Published in final edited form as:

Neurobiol Aging. 2016 January ; 37: 192–207. doi:10.1016/j.neurobiolaging.2015.10.006.

Initiation of calorie restriction in middle-aged male rats attenuates aging-related motoric decline and bradykinesia without increased striatal dopamine

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Abstract

Aging-related bradykinesia affects ~15% of those reaching age 65 and 50% of those reaching their 80s. Given this high risk and lack of pharmacological therapeutics, non-invasive lifestyle strategies should be identified to diminish its risk and identify the neurobiological targets to reduce aging-related bradykinesia. Early-life, long-term calorie restriction (CR) attenuates aging-related bradykinesia in rodents. Here, we addressed whether CR initiation at middle age could attenuate aging-related bradykinesia and motoric decline measured as rotarod performance. A 30% CR regimen was implemented for 6 months duration in 12-month old male Brown-Norway Fischer 344 F₁ hybrid rats after establishing individual baseline locomotor activities. Locomotor capacity was assessed every 6 weeks thereafter. The *ad libitum* (AL) group exhibited predictably decreased locomotor activity, except movement speed, out to 18 months of age. In contrast, in the CR group, movement number and horizontal activity did not decrease during the 6-month trial and aging-related decline in rotarod performance was attenuated. The response to CR was influenced by baseline locomotor activity. The lower the locomotor activity level at baseline, the greater the response to CR. Rats in the lower 50th percentile surpassed their baseline level of activity, whereas rats in the top 50th percentile decreased at 6 weeks and then returned to baseline by 12 weeks of CR. We hypothesized that nigrostriatal dopamine tissue content would be greater in the CR group and observed a modest increase only in substantia nigra with no group differences in striatum, nucleus accumbens, or ventral tegmental area. These results indicate initiation of CR at middle age may reduce aging-related bradykinesia and, furthermore, subjects with below average locomotor

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activity may increase baseline activity. Sustaining nigral DA neurotransmission may be one component of preserving locomotor capabilities during aging.

Keywords

Parkinsonism; caloric restriction; bradykinesia; substantia nigra; striatum; dopamine

Introduction

The risk of significant and clinically-diagnosable locomotor impairment in those 65 and older is increasing. In the United States alone, the number of retirees will double in the next 20 years. The growing elderly population is presenting an increasing challenge to the biomedical enterprise of industrialized nations. Viewed as a cardinal symptom of Parkinson's disease (PD), bradykinesia (slowness or poverty of movement and difficulty in movement initiation) is also a significant health risk to the elderly, affecting 15% by age 65 and 50% by age 80 (Bennet et al., 1996; Prettyman, 1998; Murray et al., 2004; Rosano et al., 2012). Recent evidence points to an association between social activity and motor decline (Buchman et al., 2009) as well as genetic loci contributing to vulnerability to age-related Parkinsonism (Schulman et al., 2014). This impairment greatly compromises an individual's ability to navigate the daily environment, and concomitantly the risk of severe injury greatly increases (Fleischman et al., 2007). To fully elucidate the neurobiological basis for aging-related bradykinesia, it must also be recognized that ~50% of the elderly do not exhibit signs of bradykinesia (Bennet et al., 1996; Rosano et al., 2012; Buchman et al., 2014). Therefore, it is important to frame questions that recognize that aging-related bradykinesia, while different in origin compared to PD, shares a common locomotor deficit, bradykinesia, with PD. Thus, identification of a common neurobiological basis for bradykinesia in either condition would be highly important. Second, it is imperative to understand if potential lifestyle practices may underlie the neurobiological basis as to why bradykinesia affects roughly half of the elderly, but not the other half, by age 80.

Thus, three priorities can be recognized: (1) determine if there are inherent behavioral (in this case, locomotor activity tendencies) traits that may predispose or increase risk of aging-related locomotor impairment; (2) determine if there is a neurobiological basis for predisposing individuals to locomotor impairment with aging; and (3) evaluate non-invasive lifestyle strategies that can be practically implemented to reduce the significant risk of locomotor impairment associated with aging. To address whether inherent locomotor capacity at middle age could be a predictive factor for the likelihood of bradykinesia onset, we hypothesized that a lower than average activity level at middle age would predispose that subset to aging-related bradykinesia. In established rat models of aging, studies indicate that a significant decline in locomotor activity occurs somewhere between 6 and 18 months of age, when evaluating the entire cohort for each age group (Hebert and Gerhardt, 1998; Yurek et al., 1999; Salvatore et al., 2009). However, considerable variance in individual differences in locomotor activity at a young age could predict eventual bradykinesia onset (Salvatore et al., 2009), particularly if a test subject's relative ranking in locomotor activity level at an earlier age is maintained with advancing age. Therefore, we predicted that

bradykinesia would be more evident in the bottom 50th percentile with advancing age. This tendency may portend to increased morbidity as well, since rodent studies have demonstrated that lower locomotor activity at advanced ages is predictive of earlier death (Ingram and Reynolds, 1986).

Attempts to understand the neurobiological basis of impaired locomotor function with aging have been approached from the hypothesis that deficits in dopamine (DA) neurotransmission could underlie aging-related bradykinesia. Indeed, bradykinesia is a cardinal symptom of PD itself and occurs when striatal DA or tyrosine hydroxylase loss reaches or exceeds 80% (Bernheimer et al., 1973; Hornykiewicz and Kish, 1986; Bezard et al., 2001). However, in the typical course of aging, striatal DA loss has been reported to reach a maximum of 60% in human (Kish et al., 1992; Haycock et al., 2003) or the non-human primate (Collier et al., 2007). Notably in established models of aging, there are more numerous reports indicating less than 30–40% striatal DA loss in non-human primate (Irwin et al., 1994; Gerhardt et al., 2002) and rat models of aging (Ponzio et al., 1982; Marshall and Rosenstein, 1990; Emerich et al., 1993; Yurek et al., 1998; Cruz-Muros et al., 2007; Salvatore et al., 2009; Salvatore and Pruett, 2012). The loss of striatal DA required for the onset of bradykinesia, at least in PD models, is tightly defined, as 60% loss of DA in striatum in the MPTP PD model is not associated with bradykinesia, but rather a loss exceeding 80% is seen in bradykinetic test subjects (Bezard et al., 2001). Thus, numerous studies support that age-related decreases striatal DA do not meet, let alone exceed, the threshold of striatal DA loss associated with bradykinesia onset in PD. Furthermore, more recent reports indicate that even though striatal markers of DA indicate virtually complete loss in some PD patients, the severity of locomotor impairment still increases over time (Tabbal et al. 2012; Kordower et al., 2013). Therefore, at the very least, the confluence of these results suggest that striatal DA content may not serve as a reliable index of locomotor impairment or capability.

Nigral DA function has been an oft-ignored barometer of locomotor function in such studies. In the comparatively smaller number of studies, aging-related DA loss in the substantia nigra is ~40–60%, similar to that reported in PD (Marsden, 1990; Bezard et al., 2001) and also aging in association with the onset of bradykinesia (Yurek et al., 1998; Cruz-Muros et al., 2007; Salvatore et al., 2009). Interference with DA signaling specifically in the substantia nigra has been shown to inhibit open-field locomotor activity (Berquist et al., 2003; Andersson et al., 2006; Trevitt et al. 2001). Therefore, involvement of the entire nigrostriatal pathway must be considered as the primary neural system underlying aging-related bradykinesia.

Non-invasive strategies that may reduce the risk of aging-related bradykinesia include exercise and calorie restriction (CR). Long-term CR when initiated at a relatively young age in the lifespan preserves locomotor activity in aged rodents or non-human primates to levels associated with younger cohorts (Ingram et al., 1987; Fitting et al., 2008; Weed et al., 1997). Some evidence suggests that initiation of CR at middle age may also preserve locomotor function (Kastman et al., 2010). Furthermore, CR may protect nigrostriatal function, as such intervention has shown to mitigate locomotor impairment from a neurotoxic insult with some protection against severe striatal DA loss (Maswood et al., 2004). Here we determined if a 30% reduction in calorie intake, when initiated in middle-aged Brown-Norway/Fischer

344 F₁ hybrid (BNF) male rats previously fed *ad libitum* (AL), could maintain baseline activity for 6 months out to 18 months of age, an age where locomotor and nigral DA deficits have been reported in rodents on AL diets. We also determined if any response to CR to prevent aging-related bradykinesia might be affected by the individual test subject's baseline locomotor activity at the time of intervention. Additionally, we evaluated the effects of CR on motor function via longitudinal assessment of performance in a rotarod task as well as a one-time assessment of cognitive performance as assessed in a novel location memory task at the end of the study. The other major objective was to test the hypothesis that DA levels in the nigrostriatal pathway would increase in CR rats relative to AL rats, given evidence of CR-associated protection against aging-related bradykinesia, at the conclusion of the study.

Methods

Test subjects & weight determinations

All procedures involving rats were conducted under the auspices of approved protocols at the LSU Health Sciences Center-Shreveport and Pennington Biomedical Research Center (PBRC) Institutional Animal Care and Use Committees. We used 12-month old male Brown-Norway Fischer 344 F₁ hybrid (BNF) rats obtained from the NIA-contract facility at Harlan Laboratories (Indianapolis, IN). The BNF hybrid strain exhibits aging-related changes in striatal and midbrain DA tissue content comparable to that reported in nonhuman primates and the outbred Sprague-Dawley rat (Yurek et al., 1998; Cruz-Miros et al., 2007; Salvatore et al., 2009; Pruett and Salvatore, 2013). Concomitantly, aging-related decreases in locomotor activity and function are well-documented in this strain (Spangler et al., 1994; Yurek et al., 1998; Salvatore et al., 2009; Pruett and Salvatore, 2013) as well as its positive behavioral responses to life-long CR (Fitting et al., 2008).

Body weights were determined on a weekly basis for all subjects for the duration of involvement in the study (control group, 5 measures; AL & CR groups, 24 or 25 measures after baseline). Body composition was measured by NMR (Bruker Minispec) about 2–3 days prior to euthanasia. This procedure involves placement of unanesthetized rat into a small cylinder which is placed into the calibrated NMR for about 90 sec to provide estimates of fat mass, lean estimates and water content.

