Distinct cellular and molecular environments support aging-related DNA methylation changes in the substantia nigra

Aim: We aimed to couple brain region-specific changes in global DNA methylation over aging to underlying cellular and molecular environments. **Materials & methods:** We measured two major forms of DNA methylation and analyzed *Dnmt*, *Tet* and metabolite levels in the striatum and substantia nigra (SN) over aging in healthy male mice. **Results:** The ratio of 5-hydroxymethylcytosine to 5-methylcytosine increases over aging in the SN, and 5-hydroxymethylcytosine increases preferentially in dopaminergic neurons. Additionally, this age-dependent alteration in methylation correlates with a reduction in the ratio of α -ketoglutarate to succinate in the SN. **Conclusion:** Distinct cellular and molecular environments correlate with aging-associated methylation changes in the SN, implicating this epigenetic mechanism in the susceptibility of this brain region to age-related cell loss.

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Neurons live longer than nearly all other cell types in the body. In the absence of a degenerative disease, the lifespan of most neurons is only limited by the longevity of the organism. However, some brain regions and neuronal types are more vulnerable to age-related cell death and degenerative diseases. One susceptible neuronal population is dopaminergic neurons of the substantia nigra (SN), with a 5–8% loss per decade of life in humans during normal aging [1,2]. This neuronal population is also susceptible to age-related neurodegeneration in Parkinson's disease [3]. The molecular mechanisms underlying the enhanced vulnerability of dopaminergic neurons of the SN to age-related cell loss and degenerative disease remain unclear.

Currently, the leading molecular predictor of chronological age is the genomic pattern of DNA methylation at the C5 position of cytosine (5-methylcytosine, 5mC) at CpG dinucleotide sites, which outperforms all

other biomarkers, such as cell-cycle inhibitor p16^{INK4A} expression and telomere length attrition rate [4–6]. Quantitative models based solely on DNA methylation patterns are able to predict an individual's age with an average error of only 5.2 years [4,7–10]. DNA methylation is an epigenetic mark catalyzed by DNA methyltransferases (DNMTs) that allows for the adaptability of gene expression in response to developmental or environmental factors; it plays an essential role in various biological functions such as the regulation of gene transcription and the establishment and maintenance of cellular identity [11]. Historically, DNA methylation was thought to be a stable, repressive covalent modification in mammalian cells, existing predominantly as 5mC. However, in 2009, two seminal papers confirmed the existence of another cytosine modification, 5-hydroxymethylcytosine (5hmC), which is formed from the oxidation of 5mC by the ten–eleven translocation

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(TET) family of enzymes and is generally thought to correlate with gene activation [12,13]. It has since been discovered that 5hmC can be converted to unmodified cytosine [14,15]. Thus, the DNA methylome is dynamic, and environmental influences can lead to changes in DNA methylation levels and patterns.

Age-associated alterations in DNA methylation vary in absolute number and genomic loci depending on organ type [9,10], suggesting that different tissues have different rates of changes in DNA methylation over aging. However, it is unclear whether brain regions vary in the degree of DNA methylation changes over aging, given the limited studies examining age-related changes in only a few brain regions. Early chromatography studies found that global 5mC levels in the whole brain of mice decrease with aging [5,6,16]. Recent studies employing bisulfite treatment of DNA, which allows for genome-wide profiling of DNA methylation (both 5mC and 5hmC, indistinguishably), from the cortex of human and mouse over aging have generated different results, with Day *et al.* [9] finding an increase in DNA methylation in Brodmann area 19 of the human cortex using Illumina beadchips (Illumina, CA, USA) that evaluate 26,486 CpG sites, and Lister *et al.* [17] observing stable DNA methylation levels in both the mouse or human by employing whole-genome sequencing to assess all cytosine sites in the frontal cortex. Studies focusing on age-related changes of 5mC in the hippocampus of mice using immunohistochemistry or ELISAs have also produced conflicting results, with one reporting an increase [18] and another finding no change [19], respectively. There are also contradictory results regarding 5hmC in the hippocampus over aging, with one study finding no change using a chemical labeling, affinity enrichment approach followed by sequencing (hMe-Seal) [20], while two other studies report an increase in 5hmC via ELISA [19] and immunohistochemistry [21].

