

Nigral GFR α 1 Infusion in Aged Rats Increases Locomotor Activity, Nigral Tyrosine Hydroxylase, and Dopamine Content in Synchronicity

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Abstract Delivery of exogenous glial cell line-derived neurotrophic factor (GDNF) increases locomotor activity in rodent models of aging and Parkinson's disease in conjunction with increased dopamine (DA) tissue content in substantia nigra (SN). Striatal GDNF infusion also increases expression of GDNF's cognate receptor, GFR α 1, and tyrosine hydroxylase (TH) ser31 phosphorylation in the SN of aged rats long after elevated GDNF is no longer detectable. In aging, expression of soluble GFR α 1 in the SN decreases in association with decreased TH expression, TH ser31 phosphorylation, DA tissue content, and locomotor activity. Thus, we hypothesized that, in aged rats, replenishing soluble GFR α 1 in SN could reverse these deficits and increase locomotor activity. We determined that the quantity of soluble GFR α 1 in young adult rat SN is ~3.6 ng. To replenish age-related loss, which is ~30 %, we infused 1 ng soluble GFR α 1 bilaterally into SN of aged male rats and observed increased locomotor activity compared to vehicle-infused rats up to 4 days following infusion, with maximal effects on day 3. Five days after infusion, however, neither locomotor activity nor nigrostriatal neurochemical measures were significantly different between groups. In a separate cohort of male rats, nigral, but not striatal, DA, TH, and TH ser31 phosphorylation were increased 3 days following unilateral infusion of 1 ng soluble GFR α 1 into SN. Therefore, in aged male rats, the transient increase in locomotor activity induced by

replenishing age-related loss of soluble GFR α 1 is temporally matched with increased nigral dopaminergic function. Thus, expression of soluble GFR α 1 in SN may be a key component in locomotor activity regulation through its influence over TH regulation and DA biosynthesis.

Keywords Substantia nigra · Striatum · GFR α 1 · Tyrosine hydroxylase · Dopamine · Parkinsonism · Aging · Bradykinesia

Introduction

Glial cell line-derived neurotrophic factor (GDNF) signaling is critical for dopamine (DA) neuron survival and function [1–3] and is involved in the regulation of locomotor activity. A decrease in GDNF signaling precipitates locomotor impairment while increasing GDNF improves movement. For instance, age-related locomotor impairment is accelerated in mice with heterozygous knockout of either the ligand, GDNF, or its cognate receptor, GDNF family receptor α 1 (GFR α 1) [4–6]. Conversely, direct delivery of exogenous GDNF improves locomotor functions in animal models of aging [7–11], models of Parkinson's disease (PD) [12–16], and, in some studies, the PD patient [17, 18].

The locomotor benefits afforded by GDNF therapy are thought to be mediated primarily by effects on DA and DA-regulating proteins, such as tyrosine hydroxylase (TH), in the nigrostriatal pathway. In PD models, GDNF increases expression of striatal DA tissue content and TH [16]. However, in aging models or the otherwise “intact” nigrostriatal pathway, GDNF actually decreases striatal TH expression [19–21]. Interestingly, despite these alternate effects in the striatum, GDNF similarly increases DA tissue content in the dopaminergic cell body region of the substantia nigra (SN)

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in rodent models of aging and PD [7, 9, 11–13, 22]. In addition, GDNF increases TH phosphorylation, a major means of TH activation, in the SN [20, 21]. These effects of GDNF in the SN may be directly linked to the locomotor benefit of this neurotrophic factor since modulation of nigral DA alone has been shown to influence locomotor activity generation [23–26]. Furthermore, GDNF can induce locomotor improvement even when there is no change in striatal DA tissue content [12, 13]. Thus, the effects of GDNF on DA regulation in the SN could play some role in locomotor improvement.

Unfortunately, despite the demonstrated benefits of GDNF, controversy remains as to whether GDNF should continue to be pursued as a therapeutic option due, in part, to the outcomes of studies challenging GDNF's efficacy in the clinic [27] or in particular PD models [28]. Given the current roadblocks to GDNF therapy, targeting other molecular entities involved in GDNF signaling may prove beneficial in the treatment of locomotor impairment. One such molecular entity is GDNF's own receptor, GFR α 1. After binding GDNF, GFR α 1 mediates downstream signal transduction through interactions with signaling partners such as Ret [29, 30], which is known to affect TH and DA function [31]. GDNF signaling through GFR α 1 may also be RET-independent in some cases, but does require the presence of heparin sulphate glycosaminoglycans, even in RET-dependent signaling [32]. GFR α 1 expression may even be the limiting factor in GDNF signaling [33, 34] and may mediate the long-lasting dopaminergic and locomotor improvements induced by GDNF administration. For instance, GDNF delivery induces a sustained increase in nigral GFR α 1 expression that matches long-term increases in nigral DA, TH, and TH phosphorylation [20, 21]. Notably, increased nigral GFR α 1 expression and TH phosphorylation remain elevated long after GDNF levels in the SN are no longer elevated [21].

GFR α 1 is widely expressed in the brain, with notably intense immunohistochemical staining in the SN [35]. It exists primarily in two forms: a glycosyl-phosphatidylinositol (GPI)-linked receptor attached to the outer membrane of cell surfaces with a molecular weight of ~52 kDa [29, 30, 35], and a cleaved form of ~48 kDa [35, 36] that is a fully functional and soluble receptor [33, 36]. While both forms of GFR α 1 share similar downstream signaling mechanisms, the primary difference between the two forms is that signaling via soluble GFR α 1 is slower in onset but much more sustained compared to signaling via the GPI-linked receptor [36]. The cleaved form of GFR α 1 in the SN declines with aging in association with reduced nigral TH, ser31 TH phosphorylation, DA tissue content, and locomotor activity [26, 37]. Here, we determined whether replacing the quantity of soluble GFR α 1 in the SN normally lost in aging could, at least, transiently improve locomotor activity and, in a temporally matched fashion,

increase nigral TH protein and phosphorylation as well as DA tissue content.

Methods

Test Subjects

Male Brown–Norway/Fischer 344 F₁ hybrid (BNF) rats were utilized for all three of the major experiments. Four 11-month-old BNF rats were used to characterize levels of GFR α 1 in the nigrostriatal pathway against a soluble GFR α 1 standard curve. Ten male BNF rats were used to determine the locomotor effects of bilateral infusion of soluble GFR α 1. The locomotor activity of these rats was first characterized at 25 months of age. Bilateral infusions and locomotor testing were conducted when the rats were 29–31 months old; neurochemistry was determined 5 days following infusion. Five male BNF rats ranging in age from 18 to 25 months old were used to characterize the neurochemical effects 3 days after unilateral soluble GFR α 1 infusion to the SN. For the purpose of increasing *n* for determination of GFR α 1 differences 3 days following infusion, tissue from five female BNF rats was utilized.