Calorie restriction regimen

The rats arrived at PBRC at 11 months of age. Since weaning, they had been maintained on an *ad libitum* diet (NIH-31). After arrival the rats were singly housed in plastic cages containing corn cob bedding in a SPF vivarium located within the Comparative Biology Core of at PBRC. The vivarium was maintained at 22 ± 2 °C, 70 ± 10% humidity. The light cycle was controlled automatically with lights on at 5:00 and off at 17:00. Throughout the study the rats were provided NIH-31 modified open formula diet (Catalog #7017) obtained from Harlan Teklad (Madison, WI). This diet is identical to NIH-31 diet, but contains an additional supplement of vitamin mix (30%). Use of this type of fortified diet is a generally recognized practice in the CR literature to assure that CR rats are not being deprived of essential vitamins and minerals.

At 12 months of age, the rats were randomly divided into two groups matched for baseline horizontal activity. Half the rats were maintained *ad libitum* (AL) on the fortified NIH-31 diet, while the other half rats were maintained on CR by providing the same diet at 70% of the level of food intake by the AL control group, which was adjusted as needed. Specifically, the food intake of the AL group was monitored weekly including estimates of food spillage, and a mean intake was computed, and then the estimated mean ration for the CR group was applied the following week and readjusted every week.. For the first week, the restriction was 90% of the AL group, followed by a week at 80%, and finally at 70% of this estimate the following week. Individually daily food allotments were provided to the CR rats about an hour before lights were turned off as set automatically in the vivarium. Water was provided to all animals using a Hydropac system (Seaford, DE). Body weight was measured weekly on an electronic balance.

Assessment of locomotor activity

Locomotor activity was determined in two primary ways; 1) assessment of spontaneous activity in an open-field and, 2) by rotarod. At baseline, and then again at 6, 12, 18, and 24 weeks after initiation of CR or AL in those cohorts, spontaneous locomotor activity was assessed in five separate trials of one-hour duration using automated activity chambers (VersaMax Animal Activity Monitoring System, Columbus, OH). All locomotor testing was conducted between the hours of 0830 and 1500. Testing time was randomized so that each rat was not tested at the same time each testing day. Prior to the initiation of CR, baseline locomotor activity was determined in the entire cohort of 45 12-mo old rats (15 designated for a 12-mo control group, 15 for the AL group, and 15 for the CR group, the latter two groups being maintained for an additional 6 months. The baseline testing assured that no bias in locomotor capabilities or capacity was inadvertently introduced during the assignment of individual test subjects into either the CR or AL group. Furthermore, it also allowed for subsequent analysis of whether any potential locomotor impact of CR was influenced by individual baseline locomotor performance. We previously observed that individual locomotor performance levels tend to be maintained across the lifespan of this strain of rat (Salvatore et al., 2009). Specifically, rats with the highest levels of activity at a younger age were also at the highest level when assessed 8 months later in the lifespan. By the same token, rats at the lower levels were also at the lower levels in the same time frame. Therefore, we predicted that in the AL group, rats in the lower 50th percentile of baseline locomotor performance would be more vulnerable to aging-related bradykinesia than those in the highest 50th percentile.

Spontaneous locomotor activity was assessed in chambers comprised of a 41 x 41 x 31 cm translucent plastic box with a grid of infrared beams mounted horizontally every 2.5 cm and vertically every 4.5 cm. The beams are generated from monitors connected to a VersaMax analyzer, which transduces the number of beam breaks (locomotor activity) to a dedicated computer with VersaMax software to collect and analyze the locomotor measures. Activity data were recorded during a 1-hr session, and the sum total for movement number, total distance (in cm), horizontal activity (a unitless measure of all horizontal activity), time spent moving (in sec) were recorded. Movement speed is a calculated value, taken by dividing total distance by the associated movement time (as cm/sec). Finally, a composite

movement score, representing the combination of z-scores obtained from the locomotor parameters of movement number, horizontal activity, total distance, and movement time, was also calculated. It has been previously shown that these four parameters, but not movement speed, were significantly correlated with each other (Salvatore et al., 2009), and therefore a z-score composite was also computed as a measure of relative locomotor activity differences between the AL and CR groups from baseline and at each 6 weeks out to the end of the study at 24 weeks.

The locomotor activity chambers were isolated within a separate quiet room within the Comparative Biology Core. The room was dark with the only illumination coming from a red light lamp. Prior to the initiation of any trial, the rats were transported from their vivarium to a waiting room for 15 minutes. During this time the activity chambers were cleaned with 70% ethanol, and fresh corncob bedding was placed on the chamber floor. The rats were then transported to the testing room and placed into the activity chambers where they remained undisturbed for 1 hour while activity testing was in progress. The variance in locomotor activity was examined in the first group entering the study. At baseline, the variance in horizontal activity ranged from 13.6% to 42.3% in the 10 rats assigned to this group, for an average variance of 29%.

Rotarod—Rats were also tested for motor function and coordination using an accelerating rotarod (Med-Associates, St. Albans, VT). Rotarod testing was performed after the completion of the activity tests at the baseline, 12 week, and 24 week time-points. Rotarod testing consisted of three trials separated by an approximately 30 min ITI. For each trial, the rat was placed onto the rotarod while it was stationary. The rotarod was then started at 4 rpm and accelerated to 40 rpm over 5 min. The dependent variable was the time the rat spent on the rotarod before falling which was recorded automatically by the apparatus. The maximum trial length was 5 min. The results (time spent on the rotarod before falling) from the 3 trials were then averaged to serve as the dependent variable.

Novel Object Location Task—About 10 days prior to euthanasia, rats were tested in a novel object location task. The protocol has been described previously and is sensitive to aging in mice (Wimmer et al., 2012). In general, on the first day the rats are placed into the center of a square arena for 10 minutes. On the second day, they are returned to the arena that now contained two identical objects and allowed to explore for 10 minutes. On the third day, the rats are again placed in the center of the arena with same two objects, except one had been displaced by 90 degrees in the arena. Rats were again allowed to explore with the amount to time spent in proximity to each object recorded (nose within 2 inches). The major dependent variable was the percent time spent exploring the object that had been displaced.

Neurochemistry: analysis of dopamine and regulating proteins

The analysis of DA tissue content from striatum, substantia nigra, NAc, and VTA was conducted after the study completion when the rats were 18 months of age, reflecting differences in DA tissue content after 24 weeks of CR intervention (previously AL fed until 12 months of age) against the AL control group. Given the well-established DA hypothesis for locomotion, our assessment of DA tissue content allowed for determination if any

differences in locomotor function seen at the end of a 6-month period under AL or CR conditions could be attributable to differences in DA tissue content in the nigrostriatal pathway (striatum and substantia nigra). As a control to this DA pathway, this assessment was also done in the mesoaccumbens DA pathway (NAc and VTA), representing regions that are more relevant to reward and motivated behavior.

After dissection, each brain region was kept at -70°C until sonication in 500 μl of ice-cold 0.1 M HClO_4 -EDTA buffer. Aliquots from these protein-precipitated supernatants were analyzed for content of DA by HPLC (Salvatore et al, 2012). Determination of DA was calculated based upon percent recovery of the internal standard (N-methyl-dopamine (NMDA), 20 ng/mL) in each sample, the peak height ratios obtained for both DA and NMDA in a standard and in the sample, and the fraction of the aliquot was analyzed by HPLC, representing of the total volume of the sonicated tissue. The acid buffer treatment for HPLC analysis causes precipitation of the protein content inherent in the sample. The remaining buffer is archived, and the protein pellet is sonicated in 1% SDS with 5 mM Tris buffer (pH 8.3) and 1 mM EDTA to process for determination of total protein, to which DA tissue content is normalized (Salvatore et al., 2012).

To determine the protein expression levels of proteins associated with DA regulation in striatum, we processed the protein pellet precipitated from the perchloric acid homogenized sample. To determine TH protein, we loaded $\sim 5\text{ }\mu\text{g}$ of total striatal protein in conjunction with a calibrated TH protein standard to determine ng TH protein normalized to total protein. After determination of the TH protein content (primary purchased from Millipore, Temecula, CA, cat#Ab152), TH phosphorylation stoichiometry was determined for ser19 (Phosphosolutions, Aurora, CO, cat# p1580-19), ser31 (characterization reported in Salvatore et al., 2009), and ser40 (Phosphosolutions, Aurora, CO, cat# p1580-40) using an established methodology (Salvatore et al., 2012; Salvatore and Pruett, 2012). Dopamine transporter expression was determined by loading $\sim 10\text{ }\mu\text{g}$ striatal protein using 1 $\mu\text{g}/\text{ml}$ anti-dopamine transporter goat primary antibody (Santa Cruz, sc-1433), dopamine D_1 receptor was determined using Santa Cruz primary (cat# sc-14001), and the two forms of $\text{GFR}\alpha 1$ were determined using R&D Systems (Minneapolis, MN) primary (cat#AF560). Secondary antibodies (rabbit anti-goat or swine anti-rabbit) were used for signal enhancement prior to using the detection agent [^{125}I]-protein A.

Statistics

Locomotor performance of each individual rat at 12 months of age served as the baseline measure for subsequent comparison of aging-related impact on locomotor activity. This study was designed to compare the impact of maintaining the AL fed state (AL group) versus the intervention with CR beginning at 12 months of age (noting, again, that rats in this group up until 12 months of age were AL fed). For determination of aging related effects under each diet, a one-way repeated measures ANOVA was performed to determine if significant departure from baseline activity was observed over time. For the open-field locomotor test, a post-hoc Dunnett's multiple comparison test was then conducted to determine if a significant departure from baseline was observed at each 6-week interval against the baseline. We then further determined whether an interaction of diet with aging

was observed during the study and employed a two-way repeated measures ANOVA. If significant interaction between aging and diet or an effect of diet was observed, an unpaired Student's two-tailed t-test was conducted at each 6-week period to determine at what time post-diet intervention differences in locomotor activity were detected.

For the rotarod testing, one-way repeated measures ANOVA was performed to determine if significant departure from baseline occurred over the course of the study (analyzing at 12 and 24 weeks only). Detection of significant differences in rotarod performances across time was followed by Tukey's Multiple Comparison Test to compare if differences existed at 12 and 24 weeks against baseline, as well as if differences existed between 12 and 24 weeks.

