A cytoarchitectural and chemoarchitectural analysis of the dopamine cell groups in the substantia nigra, ventral tegmental area, and retrorubral field in the mouse

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Abstract

The three main dopamine cell groups of the brain are located in the substantia nigra (A9), ventral tegmental area (A10), and retrorubral field (A8). Several subdivisions of these cell groups have been identified in rats and humans but have not been well described in mice, despite the increasing use of mice in neurodegenerative models designed to selectively damage A9 dopamine neurons. The aim of this study was to determine whether typical subdivisions of these dopamine cell groups are present in mice. The dopamine neuron groups were analysed in 15 adult C57BL/6J mice by anatomically localising tyrosine hydroxylase (TH), dopamine transporter protein (DAT), calbindin, and the G-protein-activated inward rectifier potassium channel 2 (GIRK2) proteins. Measurements of the labeling intensity, neuronal morphology, and the proportion of neurons double-labeled with TH, DAT, calbindin, or GIRK2 were used to differentiate subregions. Coronal maps were prepared and reconstructed in 3D. The A8 cell group had the largest dopamine neurons. Five subregions of A9 were identified: the reticular part with few dopamine neurons, the larger dorsal and smaller ventral dopamine tiers, and the medial and lateral parts of A9. The latter has groups containing some calbindin-immunoreactive dopamine neurons. The greatest diversity of dopamine cell types was identified in the seven subregions of A10. The main dopamine cell groups in the mouse brain are similar in terms of diversity to those observed in rats and humans. These findings are relevant to models using mice to analyse the selective vulnerability of different types of dopamine neurons.

Keywords A8 · A9 · A10 · Retrorubral field· Substantia nigra · Ventral tegmental area

Introduction

Dopamine neurons have been a focus of scientific attention ever since the discovery of dopamine as an important neurotransmitter in its own right (Carlsson et al. 1962), and the observation that it was significantly depleted in the brains of patients with Parkinson's disease (Davie 2008; Hornykiewicz and Kish 1987; Obeso et al. 2008). The first detailed description of the localisation of catecholamine-containing neurons identified 12 neuronal groups, designated as A1-A12 in the rat brain (Dahlström and Fuxe 1964). Subsequent studies detailed five more cell groups, A13-A17 (Hökfelt et al. 1984). The A8, A9, and A10 dopamine cell groups (Björklund and Dunnett 2007; Smeets and González 2000) are coincident with the retrorubral field (RRF), the substantia nigra (SN), and the ventral tegmental area (VTA), respectively. There have been few detailed studies of the cytoarchitecture and chemoarchitecture of these dopaminergic cell groups in mice (Zaborszky and Vadasz 2001). A thorough and unambiguous delineation of the various dopaminergic cell clusters in this species is not only important from an anatomical point of view, but would assist interpretations of the many experiments performed in mouse models of Parkinson's disease, particularly as there is a selective loss mainly of the ventral A9 neurons in this disease (Ma et al. 1995; Pakkenberg et al. 1991). The observation that only certain A9 cells are highly vulnerable to Parkinson's disease confirms that the A8, A9, and A10 dopamine cell groups should be considered different from each other, and that there are notable differences in the neurons within the A9 cell group. These aspects have not been previously studied in mice in any detail.

A problem hampering the detailed analysis of these areas relates to the limitations of the current terminology. Firstly, the current terminology is not clear enough, and the 'retrorubral field' (RRF or A8 cell group) is easily confused with the 'retrorubral nucleus', which is an independent, non-dopaminergic cell group in the rostral hindbrain. The problem is clearly exemplified by the fact that the term 'retrorubral nucleus' was mistakenly applied to the A8 cell group in many recent publications, despite earlier attempts to resolve this confusion by introducing the name 'retroisthmic nucleus' instead of the rather ambiguous 'retrorubral nucleus' term (Paxinos and Watson 2007). Secondly, proper delineation of the VTA regions (or A10 cell groups) is lacking in mice. At present, the VTA is the collective name of a number of distinct dopamine cell groups in the floor of the mesencephalon and in the adjacent isthmic and diencephalic tegmental areas (Dahlström and Fuxe 1964; Puelles et al. 2007). Previous

cytoarchitectural studies in rats and humans divided the A10 cell groups into five main clusters —

paranigral nucleus (PN), interfascicular nucleus (IF), parabrachial pigmented nucleus (PBP), caudal

linear nucleus (CLi), and rostral linear nucleus (RLi) (Halliday and Tork 1986; Oades and Halliday

1987; Swanson 1982). In a more recent human study (McRitchie et al. 1996), the parapeduncular

nucleus (PaP) and 'VTA' (*sensu stricto*) were added, thus creating conflict between the use of the VTA

in reference to both the entire region (in most studies) as well as to a specific cluster of

cytoarchitecturally different dopamine neurons. These different cell clusters have not been adequately

assessed and defined in the mouse brain. Finally, few subregions have been objectively defined within

the mouse SN (A9 cell groups), even though recent histogenetic and gene expression studies in mice

clearly indicate distinct SN subregions (Puelles et al. 2007). While there is a considerable diversity in

the nomenclature used for the description of the various subdivisions of the SN pars compacta of other

mammalian species (Dahlström and Fuxe 1964; Franklin and Paxinos 2008; **González-Hernández**

and Rodríguez, 2000; Halliday 2004; McRitchie et al. 1996; Paxinos and Watson 2007), at present

there is only one description of the pars compacta subdivisions in mice (Zaborszky and Vadasz 2001).

The regional vulnerability of dopamine neurons in Parkinson's disease is thought to be due to the

expression of different cellular proteins (Burns et al. 1983; German et al. 1992). In particular, **it has**

been suggested that the G-protein-activated inwardly rectifying potassium channel 2 (GIRK2) protein

and the EF-hand calcium binding protein calbindin are expressed in vulnerable (GIRK2) versus resistant

(calbindin) clusters of dopamine neurons within the A8, A9, and A10 cell groups (Alfahel-Kakunda and

Silverman 1997; Morel et al. 2002; Thompson et al. 2005). The present study, therefore, uses these

markers as well as proteins expressed only in dopamine neurons (tyrosine hydroxylase or TH and

dopamine transporter or DAT) to delineate the A8, A9, and A10 cell clusters of the mouse brain.

Materials and methods

Animals

C57BL/6J mice (8~10 weeks, 21~25 g, n=15) obtained from the Animal Resources Centre (Canning

Vale, WA, Australia) were used for the present work. All experiments were approved by the Animal

Care and Ethics Committee of The University of New South Wales, and they were carried out in

accordance with the relevant national and institutional guidelines on the care of research animals.

Tissue preparation

Mice were anesthetised with a lethal dose of **pentobarbitone sodium** (1 mg/g body weight) and perfused through the left ventricle with 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB; pH=7.4, 4 °C). The brains were postfixed in the same fixative for 2 hours at 4 °C, and cryoprotected in 30% sucrose-buffer until they sank to the bottom of the container. Serial brain sections were cut at 40 μm either coronally or sagittally using a Leica CM 1950 cryostat (Leica Mycrosystems GmbH, Wetzlar, Germany). The sections were consecutively harvested from bregma -2.30 mm to bregma -4.60 mm or from lateral 0.04 mm to lateral 1.92 mm, and consecutive sections were immunohistochemically stained.