HPLC Analysis

DA and DOPAC tissue content were measured by HPLC analysis as previously described [26, 38, 39]. Our technique allows for the processing of the protein pellet precipitated during the HPLC preparation for Western blot analysis of proteins and protein phosphorylation.

Western Blot Analysis

Tissue protein precipitates were prepared as previously described [38]. TH protein content and TH phosphorylation stoichiometry (p.s.) at ser19 and ser31 were determined by quantitative western blot against standard curves of known TH and phosphor-TH quantities from in house standards (calibrated PC12 cell extract) as previously described [26, 39]. The sources for primary antibodies were as follows: TH protein, Millipore (Cat. # AB152), ser19 phosphoTH, Phosphosolutions (Cat. # p1580-19), ser31 phosphoTH, 21st Century Biochemicals and validated previously [26, 39]. Table S1 contains information on all antibodies used. Ser40 TH phosphorylation was not assessed as the unilateral tissue dissection used for the 3-day neurochemical profile determination yielded low sample availability. We prioritized analysis first to ser31, because this site is phosphorylated by ERK1 and ERK2 [40], and these kinases are downstream of Ret [41, 42], which is a major signaling partner of GFR α 1 [29, 30]. We also prioritized TH

phosphorylation analysis to ser19, because this site is linked to Ca^{2+} -dependent depolarizing stimuli [43, 44], and GDNF itself can increase membrane depolarization [45] and ser19 phosphorylation in the SN [21].

GFR α 1 levels were also determined comparing sample aliquots of SN and striatum tissue (40 μg nominal protein load) collected from 11-month-old BNF rats ($n=4$) against a soluble GFR α 1 standard curve. Soluble GFR α 1 was acquired as a custom order from R&D Systems (Recombinant Rat GFR α 1). The resulting soluble GFR α 1 has a MW of ~ 47 kDa and the amino acid sequence of rat GFR α 1 from Asp25–Leu445 with the addition of the following sequence of amino acids at the C terminus: CSIEGR. The soluble GFR α 1 was provided in PBS (pH 7.4) at a concentration of 0.106 mg/ml. Dilutions of soluble GFR α 1 were made in Krebs bicarbonate buffer. The soluble GFR α 1 standard

curve ranged from 25 to 1,600 pg. For the standard curve, 10 μg of protein from a VTA sample from an 11-month-old BNF rat was loaded with standards to serve as a protein carrier and background GFR α 1 immunoreactivity from this VTA sample was subtracted from the standard curve.

Soluble GFR α 1 Infusion Procedure

Soluble GFR α 1 was diluted in Krebs buffer to 0.5 ng/ μl and infused at 1 $\mu\text{l}/\text{min}$ directly into the SN (relative to bregma: -5.7 AP, ± 2.5 ML, -8.5 DV) of anesthetized (pentobarbital sodium, 40 mg/kg, i.p.) BNF rats. For bilateral infusions, either 2 μl of the 0.5 ng/ μl soluble GFR α 1 solution or vehicle (Krebs' buffer) were infused into each SN. For unilateral infusions, 2 μl of the 0.5 ng/ μl soluble GFR α 1 solution was infused into one SN, while an equal volume of

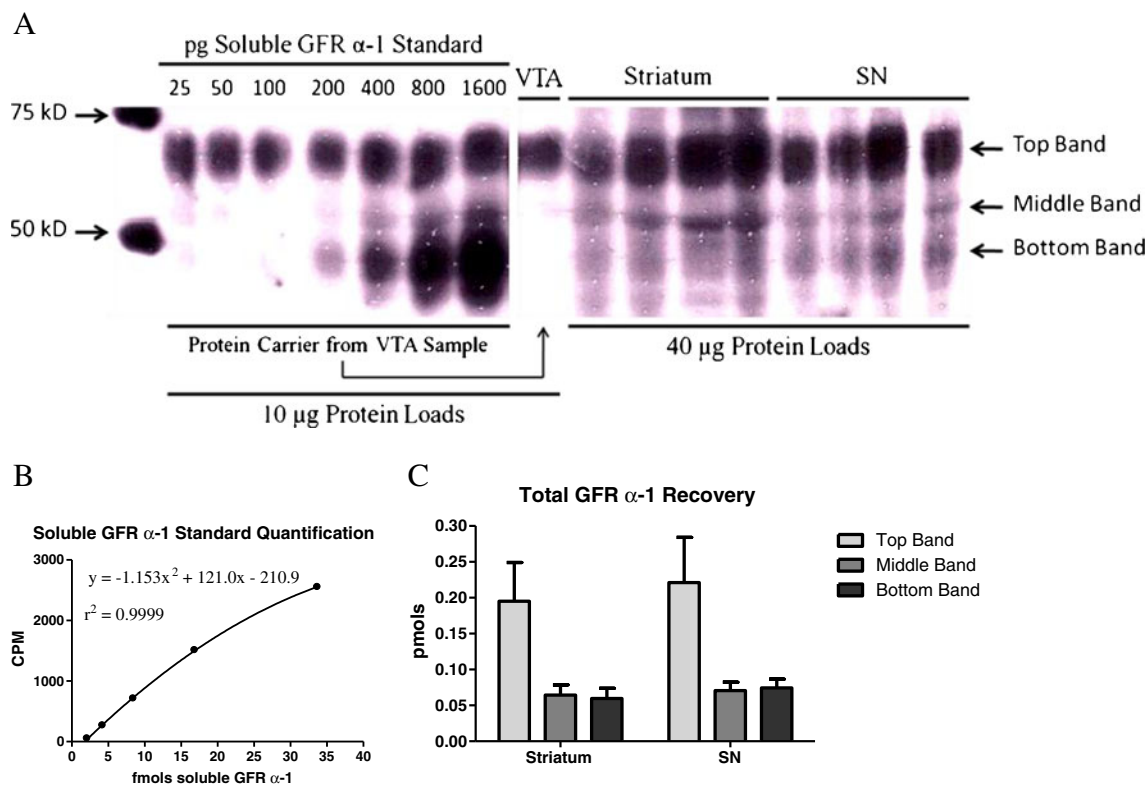


Fig. 1 Quantification of GFR α 1 in the striatum and SN. **a** Western blot immunoreactivity against GFR α 1. A soluble GFR α 1 standard curve was run in conjunction with samples from four striatum and SN tissue samples obtained from 11-month-old BNF rats. Protein loads were 40 μg . For the standard curve, a 10- μg protein load from a VTA sample (also from an 11-month-old BNF rat) was loaded as a protein carrier and also run separately to subtract immunoreactivity at the same MW as the soluble GFR α 1 standard. There were three immunoreactive bands detected in both the striatum and SN denoted as the top band (~ 60 kDa) (likely glycosylated form [37]), middle band (~ 52 kDa), and bottom band (~ 48 kDa). **b** Graph of soluble GFR α 1 standard curve. The fmol for each soluble GFR α 1 standard were graphed against immunoreactive bands corresponding to each soluble GFR α 1 standard loaded. The line represents the nonlinear regression of these data