To determine if differences in DA tissue content were observed among the four brain regions, we employed a one-tailed student's unpaired t-test. In BNF rats aging is associated with ~20% decrease in DA tissue content in the substantia nigra at 18 months of age and ~40% at 30 months of age (Salvatore et al., 2009; Salvatore and Pruett, 2012). In the striatum, reports of DA loss are much more varied, ranging from 0 to 60% (Ponzio et al., 1982; Marshall et al., 1990; Yurek et al., 1998; Cruz-Muros et al., 2007; Salvatore et al., 2009; Salvatore and Pruett, 2012). This study was powered ($n=15$ each group) to 0.41 in order to detect a 10% difference in DA tissue content between the two groups, based upon previous observations that a 20% decrease (sigma of 0.17) in DA tissue content in 18-month old rats would be observed compared to younger cohorts (Salvatore et al., 2009; Salvatore and Pruett, 2012). The use of a one-tailed t-test is justified if an effect is predicted in one direction versus another (Ruxton and Neuhäuser, 2010). Therefore, given that aging is associated with reduced nigrostriatal DA tissue content in the nigrostriatal pathway, we hypothesized that any difference in DA tissue content resulting from CR intervention would be related to an increase, not a decrease, in DA tissue content in the CR group versus the AL group. Furthermore, while we hypothesized that such an increase would be detected in the nigrostriatal pathway, it could be argued that an increase in locomotor activity from CR could be related to a modest, but significant, increase in DA tissue content in the mesoaccumbens DA pathway. This statistical analysis procedure was also used in determining striatal protein expression or phosphorylation differences existed between the AL and CR groups and for DOPAC, predicting that the turnover rate would follow any change in DA tissue content. Significance for all statistical tests was set at $p<0.05$.

Results

Longitudinal assessment of weight change in AL and CR groups

Test subject entry into either the AL or CR group was determined by evaluating baseline locomotor activity measures in 30 of the 45 rats arriving into the colony. Rats were assigned into each group such that no significant group difference existed in the mean values of movement number, distance travelled, movement time, and horizontal activity in aforementioned locomotor activity measures. AL-fed rats exhibited weight gain at 12 weeks after study initiation and continued to exhibit significant weight gain until the end of the study at 24 weeks (Fig. 1). The mean increase in body weight in the AL group from baseline until the end of the study was 7.4% (462 g at baseline to 496 g at 24 weeks). The implementation of CR predictably decreased body weight against the respective baselines

(Fig. 1). Within 2 weeks of gradual implementation of the CR regimen, there was an average of 5% loss in weight with significant decline observed at 6 weeks. This trend of weight loss continued weekly until ~12 weeks after CR implementation and remained significantly decreased until study completion at 24 weeks (Fig 1). The mean difference in body weight between the AL and CR groups was ~24% at the conclusion of the study.

In addition to body weight differences, the CR regimen also produced differences in body composition as assessed by NMR measured just prior to euthanasia at the end of the study. As noted in figure 1B, CR rats had significantly reduced amounts of lean tissue (18%), fat tissue (29%), and fluids (29%) compared to AL control group.

As the amount of food provided to the CR group was based upon consumption of the AL group, we determined if food consumption varied over the course of the 24 week study. At four time points (week 1, 2, 8, and 14), there were significant decreases in food consumption not exceeding 11% less than that consumed at baseline (123.6 ± 2.4 grams (mean $\pm$ S.E.M.) (data not shown). Thus, the significant weight gain in the AL group was not attributable to an increase in food consumption at any time-point during the 6 month long study.

Given the modest, but significant, weight gain in the AL group and weight loss in the CR group, representing a 24% difference in weight at the completion of the study, we assessed if body weight had a significant correlation to indigenous locomotor activity at baseline and during the study. We determined if such a correlation existed in the group representing 12-month old controls ($n=15$), as well as in the 30 rats selected for the AL and CR groups in the longitudinal study. In the 12-month old control group, weights ranged from 406 to 523 g, representing a range of up to a 30% in body weights between subjects of the lowest and highest weight. Within this cohort, locomotor activity ranged as follows: horizontal activity, 2108–3224 (representing a 53% difference between subjects of lowest and highest average horizontal activity); total distance, 537–1082 (representing a 101% difference between subjects of lowest and highest average total distance); movement number, 92–188 (representing a 104% difference between subjects of lowest and highest average movement number); and movement time, 77–152 (representing a 97% difference between subjects of lowest and highest average movement time). Despite this marked variance in baseline body weight and locomotor activity parameters, there were no significant correlations of body weight with horizontal activity (Pearson $r=0.24$, $p=0.32$), movement number (Pearson $r=0.29$, $p=0.29$), total distance (Pearson $r=0.30$, $p=0.28$), or movement time (Pearson $r=0.38$, $p=0.16$). This lack of significant relationship of body weight with indigenous locomotor activity was also observed at baseline in the rats segregated into either the AL or CR group for the longitudinal study of locomotor activity, including movement speed (Fig. 2A,B).

We also determined if a significant correlation existed between the percent change in body weight in each group versus the departure from baseline in horizontal activity at each 6-week interval and found no significant correlation between weight gain in the AL group and change in horizontal activity from baseline (Fig. 2C) or between weight loss in the CR group and the change in horizontal activity from baseline (Fig. 2D). We did note, however, that there was an inverse correlation of baseline weight to the decrease in horizontal activity in

the AL group at 24 weeks, such that those rats weighing the least decreased activity the most (data not shown).

Novel object location task—As observed in Fig. 3, we observed that both groups exhibited significantly increased exploration of the novel object location; however, there was not significant effect of diet on this performance. Comparing the percent time exploring the object that had been displaced, we estimated the following results: 56% in the AL group vs 57% in the CR group ($p>0.05$).

Locomotor performance results

Rotarod performance

Given that the test subjects were assigned to the AL or CR groups to represent an equal range of horizontal activity, we first confirmed that there was no significant difference in baseline rotarod performance between rats designated for the AL or CR group ($t=0.72$, $p=0.48$, two-tailed t-test), given the a priori assignment into the two groups based upon locomotor activity performance. Body weight, as assessed at the baseline, did not affect the latency to fall (data not shown (Pearson $r=-0.20$, $p=0.30$)). Twelve weeks following the initiation of the study, there was a significant decrease in rotarod performance (~34% decline in latency to fall) in the AL group (Fig. 4A). However, no significant decline in rotarod performance was observed in the CR group 12 weeks after the start of the diet regimen (Fig. 4B).

In the AL group, a further decline in rotarod performance was observed at 24 weeks compared to both baseline (~60% decline) and performance at 12 weeks (further ~40% decline) (Fig. 4A). In the CR group, there was a significant decline (~34% decrease in latency to fall) in locomotor performance on the rotarod only at 24 weeks compared to performance at baseline (Fig. 4B). Notably, the magnitude of this decline at 24 weeks in the CR group was similar to that seen 12 weeks earlier in the AL group, suggesting that CR attenuated the rate of aging-related bradykinesia observed in the AL group. Direct comparison of rotarod performances at 12 or 24 weeks between the two groups revealed significantly greater longevity of performance on the rotarod in the CR group (Fig. 4C, D).

Consistent with the finding that open-field locomotor activity was not correlative with body weight (Fig. 2), rotarod performance (latency to fall) was also not significantly related to body weight of the test subjects (data not shown); statistics, ns, Pearson correlation, $r=-0.195$, $p=0.303$).

Open field locomotor activity measures

Locomotor performance at 12 months of age represented activity levels at middle age and established the baseline for comparison of locomotor performance with aging at every 6 weeks following the implementation of the respective diet regimens out to 24 weeks, at which time the rats were ~18 months of age. Locomotor parameters were evaluated to represent the capacity to initiate movement (movement number and horizontal activity), locomotor activity over the period of testing (total distance and time-spent moving), and movement speed (calculated by total distance divided by time spent moving). The mean of

five locomotor sessions for each individual rat was taken in one week time at each time interval and then used to represent the locomotor profile for each rat. We emphasize that employing five independent measures for each test subject provides better precision in determining locomotor activity in each test subject, given the potential of daily variance in locomotor activity in individual test subjects (Salvatore et al., 2009).

Implementation of CR prevented aging-related decreases in two indices associated with movement initiation, movement number and horizontal activity (Fig. 5), which were observed in rats maintained on the AL diet. With regard to movement number, at the conclusion of the study, there was a significant decrease (~30%) in movement number against the baseline in the AL group (Fig. 5A). The impact of aging upon horizontal activity essentially mirrored the observations noted for movement number, with no significant decline in horizontal activity occurring in the CR group at any time during the study (Fig. 5B). At every 6-week interval following the CR intervention, there was a significant difference in movement number between the AL and CR groups (Fig. 5C). Furthermore, CR actually significantly increased movement number above baseline 12 weeks after implementation; however, this increase above baseline did not persist for the remainder of the study. Unlike the significant aging-related decline in movement number observed in the AL group, there was no significant decrease in movement number in CR rats at the completion of the study (Fig. 5A). Significant differences in horizontal activity, like movement number, between the AL and CR groups were detected beginning at 12 weeks after CR implementation and persisted for the duration of the study (Fig. 5D).

The movement parameter of total distance represents a combination of movement initiation and is also influenced by movement speed. In the AL group, total distance declined significantly beginning at 12 weeks until the completion of the study (Fig. 6A). In the CR group, there was a significant decline in total distance starting 6 weeks later than seen in the AL group (Fig. 6A). Whereas the decline in total distance was affected later in the CR group and the severity of the decline was consistently less at 12 weeks until the completion of the study, we did not detect a significant difference between the CR and AL group (Fig. 6C).

Movement time reflected the observations seen for total distance in that significant decreases from baseline occurred later in the CR group and that the severity of decline at 12 weeks and beyond was consistently less in the CR group (Figs. 6B,D). However, the statistical analysis did not detect a significant diet effect ($p < 0.07$), and did not indicate significant differences at any of the 6-week intervals ($p < 0.06$ at 18 and 24 weeks) (Fig. 6D). Therefore, the results indicate that while CR did prolong the time period before total distance and movement time significantly declined after 12 months, it did not completely prevent a decrease in both parameters, unlike the prevention observed for movement number and horizontal activity throughout the course of the study.

Movement speed was calculated by dividing the TM value obtained for each test subject and the corresponding DT value. Our results indicated that this variable was not significantly affected by aging between 12 and 18 months and the CR regimen did not affect movement speed at any time during the study (data not shown). Given that movement speed factors into total distance, the lack of difference in movement speed between the CR and AL groups

may have influenced the differential outcomes in total distance versus movement number and horizontal activity with regard to the observation that decreased total distance due to aging could only be attenuated, but not prevented, by CR.

