# Fractalkine Over Expression Suppresses α-Synuclein-mediated Neurodegeneration

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In Parkinson's disease, α-synuclein is known to activate microglia and this activation has been proposed as one of the mechanisms of neurodegeneration. There are several signals produced by neurons that have an anti-inflammatory action on microglia, including CX3CL1 (fractalkine). We have shown that a soluble form of CX3CL1 is required to reduce neuron loss in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice and that fractalkine agonism can reduce neuron loss in a 6-hydroxydopamine lesion model. Here, we show that fractalkine can reduce α-synuclein-mediated neurodegeneration in rats. Rats that received fractalkine showed abrogated loss of tyrosine hydroxylase and Neu-N staining. This was replicated in animals where we expressed fractalkine from astrocytes with the glial fibrillary acid protein (GFAP) promoter. Interestingly, we did not observe a reduction in MHCII expression suggesting that soluble fractalkine is likely altering the microglial state to a more neuroprotective one rather than reducing antigen presentation.

*Received 20 March 2014; accepted 23 August 2014; advance online publication 14 October 2014. doi:10.1038/mt.2014.175*

## INTRODUCTION

In Parkinson's disease (PD), α-synuclein activates microglia and this activation has been suggested as one of the mechanisms of neurodegeneration.[1–4] Microglial activation is regarded as a variable and adaptive process as microglia continuously screen their microenvironment.[5–7] It has been suggested that a continuous dialogue between neurons and microglia under basal conditions is required to maintain a normal central nervous system (CNS) state and disruption of this dialogue could trigger changes in the functional phenotype of microglia.[8] There are several signals produced by neurons that have an anti-inflammatory action on microglia, for example, CD200, CD22, CD47, and fractalkine (FKN, CX3CL1).[8,9] Within the CNS, FKN is seen as a neuroimmune regulatory protein, signaling with its sole receptor (CX3CR1) that resides on microglia.[10–15] It is believed that interaction between FKN and CX3CR1 contributes to maintain microglia in a surveillant phase however the exact microglial changes have not been determined. Functionally, FKN signaling reduces the overproduction of proinflammatory molecules such as inducible nitric oxide synthase, interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 generated by microglia.[12,13,16–20]

FKN is a transmembrane protein with a chemokine domain attached to a mucin-like stalk. The membrane-bound FKN is important for adhesion of monocytes to endothelial cells, and may also play a role in monocyte-induced endothelial cell death, at least in the periphery.[21] FKN can also be processed by cleavage with ADAM10/17 or cathepsin S to release the ectodomain and produce a secreted or soluble form of fractalkine (sFKN).[22,23] This cleavage from neuronal membranes is an early event in neuronal injury and may represent a response to quell microglial activation.[24] Evidence suggests that sFKN is important for chemotaxis and acts as a chemoattractant for both lymphocytes and monocytes.[25] The exact roles of these subtypes of FKN are not completely established in the periphery or in the CNS but it has been suggested that the membrane and soluble forms elicit different cytokine responses from immune cells.[26,27]

Within the CNS FKN has been shown to be both neuroprotective and detrimental to neurons. A cross between amyloid precursor protein (APP)/presenilin 1 (PS1) and CX3CR1$^{-/-}$ transgenic mice resulted in a decrease in amyloid burden.[28] Interestingly, a cross between CX3CR1$^{-/-}$ mice with a human tau line (hTau) resulted in an increase in tau pathology.[29] This dichotomy of actions may suggest disregarding FKN as a therapeutic approach for neurological disorders. However, we recently demonstrated that over expression of a secreted form of fractalkine using intrahippocampal gene delivery substantially reduced tau pathology, neuron loss and brain atrophy in the Tg4510 mouse model of tau deposition, but it did not alter amyloid pathology in APP/PS1 mice.[30] We have also shown that soluble fractalkine but not the membrane form is required to reduce neuron loss in the substantia nigra of MPTP-treated CX3CL1$^{-/-}$ mice.[31] Further, we have demonstrated that fractalkine receptor agonism can reduce neuron loss in a 6-hydroxydopamine (6-OHDA) PD model.[11]

Since there are currently no therapeutic treatments that are capable of reducing synuclein pathology and its associated neuron loss, we examined the potential benefits of FKN gene delivery in a synucleinopathy model. Further, we examined the over expression of both the soluble and membrane forms of FKN to see if there are any differences in neuron rescue. We report that fractalkine receptor

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agonism with the soluble FKN can rescue neuron loss in the recombinant adeno-associated virus (rAAV) mediated synuclein model of PD and warrants further investigation as a therapeutic target.