Free-floating immunohistochemistry

For histological delineation and regional 3D reconstruction, single immunolabeling was employed. Sections were pretreated with 3% H_2O_2 in 50% ethanol to block endogenous peroxidase activity, followed by preincubation in 10% goat serum. The sections were then incubated with an anti-TH antibody (Sigma) (for the detailed specification of the primary and secondary antibodies see Table 1) for 2 days at 4 °C, followed by incubation in biotinylated goat anti-mouse IgG and ExtrAvidin peroxidase (Sigma), each for 2 hours at room temperature. The peroxidase complex was visualized by incubating the sections in 0.1 M PB containing 0.05% 3,3'-diaminobenzidine (DAB), 0.1% H_2O_2, and 0.04% nickel ammonium sulphate. Sections were washed, mounted on gelatine-coated slides, counterstained with cresyl violet, dehydrated, cleared, and coverslipped using DPX mounting medium (Fluka Chemie AG, Buchs, Switzerland).

<center>**<Put Table 1 here>**</center>

To detect the colocalization of TH and other markers, double-immunofluorescence labeling was employed. The sections were blocked in 5% serum, incubated with TH- and either calbindin-, DAT-, GIRK2-, parvalbumin-, substance P-, or dopamine β-hydroxylase (DBH)- specific antibodies (Table 1) for 2 days at 4 °C, and incubated with the appropriate fluorescent secondary antibodies (Table 1) for 2 hours at room temperature. At the end of the incubation, the sections were washed, counterstained with 4´,6-diamidino-2-phenylindole (DAPI), dilactate (Invitrogen), mounted, and coverslipped with fluorescent mounting medium (Dako).

For the control experiments the same protocols were applied, but the primary antibodies were substituted by normal serum. No positive immunolabeling was observed in any sections in which the primary antibody was not applied.

Histological 3D reconstruction

Histological preparations were photographed using an Olympus AX70 microscope (Olympus, Tokyo, Japan) equipped with a SPOT digital camera (14.2 Color Mosaic; SPOT Imaging Solutions, Burroughs, MI, USA). The images were collated with Adobe Photoshop CS4 (Adobe Systems Inc, San Jose, CA, USA), and the contours of the anatomical regions of interest (ROIs) were drawn based on the coronal and sagittal sections stained with DAB and counterstained with cresyl violet. To prepare the 3D models of the relevant regions, the serial sequences of sections were aligned according to the midline and ventral bottom of each individual image, and corrected for possible displacements and distortions by moving and/or rotating them individually as necessary based on their original stereological location (Franklin and Paxinos 2008). The 3D models were assembled from the two dimensional delineations using the Amira v5.2 software (Visage Imaging, Inc., San Diego, CA, USA).

Determination of the ratios of double-labeled cells

Regional images were captured with the 40× objective of a confocal microscope (Nikon Eclipse 90i; Nikon, Tokyo, Japan). **For the visualization of the Alexa Fluor 594, Alexa Fluor 488, and DAPI signals, we employed excitation wavelengths of 561 nm, 488 nm, and 405 nm respectively. We used a different Nikon fluorescence filter block in each case; Texas Red for Alexa Fluor 594, B-2A for Alexa Fluor 488, and 'DAPI' for DAPI.** Depending on the total number of TH-immunoreactive (TH+) neurons found in a particular region, a variable number of TH+ cells were analysed (for details see Table 2). Cells double-labeled with calbindin and TH (calbindin+/TH+), cells double-labeled with DAT and TH (DAT+/TH+), and cells double-labeled with GIRK2 and TH (GIRK2+/TH+) were counted, and the ratios of double-labeled cells were calculated and expressed as *'percentage = double-labeled cell number/(TH+ cell number)%.'*

Measurement of neuronal diameter and neuronal 3D reconstruction

To determine the largest diameter of the neuronal cell bodies, consecutive coronal sections containing A8, A9, or A10 from 4 mice were examined. Only neurons situated in these regions and having clearly visible nucleoli were evaluated. The largest diameter of the selected neurons was determined manually using the AxioVision image analysis software (Carl Zeiss Inc, Oberkochen, Germany). Depending on the size of the neuronal pools, between 30 and 300 neurons were measured in each individual region.

For the 3D reconstructions of neurons, 1 μm thick Z-stack images were captured using the 40× objective of the confocal microscope. Noise reduction was achieved by median filtering (width=3), followed by the binarisation of the images based on a threshold value determined with the Otsu method (Otsu 1979). Single neurons were segmented using the 3D watershed algorithm (Malpica et al. 1997) and reconstructed with Amira v5.2.

Determination of the intensity of protein staining in individual neurons

To reveal possible relations between the expression patterns of the two most widely distributed markers (GIRK2 and TH), the intensities of the fluorescent GIRK2- and TH-specific immunolabelings within individual cells were compared. To achieve this, the Image J software (v1.42q Java, National Institute of Health, USA) was applied to 1 μm thick Z-stack images that were captured using the 60× objective of the confocal microscope. To obtain the intensity data, 8-36 randomly selected neurons were considered in each subregion of the A8, A9, and A10 cell groups. During the analysis, the optical section revealing the greatest diameter of the cell nucleus was sought to ensure that the analysis was performed in a plane precisely bisecting the neuron vertically. In the next step, the contours of the neuronal cell body and nucleus were defined, with the image analysis performed on the remaining cytoplasm only. The intensity values were normalised to the cell size (approximated as the number of pixels belonging to the individual ROIs). Throughout the manuscript arbitrary units (AU) are used to compare the intensity values characterising the expression of the relevant proteins.

Statistics

Results are given as mean±S.E.M. Statistical differences were assessed using Student's t-test. When multiple comparisons had to be performed, the Bonferroni correction was applied. A corrected p value of <0.05 was taken as statistically significant.

Results

Architecture of the A8, A9, and A10 cell groups

To ensure that the assessment of TH immunoreactivity in the investigated regions identified dopamine and not other catecholamine neurons, double-labeling experiments were performed using TH and dopamine β-hydroxylase (DBH), as the latter protein is expressed in noradrenaline and not dopamine neurons. Since no double-labeled neurons were observed in the regions of interest (data not shown), TH+ was taken as a marker of dopamine neurons in the investigated regions and used to reveal subgroups of cells in combination with other markers. Calbindin+ and DAT+ were highly variably expressed (0-99.5%) in A8, A9, and A10 dopamine neurons, whereas GIRK2+ was present in nearly all the TH+ cells within the regions investigated (for details see Table 2). Nevertheless, the TH+ and GIRK2+ intensities (normalised to the cell size as described in 'Materials and methods') showed differences within subregions of A8, A9, and A10 cell groups. Substance P+ (SP+) was found in the striatal projection to the A9 region and was used to define this region, while parvalbumin+ was observed mainly in non-dopamine neurons. Therefore, the detailed description and characterisation of the individual cell clusters and regions were performed on the basis of the distribution of these specific patterns of TH+, DAT+, SP+, parvalbumin+, calbindin+, and GIRK2+, as well as by comparing the TH+ and GIRK2+ expression intensities (Figs. 2-6) and of the proportions of dopamine neurons double-labeled for DAT+, calbindin+, and GIRK2+ (Table 2).

3D reconstruction of the A8, A9, and A10

In the present study, the coronal plane was optimal for the demarcation of A8, A9, and A10, although in some cases it was also necessary to obtain information from the sagittal plane, particularly for the 3D reconstruction of the cell clusters. The sections used for reconstruction were dehydrated by the application of fixative and cryoprotection, and the volume change induced by cryoprotection was assessed by determining the volume of the fixed brain samples before (5.2 ml/8 brains) and after (4.6 ml/8 brains) cryoprotection, revealing a volume reduction of 11.5%. The sizes of these structures in the living animal are therefore likely to be larger.

