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Tyrosine Hydroxylase Inhibition in Substantia Nigra Decreases Movement Frequency

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Abstract

Reduced movement frequency or physical activity (bradykinesia) occurs with high prevalence in the elderly. However, loss of striatal tyrosine hydroxylase (TH) in aging humans, non-human primates, or rodents does not reach the ~ 80% loss threshold associated with bradykinesia onset in Parkinson's disease. Moderate striatal dopamine (DA) loss, either following TH inhibition or decreased TH expression, may not affect movement frequency. In contrast, moderate DA or TH loss in the substantia nigra (SN), as occurs in aging, is of similar magnitude (~ 40%) to nigral TH loss at bradykinesia onset in Parkinson's disease. In aged rats, increased TH expression and DA in SN alone increases movement frequency, suggesting aging-related TH and DA loss in the SN contributes to aging-related bradykinesia or decreased physical activity. To test this hypothesis, the SN was targeted with bilateral guide cannula in young (6 months old) rats, in a within-subjects design, to evaluate the impact of nigral TH inhibition on movement frequency and speed. The TH inhibitor, α-methyl-*p*-tyrosine (AMPT) reduced nigral DA (~ 40%) 45–150 min following infusion, without affecting DA in striatum, nucleus accumbens, or adjacent ventral tegmental area. Locomotor activity in the open-field was recorded up to 3 h following nigral saline or AMPT infusion in each test subject. During the period of nigra-specific DA reduction, movement frequency, but not movement speed, was significantly decreased. These results indicate that DA or TH loss in the SN, as observed in aging, contributes as a central mechanism of reduced movement frequency.

Keywords Aging · Parkinson's · Substantia nigra · Tyrosine hydroxylase · Movement · Locomotor

The nigrostriatal dopamine (DA) pathway is critical for movement generation [1–3]. Increased DA release occurs in both substantia nigra (SN) and striatum upon movement initiation [4] and nigrostriatal neuron activity modulates the probability of future movement [5]. Systemic injection of a tyrosine hydroxylase (TH) inhibitor reduces locomotor activity [6]. To the converse, increased extracellular DA from genetic deletion of DA transporter [7] produces hyperlocomotion [8]. A general decline in motor frequency or ability to initiate movement comprises bradykinesia [9, 10], a cardinal symptom of Parkinson's disease (PD) and is also prevalent in the elderly, affecting 30–50% by age 80 [11–15]. Loss of TH at bradykinesia onset in PD is ~ 80% in striatum and 40% in the SN [16], with similar loss estimated in PD patients [17–19]. However, in aged humans, non-human primates, and rodents alike, DA or TH loss varies from 0 to 60% in striatum [20–31], never reaching the 80% threshold of TH or DA loss in striatum that generally coincides with onset of motor impairment.

It is not only in aging studies where an apparent incongruity between striatal DA function or TH expression against motor function exists. Preclinical and clinical PD studies have also revealed this potential incongruity [32–44]. Some studies show that striatal DA is associated with motor function [45–47], but these incongruities suggest that specific locomotor parameters may be affected by DA neurotransmission outside of striatum. With respect to movement initiation, even 60% striatal TH or DA loss, the most reported to date in aging studies [22, 29], does not produce bradykinesia [16] in a PD model. Decreased

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striatal DA or TH less than the PD threshold in young rats also does not reduce movement frequency [48]. Thus, although aging produces number of changes in the dynamics of DA regulation associated with the nigrostriatal pathway, including decreased DA D_1 receptor expression [48], it is possible that aging-related decreases in movement frequency may not necessarily be strictly striatal in origin.

Loss of nigral neurons is independently associated with reduced physical activity proximate to death in older adults [49]. However, the possibility that loss of DA or TH in the SN similar to that in aging could contribute to motor decline has not been systematically interrogated. However, several lines of evidence support this possibility. Nigral DA release is well-established [50, 51], and activation of DA D_1 receptors therein can increase GABA release from striatonigral neurons to inhibit GABAergic output from SN pars reticulata [52, 53]. To date, few studies have isolated nigral DA neurotransmission, autonomously from striatal DA neurotransmission, to evaluate impact on locomotor function. However, evidence supports modulation of nigral DA neurotransmission affects motor function [37, 54, 55]. Reversing the aging-related loss of TH expression and DA content in SN alone [31] increases movement frequency and vertical activity in aged rats [39]. Nigral TH or DA loss in aged rodents [26, 30, 31, 56, 59], non-human primates [24, 25, 27], and humans [17, 57, 58] is of similar magnitude (~40%) in comparison to TH loss at bradykinesia onset in primate PD [16] and in human PD [17–19]. Taken together, aging-related loss of TH in SN is a prime candidate mechanism that produces one or more of the Parkinsonian signs of aging.

The striatum and SN comprise contiguous components of the nigrostriatal neuron. However, the regulation of DA or TH expression, phosphorylation, and activity can function autonomously between these two nigrostriatal compartments [27, 31, 39, 40, 48, 59–61]. In this study, aging-related nigral DA loss was isolated from the striatum to test the hypothesis that the loss of DA in SN alone would reduce locomotor activity. Guide cannula were precisely implanted to pharmacologically inhibit TH in the SN of young rats, which are presumably devoid of age-related central or peripheral deficiencies potentially contributing to motor decline. We used a within-subjects design to evaluate locomotor activity following TH inhibition versus no inhibition in SN, as we previously evaluated in striatum [48].

Methods

Animals

Male Brown-Norway Fischer F_1 hybrid (BNF) rats ~5–6 months of age were obtained from a colony maintained by the NIA and acclimated at least 1 week prior to surgical procedures. All procedures were approved by the Animal Care and Use Committees at LSUHSC-Shreveport and UNTHSC-Fort Worth. The colony was maintained on reverse light-dark cycle (lights off at 0700, on at 1900) throughout the study. Rat surgeries were either survival surgery, to implant guide cannula used in the locomotor component of the study, or non-survival surgery, to determine the extent and duration of AMPT-mediated and SN-specific reduction of DA tissue content at four time points (45, 90, 150, or 180 min) following unilateral infusion in the SN. Rats were anesthetized by either sodium pentobarbital (50 mg/kg) or 2–3% isoflurane. Custom-made double guide cannula (PlasticsOne, Roanoke, VA) were implanted targeting SN to enable repeated bilateral infusions of sterile saline or AMPT via infusion cannula. To optimally target SN in the rostral-caudal extent, the anterior posterior (AP) coordinates to Bregma were −5.7 AP. The distance between the two stainless steel cannula was 5.0 mm, giving medial lateral coordinates of ±2.5-mm ML to target the ventral-lateral SN. The guide cannula length was 7.5 mm, terminating ~0.1–0.2 mm into the dorsal-lateral SN. The infusion cannula was 28 gauge with a +1.0-mm projection beyond the termination point of the guide cannula for a total depth of 8.5-mm DV (Fig. 1a). This "stepped" configuration restricts backflow along the cannula and maximizes the volume of distribution of infused substrates [62]. A similar setup, with termination to 8.6 mm DV, has been used previously to evaluate extracellular DA in the SN [32]. Two small screws implanted into the skull served to anchor dental cement applied to secure guide cannula. Guide cannula were slowly lowered into the brain (~0.5 mm/min) to minimize tissue damage. Recovery was at least 5 days prior to locomotor testing. Infusion cannula were introduced in awake rats via implanted guide cannula just prior to locomotor testing to deliver sterile saline (vehicle) or AMPT (in sterile saline).