## RESULTS

In this study, we set out to examine the potential therapeutic effect of increased fractalkine expression on α-synuclein-mediated neurodegeneration in rats. The model we used was the over expression of human α-synuclein via rAAV. This model can produce ~40% loss of dopaminergic neurons over a 2-month period.[32] In this study, we used the same titer described by those authors, however, we used AAV serotype 9 which has been used previously.[33] Expression of green fluorescent protein (GFP) from the rAAV9 injected into the rat substantia nigra is shown in **Figure 1c**. Experimental timeline for injections is shown in **Figure 1k** and details found in the Methods section. In conjunction with the α-synuclein virus, we coinjected either a control virus (GFP) or one of our three fractalkine constructs. Since fractalkine (FKN) is a membrane bound protein that has an ectodomain that is cleaved into a soluble chemokine, we constructed three different versions of FKN: native (nFKN), mutant membrane bound (mFKN, amino acid changes to stop cleavage), and a truncated soluble secreted form (sFKN), and it is diagrammatically represented in **Figure 1a**. Each construct including the synuclein virus was placed under the CMV-chicken β-actin hybrid promoter (CBA), as this promoter has been shown to give neuronal expression in the CNS.[34–36] Expression of our sFKN construct was observed to be predominantly neuronal as expected (**Figure 1e,g** shows anti-flag staining of sFKN). The percentage area of anti-flag positive staining for each construct is graphed in **Figure 2b**. All CBA constructs