A8, A9, and A10 cell groups were located in the ventral mouse brain within the sagittal band of the

diencephalon, mesencephalic, and isthmic territories (Fig. 1a). These regions were directly adjacent to each other forming a continuous, tightly-packed, large group of dopamine neurons, without any apparent anatomical fissure or separation between them (Fig. 1b). In three-dimensional reconstructions, the A8 cell cluster appears as a tongue-like structure, whose tapered end separates the A9 and A10 cell groups, while its broadest part is caudal to the A9 and A10 regions (Figs. 1b, 1c). The A9 cell groups form a relatively flat ventral bed of dopamine neurons (Fig. 1c) extending 1400 ± 28 μm rostrocaudally (n=8).

<Put Figure 1 and Table 2 here>

A8 dopamine cell cluster

A8 had a lower average proportion of calbindin+/TH+ neurons (19.1%) than any of the A10 subregions (41.1-97.3%; Table 2), but a higher percentage of DAT+/TH+ neurons (85.3%) than nearly all the subregions of A10 (7.1-74.1%) with the only exception of PBP (95.6%). Some A8 neurons were unusual in that they were TH+ but not DAT+ (Fig. 2e, arrow). This suggests that some A8 neurons do not have the capacity to reuptake dopamine somatodendritically. In addition, there were some neurons in the RRF that were DAT+ but not TH+ (Fig. 2e, arrowheads), suggesting that other types of RRF neurons do have the capacity for dopamine reuptake in this region. Nearly all the A8 dopamine neurons contained GIRK2+ at a medium intensity (0.061 ± 0.005; n=13; Fig. 3a). A8 neurons had significantly stronger TH+ intensities (0.19 ± 0.03; n=13) than the dopamine neurons in the SNCD ($p<0.001$), SNCL ($p<0.05$) or SNCV ($p<0.05$).

<Put Figure 2 here>

Subregions of the A9 cell group

Subregions of the A9 cell group could be identified by differences in the cell morphology, cell size, and cellular distribution within the SN. Five subdivisions of the SN (SN reticular part (SNR), SN dorsal tier (SNCD), SN ventral tier (SNCV), SN lateral cluster (SNCL), and SN medial cluster (SNCM)) were identified, including four regions (SNCD, SNCV, SNCL, and SNCM) in which dopamine neurons were concentrated. Although the different subregions of SN contained variable percentages of double-labeled calbindin+/TH+ and DAT/+TH+ neurons, nearly all the A9 dopamine neurons were GIRK2+/TH+ (Table 2). When the ratios of normalised TH+ intensity versus normalised GIRK2+ intensity were compared in A8, A9, and A10 dopamine neuron clusters, it was evident that

9

the A9 dopamine clusters had significantly lower ratios than those of the A8 and A10 dopamine neurons (p<0.001; Fig. 3b).

<Put Figure 3 here>

Substantia nigra reticular part (SNR)

SNR, a region containing only a few dopamine neurons, is the largest part of the SN, extending along its entire rostro-caudal axis. Afferent fibers containing calbindin+ and SP+ together with the neurons containing parvalbumin+ concentrated in this region and were especially useful in providing more detailed regional information on the SNR (Fig. 4). SP+ fibers and terminals were abundant, assisting with the delineation of the SNR. More densely labeled SP+ fibers were found rostromedially versus rostrolaterally (see the regions labeled with "*" in Fig. 4a, 4b). The lateral part of SNR contained numerous, densely-packed, parvalbumin+ neurons (see the regions labeled with "*" in Fig. 4c). TH+ fibers from the dopamine neurons of other SN regions were seen within the calbindin+ neuropil of the SNR (Fig. 5). The organisation of these TH+ fibers differed in the rostral and caudal parts of SNR: rostral to the exiting fibers of the oculomotor nerve, straight, dorsoventrally directed TH+ fibers were abundant (Fig. 2a); whereas caudal to the exiting fibers of the oculomotor nerve, scanty TH+ fibers curved around the medial and lateral parts of the SNR, leaving a spherical area in the middle devoid of TH+ fibers (this observation is particularly apparent in Fig. 2c).

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Substantia nigra dorsal A9 tier (SNCD)

The dorsal tier of the SN (SNCD) contained a dense collection of dopamine neurons (Figs. 2,4,5) with lower average intensity of TH+ (0.084 ± 0.008, n=36) than that of the PBP (0.122 ± 0.014, n=28; Fig. 3), although the difference did not reach the level of statistical significance. Very few dopamine neurons in SNCD were calbindin+ (1.8%), whereas nearly all the dopamine neurons in this region were DAT+ (99.5%) and GIRK2+ (99.7%). Some neurons in the most ventral layer of the SNCD had less prominent DAT+ than those situated more dorsally (arrows in Figs. 2a, 2b). The average intensity of GIRK2+ in SNCD neurons was 0.056 ± 0.005 (n=36). A dense plexus of calbindin+ fibers was found in the caudal regions of the SNCD caudal to and within the plane of the exiting 3rd nerve (see the areas indicated with "#" in Figs. 5c, 5d), but not in the SNCD rostral to the 3rd nerve (Figs. 5a, 5b).

10

Substantia nigra ventral A9 tier (SNCV)

A number of TH+ neuron clusters situated in the caudal part of the SNR were identified as the ventral tier of the SN (SNCV) (Figs. 2d, 4d, 5d, 6c). Neurons in the SNCV had the lowest average intensity of TH+ (0.055 ± 0.008, n=8) compared with other A9 subgroups. No dopamine neuron co-expressed calbindin in SNCV, but nearly all the dopamine neurons of this region were DAT+/TH+ (93.8%) and GIRK2+/TH+ (100%) — trends that made SNCV very similar to SNCD. Neurons in SNCV showed medium GIRK2+ intensity (0.050 ± 0.008). In contrast to other A9 cell clusters, a few TH+ SNCV neurons also contained parvalbumin+ (see the cells indicated by arrows in Fig. 4d).

<Put Figure 6 here>

Substantia nigra lateral A9 cluster (SNCL)

The lateral A9 cluster of dopamine neurons (SNCL) was distinguished from the SNCD by the shape of their dopamine neurons; SNCD neurons were mainly oval-shaped, whereas SNCL neurons were smaller and elongated or spherical in shape (Figs. 7a, 7b). Compared to other A9 cell clusters, the SNCL presented a considerably higher ratio of calbindin+/TH+ neurons (51.1%) and a lower ratio of DAT+/TH+ neurons (62.5%). It is worth noting that the subbrachial nucleus located directly dorsolateral to the SNCL (labeled with "+" in Fig. 5c, 5d) was also seen in these sections. One should pay particular attention when delineating this area, as the subbrachial nucleus is dominated by strongly calbindin+ neurons, thus they could easily be confused with those of the SNCL in the calbindin-labeled sections. Most of the dopamine neurons in SNCL were GIRK2+ (92.3%). SNCL neurons had medium intensities of GIRK2+ (0.063 ± 0.009; n=11) and TH+ (0.087 ± 0.013; n=11) compared to those of the other SN cell clusters.

