970)	0.992 (0.990-0.993)	0.897 (0.877-0.915)
R squared	0.928	0.983	0.805
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	481	481	404
Slope (95% confidence interval)	1.034 (1.008-1.060)	0.968 (0.957-0.979)	0.867 (0.827-0.910)
Proportionality constant ∝	1.28 ± 1.18	1.01 ± 0.03	1.10 ± 0.25
adult samples (N = 3)			
Pearson r (95% confidence interval)	0.975 (0.967-0.980)	0.996 (0.995-0.997)	0.876 (0.837-0.906)
R squared	0.950	0.992	0.767
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	223	223	180
Slope (95% confidence interval)	1.044 (1.012-1.076)	1.033 (1.021-1.046)	0.851 (0.781-0.920)
Proportionality constant ∝	1.23 ± 1.51	1.01 ± 0.02	1.01 ± 0.18
aged samples (N = 3)			
Pearson r (95% confidence interval)	0.890 (0.861-0.913)	0.988 (0.984-0.990)	0.872 (0.836-0.900)
R squared	0.792	0.976	0.872
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	258	258	224
Slope (95% confidence interval)	1.130 (1.059-1.201)	0.940 (0.9221-0.958)	0.977 (0.905-1.050)
Proportionality constant ∝	1.33 ± 0.78	1.00 ± 0.03	1.17 ± 0.27

Table S8: SN DA neuron numbers determined via unbiased stereology and via DLAP algorithm 3 on DAB-stained sections. Data and statistics for graphs in Fig. 5b. Given are the numbers of all TH-positive SN neurons, unilaterally determined for each mouse by stereology or algorithm. N represents the number of analyzed mice. P-values according to Mann-Whitney test (significant values in bold red).

		manual (Stereology)				automated			
	N	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD
	10	4789	3561-6444	4951	1079	4199	3100-6297	4317	911.8
p-value	Mann-Whitney test: 0.2176								

Table S9: SN DA neuron numbers determined via unbiased stereology and via DLAP algorithm 4 on DAB- and hematoxylin-stained sections. Data and statistics for graphs in Fig. 6b. Given are the numbers of all TH-positive SN neurons/mouse, unilaterally determined by stereology or algorithm. N represents the number of analyzed mice. P-values according to Two-way ANOVA followed by Tukey's multiple comparison (significant values in bold red).

		saline					PD-drug				p-value
	N	median	10-90 %	mean	±SD	N	median	10-90 %	mean	±SD	
manual (stereology)	9	3826	2666-4919	3959	643	12	2556	1209-3587	2504	922	**0.022**
automated (Aiforia)		6881	5749-8418	7048	823		4619	1680-5913	4108	1596	**<0.0001**
automated (Wolution)		4797	3442-5084	4614	500		2768	1314-4230	2708	975	**0.0010**
p-values	saline M vs. saline A (W), 0.7441; drug M vs. drug A (W), 0.9963; saline M vs. saline A (A), **<0.0001**; drug M vs. drug A (A), **0.0036**; saline A (W) vs saline A (A), **<0.0001**; drug A (W) vs drug A (A), **0.0156**										

Table S10: Remaining SN DA neurons after PD-drug (MPTP) treatment determined via unbiased stereology and via DLAP algorithm 4. Data and statistics for graphs in Fig. 6b. Given are the percentages of remaining TH-positive SN neurons/mouse after drug treatment relative to saline, unilaterally determined by stereology or algorithm. N represents the number of analyzed mice. P-values according to Kruskal-Wallis with Dunn's multiple comparison (significant values in bold red).

group	N	median	10-90 %	mean	±SD
manual (Stereology)	12	64.6	30.5-90.6	63.3	23.3
automated (Aiforia)		65.5	23.8-83.9	58.3	22.7
automated (Wolution)		60.0	28.5-91.7	58.7	21.1
p-values	M vs A (W), >0.9999; M vs A (A), >0.9999, A (W) vs A (A), >0.9999				

Table S11: Single cell correlation analyses between manually and DLAP algorithm 3 and 4 determined results Data and statistics for graphs in Fig. 5c/6c, according to Pearson correlation (slope of the linear regression with its own 95% confidence interval).

	juvenile	adult (Aiforia)	adult (Wolution)
Individual sections			
Pearson r (95% confidence interval)	0.796 (0.753-0.832)	0.742 (0.688-0.787)	0.791 (0.746-0.829)
R squared	0.634	0.5500	0.626
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	332	3189	319
Slope (95% confidence interval)	2.134 (1.959-2.310)	3.613 (3.251-3.974)	2.389 (2.185-2.593)
Proportionality constant ∝	2.60 ± 1.00	6.48 ± 8.43	3.86 ± 2.34
Individual sections: PD-drug			
Pearson r (95% confidence interval)		0.860 (0.833-0.882)	0.815 (0.781-0.845)
R squared		0.739	0.665
p-value		<0.0001	<0.0001