There are various factors contributing to the lack of congruity among studies. First, different methylation detection methods have vastly different levels of sensitivity. Second, methods employing bisulfite treatment cannot distinguish between 5mC and 5hmC [22], which is a necessary distinction to make given the biological differences between the two epigenetic marks, and therefore, cannot be compared with methods that evaluate 5mC or 5hmC independently. Last, the underlying cellular heterogeneity and differences in cell type composition across brain regions might contribute to the discrepancies observed. Given these incongruous findings, additional research is necessary to determine the extent to which DNA methylation changes with aging in the brain, and whether these changes are brain region-specific. It is especially important to assess age-

associated changes in DNA methylation in the SN, an area hitherto unexplored, given this region's enhanced vulnerability to aging.

Since DNA methylation integrates environmental and developmental signals for the modulation of transcriptional output, we set out to determine the degree to which global levels of 5mC and 5hmC differ across two brain regions over aging. We compare the DNA methylation status of the SN to the dorsal striatum, the region to which dopaminergic neurons of the SN project [3], to gain insight into the susceptibility of the SN to age-related cell death. To address whether changes observed in the SN are specific to dopaminergic neurons, we performed semiquantitative immunohistochemistry of 5hmC in tyrosine hydroxylase (*Th*, TH)-positive, dopaminergic and parvalbumin (*Pvalb*, PV)-positive neurons of the SN. Finally, we focused on the regulation of 5mC and 5hmC to gain insight into the underlying mechanism contributing to the differences in DNA methylation status over aging between the striatum and SN. First, we measured the expression levels of enzymes responsible for regulating 5mC and 5hmC, and second, we assessed metabolites that regulate TET function. Our findings suggest that distinct cellular and molecular environments account for the different DNA methylation states observed between the striatum and SN over aging, and therefore, implicate this epigenetic mechanism in the enhanced, age-related vulnerability of nigral dopaminergic neurons.

Materials & methods

Animal tissue

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. All mice were on the C57BL/6 genetic background. Tissues from healthy young male animals were collected at postnatal day 90 (P90), and tissues from healthy old male animals were collected at P545. Bilateral dorsal striatum and SN samples were collected as previously described [23,24]. Briefly, a mouse brain matrix (ASI-Instruments, MI, USA) was used to coronally section the brain. Bilateral dorsal striatum collection started rostrally when the anterior portion of the anterior commissure crosses the midline (Bregma 0.20 mm) and ended caudally with the loss of the connection between the dorsal third ventricle and the lateral ventricles (Bregma -0.58 mm). Bilateral SN collection started rostrally when the hippocampus completely wraps around the midbrain (Bregma -2.92 mm) and ended caudally with the disappearance of distinct blades of the dentate gyrus (Bregma -3.64 mm).

Reversed-phase HPLC coupled with tandem mass spectrometry (LC-MS/MS & MS/MS/MS)