<Put Fig. 7 here>

Substantia nigra medial A9 cluster (SNCM)

Neurons in the medial A9 cell cluster (SNCM) showed markedly stronger TH+ labeling (0.125 ± 0.10; n=20) than either SNCV ($p < 0.01$) or SNCD ($p < 0.05$). The GIRK2 expression of these neurons was also significantly stronger (0.080 ± 0.007; n=20) than that of the neighbouring SNCD (0.056 ± 0.005; n=36; $p < 0.05$). In addition, 21.8% of the TH+ neurons in the SNCM contained calbindin, and these double-labeled neurons situated mainly in the rostral SN (at the level of and rostral to the exiting oculomotor nerve). At these levels there was an increased average proportion of calbindin+/TH+ neurons (43.6%), whereas no calbindin+/TH+ neurons were found in the caudal SN. Nearly all the

dopamine neurons in SNCM were DAT+/TH+ (99.0%) and GIRK2+/TH+ (99.7%).

Subregions of the A10 cell group

The A10 cell groups were the most diverse of these dopamine cell groups. Three main groups were identified: three midline clusters in the IF, CLi, and RLi; two middle clusters (PN and PIF) overlying the interpeduncular fossa; and two lateral, wing-shaped structures, the VTAR and PBP, sitting on the mediodorsal part of the A9 SN (as demonstrated earlier in Fig. 1b). The A10 region contained highly variable ratios of calbindin+/TH+ neurons (41.1-97.3%) with lower ratios in lateral cell clusters and higher ratios observed in more medial cell clusters. Moreover, A10 accommodated rather variable ratios of DAT+/TH+ neurons (7.1-95.6%) as well, with the lowest ratios observed in the more medial cell clusters, and the highest ratios found in the more lateral cell clusters. Compared with the A8 and A9 groups, the A10 groups had the most variable ratios of GIRK2+/TH+ (50.0-94.8%) among its subregions.

Interfascicular nucleus (IF)

The dopamine neurons in IF were small and round (Fig. 7i), with intermediate levels of TH+ (0.152 ± 0.017; n=20) and GIRK2+ (0.047 ± 0.005; n=20) compared to rest of the A10 cell clusters. On the other hand, IF contained the highest ratio of calbindin+/TH+ (97.3%), the lowest ratio of DAT+/TH+ (7.1%), and a medium ratio of GIRK2+/TH+ (67.3%) neurons among the A10 subregions.

Caudal linear nucleus of the raphe (CLi)

CLi neurons presented significantly higher intensities of TH+ (0.322 ± 0.037; n=10) compared with those of the other A10 subgroups (PN, PIF, IF, PBP, and VTAR; $p<0.05$); and also showed significantly higher intensities of GIRK2+ (0.080 ± 0.013; n=10) than PN ($p<0.01$) and VTAR ($p<0.05$). CLi had a relatively high ratio of the calbindin+/TH+ neurons (85.7%) and low ratios of DAT+/TH+ (13.3%) and GIRK2+/TH+ (50.0%) neurons (Figs. 2e, 5e, 6d).

Rostral linear nucleus (RLi)

In contrast to the other dopamine cell groups, few dopamine neurons were found in RLi, though it

contained very similar ratios of calbindin+/TH+ (92.8%) and DAT+/TH+ neurons (10.3%) to those of

the CLi. The ratio of GIRK2+/TH+ neurons in RLi was 75.0%. RLi neurons had fairly high level of

TH+ and medium level of GIRK2+ (Fig. 3a).

Paranigral nucleus (PN)

The intensity of TH+ in PN A10 neurons (0.19±0.02; n=20) was higher than that observed in the PIF

(0.12±0.02; n=10), although the difference did not reach the level of statistical significance. A

relatively low proportion of PN neurons were DAT+ (36%) whereas the majority (96.7%) were

calbindin+. The ratio of GIRK2+/TH+ neurons in PN was 82.5%. Neurons in the PN had lower

intensities of GIRK2+ (0.041±0.002; n=20) than those in the nearby SNCM neurons (0.080±0.007;

n=20; $p<0.001$).

Parainterfascicular nucleus (PIF)

In PIF, 69.1% of dopamine neurons were calbindin+, 46.2% were DAT+, and 89.8% were GIRK2+.

PIF neurons had lower GIRK2+ (0.039±0.004; n=10) than those in the nearby SNCM ($p<0.01$).

Parabrachial pigmented nucleus (PBP)

TH+ neurons in the PBP had an average intensity of 0.12±0.01 (n=28). Nearly all PBP neurons were

DAT+ (95.6%) and GIRK2+ (93.4%), but only 43.0% of them were calbindin+. Most of the

calbindin+/TH+ neurons were small or middle-sized (a few examples are arrowed in Fig. 5f). In the

rostral PBP (the area lateral to the dashed lines in Figs. 2a, 4a, 5a-5c, 6a, 6b), there was a cluster of

sparsely distributed TH+ neurons adjacent to the SNCD. These dopamine neurons showed scarce

calbindin+ (see the areas marked with "*" in Figs. 5a-5c), which was in sharp contrast with the

presence of strongly calbindin+ neurons seen in the medial PBP (Figs. 5a-5c).

Ventral tegmental area rostral (VTAR)

TH+ neurons in the VTAR had similar intensity (0.119±0.013; n=11) to those in PBP, whereas the

intensity of GIRK2+ was lower (0.035±0.005 vs. 0.058±0.006; Fig. 3a). Moreover, the VTAR

contained a smaller proportion of double-labeled DAT+/TH+ cells (74.1%), but similar proportions of

double-labeled calbindin+/TH+ cells (41.1%) and GIRK2+/TH+ cells (94.8%).

Cell size in A8, A9, and A10

Although diverse cell shapes were found in each region, every region concentrated neurons with characteristic neuronal shapes and sizes (Fig. 7), as shown by cellular 3D reconstructions (Fig. 8a). Among all the subregions of A8, A9, and A10, the dopamine neurons in A8 had the largest average cellular diameter. SNCM contained mainly small dopamine neurons (longest diameter of the neurons <15 µm, Fig. 8b); SNCD contained mainly middle-sized dopamine neurons (15-21 µm); SNCV accommodated both middle-sized and large dopamine neurons (21-27 µm); IF, PN, CLi, and PIF mainly contained small dopamine neurons (Figs. 7g, 7i-7k), whereas A8 had middle-sized and large dopamine neurons as its major constituent. The average cell diameter of SNCD dopamine neurons was significantly greater than that in the SNCM, IF, PN, and PIF ($p < 0.001$ in all cases). In addition, the average cell diameter of dopamine neurons in PBP was greater than that in the IF ($p < 0.001$), PN ($p < 0.01$), and PIF ($p < 0.05$), but smaller than that in the A8 ($p < 0.001$). When the average cell diameter was calculated for each major region (A10: 17.1±0.2 µm (n=522); A9: 18.3±0.2 µm (n=413); A8: 20.5±0.6 µm (n=77)), it became evident that the A8 area accommodated the largest neurons in the entire complex ($p < 0.001$). Moreover, the cell size was somewhat variable along the rostrocaudal axis: rostral to the oculomotor nerve, SNCD, SNCL, and PBP dopamine neurons were generally smaller (15.8±0.3 µm, 14.8±0.9 µm, and 15.6±0.4 µm, respectively) than in the more caudal portions of the same areas (21.6±0.4 µm, 18.8±0.6 µm, 19.4±0.3 µm — all differences are statistically significant at the $p < 0.001$, $p < 0.05$, and $p < 0.001$ level, respectively).