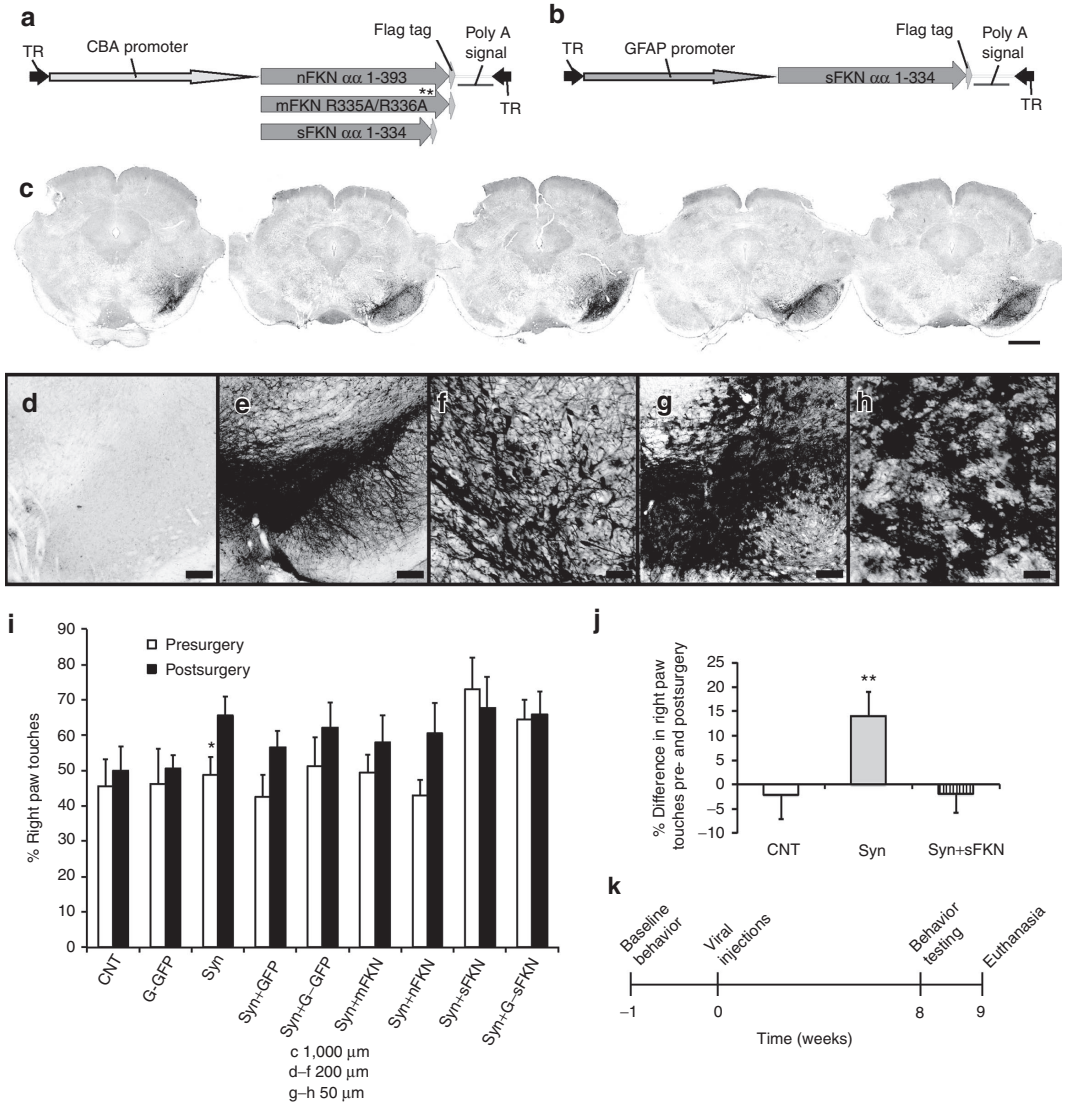


**Figure 1 Viral constructs and rAAV9 expression in the substantia nigra.** (**a**) Diagrammatic representation of FKN viral constructs; (**b**) diagrammatic representation of the GFAP promoter sFKN; (**c**) expression of GFP with an injection of rAAV9 GFP expressing virus into the rat substantia nigra; (**d**) anti-flag staining in uninjected rat substantia nigra; (**e,f**) CMV-chicken beta-actin hybrid promoter (CBA) promoter shows neuronal expression, anti-flag staining for flag-tagged sFKN; (**g,h**) glial fibrillary acid protein promoter construct shows astrocyte cell expression, anti-flag staining for flag-tagged sFKN; (**i**) cylinder test of each animal group before and 2 months after surgery; (**j**) Pooled differences in right paw usage in the cylinder test for like groups. (**k**) Timeline for injections and behavior testing. Scale bar **c** = 1,000 µm, **d–f** = 200 µm, and **g,h** = 50 µm.

delivered approximately the same amount of FKN with the exception of G-sFKN which seem to be approximately twofold lower.

After 2 months of treatment, the animals were tested behaviorally using the rotarod and cylinder tests. No significant differences were observed with the rotarod. This was not unexpected because we were aiming for a smaller lesion on only one side of the brain. In the cylinder test, there was a trend for increased right paw use within the groups that received α-synuclein except for the sFKN treated groups (**Figure 1i**). As there was no difference between like treated groups, (control, synuclein, versus synuclein + sFKN) they were combined and we observed increased right paw usage for synuclein treated groups which is abrogated in the sFKN treated animals (**Figure 1j**).

Immunohistochemical analysis of the percentage area of staining with anti-tyrosine hydroxylase (TH) antibody in the substantia nigra region showed reductions in the percentage area of TH staining for all the α-synuclein treated groups with the exception of the sFKN-treated groups (**Figure 2c**). This was confirmed with stereology counting of TH-positive neurons in the substantia nigra (**Figure 2d**). Our controls (α-synuclein and α-synuclein+GFP groups) show that we are achieving the expected amount of neuron loss (~40%) that has been previously reported.[11,32] Further, we observed similar results for both the α-synuclein only and α-synuclein+GFP, indicating that the addition of a second viral construct does not alter the delivery, expression or effect of α-synuclein-mediated neuropathology development. We also observed the same level of GFP expression between a GFP only and the α-synuclein+GFP groups (**Figure 2a**). The level of TH-positive neurons in the sFKN-treated group were similar to those of the untreated control group, suggesting that sFKN had a neuroprotective effect on α-synuclein-mediated neuron loss. We did not observe a rescue of TH-positive cells with the expression of either mFKN or nFKN.