LC-MS/MS and MS/MS/MS measurements of 5mC and 5hmC were performed blind to age and brain region. DNA was isolated using an AllPrep DNA/RNA Micro Kit (Qiagen, Hilden, Germany) and treated with RNase (Roche, Basel, Switzerland). DNA (1 µg) was then treated with nuclease P1 (0.5 U) and phosphodiesterase 2 (0.00025 U) at 37°C for 48 h in a digestion buffer (30 mM sodium acetate, 1.0 mM zinc acetate, 1 mM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), pH 5.6), followed by the addition of alkaline phosphatase (0.05 U) and phosphodiesterase 1 (0.0005 U) in another buffer (50 mM Tris-HCl, pH 8.9) for 2 h. For MS quantification of 5hmC and 5mC, 50 fmol of (1,3-¹⁵N₂, 2'-D)-5hmC and 600 fmol of (1',2',3',4',5'-D₅)-5mC were added to the digestion mixture of 50 ng genomic DNA. Enzymes were removed by chloroform extraction. The resulting aqueous layer was subjected to LC-MS/MS and MS/MS/MS analyses on an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, MA, USA) that was equipped with an ESI interface and coupled to an Agilent 1200 capillary HPLC (Agilent Technologies, CA, USA). Separation was carried out on a 0.5 × 250 mm Zorbax SB-C18 column (5 µm in particle size, Agilent Technologies, CA, USA) with a flow rate of 8.0 µl/min. A solution of 2 mM sodium bicarbonate (pH 7.0, solution A) and methanol (solution B) was used as mobile phases, and a gradient of 5 min 0–20% B and 25 min 20–70% B was employed for the separation. MS settings were as follows: electrospray voltage, 5 kV; capillary temperature, 275°C; capillary voltages, 38 V; tube lens voltages, 60 V; sheath gas flow rate, 15 arbitrary units. Selected-ion chromatograms were plotted for monitoring the transitions of *m/z* 258→142→124 for 5hmC, *m/z* 261→144→126 for [1,3-¹⁵N₂, 2'-D]-5hmC, *m/z* 242→126 for 5mC and *m/z* 247→126 for [1',2',3',4',5'-D₅]-5mC. The calibration curve for 5hmC and 5mC was constructed previously [25,26]. The numbers of moles of 2'-deoxyguanosine (dG), 5mC and 5hmC in each sample were calculated from the peak area ratios, the calibration curves and the amounts of stable isotope-labeled standards added. 5mC and 5hmC were then calculated as the percentage of dG (% dG) by dividing the moles of 5mC or 5hmC, respectively, by the moles of dG. Total methylated cytosine was calculated by adding 5mC (% dG) and 5hmC (% dG) per sample; the ratio of 5hmC to 5mC was calculated by dividing 5hmC (% dG) by 5mC (% dG); 5mC percentage of total methylated cytosine was calculated by dividing 5mC (% dG) by total methylated cytosine; and 5hmC percentage of total methylated cytosine was calculated by dividing 5hmC (% dG) by total methyl-

ated cytosine. The sample size is ten animals per group (*n* = 10 bilateral young striatum samples, *n* = 10 bilateral old striatum samples, *n* = 10 bilateral young SN samples and *n* = 10 bilateral old SN samples). To reduce biological variation, the striatum and SN samples were taken from the same animal. Additionally, RNA was taken from these same tissue samples for the quantitative RT-PCR, and therefore, a total of ten old and ten young animals (20 animals total) were used for the reversed-phase HPLC coupled with tandem mass spectrometry and quantitative RT-PCR experiments.

Semiquantitative immunohistochemistry

Mice were anesthetized with 1.25% Avertin, transcardially perfused with 4% paraformaldehyde in PBS and postfixed with 4% paraformaldehyde in PBS overnight at 4°C. Immunohistochemistry was carried out on 20 µm free-floating sections as previously described [27]. Tissue sections from the SN (see animal tissue section above for the Bregma coordinates of the SN) were incubated with a 5hmC antibody (1:1000, Active Motif [39791], CA, USA) in blocking solution overnight at 4°C. The next day, tissues were removed from the 5hmC antibody solution and incubated with either a TH antibody (1:1000, Abcam [ab76442], Cambridge, United Kingdom) or a PV antibody (1:1000, EMD Millipore [mab1572], MA, USA) for 1 h at 22–24°C. Fluorescence detection of 5hmC was performed using Alexa Fluor 488 (1:1000, Invitrogen, CA, USA) and of TH or PV using Alexa Fluor 594 (1:750, Invitrogen, CA, USA) for 1 h at 22–24°C. Images were captured and subsequently analyzed blind to age (the marked differences in neuronal morphology prevent blinding of neuronal cell types). Images were captured using a Leica confocal microscope (Leica Microsystems Inc., IL, USA) with identical settings for laser power, detector gain amplifier offset and pinhole diameter for each channel for TH-positive neurons across both ages, and the same for PV-positive neurons. TH-positive and PV-positive neurons were analyzed from the same biological samples, with four animals in the young group and four animals in the old group. Thirty-eight bilateral TH-positive neurons and 15 bilateral PV-positive neurons were analyzed per animal, giving a total of 152 TH-positive and 60 PV-positive neurons that were analyzed at each age. 5hmC fluorescence was measured using ImageJ (National Institutes of Health, MD, USA) as previously described [28]. Briefly, the freeform tool was used to calculate the area and integrated density of 5hmC for each TH-positive or PV-positive neuron with a nucleus in focus. The freeform tool was then used to measure the mean fluorescence of three background regions in close proximity to the 5hmC measured (three regions were selected to improve

accuracy). Each of the three background readings was multiplied by the area of the 5hmC tracing to give an integrated density of each of the three background readings dependent upon the size of the 5hmC tracing. These calculated integrated densities were then averaged and subtracted from the integrated density of the 5hmC tracing to get the corrected 5hmC fluorescence.