Optimal Infusion Volume to Selectively Target Substantia Nigra

We first conducted infusions with visualizing dye to optimize the infusion volume needed to infuse the SN along its rostral-caudal extent, but minimize affecting adjacent DA neuropil in the ventral tegmental area (VTA). Bromophenol blue solution was infused into SN (AP −5.7, ML ±2.5, DV 7.5, 8.0, or 8.5 mm) in volumes of 1, 2, 3, or 4 μl. Ten minutes following infusion, rats were perfused transcardially with ice-cold saline followed by 4% paraformaldehyde; the brain was removed and frozen for cryostat cutting. Our results indicated the following critical experimental outcomes: (1) the entire SN was stained when the termination depth of the cannula needle was 8.5-mm DV; termination points dorsal to 8.5 mm showed less penetration of the dye in the SN and greater susceptibility to backflow and (2) volumes of 1 μl proved to provide too little coverage along the rostral-caudal extent, and volumes of 4 μl appeared to encroach on the adjacent VTA. Volumes of 2 or

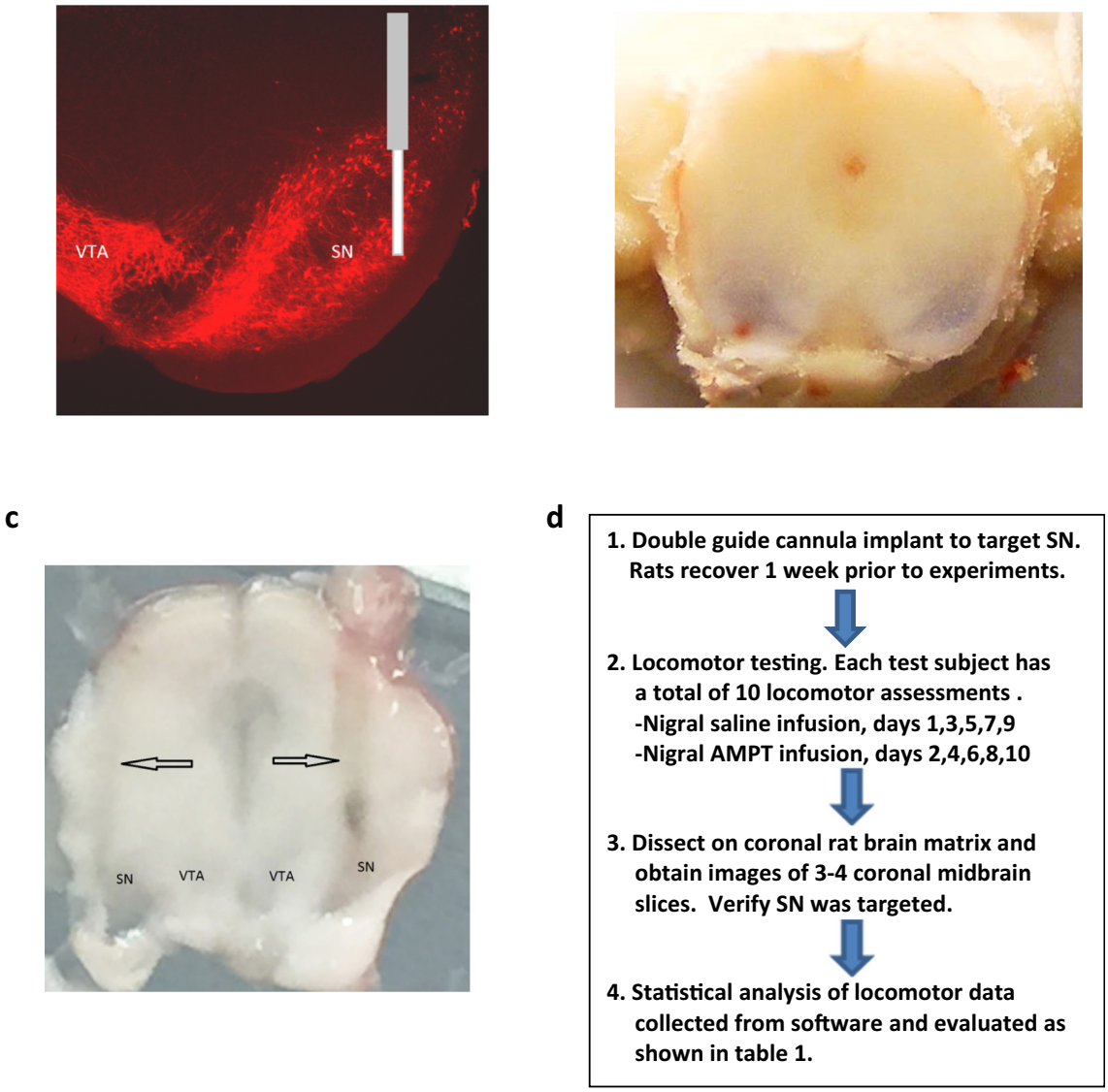

Fig. 1 Rationale for the approach to target the substantia nigra. **a** TH-positive neurons of the SN, adjacent VTA, and relation to cannula placement. The image of ser31 TH phosphorylation illustrates the dorsal and ventral tiers of the SN, clearly evident by cell body distribution and dendrites that penetrate between these two tiers throughout the SN. The densely innervated VTA is evident at the left side of the image. The stepped approach of infusions into the SN utilizes a guide cannula that terminates at the dorsal boundary of the lateral-dorsal portion of the SN, 7.5 mm below the cannula pedestal. The smaller diameter 28 gauge infusion cannula is inserted into the surgically attached guide cannula on locomotor testing days to deliver either vehicle (sterile saline) or AMPT. The infusion cannula extends 1.0 mm beyond the termination point of the guide cannula, terminating in the ventral-lateral SN. The drawn cannula assembly is reflects the targeted placement at coordinates, relative to Bregma, − 5.7 AP, 2.5 ML, and − 7.5 (guide)/− 8.5 (infusion) DV in relation to the coordinates of the representative image of the midbrain. **b** Dye infusion to illustrate coverage in targeting SN. 0.1% bromophenol blue solution was infused into anesthetized rats at 1-, 2-, 3-, or 4-μl volumes at the designated coordinates. Following a 10-min waiting period after infusion (identical to the protocol in neurochemical and locomotor experiments described herein), rats were perfused and brain was frozen for cryostat sectioning at 100-μm increments. The image in the figure depicts staining from 3- versus 2-μl infusion volumes into the SN (left and right, respectively) at 0.8-mm distance posterior from the track marks where the infusion occurred. The blue dye fills the substantia nigra in both infusion volumes, but subsequent work indicated the 3-μl infusion reduced DA tissue content not only in SN but also in VTA (Salvatore and Pruett, 2012), whereas the 2-μl infusion volume was verified to decrease DA in SN and not VTA. **c** Midbrain dissection following completion of locomotor assessment in qualified test subject. Following the completion of locomotor assessment for each test subject, dissection images were evaluated to verify that the guide cannula track marks were present above the SN (identified by arrows) with no evidence of tissue scarring or hematoma within the dark pigmented area that defines the SN