Immunohistochemical staining of Neu-N gave similar results to the anti-TH staining. Stereology counts of Neu-N staining within the substantia nigra are shown in **Figure 2e**. We observed a 40–50% reduction in the number of neurons for both the synuclein and synuclein+GFP groups. We observed a rescue of this neuron loss in the sFKN-treated group but not in the mFKN- or nFKN-treated groups. Representative images of TH staining and Neu-N staining are shown in **Figure 3**.

Since the CBA promoter expresses the sFKN in neurons and in PD neuronal damage occurs prior to diagnosis, we wanted to see if we could express sFKN from glial cells as a potential therapeutic approach and still achieve neuroprotection. We examined a sFKN construct with the astrocytic promoter for glial fibrillary acid protein (GFAP). This construct was termed G-sFKN (**Figure 1b**). As a control protein, we used green fluorescent protein (GFP) driven by the GFAP promoter (termed G-GFP). An example of

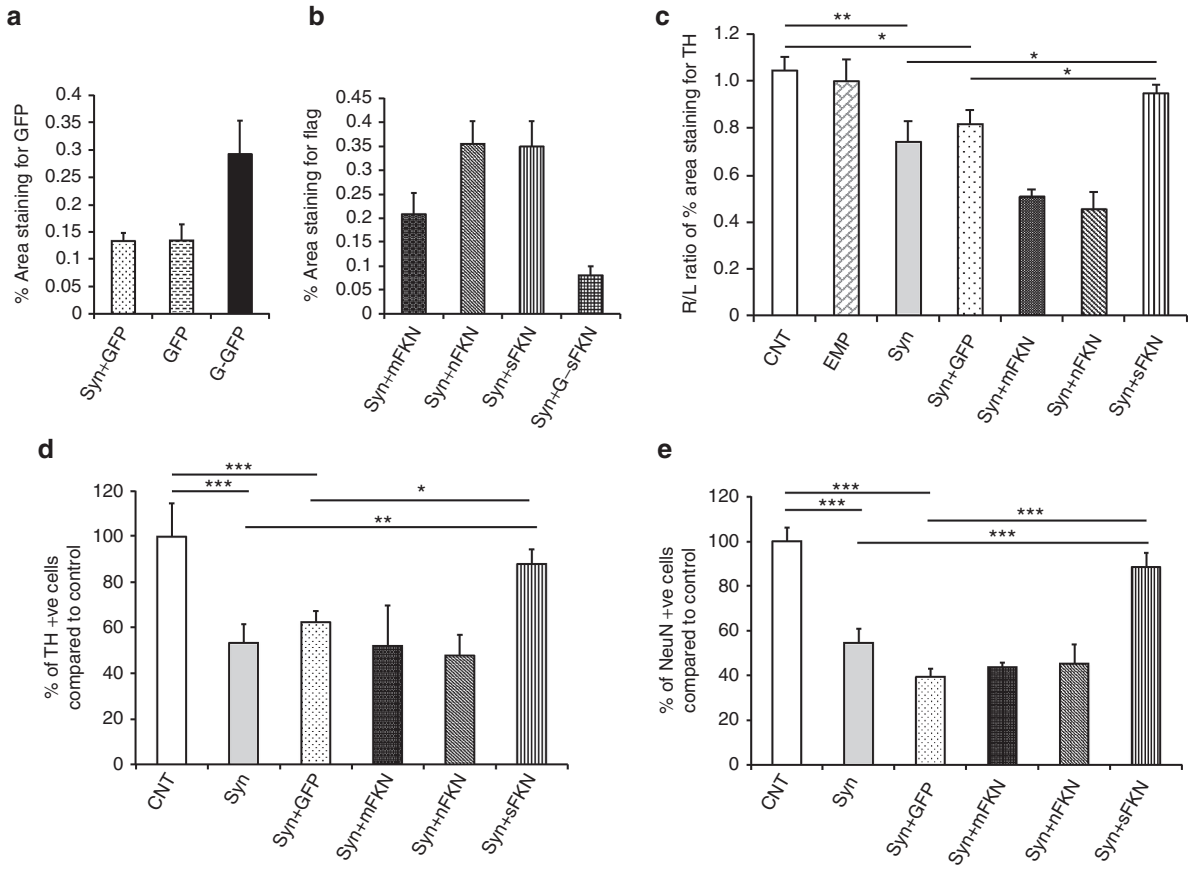


**Figure 2 sFKN reduces neuron loss.** (**a**) Level of GFP expression of CBA-GFP and glial fibrillary acid protein-GFP viruses ($n = 4$); (**b**) level of anti-flag staining for various fractalkine forms tested; (**c**) ratio of the right percentage area staining for anti-tyrosine hydroxylase (TH) in the SN compared to the untreated left substantia nigra (SN); (**d**) percentage of TH-positive cells counted by stereology compared to untreated control group; (**e**) percentage of Neu-N-positive cells counted by stereology compared to untreated control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Mean + SEM; $n = 8$.

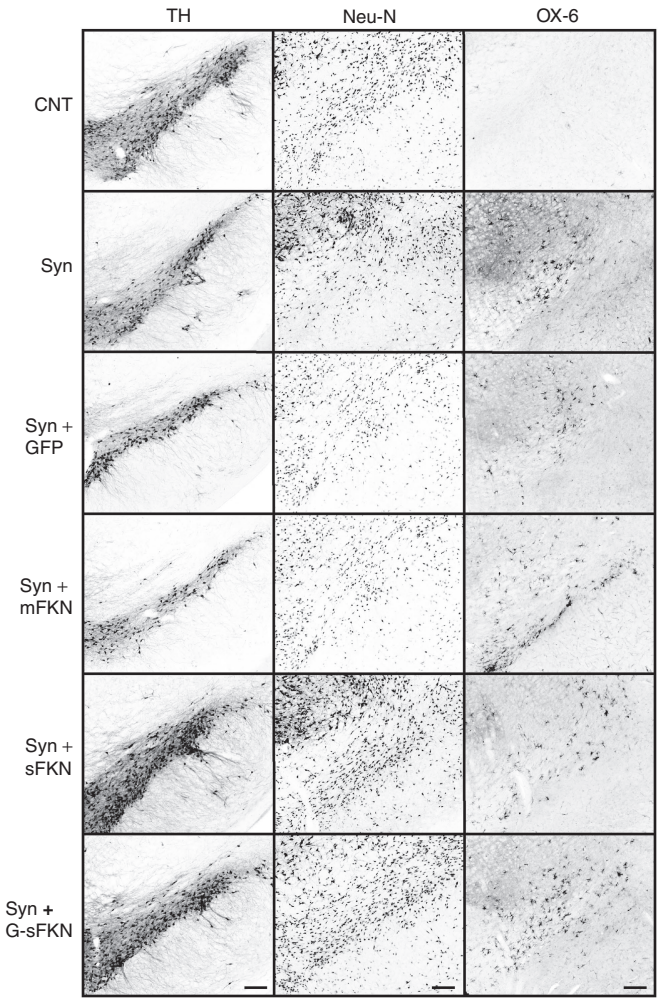