Quantitative RT-PCR

Total RNA was isolated using an AllPrep DNA/RNA Micro Kit (Qiagen, Hilden, Germany) and treated with DNase (Qiagen, Hilden, Germany). Total RNA (400 ng) was reverse transcribed by oligo-dT priming using SuperScriptIII reverse transcriptase (Invitrogen, CA, USA). Quantitative RT-PCR was performed on the resulting cDNA using TaqMan probes (Applied Biosystems, CA, USA) with primer pairs that are exon-spanning. All mRNA levels of genes of interest were normalized to *Hprt* mRNA levels. The sample size was ten animals per group (see LC-MS/MS and MS/MS/MS methods section above for more details).

Mass spectrometry measurement of metabolites

Concentrations of metabolites were determined blind to age and brain region by the Metabolomics Core at The Children's Hospital of Philadelphia, using a previously described isotope dilution approach [29,30]. Briefly, an aliquot of the sample was spiked with a mixture of ^{13}C -labeled organic acids. GC-MS measurement of ^{13}C isotopic abundance in each sample was then performed. Concentrations of metabolites in the sample were calculated as previously described [29]. Four animals are in the young striatum group, four animals are in the young SN group, six animals are in the old striatum group and six animals are in the old SN group. The striatum and SN samples were taken from the same animal, and therefore, ten animals in total were used for this analysis.

Statistics

Statistics were performed using Prism 6.0 (GraphPad Software, CA, USA) and RStudio (RStudio Inc., MA, USA). The D'Agostino & Pearson omnibus normality test was used to test normality ($p > 0.05$), and the F-test was used to test for equal variances ($p > 0.05$). The individual statistical tests performed for each experiment can be found in the figure legends.

Results

Age-related changes in DNA methylation are brain region-specific

We employed reversed-phase HPLC coupled with tandem mass spectrometry (LC-MS/MS and LC-

MS/MS/MS), along with the inclusion of stable isotope-labeled standards, to accurately measure global 5mC and 5hmC levels across aging (P90, young and P545, old) in the striatum and SN of male mice. We found that there is no statistically significant, age-dependent effect on total methylated cytosine (5mC plus 5hmC), total 5mC or total 5hmC (Figure 1A–C). However, the ratio of 5hmC to 5mC (5hmC/5mC) shows an age-dependent change, with an effect of age ($F_{1,36} = 8.358$; $p = 0.0065$) and an interaction effect ($F_{1,36} = 4.197$; $p = 0.0478$), as well as a significant increase over aging in the SN (~2% increase, $p = 0.0077$), but not in the striatum (Figure 1D). 5mC percentage of total methylated cytosine also shows an age-related change, with an effect of age ($F_{1,36} = 8.283$; $p = 0.0067$) and an interaction effect ($F_{1,36} = 4.166$; $p = 0.0486$), along with a significant decrease over aging exclusively in the SN (~2% decrease, $p = 0.008$) (Figure 1E). Additionally, 5hmC percentage of total methylated cytosine changes over aging, with an effect of age ($F_{1,36} = 8.283$; $p = 0.0067$) and an interaction effect ($F_{1,36} = 4.166$; $p = 0.0486$), together with an aging-related increase only in the SN (~2% increase, $p = 0.008$) (Figure 1F).

Cell type-specific changes in 5hmC across aging in the SN

Given the brain region-specific changes in DNA methylation observed in the SN over aging, we subsequently examined the extent to which changes in DNA methylation are specific to nigral dopaminergic neurons, the cell population that is susceptible to degeneration with advanced aging. PV-positive neurons of the SN were used as the comparison group since they are located adjacent to the dopaminergic neurons, allowing for the control of microenvironmental effects [31]. Using 5hmC immunostaining, we evaluated the levels of this epigenetic mark in dopaminergic neurons via co-staining for TH (an enzyme required for dopamine synthesis) or in PV-positive neurons via co-staining for PV in the SN of young and old mice (Figure 2A & B). Semi-quantitative analyses revealed an increase in 5hmC immunoreactivity in nigral TH-positive, dopaminergic neurons over aging ($p = 0.0003$), but not in nigral PV-positive neurons (Figure 2C).