<Put Figure 8 here>

Coronal plates of the anatomical distribution and architecture of the A8, A9, and A10 cell clusters from rostral to caudal

Plate a (bregma -2.52 mm - bregma -2.70 mm)

Three fiber bundles, the fasciculus retroflexus (fr), medial lemniscus (ml), and principal mammillary tract (pm), act as landmarks in this plate (Fig. 9a). The first appearance of SNCD and SNCL was noted in the rostral part of this block. The most rostral pole of the SNCD was seen in this plate, where it did not form a continuous cell band along the mediolateral axis, but it appeared as two disjunct regions

14

(Fig. 9a). SNCL sat near the dorsolateral extremity of SNCD. At this level, the parasubthalamic

nucleus (PSTh), situated medial to the SNCD, contained occasional TH+ neurons with long processes

projecting towards the midline (Fig. 9a-2). The rostral pole of the PBP was directly adjacent to SNCD,

located dorsally and slightly medially as well as ventrolaterally to the nigrostriatal bundle. To separate

PBP and SNCD, another marker (calbindin) was required, as the PBP region had strong calbindin+,

whereas SNCD showed little calbindin+ (Fig. 5).

Plate b (bregma -2.74 mm - bregma -2.88 mm)

The main landmark for this plate (Fig. 9b) is the fasciculus retroflexus, as it approaches the level of the

medial lemniscus. The size and shape of the TH+ neurons of PBP were variable (Fig. 7f). Neurons at

the dorsal part of PBP were mostly arranged along a mediolateral axis, while other neurons at the most

ventral part of PBP were arranged in a less organized fashion. The majority of the SNCD cells were

arranged along a mediolateral axis, and their efferents formed large bundles that intruded into the SNR.

Dorsomedial to the PBP and intermingled with the nigrostriatal fibers (ns), the VTAR cluster of TH+

neurons appeared. VTAR neurons could be differentiated from those in PBP on the basis of their sparse

distribution and dorsoventral alignment (Fig. 7e). In this plane, SNCL sat at the dorsomedial edge of

the cerebral peduncle separated from the SNCD.

Plate c (bregma -2.92 mm - bregma -3.06 mm)

The main landmark for this plate is the fasciculus retroflexus, as it encroaches on the VTAR (Fig. 9c).

PBP extended laterally in a wing-shaped fashion, and was located next to the dorsomedial margin of

the SNCD. SNCD was a continuous 3-4 cell thick band of tightly packed neurons. The alignment of the

PBP and SNCD neurons was markedly different: SNCD cells were relatively uniformly aligned along a

mediolateral axis, whereas the appearance of PBP neurons was less organized. Where the medial

lemniscus embedded into the dorsal brim of the PBP, the rostral parts of the IF, RLi, and PIF appeared.

IF sat above the caudal end of the mammillary body, and it was easily recognisable according to its

position (right on the midline), and on the basis of its densely packed, small, TH+ neurons (Fig. 7i).

RLi was also situated in the midline, right above IF. RLi was flanked by the two mamillotegmental

tracts (mtg), and unlike IF, it accommodated only a few, sparsely distributed TH+ neurons (Fig. 7h),

making its differentiation from IF straightforward. PIF appeared ventromedial to the PBP, forming a

15

narrow band. In spite of the proximity of the two structures, they could be easily separated as PIF contained markedly smaller neurons than those in PBP.

Plate d (bregma -3.10 mm - bregma -3.24 mm)

The landmark for this plate was the medial terminal nucleus of the optic tract (MT), as it appeared adjacent to the dorsomedial edge of SNCD, and embedded in the lateral wing of PBP (Fig. 9d). Along with the appearance of the medial terminal nucleus, the rostral SNCM is also visible here. The TH+ neurons of SNCM were small, and they showed a medioventral alignment. SNCM was separated from SNCD (the latter containing different sized neurons) by the MT. In this plate, TH+ neurons were occasionally seen in the SNR. VTAR was no longer present. The lateral wing of the PBP was enlarged laterally (Fig. 9d-2). At this level, the medial part of A10 was formed by IF and RLi, while the caudal PIF and rostral PN represented the middle part of A10. PN was adjacent to the ventromedial edge of PIF. Although both PN and PIF accommodated relatively small neurons, the two structures were definitely discernable, as the former area contained small, elongated, narrow neurons, whereas the latter structure was characterised by more spherical cells.

<div align="center"><Put Figure 9 (part 1) here></div>

Plate e (bregma -3.28 mm - bregma -3.42 mm)

The landmark for this plate is the medial lemniscus, which at this level is wedged deeply into the PBP, breaking the PBP into two parts (Fig. 9e). A particularly important feature of this plane was the exiting of the oculomotor nerve, which is a landmark of the mesencephalon (not present in the diencephalon or isthmus). The rostral pole of the SNCV also appears at this level, extending caudally along with the main mass of the A9. The neurons of SNCV form a distinctive cell cluster inside the SNR. Although SNCV cells were rather close to those of SNCD, the two cell groups could easily be distinguished, as SNCV cells typically aligned along a dorsoventral axis, clearly separated from the SNCD cellular band. In the middle portion of the rather long SNCD band, the TH+ neurons were rather sparsely distributed. In this plate, PIF was completely replaced by PN, and the ventral tegmental decussation (vtgx) clearly separated IF from RLi.

Plate f (bregma -3.46 mm - bregma -3.60 mm)

The landmark for this plate is again the medial lemniscus, migrating even deeper, ventral and lateral to PN (Fig. 9f). PN was adjacent to the more mediodorsally situated IF and more dorsolaterally situated PBP, respectively. The boundary between IF and PN was not clear, but these clusters could be distinguished on the basis of their different cell morphologies; the densely packed IF cells were small and round (Fig. 7i), while PN neurons had an elongated shape (Fig. 7j) and were aligned along the lateral edge of the interpeduncular nucleus (IP). PBP was reduced as the rostral part of A8 appeared. Laterally, the SNCD band is broader and shorter, while the most lateral part of SNCL swirled around into SNR. SNCM and SNCV were also present, recognisable by their respective cytoarchitectural characteristics — small neurons in the former (Fig. 7c); larger, dorsoventrally aligned, and more sparsely distributed cells in the latter (Fig. 7d).

Plate g (bregma -3.64 mm - bregma -3.78 mm)

The landmark for this plate is the large fiber bundle formed by the converging medial lemniscus and cerebral peduncle, arranged perpendicular to the transverse fibers of the pons (Fig. 9g). In this plate, there was only a small caudal remnant of RLi. PN was also smaller, being replaced by the rostral part of CLi. The rostral part of the CLi could be differentiated from RLi as a wider region containing more densely packed TH+ neurons. A largely TH negative region was noted in the middle of the CLi, surrounded by TH+ neurons. This area corresponded to the region of serotonin neurons reported by others (Bosker et al. 1997) and termed 'zygos part of the CLi'in humans (Bosker et al. 1997; Paxinos and Huang 1995). This special feature of CLi made it easily discernible. The SNCD at this level appeared as a cell clump rather than an elongated cell band. Although the alignment of neurons in SNCD was not as organised as in its more rostral regions, those adjacent to A8 could be differentiated by their soma orientation and more visible processes. The neurons in A8 were seen in a location partially obstructed by fibers belonging to the medial lemniscus passing through this area. In this plate, the medial margin of A9 was formed by SNCM, the neurons of which were smaller than those of the SNCD (Fig. 7a, 7c). SNCL was observed at the dorsolateral pole of SNR, consisting of cells showing no apparent order in their alignment.