Given that movement number, horizontal activity, total distance, and movement time all declined with advancing age, as seen in the AL group, and that CR did prevent or attenuate the rate of decline in each parameter between 12 and 18 months of age, we constructed a composite locomotor score by combining the sum of results from each of these four parameters. As more explicitly stated in the methods, this was done by calculating a z-score for each locomotor parameter at every time point (including baseline) for each test subject. The sum of these four z-scores provided a composite locomotor activity score. To justify the rationale for this approach, we first evaluated the relationship of these parameters and movement speed against movement number values obtained for all 30 test subjects at their baseline. Movement number significantly correlated with horizontal activity, total distance, and movement time, but not movement speed (Figs. 7A–D). These results are consistent with the observations that movement number, horizontal activity, total distance, and movement time each declined with aging in the AL group, but not movement speed.

The analysis of the composite locomotor score for movement number, horizontal activity, total distance, and movement time then revealed that there was a significant difference between the AL and CR groups beginning at 12 weeks following CR implementation, and this difference continued until the end of the study when the test subjects reached 18 months of age (Fig. 7E).

Relationship of baseline performance to severity of aging-related bradykinesia

Our hypothesis predicted that the test subjects with baseline activity levels in the lower 50th percentile of the 12-month cohort would be more vulnerable to aging-related bradykinesia; however, we found that this hypothesis was not confirmed. Two analyses were conducted to test this hypothesis. First, we analyzed the relationship of baseline locomotor activity against the departure from baseline and found that at 18 weeks following the start of the study, there was a significant inverse relationship of locomotor performance at baseline versus the decline from baseline during the course of aging, contrary to our prediction. Thus, we found that the greater the locomotor activity at baseline, the more rapid rate of locomotor decline, at 18 wks from the start of the study, in three of the four parameters (data not shown) Pearson correlation statistics (expressed as Pearson *r* and *p*-value) were as follows; horizontal activity (6 wks, ns; 12 wks, ns; 18 wks, -0.78 , $p=0.007$; 24 wks, ns), TSM (6 wks, ns; 12 wks, ns; 18 wks, -0.74 , $p=0.002$; 24 wks, ns), total distance (6 wks, ns; 12 wks, ns; 18 wks, -0.67 , $p=0.007$; 24 wks, ns). The *p*-value for movement number at 18 wks was 0.05 (Pearson $r = -0.51$)).

With this observation, we then segregated the rats in the AL cohort into those which were in the top 50th percentile versus those in the bottom 50th percentile at baseline. At 12 and 18 wks after the study began, there was a significant difference in the aging-related decrease in horizontal activity against individual baseline, such that rats in the top 50th percentile declined in horizontal activity against their respective baseline to a significantly greater extent than rats in the same cohort occupying the lower 50th percentile. However, this

significant difference was no longer observed at 24 wks, when they reached 18 months of age (Fig. 8A). These observations suggested that horizontal activity decreased at an earlier time period in middle age in the more active test subjects. A further analysis of horizontal activity in the bottom 50th percentile against the individual baselines revealed no significant decrease in horizontal activity during the study period, although at 24 weeks, there was a noted trend toward a decrease (Fig. 9A). The same analysis of horizontal activity in the top 50th percentile revealed significant decreases at every 6 weeks against the baseline (6 wks, $t=5.04$, $p<0.01$; 12 wks, $t=6.27$, $p<0.001$; 18 wks, $t=8.41$, $p=0.0002$; 24 wks, $t=7.13$; $p=0.0004$), with the largest incremental decrease occurring within the first 6 weeks (18% decrease) and bottoming out at 18 wks (29% decrease). Therefore, our findings here indicate that the onset of aging-related bradykinesia at middle age affects the more active test subjects at an earlier time period than less active test subjects. However, the less active cohorts in the bottom 50th percentile exhibited a trend toward decreased horizontal activity ~18 months of age.

In the CR group, aging-related bradykinesia was observed in the first 6 weeks prior to a restoration to the baseline activity levels in the rats with horizontal activity in the top 50th percentile (Fig. 9B). However, in the bottom 50th percentile, the attenuation of aging-related bradykinesia in response to CR intervention was evident within the first 6 weeks of CR (Fig. 8B, 9B). Furthermore, there was a significant increase in activity above the baseline in these rats of this particular cohort. In fact, there was a significant negative correlation of baseline locomotor activity level versus the response to CR (as percent of baseline). Therefore, the less the locomotor activity level at baseline, the greater the increase in locomotor activity after the implementation of CR. This significant negative correlation was observed beginning at 6 weeks and at every 6 weeks thereafter until the end of the study for horizontal activity, movement number, total distance, and movement time (Table 1).

Dopamine tissue content

The role of DA function in the nigrostriatal pathway in locomotor function is critically important, perhaps best demonstrated by the association of locomotor symptoms (including bradykinesia) of PD with >80% loss of DA in the striatum (Bezard et al., 2001). Our examination of DA tissue content in the striatum from rats in the current study revealed no significant increase in the CR group versus the AL group (Fig. 10A). However, there was a modest but significantly greater level of DA tissue content in the substantia nigra of CR rats compared to AL counterparts (Fig. 10B).

It may be argued that modulation of DA in the mesoaccumbens pathway could be modulated by CR, given that a deprivation of food as the calorie source could affect DA in the nucleus accumbens or VTA. However, like striatum, DA tissue content was not significantly different in the nucleus accumbens (Fig. 10C), nor in the ventral tegmental area (Fig. 10D), indicating that attenuation of aging-related bradykinesia from CR may be attributable in part to greater DA tissue content solely in the substantia nigra in this group.

The tissue levels of DOPAC essentially reflected the DA tissue content results from each region, with no significant differences between the striatum, NAc, or VTA. We observed an

nonsignificant trend toward an increase ~10% in substantia nigra of the CR group (data not shown).

Striatal proteins involved in dopamine neurotransmission

To further evaluate whether striatal proteins directly or indirectly involved with DA neurotransmission were affected by the CR regimen, we determined expression or protein phosphorylation of proteins involved at the biosynthesis step, reuptake step, or post-synaptic signaling step were affected in striatum. At the biosynthesis step, the expression of both CNS forms of the GDNF family receptor (GFR α 1) (insoluble and soluble form) was determined, as well as the expression and phosphorylation of TH protein. No significant difference was found for either form of GFR α 1 (Table 2). The CR regimen also did not affect TH protein expression nor phosphorylation (Table 2). We also did not detect a significant difference in dopamine transporter or dopamine D₁ receptor (Table 2).

Discussion

Potential lifestyle strategies to combat the significant risk of bradykinesia with advancing age need to be identified in established models of aging. The strain used here is a well-established aging model, noted for its longevity (Spangler et al., 1997; Ingram, 2000), and demonstrates decreased locomotor activity within the time period tested in this model (Spangler et al., 1997; Yurek et al., 1998; Salvatore et al., 2009). Thus, the initiation of CR at middle age was tested here as one such potential lifestyle strategy to attenuate this decrease in locomotor activity, as lifelong CR in rodents prevents aging-related bradykinesia (Ingram et al., 1987; Fitting et al., 2008). The age of CR intervention has potential human translation, as the timing of such a lifestyle practice (reducing caloric intake) could be initiated and practiced at a time when the likelihood of the risk of bradykinesia is still 10 to 20 years away in a human lifespan. Therefore, we designed the study such that rats were fed *ad libitum* up to the point of CR intervention, 12 months of age. Given that the lifespan of this strain can surpass 30 months of age, 12 months represents a human age equivalent ~35–50 years of age. We also aimed to understand and delineate the role of DA as a component of the neurobiological basis for aging-related bradykinesia. Overall, the results using this rodent model generate the following overall conclusions; 1) initiation of CR in male rats at middle-age (12 months), previously on a lifelong AL diet attenuated aging-related bradykinesia between 12 and 18 months of age; 2) weight differences between the two groups likely did not affect the differences in locomotor activity between the two groups at the end of the study, 3) we predicted that rats in the AL group with lower than average baseline locomotor activity at 12 months would decline in locomotor activity to a greater extent than their higher activity cohorts; this was not the case; 4) however, these same lower than average individuals in the CR group responded to CR intervention by exceeding their baseline activity level, unlike their higher than average activity cohorts; 5) although aging-related bradykinesia in the higher activity individuals in the CR group was attenuated, unlike that seen in rats with similar baseline activity in the AL group, these rats did not ever exceed their baseline locomotor performance; 6) the response to CR took ~6 weeks less in the lower activity cohorts of the CR group; and 7) as we hypothesized that increased DA tissue content would be seen in the CR group, we observed a modest increase in DA tissue content only in

the substantia nigra and not in striatum nor in the two mesoaccumbens regions of nucleus accumbens or VTA. The caveat to this observation is that a DA tissue readout in a 12 - month old group would be more confirmatory of CR intervention attenuating DA loss in either striatum or substantia nigra during this period of aging of 12 to 18 months.

It is noted that bradykinesia, also being a cardinal symptom of PD, in aging is not associated with >70% loss of striatal DA because, in fact, most (if not all) studies of aging indicate comparatively much less striatal DA or TH protein loss (Kish et al., 1992; Yurek et al., 1998; Collier et al., 2007; Cruz-Muros et al., 2007; Salvatore et al., 2009; Salvatore and Pruett, 2012), including those that test the human lifespan (Haycock et al., 2003). In PD models, the onset of bradykinesia is associated with a threshold of 80% TH or DA loss; even 60% striatal DA loss is not associated with bradykinesia (Bezard et al., 2001), and this level of loss represents the most, to our knowledge, that any aging study has reported (Collier et al., 2007). Indeed, aging and PD are of different etiologies and certainly present with distinctively different levels of striatal DA loss. However, at least in the context of this study, our results indicate that differences in striatal DA regulation are not associated with the distinct difference in locomotor activity observed at the end of this 6 month study.