3 μl stained the SN, 2–3 mm in the rostral-caudal extent, with but possible encroachment of dye in the area of the VTA with 3 μl (Fig. 1b. To verify, the chosen volume (2.0 μl for SN) had compartment- and target-specific reduction of DA in the SN;

the adjacent VTA was also dissected and analyzed for DA tissue content in AMPT neurochemistry experiments (as further described).

Dopamine Tissue Content Assessment of AMPT Efficacy

In a similar-aged cohort, we determined the timing and duration of AMPT to reduce DA tissue content in the SN. Twenty-six-gauge beveled needles infused sterile saline in one hemisphere and AMPT (prepared in sterile saline) at the same respective coordinates in SN in the other hemisphere. These needles remained in the targeted tissue for 10 min following infusion, and dissections occurred at four time frames (45, 90, 150 or 180 min), to be within the 180-min time period of locomotor activity assessment in the open-field. AMPT (as methyl ester HCl) purchased from Sigma-Aldrich (St. Louis, MO, cat. # M3281) or TCI-EP (Tokyo, cat. #M1373) was dissolved in sterile saline prior to infusion. The quantity of AMPT (1.4 nmol, or 2.8 nmol as the methyl ester) used was based upon previous work from ours and other laboratories [63], which has established its efficacy to reduce DA tissue content or extracellular DA levels in the midbrain or striatum via delivery into the CNS [48, 59]. AMPT also reduces stimulated DA release, as shown in voltammetry studies [64, 65], which indicates that inhibition of DA biosynthesis produces an expected decrease in DA release . We also verified our previous report that AMPT produced a nigrostriatal compartment-specific decrease in DA tissue content in SN 90 min following AMPT infusion [59]. This study also indicated that AMPT reduced DA to a similar magnitude seen in aged (30–50%) rodents and non-human primate aging studies [24, 27, 30, 39] and in the BNF strain used in this study [26, 31]. We also verified that 2-μl AMPT (at the same targeted coordinates relative to Bregma used for the locomotor experiments, (AP, −5.7; ML, 2.5; DV −8.5 mm) decreased DA tissue content only in SN and not the adjacent VTA.

DA tissue content was evaluated by electrochemical detection by HPLC, as further described in our study of striatal DA [48]. Briefly, DA was determined by interpolation against a standard curve ranging from 1.56 to 800 ng/ml, which encompassed the range of DA concentrations recovered in samples from all four DA regions when using an injection volume of 10 μl of sample recovered from tissue homogenized in perchloric acid solution.

Locomotor Testing

Experimental Design Up to 50% difference in mean baseline locomotor activity may occur among BNF rats in a cohort, along with variation in daily activity within individual rats [31, 39, 40, 48]. To account for these sources of variability, locomotor activity was evaluated once daily for a total of five assessments following infusion of sterile saline or AMPT. The infusion type was alternated in sequence (saline day 1, AMPT day 2, saline day 3, etc. (Fig. 1d)) on separate testing days until the 10 locomotor testing sessions were completed. The locomotor activity on saline infusion days control for any impact of the volume of saline alone (used to deliver AMPT on specified test days) upon the extracellular DA concentrations and represent each rat's baseline locomotor activity for comparison of locomotor parameter results following AMPT. We have previously used five testing sessions in longitudinal studies to establish differences in treatment effects on aging-related motor decline [31, 39, 40, 48, 66]. The experimental design presented the same environment for each testing session and testing was done in the colony to minimize possible influences of novel environment. This exact approach has been used to determine the impact of TH inhibition in the striatum on locomotor function [48].

Infusion Protocol Infusion cannula were 1.0 mm greater in length than the guide cannula to ensure optimal volume of distribution and to be minimally invasive. The infusion rate was 1.0 μl per min using a microsyringe pump and Hamilton syringe to operate as the medium for fluid delivery. ~400–500 μl of total volume of sterile saline or AMPT dissolved therein was introduced into two 26-gauge tubing connecting the two Hamilton syringes with the bilateral infusion cannula. Verification of infusion into the targeted CNS region was made by observing air bubble movement located in the infusion line located hear the mounted syringe on the syringe pump, being far away the infusion cannula. Saline or AMPT was delivered in 2-μl volume, at 1 μl/min, into the SN. The infusion cannula remained in the guide cannula for 10 min following infusion before removal. Open-field locomotor activity was measured for up to 180 min following infusion and conducted within the rat colony holding room to maintain the home cage environment.

Evaluation of Open-Field Locomotor Activity Rats were placed into locomotor testing chambers (Opto Varimex 4 Animal Activity Monitoring System and AutoTrack System software, Columbus Instruments, Columbus, OH) at 0 (group 1) or 30 min (group 2) after the 10 min post-infusion period (Table 1). Regardless of whether the rats were introduced into the chamber at 0 or 30 min after infusion, the magnitude of each of the three locomotor parameters in the first 20 min in the chamber was 1.6- to 6-fold greater compared to the second 20 min. This was observed following vehicle or AMPT infusion. Previous work indicates that AMPT does not affect DA release 20 min following AMPT infusion in DA terminal fields regions [63]. Therefore, given that the novelty influence on motor function occurred in the first 20 min after introduction into the locomotor chamber, we delayed

Table 1 Four epochs of evaluation of locomotor results matched to timeline of AMPT-mediated DA reduction

Epoch (time interval)	Group 1 time bins	Group 2 time bins	% DA reduction (min)
1 (0–50 min)	0–20, 21–40	31–50	NA
2 (41–90 min)	41–60, 61–80	51–70, 71–90	43% (***45***)
3 (81–150 min)	81–100, 101–120, 121–140	91–110, 111–130, 131–150	39% (***90***) 34% (***150***)
4 (141–180 min)	141–160, 161–180	151–170	No change (***180***)

After nigral vehicle-(testing days 1, 3, 5, 7, 9) or AMPT-infusion (testing days 2, 4, 6, 8, 10), rats were placed into the locomotor chambers either immediately (group 1) or 30 min later (group 2). Locomotor results were collected by automated software in 20-min time bins, as designated above by the min elapsed past infusion of vehicle or AMPT. After confirmation of successful targeting of substantia nigra (Fig. 1c), 20-min time bin results were then summated into one of four epochs to analyze locomotor activity following vehicle or AMPT infusion. These epochs were temporally aligned with AMPT-mediated DA reduction in the substantia nigra in a separate cohort of same age at four time points (45, 90, 150, and 180 min) (results shown in Fig. 2). The percent decrease in DA tissue content following nigral AMPT infusion is designated by bold italics in parentheses (in min). The sum of ambulatory counts, distance, (or the average in the case of movement speed) over the five sessions following saline or AMPT for each time bin assigned into one of four epochs generated a sum or mean value for each epoch. This value was used to evaluate locomotor function following saline or AMPT, matching the time past infusion for each rat, in repeated measures two-way ANOVA

introduction of group 2 rats into the chamber until 30 min after infusion to determine if AMPT affected locomotor function during the novelty period of locomotor activity assessment. We did not observe any difference in any locomotor parameter following AMPT versus vehicle infusion ($t = 0.669$, ns).