points. The equation for this line ($y = -1.153x^2 + 121.0x - 210.9$) was used to extrapolate the quantity of GFR α 1 in each immunoreactive band of the samples. **c** Total GFR α 1 recovery in striatum and SN. The total pmol of each GFR α 1-immunoreactive band was determined for the four striatum and SN samples (average \pm SEM). In both regions, there was significantly more total recovery of the top band with relatively equal recovery of the middle and bottom bands. Two-way repeated measures ANOVA: Source of Variation: Interaction, ns; Brain region, ns; Band, $p = 0.0002$; Bonferroni posttests: Striatum: **Top Band vs. Middle Band, $p < 0.01$; Top Band vs. Bottom Band, $p < 0.01$; Middle Band vs. Bottom Band, ns; SN: **Top Band vs. Middle Band, $p < 0.01$; Top Band vs. Bottom Band, $p < 0.01$; Middle Band vs. Bottom Band, ns

vehicle (Krebs buffer) was infused into the contralateral SN. The infusion of an equal volume of vehicle into the contralateral hemisphere or bilaterally in rats designated as controls was intended to represent inherent DA regulation in the tissue and control for any inflammatory responses arising from the penetration of the needle and the 2 μ l infusion volume.

Locomotor Testing

Open-field locomotor activity was assessed using automated activity chambers as previously described [26]. Locomotor activity was recorded for 1 h, once per day. Prior to assigning rats to experimental groups, baseline locomotor activity was measured in ten BNF rats. Five consecutive daily trials were performed when the rats were 25 months of age and another four daily trials were conducted at 28–29 months of age. These two periods of locomotor assessment were used to determine baseline locomotor activity for each animal. The rats were then placed into two groups (GFR or vehicle) which had no baseline difference in horizontal activity, movement number, total distance, and time spent moving, as these parameters correlate to nigral DA levels [26]. Rats then underwent surgery to deliver either soluble GFR α 1 or vehicle (Krebs buffer) bilaterally to the SN after one final baseline locomotor assessment and had daily locomotor assessments for 5 consecutive days.

Statistics

To analyze differences in total recovery of GFR α 1 in each of the three immunoreactive bands between the SN and the striatum, two-way ANOVA followed by Bonferroni posttests were utilized. Two-way ANOVA followed by

Bonferroni posttests were also used to analyze both time- and group-dependent differences in locomotor activity measures. To determine the effect of bilateral infusion of soluble GFR α 1 into the SN on neurochemical parameters 5 days following infusion, an unpaired two-tailed *t*-test was used comparing the values of each neurochemical parameter within each brain region between the group receiving bilateral soluble GFR α 1 infusions to the SN and the group receiving bilateral vehicle infusions to the SN. To determine the effect of unilateral infusion of soluble GFR α 1 into the SN on neurochemical parameters 3 days following infusion, a paired two-tailed *t*-test was used comparing the values of each neurochemical parameter within each brain region between the soluble GFR α 1-infused hemisphere and the vehicle-infused hemisphere for each rat.

Results

Quantification of GFR α 1 Levels In Vivo

To determine the quantity of cleaved GFR α 1 (~48 kDa) receptor lost in the SN during aging [37], we measured nigrostriatal GFR α 1 immunoreactivity in 11-month-old BNF rats against a soluble GFR α 1 standard curve ranging from 25 to 1,600 pg soluble GFR α 1 (Fig. 1a, b). Three immunoreactive bands were detected at ~48, 52, and 60 kDa (Fig. 1a). Immunoreactivity of each band was compared to the soluble GFR α 1 standard curve (Fig. 1b) to determine the amount of GFR α 1 (Fig. 1c), which was similar between the striatum and the SN (Fig. 1c). The total recovery of the GFR α 1-immunoreactive band at ~48 kDa, representing the cleaved and soluble form of GFR α 1 [37], in the SN was 0.074 ± 0.0120 pmol or ~3.6 ng. Therefore,

Table 1 Baseline and post-treatment assessment of locomotor activity parameters in aged BNF rats

Locomotor Parameter	Pre-treatment		Post-treatment (first 4 days after infusion)		Post-treatment (first 5 days after infusion)	
	% variation between treatment groups	Group order	% variation between treatment groups	Group order	% variation between treatment groups	Group order
Horizontal activity	0.18	N/A	35.2**	GFR α 1 > Veh	17.2**	GFR α 1 > Veh
Movement number	0.41	N/A	9.5*	GFR α 1 > Veh	1.5	N/A
Total distance	0.48	N/A	15.1**	GFR α 1 > Veh	8.2*	GFR α 1 > Veh
Time spent moving	0.30	N/A	13.1*	GFR α 1 > Veh	4.7	N/A
Movement speed	8.6*	GFR α 1 > Veh	0.16	N/A	1.9	N/A
Vertical activity	0.30	N/A	15.0*	GFR α 1 > Veh	5.2	N/A

Ten locomotor trials were conducted for each aged rat prior to surgery where either 1 ng soluble GFR α 1 ($n=4$) or vehicle ($n=5$) was bilaterally infused into the SN. Locomotor trials were conducted each day following this surgery for 5 days. Each locomotor parameter was assessed by two-way ANOVA to determine if there were group differences prior to or after infusion surgeries. For the sum of the first 4 days after treatment horizontal activity, movement number, total distance, time spent moving, and vertical activity were all significantly greater in the soluble GFR α 1-treated group compared to vehicle-infused group. However, for the sum of 5 days following treatment, only horizontal activity and total distance were significantly higher in the soluble GFR α 1-treated group * $p<0.05$; ** $p<0.001$

because expression of this form of GFR α 1 is reduced in the SN by ~30 % between 12 and 30 months of age, a period in the lifespan wherein bradykinesia becomes manifest in this rat strain [26, 37], we deduced that the amount of soluble GFR α 1 in the SN lost with aging was likely ~1 ng.