Unlike for the striatum, we did observe a modest elevation (~15%) in DA only in the substantia nigra of CR rats (Fig.10). We did not observe any difference in the mesoaccumbens DA regions of the nucleus accumbens or VTA (Fig. 10). We do not argue that this modest difference was the sole mechanism for the significant increase in locomotor activity in the CR group, and additional protein data to further examine the basis for this difference was not available for this study. Nonetheless this increase may represent an attenuation of normal aging-related loss of DA in this region of ~20-40%, as reported in previous studies (Ponzio et al., 1982; Marshall et al., 1990; Yurek et al., 1998; Gerhardt et al., 2002; Cruz-Muros et al., 2007; Salvatore et al., 2009; Salvatore and Pruett, 2012). Such decreases may be attributable to reduced nigral TH protein expression or cell body number (Fearnley and Lees, 1991; Emborg et al., 1998; Ross et al., 2004; Rudow et al., 2008; Sanchez et al., 2008; Salvatore et al., 2009) or TH phosphorylation (Cruz-Muros et al., 2007; Salvatore et al., 2009). Decreased TH protein expression in aging has been observed in the substantia nigra in conjunction with no TH loss in the striatum (Wolf et al., 1991; Haycock et al., 2003; Salvatore and Pruett, 2012). It is important to point out that bradykinesia in PD models is associated with ~40–50% DA loss in the substantia nigra (Marsden, 1990; Bezard et al., 2001), similar to the level of loss seen in aging. Other studies have shown that modulation of DA in the substantia nigra alone, without influence on striatal DA, can affect locomotor activity (Hoffer et al., 1994; Andersson et al., 2006; Trevitt et al., 2001; Berquist et al., 2003; Pruett and Salvatore, 2013). Thus, the findings in our present study would suggest that aging-related impairment of DA regulation in the substantia nigra may be attenuated by CR during the 6 months of aging between 12 and 18 months. To this end, it would be now critical to evaluate if CR would still produce a similar attenuation of reduced locomotor activity if initiated at an even later age (such as 18 months) and whether effects on DA in striatum would be observed in conjunction with that in the substantia nigra.

Our previous work indicated that relative differences in locomotor activity within a cohort were maintained during aging, such that the test subjects maintained their relative ranking in locomotor activity against the others (Salvatore et al., 2009). Thus, even though aging entails an overall reduction in locomotor activity, the rats moving the least in a cohort would be expected to continue to move the least during aging. This observation led us to propose that the rats in the lower 50th percentile for locomotor activity at the 12 month baseline would decline accordingly with advancing age and be most vulnerable to aging-related bradykinesia. We did not find this to be the case between 12 and 18 months of age (Fig.9A). In fact, the top 50th percentile of rats exhibited the greatest decline from their baseline and decreased to the activity level of the lower 50th percentile by 18 weeks after initiation of the study. The molecular events of the nigrostriatal pathway associated with these differences are not known, but further investigation could reveal why more active subjects are more vulnerable to bradykinesia with aging during at least the early stages of aging. To the converse, in the CR group, the lower 50th percentile responded 6 weeks earlier to CR compared to the rats in the top 50th percentile and actually exceeded their baseline level at 12 weeks after CR intervention; whereas, those rats in the top percentile did return to, but did not exceed, their baseline level (Fig. 9B). The variable impact of CR on mortality has been reported across different mouse strains and also can be influenced by age (Forster et al., 2003). Here, our results add to understanding potential benefits of CR intervention in that individual behavioral phenotype, in this case innate locomotor activity, affects the vulnerability to the onset of bradykinesia as well as possible limits to its attenuation by CR over time. Indeed, whereas the rats of the lower 50th percentile in the CR group did increase activity above baseline, they did not reach the baseline levels acquired by the top 50th percentile after 12 weeks.

Possible issues relating to our observations of CR-mediated attenuation of locomotor decline begin with discussion of events in the periphery (Horner et al., 2011). Sarcopenia is one notable age-related event and is observed in the BNF strain used in this study, but may not occur until 33 months of age in this strain (Lushaj et al., 2008). Another notable event in the periphery is the divergence in weight from baseline (Fig.1) in the AL versus CR group. However, we did not observe any significant correlation with weight and innate locomotor activity level (Fig. 2) and the same was true for rotarod performance measured at baseline. Therefore, while these peripheral issues likely do not represent the sum of confounding issues in our data interpretation, there is some evidence from other literature (in addition of our finding of increased nigral DA tissue content) to support a central source of CR-mediated attenuation of locomotor decline. First of all, CR can reduce nigrostriatal neuron loss in PD models (Duan and Mattson, 1999; Maswood et al., 2004) and attenuate abnormal function of the autonomic nervous system caused by α -synuclein (Griffioen et al., 2013). The duration of CR may require a period of longevity before beneficial effects can be realized; with 2–8 weeks CR initiated prior to 6-OHDA lesion, one group found no significant difference in nigral or striatal DA or TH expression measures (Armentero et al., 2008). In a larger scheme outside of the nigrostriatal neuron, evidence points to benefits of CR that are related to increasing adaptive responses in neurons, such as attenuation of DA receptor loss (Roth et al., 1984), increased neurotrophic factor expression, improved response to natural stressors, and proteostasis (Mattson, 2014). Exercise effects on

attenuating neurodegenerative processes or locomotor impairment have similar adaptive responses akin to that seen from CR (Ridgel et al., 2009), and notably without evidence of increased striatal DA measures in PD models (O'Dell et al., 2007; Petzinger et al., 2007). Exercise shares common properties with CR with respect to energy consumption versus availability and can not only attenuate PD-like motor symptoms (Oguh et al., 2014), but also may improve motor impairments, including aging-related bradykinesia, if initiated at midlife (Savelle et al., 2010) or even when initiated in humans ~70 years of age (Denison et al., 2013). More investigation of growth factor signaling and proteins involved in DA regulation are necessary to more precisely elucidate how DA regulation is affected by the CR intervention. Accordingly, it will be important to unravel the molecular basis for the ceiling effect of CR-mediated attenuation of bradykinesia in rats of the lower 50th percentile in baseline activity at middle age (Fig. 11B).

Summary

Bradykinesia is not only a cardinal symptom of Parkinson's disease, but is also a significant functional challenge to the elderly, with 15% being disabled at age 65 and increasing to 50% at the start of 9th decade of life. Thus, employing non-invasive lifestyle strategies to reduce the risk of aging-related bradykinesia could offer an avenue to avoid this critical challenge. Here we show evidence that CR intervention in mid-life attenuates bradykinesia in rats previously on *ad libitum* diet. Moreover, this dietary intervention also attenuated functional decline as measured in the rotarod task, but did not significantly affect performance in a cognitive task (novel object location). Our longitudinal assessment of locomotor function also revealed several novel insights on CR impact. The response to CR was influenced by the individual locomotor activity profile prior to CR intervention. Those rats at the lower end of baseline activity increased their activity levels above baseline; whereas, rats at the high end of baseline activity prior to CR initiation did return to baseline after significant decline, but did not exceed their baseline. The most pronounced impact of CR was observed at 12 weeks following CR intervention for several parameters of locomotor activity generation, thus leaving an opportunity for future work to delineate the significant molecular triggers initiated at that time point. Third, given the role of DA neurotransmission in locomotor function, we found that nigral, but not striatal, DA tissue content was slightly but significantly increased overall in the CR group above the AL group. These results give a platform for future studies for understanding the molecular basis of locomotor decline in aging, with particular attention being paid to the importance of the baseline measure before intervention and individual, rather than group, vulnerabilities to aging as well as responsiveness to potential life-enhancing interventions.

Acknowledgments

This work was funded by NIA grant R01AG040261. The funding source had no involvement in the study design, data collection, analysis, or interpretation.

References

- Andersson DR, Nissbrandt H, Bergquist F. Partial depletion of dopamine in substantia nigra impairs motor performance without altering striatal dopamine neurotransmission. *Eur J Neurosci.* 2006; 24:617–624. [PubMed: 16903863]
- Armentero MT, Levandis G, Bramanti P, Nappi G, Blandini F. Dietary restriction does not prevent nigrostriatal degeneration in the 6-hydroxydopamine model of Parkinson's disease. *Exp Neurol.* 212:548–551. [PubMed: 18508049]
- Bennet DA, Beckett LA, Murray AM, Shannon KM, Goetz CG, et al. Prevalence of Parkinsonian signs and associated mortality in a community population of older people. *New England J Med.* 1996; 334:71–76. [PubMed: 8531961]
- Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurological Sci.* 1973; 20:415–455.
- Bergquist F, Shahabi HN, Nissbrandt H. Somatodendritic dopamine release in rat substantia nigra influences motor performance on the accelerating rod. *Brain Res.* 2003; 973:81–91. [PubMed: 12729956]
- Bezard E, Dovero S, Prunier C, Ravenscroft P, Chalon S, Guilloteau D, Crossman AR, Bioulac B, Brotchie JM, Gross CE. Relationship between the appearance of symptoms and the level of nigrostriatal degeneration in a progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned Macaque model of Parkinson's disease. *J Neurosci.* 2001; 21:6853–6861. [PubMed: 11517273]
- Buchman AS, Boyle PA, Wilson RS, Fleischman DA, Leurgans S, Bennett DA. Association between late-life social activity and motor decline in older adults. *Arch Intern Med.* 2009; 169:1139–1146. [PubMed: 19546415]
- Buchman AS, Leurgans SE, Weiss A, VanderHorst V, Mirelman A, Dawe R, Barnes LL, Wilson RS, Hausdorff JM, Bennet DA. Associations between quantitative mobility measures derived from components of conventional mobility testing and Parkinsonian gait in older adults. *PLoS ONE.* 2014; 9:e86262. [PubMed: 24465997]
- Collier TJ, Lipton J, Daley BF, Palfi S, Chu Y, Sortwell C, et al. Aging-related changes in the nigrostriatal dopamine system and the response to MPTP in nonhuman primates: Diminished compensatory mechanisms as a prelude to parkinsonism. *Neurobiol Dis.* 2007; 26:56–65. [PubMed: 17254792]
- Cruz-Muros I, Afonso-Oramas D, Abreu P, Barroso-Chinea P, Rodríguez M, Gonzalez MC, Hernandez TG. Aging of the rat mesostriatal system: Differences between the nigrostriatal and the mesolimbic compartments. *Exp Neurol.* 2007; 204:147–161. [PubMed: 17112516]
- Denison HJ, Syddall HE, Dodds R, Martin HJ, Finucane FM, Griffin SJ, Wareham NJ, Cooper C, Sayer AA. Effects of aerobic exercise on muscle strength and physical performance in community-dwelling older people from the Hertfordshire cohort study: a randomized controlled trial. *J Am Geriatr Soc.* 2013; 61:1034–1036. [PubMed: 23772733]
- Duan W, Mattson MP. Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. *J Neurosci Res.* 1999; 57:195–106. [PubMed: 10398297]
- Emborg ME, Ma SY, Mufson EJ, Levey AI, Taylor MD, et al. Age-related declines in nigral neuronal function correlate with motor impairments in rhesus monkeys. *J Comp Neurol.* 1998; 401:253–265. [PubMed: 9822152]
- Emerich DF, McDermott P, Krueger P, Banks M, Zhao J, Marszalkowski J, Frydel B, Winn SR, Sanberg PR. Locomotion of aged rats: Relationship to neurochemical but not morphological changes in nigrostriatal dopaminergic neurons. *Brain Res Bull.* 1993; 32:477–486. [PubMed: 8221140]
- Fearnley JM, Lees AJ. Ageing and Parkinson's disease: Substantia nigra regional selectivity. *Brain.* 1991; 114:2283–2301. [PubMed: 1933245]
- Fitting S, Booze RM, Gilbert CA, Mactutus CF. Effects of chronic adult dietary restriction on spatial learning in the aged F344 x BN hybrid F1 rat. *Physiol Behav.* 2008; 93:560–9. [PubMed: 18035382]