**Figure 3 Representative images of anti-tyrosine hydroxylase (TH) staining, anti- Neu-N staining, and anti-OX6 (MHCII) staining for the different treatment groups.** sFKN shows reduced TH and Neu-N staining loss. Scale bar = 200 µm.

sFKN expression in astrocytes is shown in **Figure 1g,h**. Tests of GFP expression with the GFAP promoter demonstrated that it was as robust as the CBA promoter (**Figure 2a**) but in our experimental groups, the sFKN expression with the GFAP promoter was reduced compared to the CBA promoter. This may have been simply due to an error in the total amount of virus delivered.

As observed with the CBA expressed sFKN construct, we observed ablation of neuron loss associated with increased α-synuclein expression with the G-sFKN construct. Stereology cell counts for TH staining and Neu-N staining in the substantia nigra are shown in **Figure 4**. We observed identical results to the CBA promoter, with significant rescue of substantia nigra neurons compared to control groups.

We examined the level of TH staining in the striatum for selected groups. We observed a significant decrease in the level of TH staining for the Syn+GFP and Syn+mFKN groups. The Syn only group showed a decrease but it did not reach statistical significance ($P = 0.1$). Both the Syn+sFKN and Syn+G-sFKN were statistically different from the Syn+GFP group but not the control group, indicating that we had preservation of TH staining in the striatum as expected from the preservation of TH staining in the substantia nigra.