No. of XY pairs		433	433
Slope (95% confidence interval)		4.042 (3.814-4.269)	2.383 (2.223-2.544)
Proportionality constant ∝		5.00 ± 3.16	3.46 ± 2.12
Mean (for all animals) per section			
Pearson r (95% confidence interval)	0.889 (0.828-0.929)	0.946 (0.917-0.965)	0.897 (0.844-0.933)
R squared	0.790	0.895	0.805
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	73	80	80
Slope (95% confidence interval)	2.383 (2.092-2.674)	4.771 (4.402-5.139)	2.913 (2.590-3.236)
Proportionality constant ∝	2.58 ± 0.58	4.81 ± 1.01	3.20 ± 0.74
Mean per animal			
Pearson r (95% confidence interval)	0.619 (-0.018-0.898)	0.901 (0.768-0.960)	0.919 (0.808-0.967)
R squared	0.383	0.812	0.845
p-value	0.0564	<0.0001	<0.0001
No. of XY pairs	10	21	21
Slope (95% confidence interval)	0.5229 (-0.018-1.064)	1.639 (1.260-2.018)	1.057 (0.8396-1.275)
Proportionality constant ∝	0.89 ± 0.15	1.71 ± 0.29	1.13 ± 0.15

Table S12: Number of SN neurons analyzed via DLAP-5. Data and statistics for graphs in Fig. 7c, d, S5b and S6. Given are the numbers of all TH-positive SN neurons/mouse, bilaterally determined by the algorithm, the number of neurons excluded/included neurons for further DAT analysis (criteria specified in Figure S5a), and the determined number of TH-positive DAT-positive/DAT-negative SN neurons. Rostral, medial and caudal locations were determined according to the respective stereotaxic coordinates (bregma: -2.7 mm to -3.1 mm (rostral); -3.1 to -3.5 (medial); -3.5 to -3.9 (caudal)). N represents the number of analyzed mice. P-values according to Wilcoxon or Mann-Whitney tests (significant values in bold red).

	N	mean	±SD	%	mean	±SD	%	mean	±SD	%
		all detected TH+ SN neurons			excluded TH+ neurons from further DAT analysis			included TH+ neurons for further DAT analysis		
whole axis	7	4187	439	100	1171	152	28 ± 3.3	3015	374	72 ± 3.3
p-value		MWU: all vs analyzed TH+ neurons **0.006**								
		identified TH+ DAT+			identified TH+ DAT-			all included TH+ neurons		
whole axis		2876	340	95.5 ± 1.5	139	54	4.5 ± 1.5	3015	374	100
rostral		941	199	98.8 ± 0.6	12	8	1.2 ± 0.6	953	203	100
medial		1067	208	94.7 ± 2.2	60	29	5.3 ± 2.2	1127	225	100
caudal		838	120	92.6 ± 2.4	67	25	7.4 ± 2.4	905	129	100
p-value		Wilcoxon: all analyzed TH+ vs analyzed TH+ DAT+ **0.0156** Chi-square: rostral vs medial, 0.0973 , rostral vs caudal, **0.0304** , caudal vs medial, 0.5515								

Table S13: Semi-quantitative DAT immunofluorescence per mouse SN DA neuron, determined via DLAP algorithm 5. Data and statistics for graphs in Fig. S5c. n represents the number of total analyzed SN DA neurons or the number background images.

	SN DA neurons					background					threshold
mouse No	n	median	10-90 %	mean	±SD	median	n	10-90 %	mean	±SD	BG-RF+2SD
1	3407	3.4	2.2-4.5	3.3	0.9	1.0	56	0.5-1.5	1.0	0.4	1.8
2	2945	3.6	2.4-4.9	3.6	1.0	0.9	58	0.5-1.4	1.0	0.4	1.8
3	2969	3.6	2.3-4.8	3.6	1.0	0.9	56	0.5-1.6	1.0	0.4	1.8
4	3129	3.4	2.1-4.7	3.4	1.0	0.9	60	0.5-1.6	1.0	0.5	1.9
											BG-RF+1.5SD
5	2715	2.9	2.1-3.7	2.9	0.6	1.1	58	0.4-1.6	1.0	0.4	1.9
6	2314	2.6	1.8-3.6	2.6	0.7	0.9	54	0.6-1.6	1.0	0.4	1.8
7	3417	2.6	1.8-3.5	2.6	0.7	0.9	54	0.5-1.6	1.0	0.4	1.9

Table S14: SN neuron size and calbindin$_{d28k}$ (CB) co-expression. Data and statistics for graphs in Fig. 7e and 9b. Sizes were determined for all DAT-pos, and DAT-neg. TH-pos. SN neurons according to via DLAP-5 determined cell body areas. For CB, the mean numbers of CB-pos. and CB-neg. neurons / animal, and the relative % of CB and TH co-expression are given. N represents the number of analyzed mice, n represents the number of analyzed neurons. P-values according to Two-way ANOVA followed by Tukey's multiple comparison (cell size) or Chi-square (CB) test (significant values in bold red).