- Fleischman DA, Wilson RS, Schneider JA, Beinias JL, Bennet DA. Parkinsonian signs and functional disability in old age. *Exp Aging Res.* 2007; 33:59–76. [PubMed: 17132564]
- Forster MJ, Morris P, Sohal RS. Genotype and age influence the effect of caloric intake on mortality in mice. *FASEB J.* 2003; 17:690–692. [PubMed: 12586746]
- Gerhardt GA, Cass WA, Yi A, Zhang Z, Gash DM. Changes in somatodendritic but not terminal dopamine regulation in aged rhesus monkeys. *J Neurochem.* 2002; 80:168–177. [PubMed: 11796755]
- Griffioen KJ, Rothman SM, Ladenheim B, Wan R, Vranis N, Hutchison E, Okun E, Cadet JL, Mattson MP. Dietary energy intake modifies brainstem autonomic dysfunction caused by mutant α -synuclein. *Neurobiol Aging.* 2013; 34:928–935. [PubMed: 22883907]
- Haycock JW, Becker L, Ang L, Furukawa Y, Hornykiewicz O, Kish SJ. Marked disparity between age-related changes in dopamine and other presynaptic dopaminergic markers in human striatum. *J Neurochem.* 2003; 87:574–585. [PubMed: 14535941]
- Hebert MA, Gerhardt GA. Normal and drug-induced locomotor behavior in aging: comparison to evoked DA release and tissue content in Fischer 344 rats. *Brain Res.* 1998; 797:42–54. [PubMed: 9630504]
- Hoffer BJ, Hoffman AF, Bowenkamp KE, Huettl P, Hudson J, Martin D, Lin LF, Gerhardt GA. Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. *Neurosci Lett.* 1994; 182:107–111. [PubMed: 7891873]
- Horner AM, Russ DW, Biknevicius AR. Effects of early-stage aging on locomotor dynamics and hindlimb muscle force production in the rat. *J Exp Biol.* 2011; 214:3588–3595. [PubMed: 21993787]
- Hornykiewicz, O.; Kish, SJ. Biochemical pathophysiology of Parkinson's disease. In: Yahr, MD.; Bergmann, KJ., editors. *Advances in Neurology*. Raven Press; New York: 1986.
- Ingram DK, Reynolds MA. Assessing the predictive validity of psychomotor tests as measures of biological age in mice. *Exp Aging Res.* 1986; 12:155–162. [PubMed: 3830235]
- Ingram DK, Weindruch RH, Spangler EL, Freeman JR, Walford RL. Dietary restriction benefits learning and motor performance of aged mice. *J Gerontol.* 1987; 42:78–81. [PubMed: 3794202]
- Ingram DK. Age-related decline in physical activity: generalization to non-humans. *Med Sci Sports Exerc.* 2000; 32:1623–1629. [PubMed: 10994915]
- Irwin I, DeLanney LE, McNeill T, Chan P, Forno LS, et al. Aging and the nigrostriatal dopamine system: a non-human primate study. *Neurodegeneration.* 1994; 3:251–265. [PubMed: 7531106]
- Kastman EK, Willette AA, Coe CL, Bendlin BB, Kosmatka KJ, McLaren DG, Xu G, Canu E, Field AS, Alexander AL, Voytko ML, Beasley TM, Colman RJ, Weindruch RH, Johnson SC. A calorie-restricted diet decreases brain iron accumulation and preserves motor performance in old rhesus monkeys. *J Neurosci.* 2010; 30:7940–7947. [PubMed: 20534842]
- Kish SJ, Shannak K, Rajput A, Deck JH, et al. Aging produces a specific pattern of striatal dopamine loss: Implications for the etiology of idiopathic Parkinson's disease. *J Neurochem.* 1992; 58:642–648. [PubMed: 1729408]
- Kordower JH, Olanow CW, Dodiya HB, Chu Y, Beach TG, Adler CH, Halliday GM, Bartus RT. Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain.* 2013; 136:2419–2431. [PubMed: 23884810]
- Lushaj EB, Johnson JK, McKenzie D, Aiken JM. Sarcopenia accelerates at advanced ages in Fisher 344x Brown Norway rats. *J Gerontol A BiolSci Med Sci.* 2008; 63:921–927.
- Marsden CD. Parkinson's disease. *The Lancet.* 1990; 335:948–952.
- Marshall JF, Rosenstein AJ. Age-related decline in rat striatal dopamine metabolism is regionally homogeneous. *Neurobiol Aging.* 1990; 11:131–137. [PubMed: 2348901]
- Maswood N, Young J, Tilmont E, Zhang Z, Gash DM, Gerhardt GA, Grondin R, Roth GS, Mattison JA, Lane MA, Carson RE, Cohen RM, Mouton PR, Quigley C, Mattson MP, Ingram DK. Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease. *Proc Natl Acad Sci.* 2004; 100:18171–18176.
- Mattson MP. Interventions that improve body and brain bioenergetics for Parkinson's disease risk reduction and therapy. *J Parkinsons Dis.* 2014; 4:1–13. [PubMed: 24473219]

- Murray AM, Bennet DA, Mendes de Leon CF, et al. A longitudinal study of Parkinsonism and disability in a community population of older people. *J Gerontol Medical Sciences*. 2004; 59A: 864–870.
- O'Dell SJ, Gross NB, Fricks AN, Casiano BD, Nguyen TB, Marshall JF. Running wheel exercise enhances recovery from nigrostriatal dopamine injury without inducing neuroprotection. *Neuroscience*. 2007; 144:1141–1151. [PubMed: 17157992]
- Ogihara O, Eisenstein A, Kwasny M, Simuni T. Back to the basics: Regular exercise matters in Parkinson's disease: Results from the National Parkinson Foundation QII Registry study. *Parkinsonism and Rel Disord*. 2014; 20:1221–1225.
- Petzinger GM, Walsh JP, Akopian G, Hogg E, Abernathy A, Arevalo P, Turnquist P, Vuckovic M, Fisher BE, Togasaki DM, Jakowec MW. Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury. *J Neurosci*. 2007; 27:5291–5300. [PubMed: 17507552]
- Ponzio F, Calderini G, Lomuscio G, Vantini G, Toffano G, Algeri S. Changes in monoamines and their metabolite levels in some brain regions of aged rats. *Neurobiol Aging*. 1982; 3:23–29. [PubMed: 6178985]
- Prettyman R. Extrapyramidal signs in cognitively intact elderly people. *Age and Ageing*. 1998; 27:557–560. [PubMed: 12675093]
- Pruett BS, Salvatore MF. Nigral GFR α -1 infusion in aged rats increases locomotor activity, nigral tyrosine hydroxylase, and dopamine content in synchronicity. *Mol Neurobiol*. 2013; 47:988–999. [PubMed: 23321789]
- Ridgel AL, Vitek JL, Alberts JL. Forced, not voluntary, exercise improves motor function in Parkinson's disease patients. *Neurorehabil Neural Repair*. 2009; 23:600–608. [PubMed: 19131578]
- Rosano C, Bennett DA, Newman AB, Venkatraman V, Yaffe K, Harris T, Kritchevsky S, Aizenstein HJ. Patterns of focal gray matter atrophy are associated with bradykinesia and gait disturbances in older adults. *J Gerontol: Medical Sciences*. 2012; 67:957–962.
- Ross GW, Petrovich H, Abbott RD, Nelson J, Markesbery W, Davis D, Hardman J, Launer L, Masaki K, Tanner CM, White LR. Parkinsonian signs and substantia nigra neuron density in descendants elders without PD. *Ann Neurol*. 2004; 56:532–539. [PubMed: 15389895]
- Roth GD, Ingram DK, Joseph JA. Delayed loss of striatal dopamine receptors during aging of dietarily restricted rats. *Brain Res*. 1984; 300:27–32. [PubMed: 6733465]
- Rudow G, O'Brien R, et al. Morphometry of the human substantia nigra in ageing and Parkinson's disease. *Acta Neuropathol*. 2008; 115:461–470. [PubMed: 18297291]
- Ruxton GD, Neuhauser M. When should we use one-tailed hypothesis testing? *Methods in Ecology and Evolution*. 2010; 1:14–117.
- Salvatore MF, Pruett BS, Spann SL, Dempsey C. Aging reveals a role for nigral tyrosine hydroxylase ser31 phosphorylation in locomotor activity generation. *PLoS ONE*. 2009; 4(12):e8466. [PubMed: 20037632]
- Salvatore MF, Pruett BS. Dichotomy of tyrosine hydroxylase and dopamine regulation between somatodendritic and terminal field areas of nigrostriatal and mesoaccumbens pathways. *PLoS ONE*. 2012:e029867.
- Salvatore MF, Pruett BS, Fields V, Dempsey C. Comprehensive profiling of dopamine regulation in substantia nigra and ventral tegmental area. *J Vis Exp*. 2012
- Sanchez HL, Silva LB, Portiansky EL, Herenu CB, Goya RG, Zuccolilli GO. Dopaminergic mesencephalic systems and behavioral performance in very old rats. *Neuroscience*. 2008; 154:1598–1606. [PubMed: 18554807]
- Savelle SL, Koistinen P, Tilvis RS, Strandberg AY, Pitkala KH, Salomaa VV, Miettinen TA, Strandberg TE. Physical activity at midlife and health-related quality of life in older men. *Arch Intern Med*. 2010; 170:1171–1172. [PubMed: 20625028]
- Shulman JM, Yu L, Buchman AS, Evans DA, Schneider JA, Bennett DA, De Jager PL. Association of Parkinson disease risk loci with mild parkinsonian signs in older persons. *JAMA Neurol*. 2014; 71:429–435. [PubMed: 24514572]

- Spangler EL, Waggie KS, Hengemihle J, Roberts D, Hess B, Ingram DK. Behavioral assessment of aging in male Fischer 344 and Brown Norway rat strains and their F1 hybrid. *Neurobiol Aging*. 1994; 15:319–328. [PubMed: 7936056]
- Tabbal SD, Tian L, Karimi M, Brown CA, Loftin SK, Perlmutter JS. Low nigrostriatal reserve for motor parkinsonism in nonhuman primates. *Exp Neurol*. 237:355–362. [PubMed: 22836146]
- Trevitt JT, Carlson BB, Nowend K, Salamone JD. Substantia nigra pars reticulata is a highly potent site of action for the behavioral effects of the D1 antagonist SCH23390 in rat. *Psychopharmacol*. 2001; 156:32–41.
- Weed JL, Lane MA, Roth GS, Speer DL, Ingram DK. Activity Measures in Rhesus Monkeys on Long-Term Calorie Restriction. *Physiology and Behavior*. 1997; 62:97–103. [PubMed: 9226348]
- Wimmer ME, Hernandez PJ, Blackwell J, Abel T. Aging impairs hippocampus-dependent long-term memory for object location in mice. *Neurobiol Aging*. 2012; 33:2220–2224. [PubMed: 21872364]
- Wolf ME, LeWitt PA, Bannon MJ, et al. Effect of aging on tyrosine hydroxylase protein content and the relative number of dopamine nerve terminals in human caudate. *J Neurochem*. 1991; 56:1191–1200. [PubMed: 1672141]
- Yurek M, Hipkens SB, Hebert MA, Gash DM, Gerhardt GA. Age-related decline in striatal dopamine release and motoric function in Brown Norway/Fischer 344 hybrid rats. *Brain Res*. 1998; 791:246–256. [PubMed: 9593919]

A.