Group 1 and group 2 data were collapsed and analyzed so that the duration of time following vehicle or AMPT infusion was matched to evaluate locomotor parameters. Given that the introduction into the chamber differed by 30 min between the two groups and that locomotor data was collected in 20-min bins by the software, there was a 10-min overlap (Table 1) in four epochs used for evaluation. We had previously established that AMPT can reduce DA tissue content in the SN and not striatum, at 90 min following infusion [60]. In this study, time periods of 45, 90, 150, and 180 min were used to evaluate the impact of AMPT-mediated nigra-specific DA reduction in another cohort to increase the accuracy of matching the time of onset and longevity of DA reduction specifically in the SN against the locomotor outcomes. Locomotor results from the 20 min time bins were therefore combined into one of four time intervals (or epochs) to be congruent with the four assessments of DA tissue content conducted in the separate cohort (Table 1). These four time intervals of locomotor analysis were as follows (Table 1); epoch 1: 0–50 min, epoch 2: 41–90 min, epoch 3: 81–150 min, and epoch 4: 141–180 min.

The 30-min delay in entering rats into the chamber (group 2) did not affect ambulatory counts following vehicle infusion over time compared to group 1 (statistics: 2 way ANOVA matched for test subjects; group 1 vs group 2 $F_{(1,\ 7)} = 0.034$, $p = 0.86$; epoch $F_{(3,\ 21)} = 56.77$, $p < 0.0001$). No significant differences in ambulatory counts were observed between the two groups at any of the four epochs following vehicle infusion.

Target Validation after Completion of Locomotor Testing After completion of locomotor testing, accurate targeting of the guide cannula to the SN was verified before statistical analysis of the collected locomotor data for each rat. Using a rodent brain matrix, coronal slices of the targeted tissue were obtained to capture images of two to four coronal slices of the midbrain and later evaluated using the following two criterion: (1) evidence of track marks dorsal to the SN typically found in the overlying cortical tissue or below and (2) no evidence of hematoma or scarring within the SN for either hemisphere (Fig. 1c). 11 rats passed criterion for valid targeting of the SN without evidence of scarring or hematoma within the SN. Application of the Grubb's outlier test (alpha at 0.05) to locomotor parameter results and % difference in ambulatory counts in AMPT versus vehicle in each of the 11 rats eliminated two rats. The statistical evaluation of locomotor results represents nine test subjects in a repeated measures experimental design.

Statistics:

Locomotor Assessment A repeated measures two-way ANOVA was used to analyze each parameter (ambulatory counts, movement speed, and total distance) to determine three outcomes: (1) whether significant differences in the magnitude of each parameter occurred over the designated time intervals following infusion, (2) an overall treatment effect of AMPT vs vehicle over all time intervals, and (3) evidence of interaction of time following infusion × AMPT. The within-subjects design matched the mean of the sum of locomotor parameter results obtained from the time bins (as shown in Table 1) over the five assessments following time elapsed past infusion of saline versus AMPT for each of the four epochs following infusion for each test subject. A post hoc

two-tailed paired t test evaluated each locomotor parameter following nigral AMPT versus vehicle infusion within each of four epochs (shown in Table 1).

We also evaluated if the conduct of the five locomotor assessments following vehicle or AMPT affected the locomotor outcome differences following AMPT versus vehicle infusion for ambulatory counts. The values for mean or sum of ambulatory counts obtained following vehicle and AMPT infusion, during the period of confirmed nigral DA reduction by AMPT (41–150 min), for each testing day was evaluated by two-way repeated measures ANOVA with testing day (1–5) compared against overall treatment.

DA Reduction Assessment The studies were designed such that one hemisphere of the targeted region received AMPT and the contralateral hemisphere received saline. DA tissue content levels were compared in the AMPT-infused hemisphere of the SN, the adjacent DA neuron center (VTA), striatum, and nucleus accumbens versus the DA tissue content result obtained from the saline-infused side. A paired t test determined whether significant differences were observed in all four DA regions (striatum, SN, nucleus accumbens, VTA) following the infusion. The Grubb's test identified any outlier in the neurochemistry or locomotor data sets with alpha set to < 0.05. All statistical determinations used GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com.

Results

Nigrostriatal Compartment Specificity and Longevity of AMPT-Mediated DA Reduction in SN

The extent and duration of nigral AMPT infusion to reduce DA tissue content was evaluated at four time points (45, 90, 150, and 180 min). DA tissue content in AMPT-infused SN was reduced at 45 (Fig. 2a), 90 (Fig. 2b) (repeating our previous observation [59], and at 150 min (Fig. 2c). However, AMPT was no longer effective to reduce DA in the SN 180 min following nigral infusion (Fig. 2d). Therefore, AMPT delivery to the SN was confirmed to reduce DA tissue content 34–43% at 45–150 min following infusion.

The reduction of DA tissue content was specific for the SN, as DA tissue content was not affected in the striatum, adjacent VTA, or nucleus accumbens 90 min following infusion (Fig. 2e) or at 150-min post-nigral AMPT infusion in the dorsal striatum ($t = 1.71$, $p = 0.16$, df 4) or VTA ($t = 0.38$, $p = 0.73$, df 3).

These data establish the duration and specificity of DA reduction by AMPT-mediated TH inhibition in the SN of young adult rats, with the SN-specificity in agreement with previous results [59]. The magnitude of DA reduction (~ 40%) in the SN by AMPT was also similar to that reported in aged BNF or Sprague-Dawley rats [26, 30, 31], as well as non-human primates and TH loss in human. Furthermore, the quantity of DA recovered in the SN following nigral AMPT infusion at 45, 90, and 150 min was similar to that of aged BNF rats (5 ng/mg protein) [31, 39].

Nigral AMPT and Impact on Locomotor Function: Ambulatory Counts

Over the duration of locomotor evaluation, bilateral AMPT infusion into the SN decreased movement frequency (as ambulatory counts) compared to movement frequency following nigral vehicle (saline) infusion (Fig. 3a). Movement frequency was significantly reduced in two of the four epochs (41–90 and 91–150 min following nigral infusion) (Fig. 3a), which coincided with the timeline of AMPT-mediated DA reduction in the SN (Fig. 2).