Plate h (bregma -3.82 mm - bregma -3.96 mm)

17

The landmarks for this plate are the cross sections of the superior cerebellar peduncles (scp), which were adjacent to and more lateral than CLi (Fig. 9h). The dorsal raphe nucleus (DR) was situated dorsal to the CLi. Although DR contains serotonin neurons, it also contains a population of catecholamine synthesizing neurons. PBP was sparsely populated and it was very much reduced in volume compared to its appearance in more rostral sections. SNCM, SNCV, and SNCL were not present in this plane, giving way to four neuronal clusters, corresponding to CLi, PBP, A8, and SNCD from medial to lateral, respectively. The neurons of A8 were differentiable from those of the SNCD; at this particular level, the A8 cells were smaller and more densely packed along a mediolateral axis, whereas SNCD neurons were bigger, more sparsely distributed, and formed a curved band with a primarily dorsoventral orientation (Fig. 9h).

<center>**<Put Figure 9 (part 2) here>**</center>

Plate i (bregma -4.00 mm - bregma -4.14 mm)

The landmark for this plate is the decussation of the superior cerebellar peduncle (xscp), forming a large and easily recognisable fiber trunk, situated directly beneath CLi (Fig. 9i). The DR was expanded in this plate, while CLi was reduced in cross-section. The two structures could be easily differentiated from each other, as DR accommodated densely packed and prominent TH+ somata and projections, whereas CLi contained loosely packed neuronal cell bodies. A8 forms an elongated, olive-shaped, disparate structure, which is separated from CLi by the xscp. A8 contained neurons of different sizes (Fig. 7l) and the dopamine neurons were sparser in its most medial and lateral portions (Fig. 9i). Neither the caudal SNR, nor SNCD was present in this plate.

Plate j (bregma -4.18 mm - bregma -4.60 mm)

The landmarks for this most caudal plate were the appearance of the tectospinal tract (ts), the short medial longitudinal fasciculus (mlf), and the mediolaterally expanding decussation of the superior cerebellar peduncle (Fig. 9j). The density of TH+ neurons decreased in the more caudal portions of A8. CLi was further reduced in size.

<center>**<Put Figure 9 (part 3) here>**</center>

Discussion

Gene targeting methods applied on mice are now becoming increasingly important in studies concerning the pathogenesis and treatment of Parkinson's disease, in which there is selective dopamine neuron loss in A9. Unfortunately, proper interpretation of the relevant studies have been severely hampered by the lack of unambiguous and universally accepted terminology for this region, and by the absence of a detailed description of all the subregions of the murine A8, A9, and A10. **In the present work, we have carefully delineated the A8, A9, and A10 areas in adult C57BL/6J mice using immunofluorescence and immunohistochemistry, and we provide a detailed description of the cytoarchitectural and chemoarchitectural characteristics of each subregion. We found that the main dopamine cell groups of the mouse brain are similar to those observed in rats and humans in terms of diversity, and we show that the expression patterns of some of the biomarkers tested are markedly different in A8, A9, and A10. The detailed anatomical and histological features described here provide a clear and unambiguous nomenclature of the subdivisions of these dopamine cell groups. In addition, our results may help in the adequate interpretation of the functional data, and they raise the possibility that some of the unique expression patterns might be associated with the selective vulnerability of different types of dopamine neurons in disease states.**

Proteins identifying A8, A9, and A10 dopamine neurons

Although TH is a commonly used marker for labeling dopamine neurons, as an upstream enzyme participating in the biosynthesis of catecholamines, it is also present in noradrenaline neurons. For this reason, the application of DBH labeling is usually necessary to differentiate between dopamine and other catecholamine neurons, such as those containing noradrenaline (Björklund and Dunnett 2007). In the present work, no A8, A9, or A10 neurons co-expressed TH and DBH, confirming previous reports that these TH+ neurons are all dopaminergic (Dahlström and Fuxe 1964; Hökfelt et al. 1984).

Calbindin-D-28k is an EF-hand homology protein binding Ca^{2+} with dissociation constants in the micromolar range (Persechini et al. 1989). It has been found that in Parkinson's disease (German et al. 1992; Yamada et al. 1990) and in animal models (German et al. 1992; Lavoie and Parent 1991) induced with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP is a neurotoxin producing alterations in intracellular Ca^{2+} concentration), a preferential cell loss occurs with those dopamine

neurons lacking calbindin-D-28k most vulnerable. In the present study, different A10 subregions showed medium and high ratios of calbindin+/TH+ neurons, A8, SNCD and SNCV of the A9 had low ratios, and the SNCM (rostral to or at the exit of the oculomotor nerve fibers) and SNCL showed medium ratios. These findings are similar to those in earlier reports in mouse (Liang et al. 1996) and rat (Alfahel-Kakunda and Silverman 1997; Haber et al. 1995). Based on the distribution pattern of the calbindin-D-28k immunoreactivity, our results **might** suggest **some** heterogeneity in the vulnerability of the different subregions of the A8, A9, and A10 dopamine cell groups.

DAT is responsible for the reuptake of dopamine after its release, and is expressed on the soma, dendrites, and axons of most dopamine neurons, although in different glycosylated (mature) forms (Cruz-Muros et al. 2009). In the present study, an antibody recognising an epitope situated at the N-terminus of DAT was employed. Application of this antibody revealed a mismatch between the expression of DAT and TH, especially in many A10 cell clusters (IF, RLi, CLi, PN, and PIF), where less than half of the cells were immunoreactive for this protein. This finding suggests that a substantial portion of A10 dopamine neurons may not be involved in the reuptake of dopamine through their somatodendritic domains. This may be an additional factor contributing to the decreased vulnerability of these dopamine neurons (Afonso-Oramas et al. 2009; González-Hernández et al. 2004).

GIRK2 was reported to control the membrane excitability of DA neurons (Chung et al. 2005). It should be noted that most reports concerning GIRK2 expression in mice suggest that only A9 dopamine neurons express this protein (Chung et al. 2005; Schein et al. 1998). In contrast to these reports, we found a relatively widespread expression of GIRK2 in the A8 and A10 subregions as well, although the expression intensities were highly variable, as evidenced by the GIRK2+ intensities observed in the individual subregions. The discrepancy between the present and earlier reports on GIRK2 expression could be the result of interspecies differences, or it may be the consequence of less stringent anatomical criteria used in previous reports to define the boundaries of the SN. It should be emphasized that in the present study constant evaluation criteria were maintained and applied when the labeling intensities were assessed. More importantly, all the sections were only exposed to laser light when images were captured for analysis, and all images were scanned with the same sampling parameters (e.g. the same pixel dwell time, laser intensity, and voxel size were applied, and all images were captured at an optical

thickness of 1 μm). In addition, to avoid the effects of scattering signal, the absolute intensities were always corrected to cell size. Keeping these efforts in mind, our study shows that A9 is the only region where a fairly constant GIRK2 expression is combined with relatively low levels of TH expression. It is tempting to conclude that a given level of GIRK2 expression combined with high levels of TH is associated with cell survival, whereas neurons demonstrating a similar GIRK2 expression levels in the presence of significantly lower levels of TH are more prone to neural injury, and are thus more vulnerable when Parkinson's disease develops.