Locomotor Effects of Bilateral Infusion of Soluble GFR α 1 into the SN of Aged Rats

Decreased nigral GFR α 1 is linked to reduced TH and DA in the SN [37] and reduced nigral DA or DA signaling is associated with decreased locomotor activity [23–26]. More specifically, nigral DA levels correlate to horizontal activity, movement number, total distance, and time spent moving, but not locomotor speed [26]. Therefore, we hypothesized that replenishing the age-related loss of soluble GFR α 1 would increase these locomotor measures in aged rats compared to age-matched controls of equal overall locomotor capability (Table 1). Thus, 1 ng of soluble GFR α 1, the approximate amount of soluble GFR α 1 predicted to be lost in aging (Fig. 1), was infused bilaterally to each SN, while control rats received bilateral vehicle infusions into each SN. In the first 4 days after surgery, the group receiving soluble GFR α 1 had significantly greater horizontal activity ($p < 0.0001$), movement number ($p = 0.033$), total distance ($p = 0.0096$), time spent moving ($p = 0.018$), and vertical activity ($p = 0.0074$) than the vehicle-treated group (Table 1, Fig. 2). In particular, there was a significant separation between the two groups' horizontal activity on day 3 (Fig. 2a) and movement number on day 4 following infusion.

Locomotor increases induced by soluble GFR α 1 infusion into the SN were transient and were no longer uniformly present on the fifth day following the infusion surgery (Fig. 2b, Table 1). In agreement with our previous finding that movement speed does not correlate with nigral DA levels [26], no significant difference in movement speed was observed after GFR α 1 infusion (Table 1, Fig. 2c).

Neurochemical Effects 5 Days After Bilateral Nigral Infusion of Soluble GFR α 1

Immediately after the locomotor trial performed on the fifth day following infusion surgery, dopaminergic tissue was collected. DA levels, TH levels, DA per TH, and DA turnover were not significantly different between the two treatment groups for any of the dopaminergic brain regions collected (Table 2). This lack of a difference in any DA parameter 5 days following infusion of 1 ng soluble GFR α 1 to the SN temporally matches the lack of a difference in locomotor activity between the soluble GFR α 1- and vehicle-infused rats at day 5 (Fig. 2).

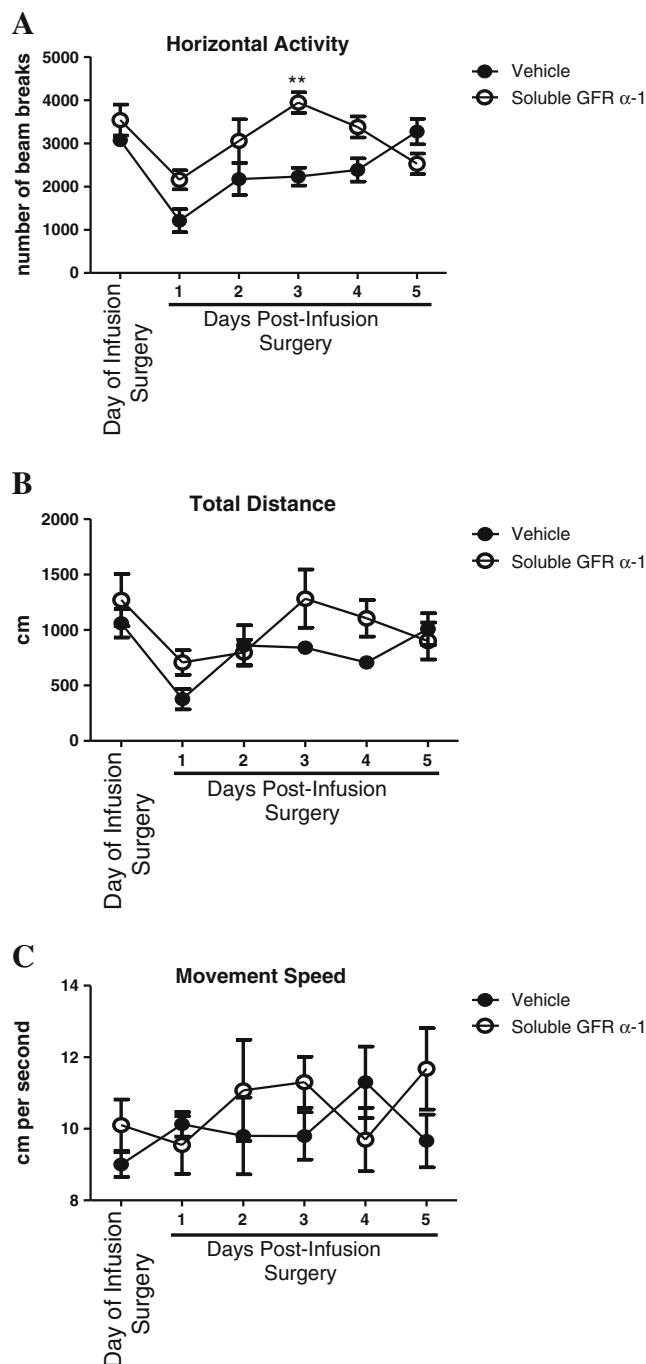


Fig. 2 Locomotor activity on the day of and for 5 consecutive days after nigral infusion of 1 ng soluble GFR α 1 or vehicle into aged rats. After establishment of baseline locomotor activity, rats were assigned to treatment groups (GFR or vehicle) and had equivalent locomotor activity parameters (Table 1). Then, 1 ng soluble GFR α 1 ($n = 4$) or vehicle ($n = 5$) was bilaterally infused into the SN of aged rats and locomotor activity was assessed daily for 5 days. Displayed are the group means \pm SEM on each day of locomotor testing for horizontal activity (a), total distance (b), and movement speed (c). As indicated in Table 1, all movement parameters except movement speed were significantly greater in the soluble GFR α 1-treated group in the first 4 days following the surgery. Bonferroni post-tests revealed that there were also significant differences for some of the individual days following infusion surgery. Horizontal activity (a) was found to be significantly higher in the soluble GFR α 1-treated group on the third day following infusions (** $p < 0.01$)

Table 2 Neurochemical measures 5 days following bilateral infusion of soluble GFR α 1 or vehicle to the SN of aged rats