During the testing session, there was a significant decrease in ambulatory counts as a function of time after infusion (Fig. 3a). Between epochs 2 and 3, ambulatory counts decreased 35% in epoch 3 versus epoch 2 following the bilateral infusion of vehicle (saline) ($t = 4.19$, $p = 0.003$, $df = 8$). However, despite this decrease in movement frequency during the third epoch, ambulatory counts were still significantly reduced following nigral AMPT infusion versus the respective movement frequency following vehicle within the third epoch. Within the 41–150-min timeline of confirmed DA reduction in the SN, movement frequency was reduced 35% following nigral AMPT infusion (Fig. 3b).

Given the experimental design had five locomotor sessions following saline or AMPT infusion to account for possible daily variability in locomotor activity, we evaluated if the number of testing sessions affected the AMPT effect observed. There was a significant effect of testing session number over the course of experiments, accounting for 27% of the total variation by either the average mean or sum of ambulatory counts (by mean $F_{(4, 32)} = 10.67$, $p < 0.0001$; by sum $F_{(4, 32)} = 10.17$, $p < 0.0001$). AMPT infusion accounted for 9–10% of the total variation (by mean $F_{(1, 8)} = 20.77$, $p = 0.0019$; by sum $F_{(1, 8)} = 24.68$, $p = 0.0011$). There was no significant interaction between testing session number × AMPT infusion. The mean sum of ambulatory counts following AMPT infusion was reduced by 47, 49, 36, 20, and 24% against ambulatory counts following vehicle infusion over the 5 testing days, respectively.

Nigral AMPT and Impact on Locomotor Function: Movement Speed

Contrary to the observation of reduced movement frequency following nigral AMPT vs vehicle infusion, average movement speed was not affected by nigral AMPT infusion compared with that following vehicle infusion at any point in time of evaluation (Fig. 4a). These results suggest that nigral TH

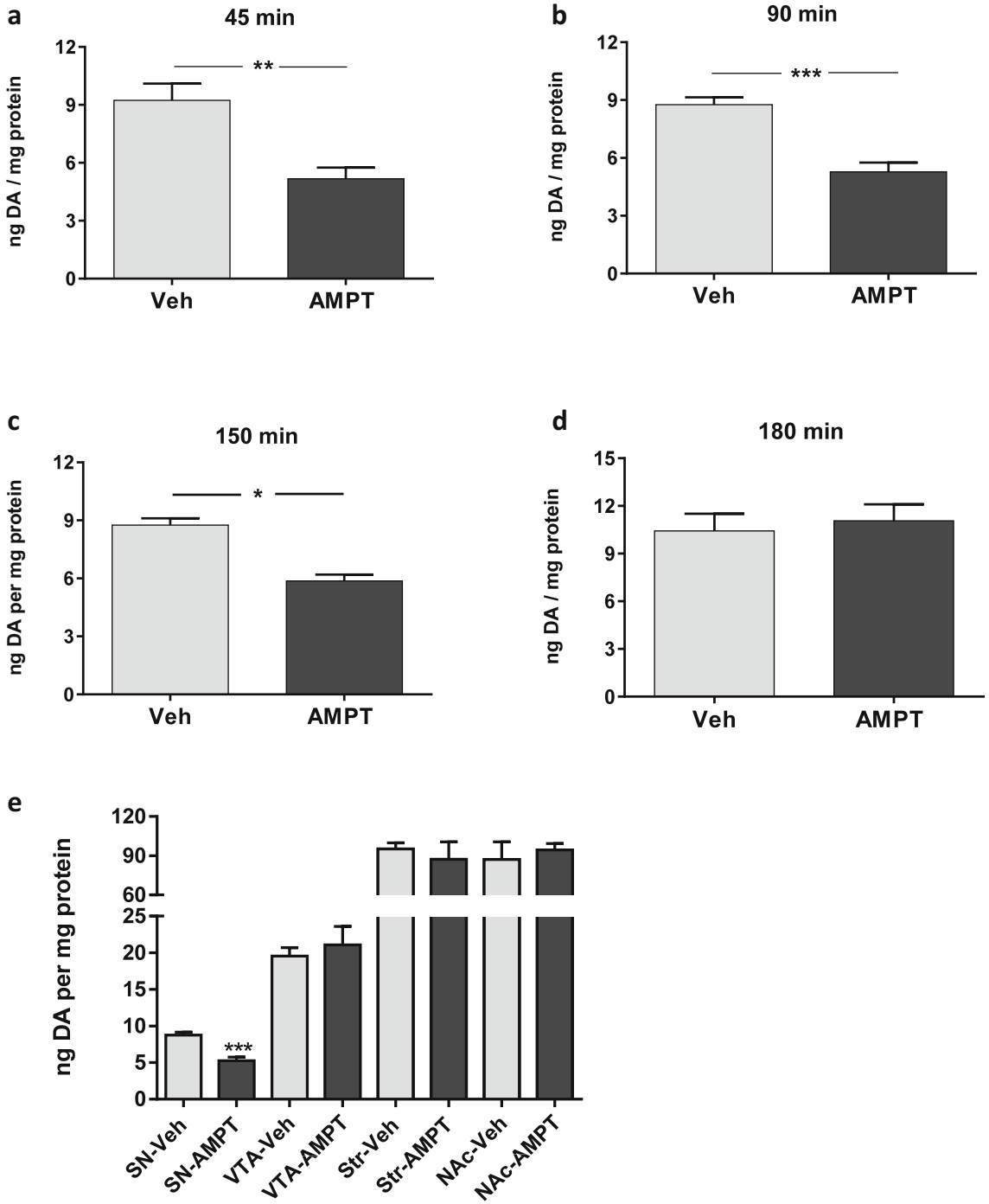

Fig. 2 Timeline of nigral AMPT infusion impact on DA tissue content: DA values are expressed as ng DA/mg protein. **a** SN: 45 min after AMPT infusion. 1.4-nmol AMPT decreased DA tissue content in the SN by 43% 45 min following infusion ($t = 3.78$, $^{**}p = 0.002$, df = 13). **b** SN: 90 min after AMPT infusion. 1.4-nmol AMPT decreased DA tissue content in the SN by 39% overall 90 min following infusion (vehicle 8.8 ± 0.4; AMPT 5.3 ± 0.6) ($t = 5.75$, $^{***}p = 0.0007$, df = 7). **c** SN: 150 min after AMPT infusion 1.4-nmol AMPT decreased DA tissue content in the SN by ~ 34% overall 150 min following infusion (vehicle 8.8 ± 0.4; AMPT 5.3 ± 0.6) ($t = 5.79$, $^{*}p = 0.010$, df = 3). **d** SN: 180 min after infusion 1.4-nmol AMPT was no longer effective to reduce DA tissue content in the SN after 180 min following infusion (vehicle 10.4.8 ± 1.1; AMPT 11.1 ± 1.0) ($t = 0.43$, ns, df = 6). **e** Specificity of nigral DA tissue content reduction following nigral AMPT infusion. AMPT infusion into the SN decreased DA only in the SN (statistical outcome shown in this figure, part **b**) and not in the adjacent VTA ($t = 0.56$, ns, df = 7), the striatum ($t = 0.62$, ns, df = 7), or nucleus accumbens ($t = 0.66$, ns, df = 7)

function is dissociated from the locomotor parameter of movement speed. In other longitudinal assessments of motor function in aging rats, movement frequency and speed have also been shown to be dissociated from each other [31, 39, 40, 48].