Comparison of the terminology applied for mouse, rat, and human

In an earlier study (González-Hernández and Rodríguez, 2000) on the basis of the chemical profile of the neurons situated in the rat substantia nigra, five dopaminergic neuron groups were identified; they were named the dorsomedial portion of the substantia nigra pars compacta (dmSNC), the substantia nigra pars lateralis (SNL), the rostral region of the substantia nigra pars compacta (rSNC), the lateral part of the substantia nigra pars compacta (lSNC), and the dopamine cell groups in the caudal part of the substantia nigra pars compacta and pars reticulata (cSNC-SNR). After a careful comparison of these findings with our results, we have concluded that the dopamine neurons in cSNC-SNR, rSNC, and SNL might be homologous with those of the SNCV, SNCD, and SNCL, respectively, as defined in the present study. Furthermore, we cannot exclude the possibility that the dmSNC and lSNC identified in the above rat study may correspond to SNCM and SNCD of our own findings with some of the ventral A10 neurons included.

Based on the nigrostriatal projections (Heimer 2003; Joel and Weiner 2000), DAT protein expression (Afonso-Oramas et al. 2009), dopamine and vesicular monoamine transporter expression patterns (González-Hernández et al. 2004), and the degeneration pattern in Parkinson's disease (Braak et al. 2004; Damier et al. 1999), the A9 cell group has been divided into rostromediodorsal (RMD) and caudolateroventral (CLV) regions. In humans, the RMD and CLV regions are referred to as the beta and alpha parts of the SN, respectively (Olszewski and Baxter 1954). In rats, on the other hand, the RMD region of the A9 cell group corresponds to the dorsal tier of the SN; whereas the CLV part of A9 cell group is identified as the ventral and displaced ventral tiers of the SN (Gerfen et al., 1987). On the basis of the data provided in the present work, we suggest that the dorsal, ventral, and displaced ventral

tiers of the rat A9 cell group (as defined by Gerfen et al.) correspond to the murine lateral PBP, SNCD, and SNCV, respectively. This is based on the boundaries of the SN as identified by the SP+ fiber projections from the striatum (see also (McRitchie et al. 1996)), and on the significant differences in the cell types involved. In the present study, the displaced ventral A9 dopamine neurons form a separate tier, as seen in other species in the caudal SN, while the larger ventral A9 tier is equivalent to the A9 dorsal tier identified in other comparative studies (McRitchie et al. 1996; Paxinos and Huang 1995; Paxinos and Watson 2007).

The SN is a heterogeneous structure both developmentally and histologically

At all coronal levels, the SN has two major parts, the compact dopamine cell groups (SNCD, SNCM, SNCV, and SNCL) and the SNR. Less well known are the reports of developmental and anatomical heterogeneity within the A9 dopamine cell group (Gerfen et al. 1987). The A9 dopamine neurons in the mouse SN align in dorsal, lateral, medial, and ventral laminae — this laminar arrangement of neurons is particularly obvious in the 3D reconstructions. In the present study, we found that the mouse SNCV contains only a few dopamine cells. Compared with the densely packed dopamine cells seen in the SNCD, the SNCV accommodates much smaller dopamine neuron clusters, similar to that shown in the rat (Paxinos and Watson 2007). Furthermore, an early study directly comparing the size of the A9 dorsal versus the ventral tiers of the SN showed that the A9 ventral tier was smaller in rats than in primates (Hardman et al. 2002). In the present study, dopamine neurons in SNCM always possessed smaller average cell diameters than those of the other A9 dopamine neuron clusters when investigated along the same rostro-caudal axis. The neurons present in SNCM were round and densely clustered, presenting the highest TH expression intensity among the A9 cell clusters. In addition, this is the first time that SNCM has been recognized to extend from the exiting of oculomotor root to nearly the caudal end of the A9 SN. SNCL contained variable shaped neurons, including very small spherical, small fusiform, and middle-sized olive-shaped cells. Similar to previous reports (Afonso-Oramas et al. 2009; McRitchie et al. 1996; Thompson et al. 2005), SNCL contained the highest ratio of calbindin+/TH+ cells of all the A9 subregions, but the lowest ratio of DAT+/TH+ and GIRK2+/TH+ cells.

Marchand and Poirier (Marchand and Poirier 1983) showed that "early-generated" neurons settle in the rostral half of the SN, whereas neurons appearing at later stages of development settle in more caudally

located areas. The same chronological order of SN development was observed in rats, and the rostrocaudal landmark of this developmental difference is the exiting fibers of the oculomotor nerve (Marchand and Poirier 1983). The oculomotor fibers exit between the isthmus and mesencephalon (Puelles et al., 2007), suggesting that the SN is generated from three separate neuromeres — diencephalon, mesencephalon, and isthmus.

By keeping the available developmental information in mind, we carefully examined the cellular characteristics in the rostral and caudal parts of SN by taking the exiting oculomotor nerve fibers as the landmark separating these developmentally different regions (see above). This comparison revealed four major points: (1) dendrites from the A9 TH+ neurons in the rostral SNR were straight, running in a dorsomedial to ventrolateral direction, whereas in the caudal portion these dendrites were curved, running in the peripheral parts of the SNR and leaving its central regions free of TH+ dendrites; (2) the average size of some A9 TH+ neurons (situated in the SNCD and SNCL) was bigger in the caudal than in the rostral SN; (3) the SNCV was confined to the caudal part of the SN; and (4) calbindin+/TH+ neurons could only be found rostral to or at the level of exiting oculomotor nerve roots.

The A10 cell group is markedly heterogeneous

Seven separate cytoarchitectural and chemoarchitectural regions in the mouse brain contained A10 dopamine neurons. This is similar to the heterogeneity observed in other species (McRitchie et al. 1996; Paxinos and Huang 1995). The IF, CLi, and RLi are recognised here as medial parts of the A10 dopamine cell group, although the types of dopamine neurons accommodated in these nuclei are clearly different from those found more laterally, especially in the PBP. First, these medial A10 nuclei contained appreciably more dopamine neurons with calbindin+; second, these medial A10 nuclei had less dopamine neurons with DAT+; third, the medial A10 nuclei had less dopamine neurons with GIRK2+; finally, the cell size in the IF nucleus was smaller than in any other A10 nucleus.

The PN and PIF were two clusters easily recognized by their locations (underlying the interpeduncular fossa), and by the size of their neurons, as they were smaller when compared with those in the PBP. The neurons in PN were more elongated, whereas the neurons in PIF tended to be smaller and more spherical in shape. PIF is more rostral to PN, and there are higher ratios of calbindin+/TH+ neurons but lower ratios of DAT+/TH+ neurons in PN and PIF compared with those in PBP.

The lateral A10 regions (VTAR and PBP) are located on the mediodorsal part of the A9 SN. It should be pointed out that PBP has been erroneously delineated as the dorsal tier of SN in a surprisingly high number of previous studies. However, major differences in the architecture of these two cell clusters clearly identify PBP as a separate A10 structure. First, PBP has much higher ratio of calbindin+/TH+ neurons than that of the SNCD; second, PBP neurons demonstrate higher TH+ intensity; third, the dopamine cells in the dorsal tier of SN regularly align mediolaterally, whereas the dopamine cells in the PBP are aligned in various directions and they are immersed in diverse fibers. More than that, it has been described that the dorsal tier of the rat SN (the ventral tier of Fallon and Loughlin (1995)) projects exclusively to the sensorimotor part of the caudate-putamen, whereas VTAR and PBP (the dorsal tier of Fallon and Loughlin (1995) in their terminology) project widely to both limbic and cortical forebrain regions. This observation suggests a functional discrepancy between PBP and the dorsal tier of SN, along with a possible homology between PBP and the other VTA subregions. In the present work, VTAR extends the current nomenclature system to describe the rostrodorsal VTA region, which contains less densely packed dopamine cells with a lower proportion of DAT+/TH+ neurons when compared with PBP.