Dependent measure	Brain region	Bilateral vehicle infusion to SN	Bilateral soluble GFR α -1 infusion to SN	<i>p</i> value of unpaired two-tailed <i>t</i> -test
ng DA per mg protein	Striatum	318.2 \pm 12.09	341.0 \pm 8.51	0.17
	SN	9.5 \pm 0.55	8.2 \pm 0.92	0.27
	NAc	150.5 \pm 14.56	151.2 \pm 10.98	0.97
	VTA	33.1 \pm 2.85	30.0 \pm 3.88	0.55
ng TH per μ g protein	Striatum	0.53 \pm 0.080	0.64 \pm 0.091	0.41
	SN	0.051 \pm 0.0055	0.046 \pm 0.0093	0.71
	NAc	0.16 \pm 0.037	0.15 \pm 0.035	0.91
	VTA	0.28 \pm 0.032	0.28 \pm 0.045	0.99
ng DA per ng TH	Striatum	0.65 \pm 0.105	0.58 \pm 0.079	0.60
	SN	0.20 \pm 0.013	0.20 \pm 0.025	1.00
	NAc	1.04 \pm 0.115	1.11 \pm 0.172	0.76
	VTA	0.13 \pm 0.013	0.12 \pm 0.017	0.58
pmol DOPAC per pmol DA	Striatum	0.075 \pm 0.0029	0.076 \pm 0.0058	0.94
	SN	0.10 \pm 0.009	0.12 \pm 0.004	0.17
	NAc	0.13 \pm 0.006	0.13 \pm 0.009	0.76
	VTA	0.15 \pm 0.007	0.16 \pm 0.009	0.50

DA per protein, TH per protein, DA per TH, and DOPAC per DA (a measure of DA turnover) were measured in the striatum, SN, NAc, and VTA of aged rats 5 days after direct bilateral infusion of 1 ng soluble GFR α 1 ($n=4$) or vehicle ($n=4$) into the SN. The data reflect the mean \pm SEM for each group and brain region. The measures were compared for each brain region between the vehicle and GFR α 1-treated groups using an unpaired two-tailed *t*-test. None of these measures was significantly different between the treatment groups for any of the brain regions

Neurochemical Effects 3 Days After Unilateral Nigral Infusion of Soluble GFR α 1

In a different cohort of 18- to 25-month-old BNF rats, we infused 1 ng soluble GFR α 1 unilaterally into the SN (an equal volume of vehicle was infused into the contralateral SN) to determine if the maximal differences in horizontal activity seen 3 days after bilateral infusion of soluble GFR α 1 into the SN (Fig. 2a) could be temporally matched to increased nigral DA and TH expression. Unilateral nigral infusion of 1 ng soluble GFR α 1 increased DA levels by 39 % (Fig. 3a) and DOPAC levels (Fig. 3b) in the SN compared to the contralateral vehicle-infused SN, but was without effect in the striatum, NAc, or adjoining VTA. Increased nigral DA was matched by a \sim 47 % increase in nigral TH protein (Fig. 4b, d), with no effect in striatum (Fig. 4a, c).

GFR α 1 Quantification After Nigral Infusion

Because of the impact of nigral GFR α -1 infusion on locomotor activity and DA modulation in the SN, we sought to determine if the time course of increased locomotor activity and nigral DA matched increased recovery of the receptor following its infusion. A trend toward an increase in the \sim 48 kDa band of GFR α 1 was observed in the GFR α 1-infused SN 3 days following infusion, and with the addition

of nigral samples from a separate cohort of rats, there was significantly greater (\sim 27 %) GFR α 1 recovery in the GFR α 1-infused SN compared to endogenous levels in the vehicle-infused SN 3 days following infusion (Fig. 5a). However, GFR α 1 levels were not significantly different between the groups 5 days following infusion (Fig. 5b), indicating that soluble GFR α 1 was either washed out or degraded by this time point.

TH Phosphorylation

TH phosphorylation at ser19 is a Ca²⁺-dependent process tied to neuronal excitability [43, 44], and GDNF can increase DA neuron excitability and Ca²⁺ influx [45]. Infusion of 1 ng soluble GFR α 1 into the SN was without effect on p.s. at ser19 in either the striatum or SN (Fig. 6a). Thus, the infusion of soluble GFR α 1 likely did not cause any changes in Ca²⁺ influx or excitability of DA neurons.

Soluble GFR α 1 infusion did significantly increase TH p.s. at ser31 in SN \sim 23 % (Fig. 6b). TH is phosphorylated at ser31 by ERK1 and ERK2 [40], which is downstream of Ret activation [41, 42]. Thus, the increase in nigral ser31 p.s. is likely to be indicative of increased activation of Ret due to interactions with the infused soluble GFR α 1. Lack of sample availability prevented the assessment of TH phosphorylation at ser40.

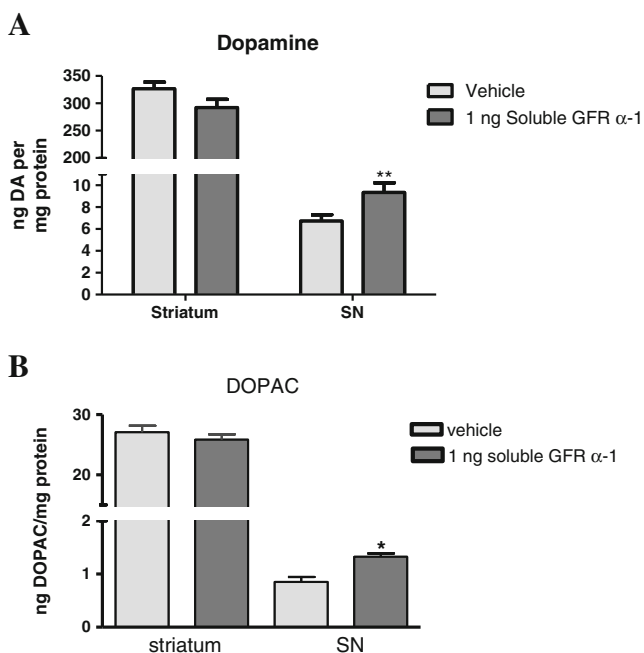


Fig. 3 Nigrostriatal DA and DOPAC tissue content 3 days following unilateral infusion of soluble GFR α 1 into the SN of aged rats. **a** Nigral DA was significantly increased 39 % (** $p=0.0088$, paired two-tailed t -test). Striatal DA was unaffected by infusion of 1 ng soluble GFR α 1 into the SN ($p=0.24$, paired two-tailed t -test). **b** Nigral DOPAC was significantly increased 56 % (* $p=0.05$), paired two-tailed t -test). Striatal DOPAC was unaffected by infusion of 1 ng soluble GFR α 1 into the SN ($p=0.24$, paired two-tailed t -test). Data represent the mean \pm SEM for each hemisphere (vehicle- or soluble GFR α 1-infused hemisphere) and brain region

Discussion

Locomotor and Neurochemical Effects of Replenishing Soluble GFR α 1 in the Aged SN