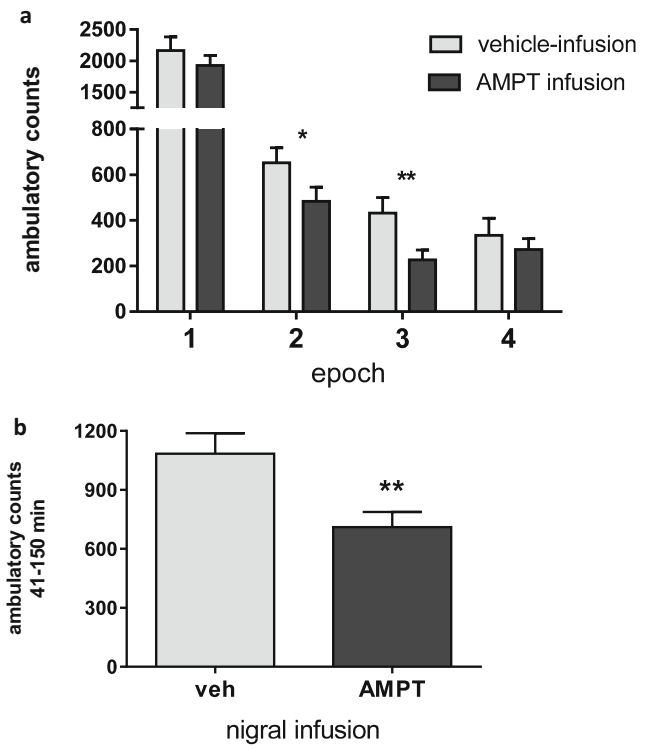

Fig. 3 Nigral AMPT infusion impact on locomotor function: movement frequency. **a** Ambulatory counts over entire locomotor assessment period. A significant difference in ambulatory counts was observed following nigral infusion of AMPT versus ambulatory counts following infusion of vehicle (veh). Data represent the sum of ambulatory counts obtained over five trails for each rat following infusion of either veh or AMPT. Repeated measures two-way ANOVA results: Time following infusion: ($F_{(3,\ 24)} = 83.37$, $p < 0.0001$). No interaction time following infusion X AMPT ($F_{(3,\ 24)} = 1.11$, ns). Ambulatory counts following AMPT infusion: ($F_{(1,\ 8)} = 7.23$, $p = 0.0275$). Time intervals in respective epoch; epoch 1, 0–50 min ($t = 1.69$, df = 8, $p = 0.13$); epoch 2, 41–90 min ($t = 2.47$, df = 8, $^{*}p = 0.039$); epoch 3, 81–150 min ($t = 4.93$, df = 8, $^{**}p = 0.001$); epoch 4, 141–180 min ($t = 1.13$, df = 8, $p = 0.29$). Post hoc results determined by two-tailed paired *t* test. **b** Mean ambulatory counts, 41–150 min following infusion. During epochs 2 and 3, movement frequency was decreased ~35% overall following AMPT versus that following infusion ($t = 4.19$, $^{**}p = 0.003$, df = 8, two-tailed paired *t* test)

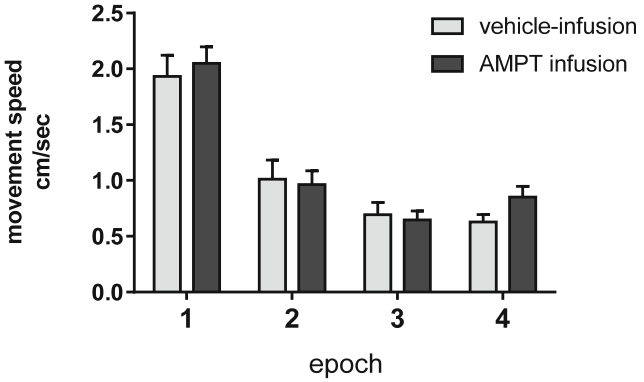

Fig. 4 Nigral AMPT infusion impact on locomotor function: analysis of movement speed over entire locomotor assessment period. Movement speed (cm/s) was unaffected by nigral AMPT infusion over the entire locomotor assessment period. Repeated measures two-way ANOVA results: time following infusion: ($F_{(3,\ 24)} = 65.83$, $p < 0.0001$). Interaction; time following infusion X AMPT ($F_{(3,\ 24)} = 2.30$, $p = 0.103$). Movement speed following AMPT infusion: ($F_{(1,\ 8)} = 0.38$, ns). Time intervals in respective epoch; epoch 1, 0–50 min ($t = 0.71$, df = 8, $p = 0.50$); epoch 2, 41–90 min ($t = 0.49$, df = 8, $p = 0.64$); epoch 3, 81–150 min ($t = 0.39$, df = 8, $p = 0.71$); epoch 4, 141–180 min ($t = 1.96$, df = 8, $p = 0.09$). *Post hoc* results determined by two-tailed paired *t* test

Nigral AMPT and Impact on Locomotor Function: Total Distance

Total distance is also an index of locomotor activity and is decreased in aged rats [26, 31, 39]. The total distance covered by rats following nigral AMPT infusion over the entire 180 min of evaluation was not significantly reduced compared to their total distance covered following vehicle infusion (Fig. 5a). However, there was a significant reduction in total distance covered following nigral AMPT infusion in epochs 2 (41–90 min) and 3 (81–150 min) (Fig. 5a). During this time period of confirmed DA reduction in the SN, overall total distance covered was reduced 34% following nigral AMPT infusion versus vehicle infusion in the same test subjects (Fig. 5b).

Guide Cannula Placement and Locomotor Function

The possibility that the guide cannula implants affected locomotor function at this age was evaluated by comparing locomotor activity following vehicle infusion against locomotor activity in a naïve cohort (without intervention or treatment of any kind) of similar age. Baseline locomotor activity following nigral vehicle infusion was not significantly different from locomotor performance from surgically naïve rats of similar age (Fig. 6). Therefore, the surgery and saline infusions did not produce a significant deviation from locomotor performance that would be expected in a rat of this strain at similar age.