DAT / CB co-expression of TH+ neurons	**n** **N**	relative % (co-expression-type)		cell size [µm²]		p-value (cell size)
		mean	±SD	mean	±SD	
TH+ DAT+	20211 7	95.4	1.6	213.6 213.8	78.1 12.9	**<0.0001**
TH+ DAT-	972 7	4.6	1.6	144.5 143.7	60.8 13.2	
TH+ CB+	845 3	10.0	1.0	170.9 170.0	70.1 12.5	**<0.0001**
TH+ CB-	7601 3	90.0	1.0	204.6 203.7	72.8 10.4	
		% of all DAT+				
TH+ DAT+ CB+	703 3	8.7	0.8	183.2 181.9	67.8 8.5	**<0.0001**
TH+ DAT+ CB-	7348 3	91.3	0.8	206.3 205.4	72.6 10.1	
		% of all DAT-				
TH+ DAT- CB+	142 3	37.4	7.3	109.7 109.8	44.3 9.7	**0.0117**
TH+ DAT- CB-	253 3	62.6	7.3	154.9 153.0	58.8 13.6	

Table S15: Semi-quantitative immunofluorescence per mouse SN DA neuron, determined manually and via DLAP algorithm 6. Data and statistics for graphs in Fig. 10b. Number of total analyzed neurons is given by n, N represents the number of analyzed mice. P-values according to Kruskal-Wallis with Dunn's multiple comparison and Mann-Whitney test (significant values in bold red).

TH										
		manual				automated				p-value
ROI	n	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
cell body	94	272.8	125.6-534.1	307.9	145.4	284.4	134.4-542.0	317.8	147.3	0.6029
membrane		181.2	81.44-357.1	203.4	101.8	162.0	79.78-316.2	180.3	86.10	0.1707
nucleus		39.52	24.33-75.87	45.78	22.51	46.93	26.80-88.50	52.00	24.14	**0.0334**
p-values	Kruskal-Wallis: M; cell body vs nucleus, <0.0001; cell body vs membrane, <0.0001; A; cell body vs nucleus, <0.0001; cell body vs membrane, <0.0001;									
Kv4.3										
		manual				automated				p-value
ROI	n	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
cell body	94	2.3	1.4-3.8	2.5	1.0	2.3	1.3-3.9	2.6	1.1	0.5862
membrane		9.0	5.1-15.7	9.9	4.0	6.6	3.9-11.5	7.2	3.0	**<0.0001**
nucleus		0.6	0.3-1.2	0.6	0.4	0.6	0.3-1.1	0.7	0.4	0.9270
p-values	Kruskal-Wallis: M; membrane vs cell body, <0.0001; membrane vs nucleus, <0.0001; A; membrane vs cell body, <0.0001; membrane vs nucleus, <0.0001;									
DAPI										
		manual				automated				p-value
ROI	n	median	10-90 %	mean	±SD	median	10-90 %	mean	±SD	
cell body	94	1.9	1.0-3.2	2.1	1.1	1.6	0.9-2.8	1.8	0.9	0.0765
membrane		0.8	0.5-2.0	1.0	0.6	0.8	0.5-3.4	1.3	1.0	0.3500
nucleus		9.3	6.3-13.5	9.8	2.8	9.3	6.3-13.7	9.7	2.8	0.8212
p-values	Kruskal-Wallis: M; nucleus vs membrane, <0.0001; nucleus vs cell body, <0.0001; A; nucleus vs membrane, <0.0001; nucleus vs cell body, <0.0001;									

Table S16: Single cell correlation analyses between manually and DLAP-6 determined results. Data and statistics for graphs in Fig. 10c., according to Pearson correlation (slope of the linear regression with its own 95% confidence interval).

	Cytoplasm: TH	Membrane: Kv4.3	Nucleus: DAPI
Pearson r (95% confidence interval)	0.995 (0.992-0.997)	0.966 (0.949-0.977)	0.996 (0.994-0.008)
R squared	0.990	0.933	0.993
p-value	<0.0001	<0.0001	<0.0001
No. of XY pairs	94	94	94
Slope (95% confidence interval)	1.01 (0.989-1.029)	0.737 (0.696-0.777)	0.986 (0.969-1.00)
Proportionality constant ∝	0.96 ± 0.05	1.38 ± 0.15	1.01 ± 0.03

Table S17: Relative immunofluorescence-signal quantification for Kv4.3 in SN DA neuron membranes from WT and Kv4.3 KO mice, determined via DLAP-6. Data and statistics for graphs in Fig. 10e & S7. Number of analyzed neurons is given by n, N represents the number of analyzed mice. P-values according Mann-Whitney test (significant values in bold red).

WT					Kv4.3 KO					p-value
n N	median	10-90 %	mean	±SD	n N	median	10-90 %	mean	±SD	
179	7.2	4.4-11.5	7.7	2.9	200	0.7	0.21-1.5	0.8	0.5	**<0.0001**
2	7.7	7.2-8.2	7.7	0.7	2	0.9	0.5-1.3	0.9	0.6	

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