Our previous work indicates that age-related reductions in initiated locomotor activity are associated with decreased nigral DA tissue content, TH protein, and ser31 TH phosphorylation [26]. These decreases, in turn, are linked to an age-related reduction in the cleaved form of GFR α 1 in the SN [37]. We, therefore, hypothesized that decreased expression of the cleaved and soluble form of GFR α 1 in the SN is a deficient molecular link upstream of age-related reductions in nigral DA and, consequently, locomotor activity. We now demonstrate that locomotor activity in aged rats can be transiently increased following nigral infusion of soluble GFR α 1 in the quantity normally lost with aging. This locomotor effect was temporally matched with increased nigral DA along with increased TH protein expression and ser31 phosphorylation (Figs. 3, 4b, d and 6b). Furthermore, not only did infusion of soluble GFR α 1 into the SN increase nigral DA, TH, and ser31 phosphorylation of TH, measures which all normally decline with aging [26, 39], it increased

these measures to nearly the same magnitude that aging has been shown to reduce them (Table 3). These observations suggest GFR α 1 may normally maintain DA biosynthesis capacity in the SN, and adequate expression may preserve normal locomotor activity. It also raises questions as to why expression of the soluble form of GFR α 1 declines with aging.

Bilateral infusion of 1 ng soluble GFR α 1 to the SN maximally increased horizontal activity 3 days following infusion (Fig. 2a). Out to 4 days past infusion, movement number, total distance, and time spent moving, measures previously shown to correlate with nigral DA levels [26], as well as vertical activity were also significantly increased compared to vehicle-infused controls (Table 1). On the other hand, movement speed, a movement parameter shown not to correlate with nigral DA levels [26], was not significantly different at any time point after soluble GFR α 1 infusion (Table 1, Fig. 2c). The transient locomotor effects induced by soluble GFR α 1 infusion were temporally correlated with increased nigral DA levels (Figs. 2 and 3), both with regard to the increases seen in each at 3 days and eventual lack of effects 5 days following infusion (Fig. 2; Tables 1 and 2). Furthermore, these effects coincided with the time course during which nigral GFR α 1 levels were themselves elevated after infusion (Fig. 5). The transient nature of the effects induced by nigral GFR α 1 infusion suggests that sufficient and sustained expression of GFR α 1 in the SN may be required for continued locomotor activity improvement or maintenance during aging.

Comparison of Soluble GFR α 1 Effects Versus GDNF Administration

Both GDNF and soluble GFR α 1, as seen in our work, similarly increase nigral DA and locomotor activity. In aged rats and non-human primates, GDNF administration increases locomotor activity [7–11]. In fact, 10 μ g GDNF to the SN can increase total distance out to one [7] or 3 weeks [9]. Furthermore, GDNF-mediated increases in locomotor activity are associated with elevated DA tissue content in the SN but not the striatum [7, 9, 11–13]. The increase in nigral DA induced by either GDNF or soluble GFR α 1 likely arises, at least in part, through increased ser31 TH phosphorylation, which is seen following nigral infusion of soluble GFR α 1 (Fig. 6b) or striatal delivery of GDNF [20, 21].

There is, however, a notable contrast between the effects of GDNF compared to GFR α 1 on TH expression in the “intact” nigrostriatal pathway. While nigral TH levels were increased following unilateral infusion of 1 ng soluble GFR α 1 (Fig. 4d), GDNF infusion to intact nigrostriatal pathways (such as in aged rats) decreases striatal TH expression [19–21, 46]. In models of Parkinson’s disease,