Discussion

Aging brings a number of changes in central and peripheral mechanisms that are associated with locomotor function. Identifying which of these central mechanisms contribute to impaired mobility during aging is a critical research need [67], given its high prevalence and association with loss of independent living, disabilities, and mortality [68, 69]. To identify the aging-related changes associated with locomotor impairment, it is necessary to systematically isolate candidate mechanisms and evaluate the impact of impairing their expression or activity upon specific parameters of locomotor activity that are affected in aging. Here, we isolated one hypothesized mechanism, aging-related loss of TH protein in the SN, by inhibiting TH activity in the SN of a young rat, which would be devoid of any number of aging-related changes that could

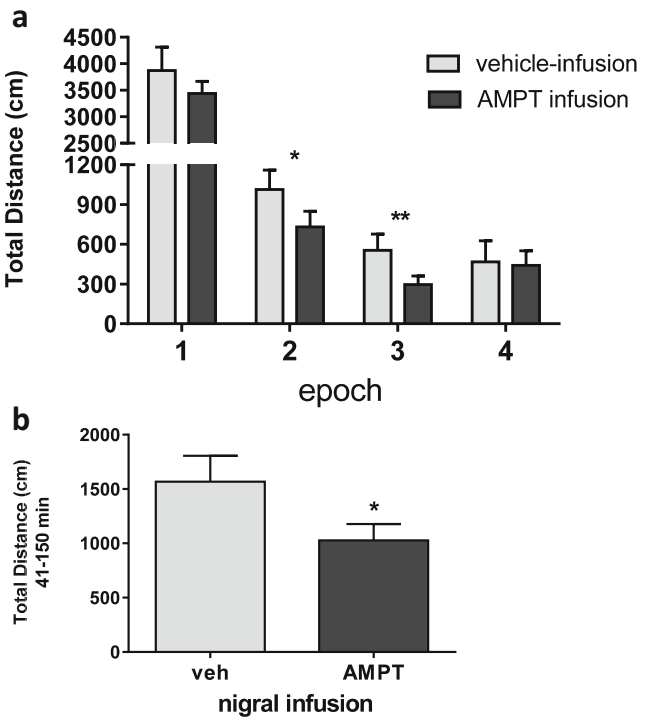

Fig. 5 Nigral AMPT infusion impact on locomotor function: analysis of total distance. **a** Total distance over entire locomotor assessment period. Data represent the mean of sum of total distance covered over five trails for each rat following infusion of either veh or AMPT. Total distance following nigral infusion of AMPT versus total distance following infusion of vehicle (veh) was not significantly different over the entire 180 min of assessment. Repeated measures two-way ANOVA results: Time following infusion: ($F_{(3,\ 24)} = 106.4$, $p < 0.0001$). No interaction time following infusion X AMPT ($F_{(3,\ 24)} = 1.52$, ns). Total distance following AMPT infusion: ($F_{(1,\ 8)} = 3.51$, $p = 0.10$). There was significant reduction in total distance covered in epochs 2 and 3. Time intervals in respective epoch; epoch 1, 0–50 min ($t = 1.39$, df = 8, $p = 0.20$); epoch 2, 41–90 min ($t = 2.42$, df = 8, $^{*}p = 0.042$); epoch 3, 81–150 min ($t = 3.46$, df = 8, $^{**}p = 0.009$); epoch 4, 141–180 min ($t = 0.27$, df = 8, $p = 0.80$). *Post hoc* results determined by two-tailed paired *t* test. **b** Mean total distance, 41–150 min following infusion. During epochs 2 and 3, rat covered ~ 34% less distance following AMPT versus that following infusion ($t = 3.11$, $^{*}p = 0.014$, df = 8, two-tailed paired *t* test)

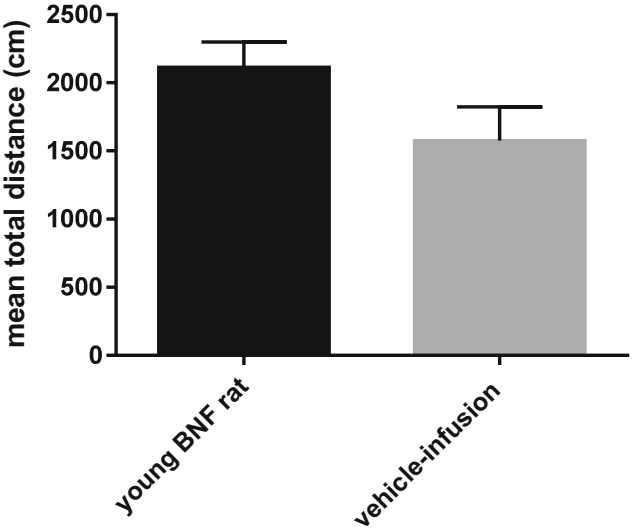

Fig. 6 Comparison of locomotor activity to surgically-naïve rats of the same age. Guide cannula implant and infusion of sterile saline (vehicle) did not significantly affect locomotor activity that would be expected in a similarly aged rat of the same strain. The mean total distance covered between the two cohorts in 1-h time was evaluated. The surgical implantation of guide cannula and five infusions of vehicle (identified as vehicle infusion) did not significantly affect distance covered when compared against their similarly-aged and surgically-naïve cohort (young BNF rat). Statistical results: two-tailed *t* test, $t = 1.54$, $p = 0.14$, df = 16. Variance between the two groups was not significantly different ($p = 0.22$)

impair locomotor activity and thereby bias our results. Furthermore, given that mesoaccumbal DA function can affect locomotor behavior [70, 71], we verified that our targeting strategy to the reduce DA in the SN did not affect DA in the adjacent VTA or the nucleus accumbens (Fig. 2e). The within-subjects design permitted simultaneous assessment of three locomotor parameters to compare movement frequency measured as ambulatory counts), movement speed, and distance following nigra-specific TH inhibition with AMPT versus following nigral saline infusion (representing individual baseline locomotor function). The guide cannula placement did not affect motor function that would be expected for this age group (Fig. 6). The three locomotor activity parameters represent well-established aging-related locomotor impairments that are well-documented in humans [11–15, 49, 68, 69]. The BNF rat strain used emulates aging-related locomotor decline seen across rodents, non-human primates, and humans with decreased movement initiation frequency or ability [72], exhibiting motor decline beginning in the middle to latter half of the lifespan [40, 48, 66] and further decreasing out to 30 months of age [26, 31]. While recognizing that aging produces a number of changes in DA neurotransmission and in other CNS components, nigral neuron loss is independently associated with physical activity proximate to death [49]. Our results support the possibility that aging-related loss of TH protein or DA in the SN, possibly due to neuron loss [49], is a contributing neurobiological mechanism of aging-related decreases in movement frequency.