As well as neuronal markers we examined animals for glial cell activation. Anti-GFAP staining in all groups showed no significant changes, suggesting that we are not significantly increasing astrocyte activation (**Figure 5b**). We examined microglial activation as indicated by increased MHC II, using an anti-OX-6 antibody. OX-6 staining was increased in the synuclein treated animals compared to controls as expected. However, we did not see a reduction in the number of OX-6 (MHC II) positive microglia with the treatment of sFKN as we would have predicted (**Figure 5c**). In fact, the sFKN seems to have an increased level of MHC II. Similar results were observed for anti-CD45 staining (results not shown).

## DISCUSSION

Disruption of fractalkine signaling has been shown to be detrimental in a number of neurological disorders including PD.[29,37] We have previously examined the importance of FKN signaling in two toxic models of PD, MPTP and 6-OHDA.[11,31] In both models, we observed neuroprotection with FKN agonism. However, no one has previously examined the benefits of FKN in a synuclein expression model. As stated previously, FKN exists as a membrane bound chemokine that is cleaved to form a soluble extracellular chemokine, therefore, we expressed different forms of FKN (native, soluble and membrane bound) to examine the efficacy of therapies with the different states of FKN. Our data suggest that sFKN is neuroprotective against α-synuclein-mediated damage but the native and membrane forms were not effective.

We observed that sFKN but not the membrane forms of FKN are capable of reducing the dopaminergic neuron loss caused by human α-synuclein over expression. Interestingly, we would have predicted that the native form would act similarly to the soluble because it can be cleaved into the soluble form. Since we did not observe similar results, we might predict that the cleavage of nFKN is limited by the amount of protease enzymes available or the regulation of those enzymes' activity. We previously observed in the MPTP PD model that the mFKN was not capable of reducing MPTP-induced neuron loss like the sFKN.[38] This may suggest that the membrane and secreted forms of FKN have alternative functions with in the CNS as has been suggested in the periphery.[26,27] One possible explanation could be altered receptor signaling with the sFKN because the secreted form allows for internalization of the receptor.[39]

As a targeted gene therapeutic it may be difficult to target neuronal cells *in vivo* where neuronal degeneration is occurring. Therefore, we wanted to examine if sFKN expression in glial cells could produce the same benefits as expression from neurons. This would have a distinct benefit in brain regions with existing neurodegeneration such as the substantia nigra. We expressed sFKN with the GFAP promoter. This promoter has previously been used successfully for astrocyte specific expression with AAV.[40,41] We observed the same neuroprotection with this construct as we did with the CBA promoter (**Figure 3**). This was not unexpected because we would have predicted the same amount of expression and diffusion of the chemokine from glial cells as from neurons.