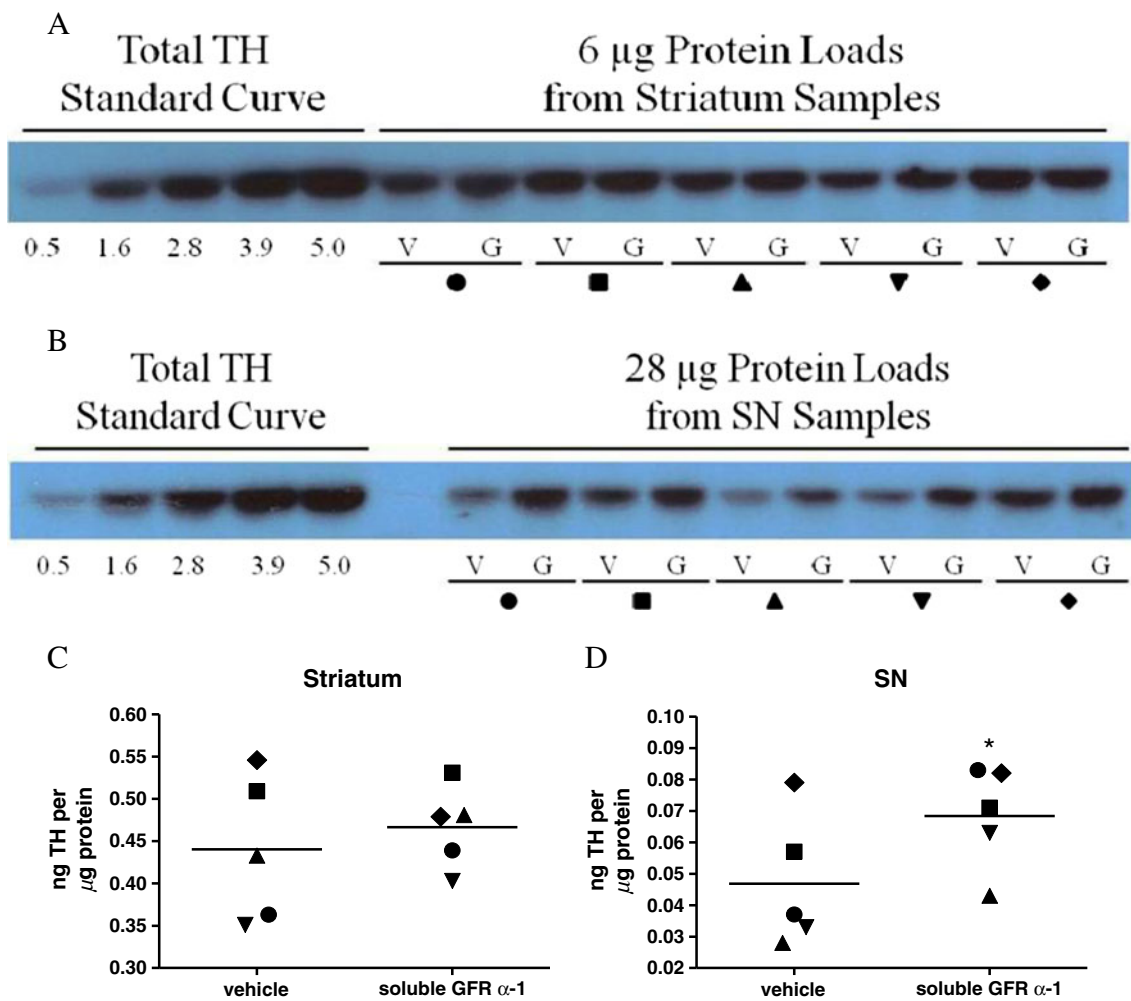


Fig. 4 Nigrostriatal TH protein expression levels 3 days following unilateral infusion of soluble GFR α 1 into the SN of aged rats. TH protein expression in striatum (**a**, **c**) and substantia nigra (**b**, **d**) represented against TH protein standard (**a**, **b**) and as individual differences 3 days following nigral GFR (1 ng) infusion versus vehicle (**c**, **d**). This analysis was carried out on five rats each denoted by a different symbol (filled circle, filled square, filled upright triangle, filled inverted triangle, filled diamond). The vehicle-treated hemisphere is denoted by a V and the soluble GFR α 1-treated hemisphere is denoted by a G. Data in **c**, **d** represent the mean \pm SEM for each hemisphere (vehicle- or

soluble GFR α 1-infused hemisphere) and brain region. **a** Western blot of striatal TH. The 6 μ g protein loads from striatal samples were compared against a TH standard curve ranging from 0.5 to 5.0 ng. **b** Western blot of nigral TH. The 28- μ g protein loads from nigral samples were compared against a TH standard curve ranging from 0.5 to 5.0 ng. **c** Striatal TH levels. Striatal TH was unaffected by infusion of 1 ng soluble GFR α 1 into the SN ($p=0.35$, paired two-tailed t -test). **d** Nigral TH levels. Nigral TH was significantly increased 47 % ($*p=0.044$, paired two-tailed t -test)

though, wherein there is substantial loss of TH, GDNF does increase striatal TH expression [13–16], suggesting that basal TH expression levels may predict responsiveness to GDNF such that TH is only increased by GDNF when basal TH levels are below normal. Since little if any loss of TH protein has been reported in the striatum of aged rats [26, 39] or humans [47], this may explain why delivery of GDNF to intact systems reduces striatal TH expression. However, the BNF rats utilized in this study were at ages for which nigral TH is reduced [26, 39]. Thus the increase in nigral TH seen following infusion of GFR α 1 in this study may be more analogous to the effect GDNF has of increasing TH expression in Parkinson's disease models.

Another major difference between GDNF and soluble GFR α 1 administration is the longevity of their effects. Namely, the neurochemical and locomotor effects following infusion of 1 ng soluble GFR α 1 to the SN occur for a much shorter period of time than those following GDNF administration. However, this temporal difference may be accounted for by the long-lasting impact GDNF has upon GFR α 1 expression and the difference in the quantities of GDNF typically infused into brain compared to the amount of soluble GFR α 1 infused in this study. Normal levels of GDNF in the SN are 0.003 ng per mg protein in the rhesus monkey [48] and in the range of 0.01–0.06 ng per mg protein in the rat [19, 49], while levels of cleaved GFR α 1

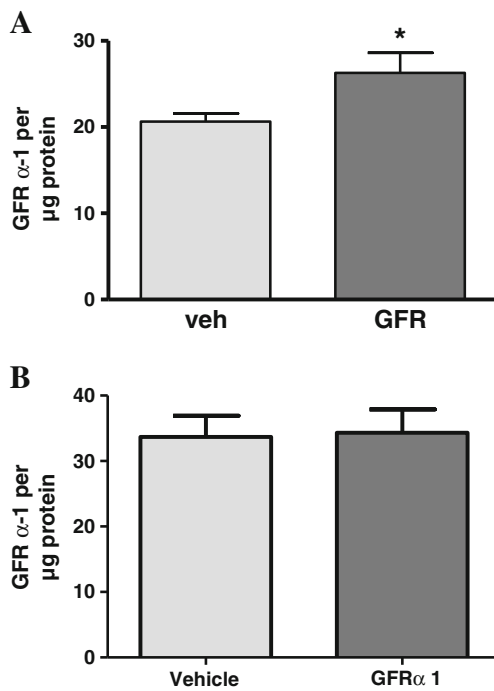


Fig. 5 GFR α 1 recovery in SN. The difference in GFR α 1 recovery in the SN between vehicle control hemisphere and the contralateral hemisphere infused with 1 ng soluble GFR α 1 (48 kDa), at **a** 3 days after infusion ($*p < 0.05$, $n = 10$, paired two-tailed t -test) and **b** 5 days after infusion (ns, $n = 4$, unpaired two-tailed t -test)

in the rat SN are ~100-fold greater, being 5.7 ± 0.50 ng per mg protein by our calculation (Fig. 1). Given that the average total protein recovery from the SN samples used to quantify GFR α 1 levels was 0.63 ± 0.098 mg, this suggests that the quantity of GDNF in the rat is ~0.01–0.04 ng per SN, compared to the total amount of cleaved GFR α 1, which is ~3.6 ng. As a point of reference to this *in vivo* data on GDNF expression, GDNF studies often infuse 10–100 μ g GDNF [7–9, 20, 21], which is 250,000–10,000,000 times greater than endogenous levels. Conversely, in this study we infused 1 ng soluble GFR α , which is only a third of the total amount of cleaved GFR α 1 recovered from the rostral–caudal extent of the SN from 11-month-old BNF rats. Thus, our infusion of soluble GFR α 1 is within physiological range and was intended to transiently increase nigral GFR α 1 levels to levels seen in younger rats.