B.

Figure 1. Weight change and body composition during study

A. weight change Test subjects in the AL and CR groups weighed 462 ± 8 and 448 ± 10 grams, respectively, at the start of the CR intervention. There was no significant difference in these weights ($p=0.24$, $t=1.18$). In the AL group, there were significant differences in weight during the 24 wks of the study (repeated measures one-way ANOVA, $p<0.0001$, $F=167$), with significantly increased weight observed at 12 ($***p<0.0001$, $q=4.96$), 18 ($***p<0.0001$, $q=13.2$), and 24 weeks ($***p<0.0001$, $q=21.60$) compared to respective individual baseline weights at 12 months of age. In the CR group, there were significant differences in weight during the 24 wks of the study (repeated measures one-way ANOVA,

$p < 0.0001$, $F = 55.8$), with significantly decreased weight observed at 6 ($***p < 0.0001$, $q = 8.65$), 12 ($***p < 0.0001$, $q = 10.82$), 18 ($***p < 0.0001$, $q = 13.01$), and 24 weeks ($***p < 0.0001$, $q = 12.34$) compared to respective individual baseline weights at 12 months of age. **B. body composition** Body composition measured by NMR at 24 weeks on diet. Measures of lean mass ($t = 7.82$, $p < 0.0001$), fat mass ($t = 7.27$, $p < 0.0001$), and fluid mass ($t = 7.56$, $p < 0.0001$) were all significantly greater in the AL group vs. the CR group.

Figure 2. Weight and relationship to locomotor activity parameters at baseline (A,B) and at study completion (B,C)

A. Rats assigned to AL group. At the start of the longitudinal assessment of locomotor activity in rats assigned to the AL group, the weight of individual subjects was analyzed against locomotor parameters of horizontal activity and movement number (data shown), and total distance, movement time, and movement speed (data not shown). Weight in these subjects did not correlate with any locomotor parameter measured (horizontal activity, Pearson $r = -0.399$, $p = 0.14$; movement number, Pearson $r = -0.199$, $p = 0.47$; total distance, Pearson $r = -0.204$, $p = 0.47$; movement time, Pearson $r = -0.228$, $p = 0.41$; movement speed, Pearson $r = 0.098$, $p = 0.73$). **B. Rats assigned to CR group.** At the start of the longitudinal assessment of locomotor activity in rats assigned to CR group, the weight of individual subjects was analyzed against locomotor parameters of horizontal activity and movement number (data shown), total distance, movement time, and movement speed (data not shown). Weight in these subjects also did not correlate with any locomotor parameter measured (horizontal activity, Pearson $r = -0.333$, $p = 0.25$; movement number, Pearson $r = -0.2829$, $p = 0.33$; total distance, Pearson $r = -0.407$, $p = 0.15$; movement time, Pearson $r = -0.398$, $p = 0.16$; movement speed, Pearson $r = -0.375$, $p = 0.18$). **C. AL group, 24 weeks.** The percent change in weight against the baseline at study completion was analyzed against the percent change in horizontal activity at study completion (24 weeks). There was no significant correlation observed with this relationship (Pearson $r = -0.171$, $p = 0.54$). **D. CR**

group, 24 weeks. The percent change in weight against the baseline at study completion was analyzed against the percent change in horizontal activity at study completion (24 weeks). There was no significant correlation observed with this relationship (Pearson $r = -0.251$, $p=0.38$).

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Figure 3. Location Recognition Task

We observed a significant effect on memory in both groups of rats, as each group exhibited significantly greater exploration of the novel location during the test trial compared to the training trial. However, CR had no significant effect. The results supported the significant main effect of training, $F(1,27) = 13.9$, $p=0.002$, but the main effect of diet and the diet by time interaction were not significant. Analysis by 2-way repeated measures ANOVA.

Figure 4. Rotarod performance measures at 12 and 24 weeks

The test subjects were tested for baseline performance on a rotarod during the same week of assessment of open-field locomotor performance. Assignment to *ad libitum* or calorie restricted group was based upon prioritizing to equalizing locomotor performance. Based upon this priority, we did not observe a significant difference in rotarod performance between the two groups (ns, $p=0.478$, Student's unpaired t-test). **A. *ad libitum* (AL) group.** Latency to fall on the rotarod test was significantly different in the AL group (one-way ANOVA repeated measures, $p<0.0001$, $F=25.79$), with decreased performance at 12 (** $p<0.01$, $q=5.71$) and 24 weeks (** $p<0.001$, $q=10.13$) compared to individual baseline performances at the start of study at 12 months of age). There was also a significant decrease in performance between 12 and 24 weeks in this group ($q=4.41$ * $p<0.05$). **B. calorie restricted (CR) group** Latency to fall on the rotarod test was significantly different in the CR group (one-way ANOVA repeated measures, $p<0.0084$, $F=5.78$), with decreased performance at 24 weeks (** $p<0.01$, $q=4.77$), but not earlier at 12 weeks (ns, $q=1.87$) as compared to individual baseline performances at the start of study at 12 months of age. There was no significant decrease in performance between 12 and 24 weeks in this group (ns, $q=2.90$). Tukey's Multiple Comparison Test. **C. Rotarod performance at 12 weeks following CR intervention.** Rats in the AL group had a significantly decreased latency to fall compared to the rats in the CR group (* $p<0.05$, $t=2.24$, unpaired two-tailed Student's t-test). **D. Rotarod performance at 24 weeks following CR intervention.** At the

conclusion of the study, rats in the AL group had a significantly decreased latency to fall compared to the rats in the CR group ($*p<0.05$, $t=2.14$, unpaired Student's t-test).

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Figure 5. Impact of CR vs AL diet on movement number and horizontal activity from 12 to 18 months of age

A. Assessment of movement number against baseline in AL and CR groups between 12 and 18 months of age. In the AL group, movement number declined against individual baseline values beginning at 6 weeks and thereafter for the duration of the study (repeated measures one-way ANOVA ($p < 0.0001$, $F = 15.70$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks ($p < 0.05$, $q = 2.79$), baseline v 12 wks ($p < 0.0001$, $q = 4.61$), baseline v 18 wks ($p < 0.0001$, $q = 5.77$), baseline v 24 wks ($p < 0.0001$, $q = 7.33$). In the CR group, movement number did not decline against individual baseline values at 24 weeks, and there was a significant increase from baseline values observed at 12 week (repeated measures one-way ANOVA ($p < 0.05$, $F = 2.93$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks (ns, $q = 0.80$), baseline v 12 wks ($p < 0.05$, $q = 3.09$), baseline v 18 wks (ns, $q = 2.20$), baseline v 24 wks (ns, $q = 1.14$). Significant departures from baseline are indicated as a numerical percentage value in the graph. **B. Assessment of horizontal activity against baseline in AL and CR groups between 12 and 18 months of age.** In the AL group, horizontal activity declined against individual baseline values beginning at 6 weeks and thereafter for the duration of the study (repeated measures one-way ANOVA ($p < 0.0001$, $F = 12.73$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks ($p < 0.05$, $q = 2.64$), baseline v 12 wks ($p < 0.01$, $q = 3.68$), baseline v 18 wks

($p < 0.0001$, $q = 5.28$), baseline v 24 wks ($p < 0.0001$, $q = 6.57$). In the CR group, horizontal activity did not decline against individual baseline throughout the course of the study (repeated measures one-way ANOVA ($p = 0.076$)). **C. Evaluation of interaction of diet impact and aging on movement number.** To determine if there was interaction of the CR diet with aging, movement number was evaluated by two-way repeated measures ANOVA. A significant interaction between aging and diet on was observed ($F_{4,27} = 11.39$, $p < 0.0001$). There was a significant effect of diet on during aging between AL and CR groups ($F_{1,27} = 18.2$, $p = 0.0002$). Students t-test at each 6 weeks indicated significant differences in between the AL and CR groups at each 6 weeks; 6 weeks ($p < 0.05$, $t = 2.20$), 12 weeks ($p < 0.0001$, $t = 5.79$), 18 weeks ($p < 0.0001$, $t = 4.96$), and 24 weeks ($p < 0.0001$, $t = 5.23$). **D. Evaluation of interaction of diet impact and aging on horizontal activity.** To determine if there was interaction of the CR diet with aging, a significant interaction between aging and diet was observed ($F_{4,27} = 7.41$, $p < 0.0001$). There was a significant effect of diet during aging between AL and CR groups ($F_{1,27} = 5.12$, $p < 0.05$). Students t-test at each 6 weeks indicated significant differences between the AL and CR groups beginning at 12 weeks; 6 weeks (ns, $t = 0.66$), 12 weeks (** $p < 0.01$, $t = 3.41$), 18 weeks (** $p < 0.01$, $t = 3.35$), and 24 weeks (** $p < 0.01$, $t = 3.28$).