To determine the components of striatal or nigral DA neurotransmission affecting specific parameters of motor function, the experimental approach must modulate DA neurotransmission in one nigrostriatal compartment without affecting the other [37, 39]. To that end, our study approach required autonomy of TH regulation between the SN and striatum as a critical necessity for evaluating impact on parameters of motor function. We have previously reported that TH inhibition by AMPT in either striatum or SN reduces DA only in the targeted region [48, 59]. We confirmed the compartmental specificity of DA reduction in the SN following TH inhibition, with an estimated longevity for ~ 100 min (Fig. 2). Although this intervention is transient, the experimental approach essentially recreates what has been reported in aging. This

autonomy of TH function or expression in the SN is also seen in dietary or exercise intervention studies [40, 48, 73]. Recovery of locomotor activity or prevention of aging-related decline in movement frequency, with increased nigral, but not striatal, DA, and TH expression, has also been reported in preclinical studies of aging and PD [33, 34, 39, 48]. Therefore, although the nigrostriatal pathway has contiguous functions, such as coincident release of DA [4], in association with movement initiation, isolation of one nigrostriatal compartment from another may indicate which motor parameters are affected by striatal or nigral DA neurotransmission.

Movement frequency was continually recorded by the automated software by beam breaks to generate ambulatory counts. The software does not differentiate between individual movement initiation events and the movement that continues after its initiation. Assessment of rearing behavior, number of hindlimb steps in a cylinder, or adjusting steps are specific sensorimotor function tests that can isolate movement initiation from movement continuing after its initiation. Although we did not assess these behaviors in this study, the available data from other evaluations of parameters of motor function in PD models indicate that differences in locomotor activity (recorded as beam breaks) versus rearing behavior and number of hindlimb steps among experimental groups are nearly identical [74, 75].

It may be argued that compensatory mechanisms could occur in striatum that delay or mitigate motor impairment in PD, but not in aging. Thus, if there are such mechanisms, 80% TH loss in striatum in PD may be compensated and be akin to less TH loss, as seen in aging. However, activation of such compensatory mechanisms occur after motor impairment in a PD model, with > 80% TH loss in striatum [16]. In the 6-OHDA model, DA loss exceeds TH loss in striatum, but the converse occurs in the SN [60]. Genetic knockout of the DA transporter also produces similar differences in TH expression and phosphorylation between striatum and SN [61]. There is greater reliance upon de novo catecholamine biosynthesis in SN to maintain DA tissue content [59]. The autonomy of TH regulation between striatum and SN strengthens the possibility that correlations of nigral TH loss with motor function established in aging studies in rats [26, 30, 31, 57, 60], non-human primates [24, 25], and human [17, 49, 57, 58] represent a mechanism of aging-related motor decline.

Loss of DA biosynthesis is not the only step in DA neurotransmission in the SN where locomotor function may be affected in aging. Blockade of the post-synaptic DA D_1 receptor signaling also decreases locomotor activity [4, 54]. Consistent with these observations, we have recently reported a decrease in DA D_1 receptor expression in both striatum and SN occurs between 12 and 18 months of age in association with decreased movement frequency [48]. It may be argued that decreased D_1 receptor expression in striatum alone could have contributed to motor decline in this time frame. However, in a calorie-restricted group in this study, wherein aging-related motor decline was prevented, DA and TH expression were unexpectedly decreased in striatum, without effect on D_1 receptor expression. Conversely, an increase in both DA and TH expression was occurred in the SN of the aforementioned calorie restricted group, suggesting that this increase offset the decrease in locomotor activity presumably caused by loss of DA D_1 receptors in the SN [48]. Our current and previous results [39] support this possibility. Therefore, loss of D_1 receptor in the SN may be one of the first central mechanisms occurring in the lifespan contributing to aging-related motor decline.

Our approach in targeting the SN was to emulate loss of DA during aging in this nigrostriatal compartment alone and not the adjacent VTA. This infusion covered both tiers of DA neurons (Fig. 1b), and DA was reduced in the entire SN, given our dissection. Our approach also had to ensure that infused substrate targeted the ventral-lateral tier (Fig. 1a), still reduced DA in the central SN, but did not reduce DA in the VTA, with minimal reflux of the infused substrate out of the SN. We used infusion cannula with a 1.0-mm projection beyond the guide cannula to optimize diffusion within the SN (Fig. 1a), an approach supported by earlier work [62]. There is evidence that both the dorsal and ventral tiers of the SN are affected in aged humans [14, 17]. One study revealed loss of pigmented neurons in the SN ~ 33% between 20 and 90 years of age [17], with comparatively greater loss in the dorsal tier in aging and more loss in the ventral tier in PD. More recent reports, however, indicate that nigral neurons in the medial and lateral ventral quadrant are reduced in aged human [14] and in primates [76].

Deficits in nigral DA neurotransmission are generally not considered when evaluating locomotor impairment, despite evidence of incongruity between changes in locomotor function against striatal DA-related measures in PD and aging models alike [27, 28, 32–44, 46]. There are well-controlled studies which have shown a calibrated metric of striatal TH and DA loss against locomotor outcomes [45, 77, 78], specifically indicating that impairment of reaction time (an index of movement initiation) correlates with severity of striatal DA loss, from 60% to > 95% [77, 78]. Given the autonomy of TH regulation between striatum and SN, including following MPTP- or 6-OHDA lesion [16, 60], the contribution of nigral TH loss in impaired movement initiation is yet unknown, although our study represents a significant step forward to evaluate its influence. Bezard and colleagues reported 80% TH loss in striatum and 40% TH loss in the SN at the onset of bradykinesia in a primate PD model [16]. Our previous attempt to reduce striatal DA content in striatum to this extent (> 80%) by AMPT-mediated TH inhibition proved to not be possible, but striatal DA reduction to the average extent seen in aging (30%) did not affect movement frequency [48]. DA neurotransmission in each compartment could influence different components of motor function. Accordingly, our study revealed that motor speed was not affected by decreased DA in the SN (Fig. 4). Still, our study outcome, and many others,

emphasizes the need to evaluate DA function in both nigrostriatal compartments to parse out their respective influences upon motor parameters. Nigra-specific manipulations can locally modulate basal ganglia function and motor function [52, 53, 79], including cholinergic modulation [80, 81], as first reported by Andersson and colleagues [55]. A recent PD case study reported that a PD patient receiving fetal mesencephalic grafts in putamen demonstrated no clinical benefit over 16 years post-transplantation, despite evidence of a robust TH expression therein [41]. Taken together, these results serve as additional rationale to interrogate nigral DA function for a role in motor outcomes.

In conclusion, TH inhibition in the SN in young rats produced a decrease in DA, similar to that reported in aged rats, non-human primates, or humans, in temporal congruence with a significant decrease in movement frequency. The within-subjects experimental design controlled for inherent locomotor activity within each test subject to evaluate locomotor function during SN-specific reduction of DA following AMPT-mediated TH inhibition. These results suggest that age-related nigral TH or DA loss alone, a molecular deficit well-established from rodent to human, is one of several likely mechanisms that contribute to deficits in movement initiation observed in aging. As similar nigral TH loss occurs at bradykinesia onset in PD, with continual decline thereafter, these data also suggest that some aspects of locomotor impairment in PD may have the same origin.

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Author Contribution MFS conceived the work, analyzed the data, wrote the manuscript, and received funding to conduct the work. TRM, MAC, and BSP conducted the studies. TRM and MAC processed and analyzed the data. DMA provided critical comments and editing.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that there are no competing interests.

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