Dnmt & *Tet* expression over aging in the striatum & SN

Since DNA methylation status is mediated by two families of enzymes, DNMTs and TETs, we measured mRNA levels of the family members that are highly expressed in the brain: *Dnmt1* and *Dnmt3a*, which methylate cytosine to 5mC, and *Tet2* and *Tet3*, which oxidize 5mC to 5hmC. *Dnmt1* ($p = 0.0432$) and *Dnmt3a* ($p = 0.0185$)

Figure 1. Age-associated changes in global DNA methylation levels over aging in the substantia nigra, but not striatum. Measurement of global total methylated cytosine (5mC plus 5hmC; Kruskal–Wallis rank sum test) (A), 5mC (Kruskal–Wallis rank sum test) (B), 5hmC (Kruskal–Wallis rank sum test) (C), the ratio of 5hmC to 5mC (two-way ANOVA with pairwise comparisons with Bonferroni correction, $**p < 0.01$) (D), 5mC percentage of total methylated cytosine (two-way ANOVA with pairwise comparisons with Bonferroni correction, $**p < 0.01$) (E) and 5hmC percentage of total methylated cytosine (two-way ANOVA with pairwise comparisons with Bonferroni correction, $**p < 0.01$) (F) by LC–MS/MS and LC–MS/MS across aging in the two brain regions. Solid bars indicate the young cohort, and dotted bars indicate the old cohort (mean $\pm$ standard error of the mean; $n = 10$ per group).

5hmC: 5-hydroxymethylcytosine; 5mC: 5-methylcytosine; dG: 2'-deoxyguanosine.

Figure 2. Selective increase in 5-hydroxymethylcytosine over aging in TH-positive, dopaminergic neurons, but not in PV-positive neurons, of the substantia nigra. Immunohistochemistry in substantia nigra sections of young and old mice showing 5hmC (green) in TH-positive, dopaminergic neurons (red) (A) and 5hmC in PV-positive neurons (red) (B). Cells that were analyzed are marked with a white arrowhead. (C) Quantification of 5hmC fluorescence intensity in young and old PV-positive and TH-positive, dopaminergic neurons. Solid bars indicate the young cohort, and dotted bars indicate the old cohort (mean $\pm$ standard error of the mean; $n = 152$ neurons from four animals (38 neurons per animal) for TH-positive, dopaminergic neurons per age group; $n = 60$ neurons from four animals (15 neurons per animal) for PV-positive neurons per age group; *** $p = 0.0003$; Dunn's multiple comparison test).

5hmC: 5-hydroxymethylcytosine; PV: Parvalbumin; TH: Tyrosine hydroxylase.

expression significantly increases in the striatum, but not in the SN, over aging (Figure 3A & B). *Tet2* and *Tet3* expression levels, on the other hand, do not change in either brain region over aging (Figure 3C & D).

Metabolites in the striatum & SN across aging

We next investigated the potential contribution of metabolites to the observed differences in global DNA methylation status between the striatum and SN over

Figure 3. An age-dependent increase of *Dnmt* expression is specific to the striatum. Relative changes in mRNA levels of *Dnmt1* (striatum, two-tailed t-test with Welch's correction; SN, two-tailed t-test) (A), *Dnmt3a* (striatum, Mann–Whitney test; SN, two-tailed t-test with Welch's correction) (B), *Tet2* (two-tailed t-test) (C) and *Tet3* (striatum, two-tailed t-test; SN, two-tailed t-test with Welch's correction) (D) as measured by quantitative RT-PCR with the young group serving as the reference for each brain region. Solid bars indicate the young cohort, and dotted bars indicate the old cohort (mean $\pm$ standard error of the mean; $n = 10$ per group; * $p < 0.05$). *Dnmt*: DNA methyltransferase; SN: Substantia nigra; *Tet*: Tet methylcytosine dioxygenase.