A potentially unique feature of GDNF is that it may induce a long-term increase in GFR α 1-mediated signaling. Notably, GDNF increases the expression of GFR α 1 [20, 49] specifically within the SN up to 30 days following a 100- μ g infusion of GDNF into the ipsilateral striatum [20]. This sustained elevation of nigral GFR α 1 levels is seen despite the fact that nigral GDNF levels themselves are elevated at 1 day, but not 7 days, following striatal GDNF infusion [21]. Thus, the prolonged increases in DA function and locomotor activity induced by supra-physiological levels of GDNF

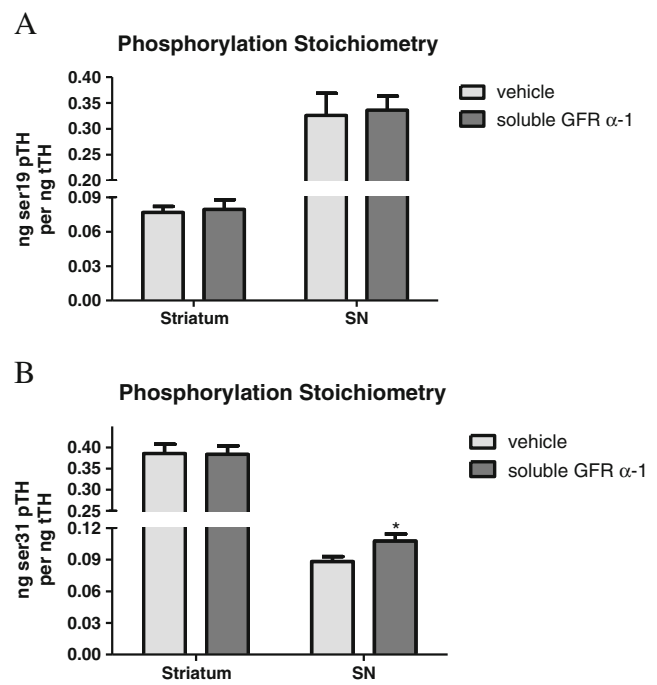


Fig. 6 TH phosphorylation in the nigrostriatal pathway 3 days following unilateral infusion of soluble GFR α 1 into the SN of aged rats. The effect of unilateral infusion of 1 ng soluble GFR α 1 into the SN of aged rats on TH phosphorylation stoichiometry. Data represent the mean \pm SEM for each hemisphere (vehicle- or soluble GFR α 1-infused hemisphere). **a** Phosphorylation stoichiometry at ser19 was unchanged in the striatum ($p = 0.54$, paired two-tailed t -test) or the SN ($p = 0.75$, paired two-tailed t -test) 3 days following nigral infusion of soluble GFR α 1. **b** Phosphorylation stoichiometry at ser31 was unchanged in the striatum ($p = 0.81$, paired two-tailed t -test) but was significantly increased by 23 % in the SN ($*p = 0.0105$, paired two-tailed t -test) 3 days following nigral infusion of soluble GFR α 1

may result primarily from a sustained increase in GFR α 1 expression.

Implications

Age-related loss of soluble GFR α 1 in the SN could be a critical molecular event precipitating bradykinesia in aging, particularly since levels of striatal GDNF [50] and nigral RET [51] are maintained with aging. Our findings show that a rather narrow window of receptor quantity (~1 ng) is associated with TH protein expression, TH phosphorylation at ser31, and DA tissue content in the SN. Given that nigral DA may affect locomotor capabilities [23–26], it stands to reason that sustaining GFR α 1 expression in the SN could represent a new strategy for treating locomotor impairment. However, we point out that differentiating between the expression of soluble and GPI-linked forms of GFR α 1 may be critical in this regard, particularly since a recent study suggests that there is no loss of nigral GFR α 1 in a population of aged Asian Indians [52]. While this may seem at odds with our finding of reduced soluble GFR α 1 in the

Table 3 Neurochemical changes in the SN induced by aging, 12 to 30 months (mos) and 3 days following infusion of soluble GFR α 1 to the SN

Dependent Measure	Age-related changes in the SN of BNF rats (Salvatore et al. [26])			Impact in SN 3 days after unilateral GFR α 1 infusion in SN of aged rats		
	12 mos	30 mos	% Change	Vehicle	1 ng soluble GFR α 1	% Change
ng DA	7.6	4.9	↓ 36 %	6.7	9.3	↑ 39 %
ng TH	0.053	0.028	↓ 47 %	0.047	0.069	↑ 47 %
ser31pTH	0.10	0.074	↓ 30 %	0.088	0.11	↑ 23 %

Unilateral infusion of 1 ng soluble GFR α 1 into the SN of aged animals increases DA per protein, TH protein expression (as ng TH per μ g protein), and ser31TH phosphorylation (ser31 pTH) in the SN

SN with aging [37], one reason for this apparent dissimilarity may be that in this study GFR α 1 expression was determined by immunohistochemistry, which may have only measured the GPI-linked form of GFR α 1 as soluble GFR α 1 could potentially be washed out during tissue preparation. We also have reported no age-related loss of the full-length (~52 kDa), presumably GPI-linked, form of GFR α 1 in the SN [37]. Also, this particular population of Asian Indians is less prone to Parkinson's disease [52]. As such, preservation of nigrostriatal function with aging may be unique to this population. Indeed, while bradykinesia becomes increasingly prevalent with aging, half of the population over age 80 years does not exhibit bradykinesia [53, 54]. Thus, loss of soluble GFR α 1 may represent a molecular deficit, which predisposes the aging population to developing bradykinesia.

Recent work by Bartus et al. [55] has prompted consideration of targeting growth factors to the SN in order to overcome the deficits in the retrograde transport of proteins that occur in neurodegenerative disorders. Here, we show that targeted infusion of the soluble GDNF receptor, GFR α 1, to the SN can transiently increase locomotor activity in a manner that is temporally matched with increased DA tissue content, TH expression, and TH ser31 phosphorylation in the SN and, notably, independent of any such increases in the striatum. However, there are questions that remain unanswered going forward. First and foremost, we stress that our study took place in the temporal window immediately following infusion surgery wherein locomotor activity was decreased below the presurgery baseline level (Fig. 2), and during this time frame, deficits in locomotion and nigral DA function were only transiently increased following a one-time infusion of soluble GFR α 1. Therefore, our future work will examine the impact of GFR α 1 infusion on a more protracted basis, wherein post-surgical effects on locomotor function are diminished in both control and treatment groups. Second, the cellular source of soluble GFR α 1 in the SN is unknown as is the mechanism of how expression of this receptor decreases with aging. Both dopaminergic and GABAergic neurons [56] as well as astrocytes [57, 58] and microglia [59] can express this receptor, so determining if any of these cell types are the primary source of

soluble GFR α 1 in the SN will be a necessary next step. Third, we need to determine if the transient increase in nigral TH expression induced by soluble GFR α 1 infusion is mediated by an increase in the number of TH-positive neurons or simply an upregulation of TH in those neurons already expressing TH. Indeed, increasing TH expression or function in the SN could be a means to increase locomotor activity, possibly through its impact upon nigral TH expression or activity and resulting increased DA [60]. Our findings have revealed soluble GFR α 1 as a molecular substrate involved in the maintenance of nigral DA levels, demonstrated that increasing its expression in the SN can increase locomotor activity, and identified this soluble receptor as a possible target to treat impaired locomotor activity.

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