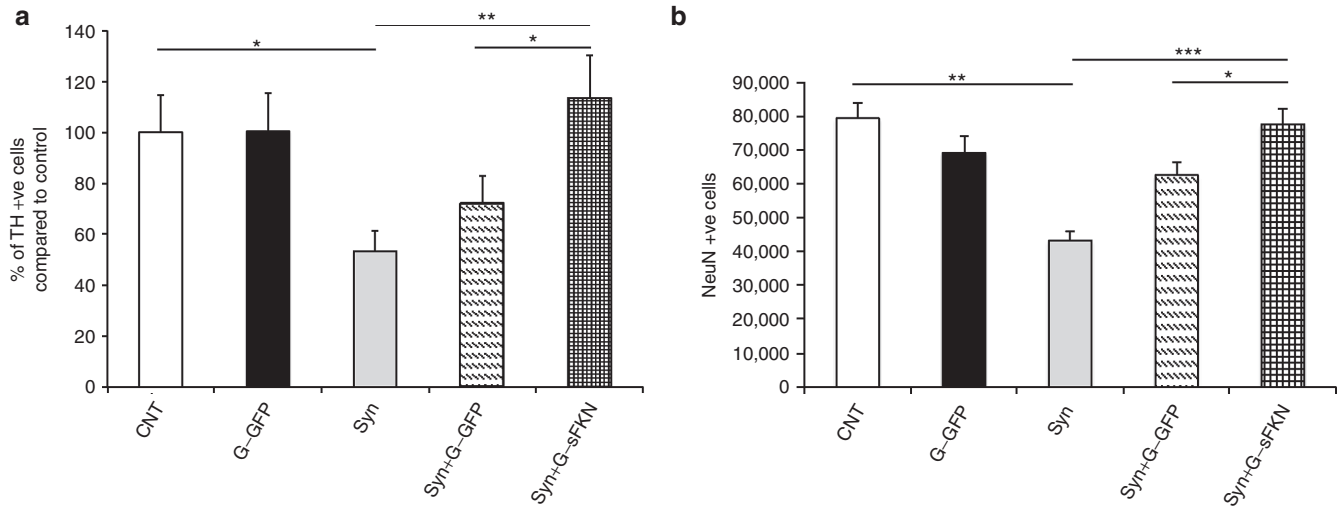


**Figure 4 Astrocyte produced sFKN reduces neuron loss as well as neuronal expressed sFKN.** (**a**) Percentage of tyrosine hydroxylase-positive cells counted by stereology compared to untreated control group; (**b**) Neu-N-positive cells counted by stereology compared to untreated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Mean + SEM; $n = 8$.

Based on our previous results with the MPTP mouse model, we predicted that we would see a reduction in microglial activation (as reflected by CD68 expression) and it is this reduction that would reduce neurodegeneration. However, interestingly, we did not observe a reduction in microglial activation based on two common microglial markers (MHC II and CD45). In fact, the sFKN seems to have increased the level of MHC II (**Figure 5**). In a pilot study ($n = 2–3$), we previously observed a low level increase in MHC II staining with sFKN expression for one month, similar to levels observed with GFP expression (data not shown). It is currently unclear what the significance of this increase in MHC II following sFKN expression is and further investigation is warranted. Some possible explanations are microglial activation from the surgical procedure or reaction to the flag epitope attached to the sFKN.

Although this MHC II increase is contradictory to our hypothesis, one likely explanation would be that we are altering the microglial state with the addition of sFKN rather than decreasing the level of antigen presentation as reflected by MHC II, as this marker can be raised in both M1 and M2 conditions. We would predict that this altered state would be less pro-inflammatory and more neuroprotective. We have previously published on biochemical changes observed by microarray analysis with microglia activated to either an M1 or M2 activation state.[42] We observed that MHC II staining was similar between both M1 and M2 activated microglia, however, markers such as MARCO and FIZZ1 among others were markedly different between the two states. This supports a hypothesis of altered activation without an alteration in MHC II activation. Closer examination of some of these alternative markers is required to see if we are indeed changing the activation state of the microglia to a more anti-inflammatory (M2) or neuroprotective state. This was not achievable in this study due to the limited number of available sections containing the substantia nigra for immunohistochemical staining. An alteration to M2 would also result in a reduction of proinflammatory cytokines (*e.g.*, IL-1β, TNF-α, and IL-6) and potentially an increase in anti-inflammatory cytokines (*e.g.*, IL-10 and IL-4). It may be possible that FKN is also altering the release of factors other than cytokines from microglia. Microglia have been shown to produce a number of growth factors that are neuroprotective, such as TGF-β1, TGF-β2, IGF-1, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and nerve growth factor (NGF).[43,44] Neurotrophic factors such as GDNF and neurturin (NTRN) have been the focus of therapeutic intervention trials for PD. Although presenting excellent results in some animal models, individual trophic factors have yet to prove effective in PD patients,[45] or in animal models involving synuclein expression.[46,47] Perhaps, a combination of growth factors is required to achieve a significant therapeutic benefit. We are currently examining both the cytokine and growth factor profiles of animals treated with synuclein or synuclein plus fractalkine in a new cohort of animals, as we believe these factors will be critical in the further understanding of PD pathology.