aging. The metabolite α -ketoglutarate (α -KG) is an obligatory cofactor for TET function [13]. TETs use molecular oxygen to catalyze oxidative decarboxylation of α -KG, creating a highly reactive intermediate that converts 5mC to 5hmC, as well as generating carbon dioxide and succinate as byproducts [32]. Recently, it was discovered that the intracellular ratio of α -KG to succinate (α -KG/succinate) regulates TET activity, which in turn alters DNA methylation levels [33]. Therefore, we evaluated the ratio of α -KG/succinate over aging in both brain regions by using a highly quantitative method that employs GC–MS and isotope-labeled standards. In addition to α -KG and succinate, we measured lactate, citrate and fumarate to ensure that any differences observed in the α -KG/succinate ratio were not due to changes in metabolite flux through the mitochondrial tricarboxylic acid cycle. We found that the levels of all metabolites measured do not statistically differ across aging in either brain region (Figure 4A–E & G–K). However, we

found that although the α -KG/succinate ratio remains unchanged across aging in the striatum (Figure 4F), it significantly decreases in the SN (Figure 4L).

Discussion

Although few in number, previous studies suggest that changes in global 5mC and 5hmC levels occur in the brain over the aging process [9,16,17,19–21]; however, the findings have been inconsistent, likely due to the inherent differences in the methods employed for detecting 5mC and 5hmC, and the brain regions assessed. Given the different biological functions of 5mC and 5hmC, and the discrepancies regarding age-associated changes of these two major forms of DNA epigenetic modifications in the brain, we used a highly sensitive LC–MS/MS and LC–MS/MS/MS method to measure 5mC and 5hmC levels from the striatum and SN at two different ages (P90, young and P545, old). We specifically chose the SN given its enhanced vulnerability to aging, and the striatum was

Figure 4. Decrease in the α -ketoglutarate to succinate ratio in the substantia nigra, but not striatum, over aging. Quantification of lactate (two-tailed t-test) (A & G), citrate (two-tailed t-test) (B & H), fumarate (two-tailed t-test with Welch's correction) (C & I), α -KG (two-tailed t-test) (D & J), succinate (two-tailed t-test) (E & K) and α -KG to succinate ratio (two-tailed t-test) (F & L) as measured by MS with isotope-labeled standards. Solid bars indicate the young cohort, and dotted bars indicate the old cohort (mean $\pm$ standard error of the mean; n = 4 for young group and n = 6 for old group; *p < 0.05). α -KG: α -ketoglutarate.

selected as the brain region for comparison since this is the area to which dopaminergic neurons of the SN project [3].

We found selective changes in these two epigenetic marks over aging in the SN, but not striatum. Specifically, we observed an increase in the ratio of 5hmC/5mC, which correlated with a 2% decrease in the 5mC percentage of total methylated cytosine and a 2% increase in the 5hmC percentage of total methylated cytosine. This suggests that although the absolute levels of 5mC and 5hmC do not significantly change, the percentage of sites that are converted from 5mC to 5hmC increases in the SN over aging. Although a 2% change in DNA methylation seems marginal, this could account for a change at approximately 1 million cytosines, given that there are approximately 50 million cytosines that are methylated in the mouse genome [17], and therefore, the changes observed are substantial and potentially biologically relevant. When we examined two neuronal types in the SN, we found a significant increase in 5hmC in the TH-positive, dopaminergic population, but not in neighboring PV-positive neurons. Despite the limitation of a small sample size ($n = 4$ animals per group in the cell type-specific analyses), these findings suggest that changes in global levels of 5hmC during aging are both brain region- and cell type-specific. Since 95% of the striatum is composed of GABAergic medium spiny neurons [34] and since PV-positive neurons of the SN are GABAergic, our findings suggest that GABAergic neurons maintain their DNA methylation status across aging, independent of the brain region. This study elucidates a novel age-associated molecular change in the vulnerable dopaminergic population and indicates that changes in 5hmC are contingent upon the intracellular molecular environment, rather than extracellular environment (i.e., brain region).