Applicability of the present findings to other mouse strains

The present study was performed on C57BL/6J mice, which is the most often employed strain in the fields of both neuroscience and transgenic engineering, so the results presented here may have particular significance for murine studies concerning either the function or pathology of dopaminergic neurons (e.g. Parkinson's disease, schizophrenia, and addiction) that use this strain. It must be recognised, however, that interstrain differences (such as differences in the number of SN neurons) exist (Zaborszky and Vadasz 2001). Nevertheless, after mapping TH *in situ* hybridization data in 7 mouse strains preserved in the Allen Institute online database (Lein et al. 2007; Ng et al. 2009), we found that all the strains contained every subregion of the A8, A9, and A10 dopamine cell groups identified in the C57BL/6J mice studied. As both cell size and shape are useful criteria when distinguishing different A8, A9 and A10 dopamine cell clusters, these clues may assist delineation of the appropriate areas in other strains or even in other species as well. Moreover, the major characteristics provided about each dopamine region of A8, A9, and A10 cell groups in the present

study may serve as a reference when mapping the same or similar regions, irrespective of the types of

mouse strains employed.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Figure Legend

Fig. 1 3D reconstruction of A8, A9, and A10

Lateral view of A8, A9, and A10 relative to the rest of the mouse brain (a), integrated view of A8, A9, and A10 (b), and individual views of A8, A9, and A10 (represented as medial and lateral A10) (c). Note the orientation of the 3D panels that is indicated in the lower left corner of each panel. *Scale bar* 1 mm (a); 200 µm (b and c).

Fig. 2 Co-localization of DAT+ and TH+ in A8, A9, and A10

Distribution of the TH+ (left panels) and DAT+ (middle panels) in the mouse A8, A9, and A10, along with the overlay images (right panels) (a-e). **<u>The overlay images show the distribution of the cell nuclei as well (blue signal, DAPI labeling).</u>** The panels (a-e) are arranged along the rostro-caudal axis. *Scale bar* 100 µm (a-e).

Fig. 3 Comparison of GIRK2+ and TH+ in A8, A9, and A10

Normalised fluorescence intensities of GIRK2+ and TH+ in A8 and subregions of A9 and A10 (mean ± SEM) (a), the ratios of the TH+ and GIRK2+ fluorescence intensities in A8, A9, and A10 (mean ± SEM; the number of cells considered is indicated at the bottom of each bar), *** p<0.001 (b).

Fig. 4 Expression of SP+ and parvalbumin+ in SN

SP+ and TH+ in the rostral SN (a-b), parvalbumin+ and TH+ in the SN along its rostro-caudal axis (c), high power views of SNCV demonstrating simultaneous TH+ and parvalbumin+ (d). **<u>Figure c and the overlay images show the distribution of the cell nuclei as well (blue signal, DAPI labeling).</u>** *Scale bar* 100 µm (a-c); 40 µm (d).

Fig. 5 Co-localization of calbindin+ and TH+ in A8, A9, and A10

Distribution of TH+ (left panels) and calbindin+ (middle panels) in the mouse A8, A9, and A10, along with the overlay images (right panels) (a-e). The panels (a-e) are arranged along the rostro-caudal axis. High power views of part of the PBP (indicated with the rectangular box in (b)) are also presented (f).

The overlay images show the distribution of the cell nuclei as well (blue signal, DAPI labeling).

Scale bar 100 μm (a-e); 20 μm (f).

Fig. 6 Co-localization of GIRK2+ and TH+ in A8, A9, and A10

Distribution of the TH+ (left panels) and GIRK2+ (middle panels) in the mouse A8, A9, and A10, along with the overlay images (right panels) (a-d). **The overlay images show the distribution of the cell nuclei as well (blue signal, DAPI labeling).** The panels (a-d) are arranged along the rostro-caudal axis. *Scale bar* 100 μm (a-d).

Fig. 7 Morphology of dopamine cells in A8, A9, and A10

High magnification images demonstrating the morphology of the dopamine cells in various parts of the A8, A9, and A10 regions (a-l). **The cells are visualized by their TH immunoreactivity (dark brown signal) with Nissl-counterstaining (blue signal)**. *Scale bar* 20 μm (a-l).

Fig. 8 Size and 3D reconstruction of representative examples of A8, A9, and A10 cells

For each region, the most representative neuronal shape is presented in 3D and the mean values of the greatest neuronal diameters characteristic of the different regions are shown (a). Distribution of the cell size in the investigated regions (b). *Scale bar* 10 μm (a).

Fig. 9 Demarcation of A8, A9, and A10

Rostro-caudal coronal sections demonstrating the distribution of TH+ (a-j). The panels in the left column are Nissl-counterstained low magnification images (left half of the sections), and schematic drawings (right half of the sections), giving a general overview of the distribution and general appearance of the dopamine neuronal clusters in the sections. The images in the right column show the cytoarchitectural details of the individual regions. All the sections used in this figure were consecutively collected from the same mouse brain. The distance between two consecutive plates was 80 μm. *Scale bar* 500 μm (left column in a-j) and 100 μm (right column in a-j).

Table 1 Characteristics of the primary and secondary antibodies

Table 2 Percentage of dopamine neurons containing calbindin-D-28k, DAT, or GIRK2 in C57BL/6 mice

Abbreviations

3n	Oculomotor nerve
AU	Arbitrary unit
CLi	Caudal linear nucleus of the raphe
CLV	Caudolateroventral region of A9
cp	Cerebral peduncle
cSNC-SNR	Caudal part of the substantia nigra pars compacta and pars reticulata
DAB	3,3'-diaminobenzidine
DAPI	4'-6-diamidino-2-phenylindole
DAT	Dopamine transporter
DBH	Dopamine β-hydroxylase
dmSNC	Dorsomedial portion of the substantia nigra pars compacta
DR	Dorsal raphe nucleus
fr	Fasciculus retroflexus
GIRK2	G-protein-activated inwardly rectifying potassium channel 2
IF	Interfascicular nucleus
IP	Interpeduncular nucleus
lSNC	Lateral part of the substantia nigra pars compacta
ml	Medial lemniscus
mlf	Medial longitudinal fasciculus
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MT	Medial terminal nucleus of the optic tract
mtg	Mammillotegmental tract
ns	Nigrostriatal bundle
PaP	Parapeduncular nucleus
PBP	Parabrachial pigmented nucleus
PIF	Parainterfascicular nucleus

pm	Principal mammillary tract
PN	Paranigral nucleus
PSTh	Parasubthalamic nucleus
RLi	Rostral linear nucleus
RMD	Rostromediodorsal region of A9
ROI	Region of interest
RRF	Retrorubral field
rSNC	Rostral region of the substantia nigra pars compacta
scp	Superior cerebellar peduncle
SN	Substantia nigra
SNCD	Substantia nigra compact part dorsal tier
SNCL	Substantia nigra compact part lateral tier
SNCM	Substantia nigra compact part medial tier
SNCV	Substantia nigra compact part ventral tier
SNL	Substantia nigra pars lateralis
SNR	Substantia nigra reticular part
SP	Substance P
TH	Tyrosine hydroxylase
ts	Tectospinal tract
VTA	Ventral tegmental area
VTAR	Ventral tegmental area rostral part
vtgx	Ventral tegmental decussation
xscp	Decussation of the superior cerebellar peduncle