Figure 6. Impact of 24 weeks CR diet on total distance and movement time (from 12 to 18 months of age)

A. Assessment of total distance against baseline in AL and CR groups between 12 and 18 months of age. In the AL group, total distance (cm travelled in 1 hour session) declined against individual baseline values beginning at 12 weeks and thereafter for the duration of the study (repeated measures one-way ANOVA ($p < 0.0001$, $F = 16.65$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks (ns, $q = 2.38$), baseline v 12 wks ($p < 0.0001$, $q = 4.04$), baseline v 18 wks ($p < 0.0001$, $q = 5.98$), baseline v 24 wks ($p < 0.0001$, $q = 7.29$). In the CR group, total distance declined against individual baseline beginning at 18 weeks (repeated measures one-way ANOVA ($p < 0.01$, $F = 4.14$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks (ns, $q = 2.45$), baseline v 12 wks (ns, $q = 1.26$), baseline v 18 wks ($p < 0.05$, $q = 2.72$), baseline v 24 wks ($p < 0.01$, $q = 3.73$). Significant departures from baseline (as percent change from baseline) are indicated by numerical value in the graph. **B. A. Assessment of movement time against baseline in AL and CR groups between 12 and 18 months of age** In the AL group, movement time declined against individual baseline values beginning at 6 weeks and thereafter for the duration of the study (repeated measures one-way ANOVA ($p < 0.0001$, $F = 20.06$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks ($p < 0.05$, $q = 2.91$), baseline v 12 wks ($p < 0.0001$, $q = 4.62$), baseline v 18 wks ($p < 0.0001$, $q = 6.67$), baseline v 24 wks ($p < 0.0001$, $q = 8.05$). In the CR group, movement time declined against individual baseline beginning at 18 weeks (repeated measures one-way ANOVA ($p < 0.01$, $F = 3.57$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks (ns, $q = 2.29$), baseline v 12 wks (ns, $q = 1.22$),

baseline v 18 wks ($p<0.05$, $q=2.64$), baseline v 24 wks ($p<0.01$, $q=3.44$). Significant departures from baseline (as percent change from baseline) are indicated by numerical value in the graph. **C. Evaluation of CR diet impact and aging on total distance.** No significant interaction between aging and diet was observed ($F_{4,27}=1.54$, ns) or effect of diet during aging between AL and CR groups ($F_{1,27}=1.16$, ns). **D. Evaluation of CR diet impact and aging on movement time.** No significant interaction between aging and diet was observed ($F_{4,27}=1.98$, $p=0.07$) or effect of diet during aging between AL and CR groups ($F_{1,27}=1.79$, ns).

Figure 7. Relationship of movement parameters with movement number and composite locomotor score assessment
A. movement number v. horizontal activity ($n=30$, $p<0.0001$, Pearson $r=0.799$). **B. movement number v. total distance** ($n=30$, $p<0.0001$, Pearson $r=0.801$). **C. movement number v. movement time** ($n=30$, $p<0.0001$, Pearson $r=0.840$). **D. movement number v. movement speed.** ($n=30$, ns, Pearson $r=0.332$). **E. Composite analyses of relative longitudinal locomotor activity, AL vs. CR.** Given the correlation of movement number with horizontal activity, total distance, and movement time, a composite locomotor activity

score was calculated for each test subject for these locomotor parameters at baseline and at every 6 weeks. The composite score was obtained for each test subject by first obtaining a z-score for each parameter (combining the AL and CR raw values to obtain a normalized differential among the test subjects) at each time point. The sum of these z-scores was calculated to represent overall locomotor activity for each rat for each time of locomotor analysis conducted throughout the study. There was a significant interaction between aging and diet ($F_{4,112}=3.66$, $p<0.01$) and a significant effect of CR on the composite locomotor activity score during aging ($F_{1,28}=5.69$, $p<0.05$). Student's unpaired two-way t-test was then applied *post-hoc* to determine if significant differences existed between the AL and CR groups at each time point. Results are as follows; Baseline, ns, $p=0.83$, $t=0.209$; 6 wks, ns, $p=0.41$, $t=0.834$; 12 wks, $**p=0.003$, $t=3.22$; 18 wks, $**p=0.003$, $t=3.20$; 24 wks, $**p=0.004$, $t=3.10$. A repeated measures two-way ANOVA was used for this assessment.

Figure 8. Baseline locomotor activity vs locomotor decline

Within the AL and CR groups, the baseline activity the bottom 50% versus the top 50% in each cohort was used to examine if aging would differentially affect locomotor activity decline between these two cohorts and whether the response to CR intervention would be influenced by these baseline differences. **A. AL group.** The prediction in the AL group was that rats in the bottom 50th percentile would be more prone to decreased locomotor activity earlier and more severe, but this was not the case. At 12 and 18 weeks following the start of the study, there was significantly greater decreased horizontal activity in the top 50th percentile. Statistical analysis used all unpaired t-tests comparing percent decrease from baseline between each subgroup in the cohort; 6 wks, ns, $t=0.145$; 12 wks, $p=0.0039$, $t=3.56$; 18 wks, $p=0.0004$, $t=4.91$; 24 wks, ns, $t=1.62$). **B. CR group.** At 6 and 12 wks following CR intervention, the bottom 50th percentile at baseline in the cohort exhibited significantly increased departure from baseline horizontal activity compared to the top 50th percentile. 6 wks, $p=0.007$, $t=3.24$; 12 wks, $p=0.028$, $t=2.49$; 18 wks, ns, $t=1.80$; 24 wks, ns, $t=1.41$).

Figure 9. Departure from baseline in top vs. bottom 50th percentile between 12 and 18 months of age

A. AL group. In the AL group, the top 50th percentile showed significant decline in horizontal activity at 6 weeks, and every 6 weeks thereafter, from the start of the study when the rats were 12 months of age (repeated measures one-way ANOVA ($p < 0.0001$, $F = 20.06$). Post-hoc results (Dunnett's Multiple Comparison test); baseline v 6 wks ($p < 0.001$, $q = 4.46$), baseline v 12 wks ($p < 0.001$, $q = 5.82$), baseline v 18 wks ($p < 0.001$, $q = 7.73$), baseline v 24 wks ($p < 0.001$, $q = 7.61$). The bottom 50th percentile did not show a significant decline overall between 12 and 18 months of age (repeated measures one-way ANOVA ($p < 0.105$, $F = 2.16$)), although a trend toward a decrease from baseline was observed at 24 weeks. **B. CR group.** In the CR group, the top 50th percentile showed significant decline in horizontal activity (repeated measures one-way ANOVA ($p = 0.056$, $F = 2.68$) at 6 weeks (Dunnett's

Multiple Comparison test); baseline v 6 wks ($p<0.05$, $q=3.09$), but not for the remainder of the CR intervention as there was no significant departure from baseline at 12, 18, or 24 weeks, baseline v 12 wks (ns, $q=0.69$), baseline v 18 wks (ns, $q=1.00$), baseline v 24 wks (ns, $q=1.32$). The bottom 50th percentile did not show a significant decline from baseline at any 6 week interval from the start of CR, but actually showed an increase above baseline between 12 and 18 months of age (repeated measures one-way ANOVA ($p=0.047$, $F=2.84$) at 12 weeks from the start of CR (Dunnett's Multiple Comparison test); baseline v 12 wks ($p<0.05$, $q=2.97$).

Figure 10. Dopamine tissue content

All results are expressed as ng DA per mg protein and statistical analysis is by one-tailed unpaired t-test, as stated in the methods. **A. Striatum** DA tissue content was not significantly greater in the CR group compared to the AL group. **B. substantia nigra** DA tissue content was significantly greater in the CR group vs the AL group (* $p < 0.05$, $t = 1.81$, $df = 27$). **C. Nucleus Accumbens** DA tissue content was not significantly greater in the CR group compared to the AL group. **D.** DA tissue content was not significantly greater in the CR group compared to the AL group.

Table 1

Relationship of initial baseline locomotor activity in response to CR

<i>parameter</i>	6 weeks	12 weeks	18 weeks	24 weeks
Horizontal activity	-0.823, $p=0.0002$	-0.730, $p=0.002$	-0.569, $p=0.034$	-0.669, $p=0.009$
Movement number	-0.722, $p=0.002$	-0.752, $p=0.001$	-0.604, $p=0.022$	-0.701, $p=0.005$
Movement time	-0.733, $p=0.002$	-0.607, $p=0.016$	-0.619, $p=0.018$	-0.541, $p=0.046$
Total distance	-0.752, $p=0.001$	-0.571, $p=0.026$	-0.628, $p=0.016$	-0.639, $p=0.014$

In CR group, there was a significant negative correlation of baseline locomotor activity level versus the response to CR (as percent of baseline). The less the locomotor activity level at baseline, the greater the increase in locomotor activity after CR implementation. This significant negative correlation was observed beginning at 6 weeks and at every 6 weeks thereafter until the end of the study for horizontal activity, movement number, movement time, and total distance (Table 1).

Table 2
Striatal protein and protein phosphorylation

protein	AL	CR
Tyrosine hydroxylase (TH) ng TH /μg total protein	0.517 $\pm$ 0.021	0.474 $\pm$ 0.027
Ser¹⁹ TH	0.041 $\pm$ 0.004	0.042 $\pm$ 0.003
Ser³¹ TH	0.200 $\pm$ 0.008	0.180 $\pm$ 0.010
Ser⁴⁰ TH	0.061 $\pm$ 0.003	0.065 $\pm$ 0.003
DA transporter	780 $\pm$ 65	756 $\pm$ 75
DA D₁ receptor	1532 $\pm$ 105	1431 $\pm$ 148
GFRα1 insoluble	1244 $\pm$ 145	1217 $\pm$ 154
GFRα1 soluble	5.8 $\pm$ 0.6	5.6 $\pm$ 0.8

To determine impact of CR upon proteins involved in the regulation of dopamine neurotransmission in striatum, measures at the biosynthesis step (TH protein expression and phosphorylation), uptake, post-synaptic signaling, and upstream GDNF signaling (expression of the two isoforms of GFR α 1) were determined. No significant difference between the two groups was detected for any dependent measure. However, a trend toward a decrease in ser³¹ phosphorylation was noted in the CR group ($p=0.11$). Results for TH protein are expression in ng total TH per μ g protein, TH phosphorylation is expressed as phosphorylation stoichiometry against a calibrated standard curve (Salvatore and Pruett, 2012), and soluble GFR α 1 is expressed as pg soluble GFR per μ g protein (quantified against a standard curve of purified soluble GFR α -1 (Pruett and Salvatore, 2013). No significant differences in DA transporter nor DA D₁ receptor were evident between the two groups (results presented as immunoreactivity normalized to total protein).