Therefore, we subsequently examined the molecular environment that could underlie the difference in DNA methylation status between the striatum and SN over aging and made two discoveries. First, we found an age-associated increase in both *Dnmts* that are highly expressed in the brain, *Dnmt1* and *Dnmt3a*, in the striatum, but not the SN. It is plausible that the increase in *Dnmts* over aging in the striatum contributes to the stability of the ratio of 5hmC/5mC, unlike the SN. Our second finding was the decrease in the α -KG/succinate ratio in the SN, but not striatum, albeit with a small sample size ($n = 4$ for the young group and $n = 6$ for the old group). This suggests that brain region-specific metabolic changes over aging could contribute to region-specific changes in DNA methylation. Since we measured steady state levels of

metabolites, this finding supports a model in which TETs are more active in the SN of old animals given the relative depletion of the α -KG pool, which is used by TETs to oxidize 5mC to 5hmC, and an increase in succinate, which is a byproduct of TET activity. Importantly, this result is consistent with our finding of an increase in the 5hmC/5mC ratio over aging in the SN, but not striatum.

Additional research is necessary to define a potentially causative role of the enzymatic expression and/or metabolic ratio alterations with the age-related differences in DNA methylation status between the striatum and SN. There are other regulators of TETs that were not assessed in this work that could also contribute to the brain region-specific DNA methylation differences across aging, such as iron levels, ascorbic acid levels and calpain activity [13,35–37]. Additionally, determining the genomic loci of these methylation changes, and potential consequential alterations in gene expression [38], will aid in elucidating how normal aging predisposes nigral dopaminergic neurons to cell death.

Conclusion

This work indicates that an increase in TET activity, as indicated by the alteration in the α -KG/succinate ratio, may account for the selective, age-dependent increase in the ratio of 5hmC/5mC in the SN. This work also supports that a brain region less susceptible to aging, like the striatum, is able to employ protective mechanisms, such as increasing *Dnmt* expression, to stabilize DNA methylation profiles over aging. Taken together, our findings suggest that distinct cellular and molecular environments support the different DNA methylation states observed between the striatum and SN over aging, and specifically within dopaminergic neurons, implicating this epigenetic mechanism in the enhanced vulnerability of this neuronal population over aging.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

Executive summary

There are age-associated changes in DNA methylation in the substantia nigra, but not striatum

- Aging does not significantly affect any of the DNA methylation parameters assessed in the striatum.
- In the substantia nigra, on the other hand, the ratio of 5-hydroxymethylcytosine to 5-methylcytosine (5hmC/5mC) significantly increases over aging, suggesting that although the absolute levels of 5mC and 5hmC do not significantly change, the percentage of sites that are converted from 5mC to 5hmC increases.

There is a selective increase in 5hmC over aging in tyrosine hydroxylase-positive, dopaminergic neurons, but not parvalbumin-positive neurons, of the substantia nigra

- Our results indicate that changes in 5hmC over aging are cell type-dependent and contingent upon the intracellular molecular environment, rather than the extracellular environment (i.e., brain region).

There is an age-dependent increase in *Dnmt* expression in the striatum, but not substantia nigra

- In the substantia nigra over aging, *Tet2*, *Tet3*, *Dnmt1* and *Dnmt3a* mRNA levels remain unchanged.
- In the striatum, although *Tet2* and *Tet3* mRNA levels are stable across aging, *Dnmt1* and *Dnmt3a* levels increase, suggesting that an increase in *Dnmts* contributes to the stability of the ratio of 5hmC/5mC over aging, unlike the substantia nigra.

We found a substantia nigra-selective reduction in the α -ketoglutarate to succinate ratio over aging

- Although the α -ketoglutarate to succinate ratio remains unchanged across aging in the striatum, it significantly decreases in the substantia nigra.
- Since we are measuring steady state levels of the metabolites, this finding supports a model in which ten-eleven translocation (TET) enzymes are more active in the substantia nigra of old animals given the relative depletion of the α -ketoglutarate pool, which is used by TETs to oxidize 5mC to 5hmC, and an increase in succinate, which is a byproduct of TET activity.

Conclusion

- Our findings suggest that distinct cellular and molecular environments account for the different DNA methylation states observed between the striatum and substantia nigra over aging, and therefore, implicate this epigenetic mechanism in the enhanced vulnerability of nigral dopaminergic neurons over aging.

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