In our previous work in the PD MPTP mouse model, we were examining the expression of different fractalkine forms on a fractalkine null animal ($CX3CL1^{-/-}$). This gave us the unique ability to specifically examine whether the membrane or soluble forms of fractalkine contribute anti-inflammatory activity with in the CNS. We observed that the sFKN but not the membrane was critical in reducing excess neuron loss due to MPTP treatment. In this study, it is important to note that we show efficacy with the sFKN in a model where basal FKN is present. This demonstrates that increasing FKN agonism over basal levels can reduce neuron loss in a synuclein-mediated neurodegeneration model of PD. This model would more closely recapitulate what would be observed in the human condition than the toxic model. However, it should be noted that in this study we had to inject both the α-synuclein and FKN virus at the same time due to the rapid nature of the synuclein neuropathy in this model and the alternative would require two intracranial surgical injections within a couple weeks of each other. This model system therefore does not allow for some neuron loss to occur prior to increased FKN agonism as both transgenes are expressed simultaneously. Examination of FKN expression in alternative models such as the PDGF or Thy1 promoter driven

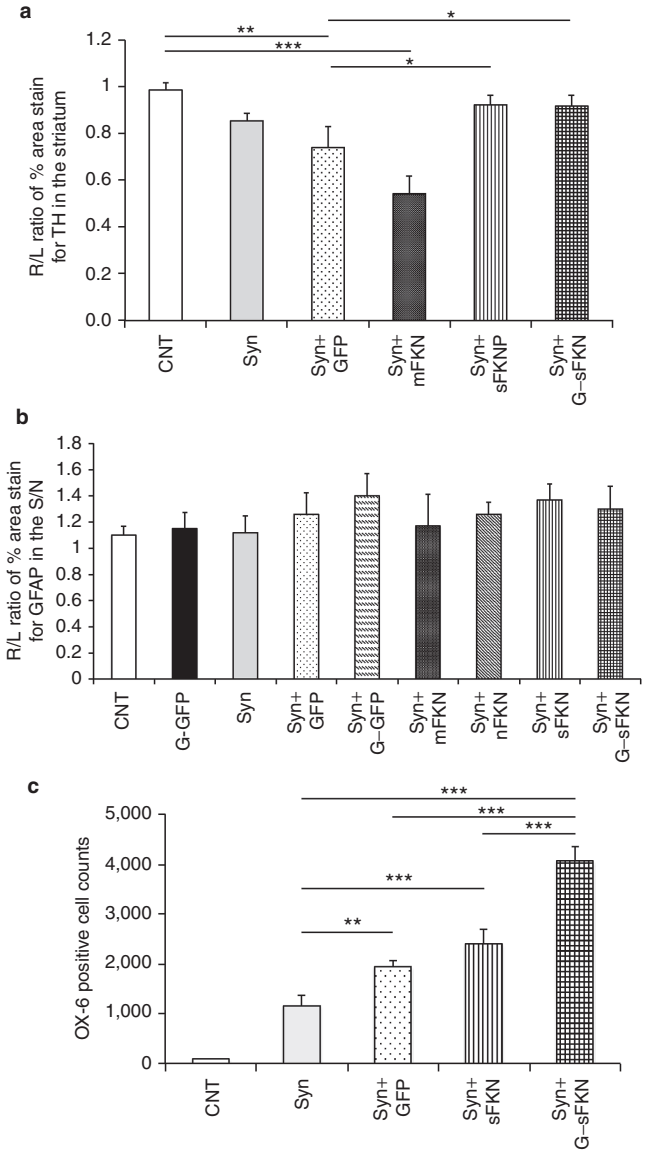


**Figure 5 Levels of anti-TH, anti-GFAP and anti-OX-6 staining.** (**a**) Right:left ratio of percentage area staining with anti-tyrosine hydroxylase antibody in the striatum. (**b**) Right:left ratio of percentage area staining with anti-glial fibrillary acid protein antibody in the S/N. (**c**) Anti-OX-6-positive cell counts shows that FKN does not reduce MHC II-positive microglia. Graph of stereology cell counts shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Mean + SEM; $n = 6$–8.

α-synuclein transgenic mice, may offer a model system where existing neuron loss has occurred prior to sFKN treatment. These models may more closely reflect the states of pathology of patients but it should be noted that these models also have limitations.[48] Importantly, our model does demonstrate the inhibition of pathology development in the presence of increased CX3CR1 agonism and we believe that these data validate the potential of fractalkine receptor agonism as a novel therapeutic target for drug discovery in the treatment of synucleinopathies such as PD. Further, we believe this study demonstrates a novel and effective gene therapeutic approach to reducing synuclein mediated pathology and neuron loss seen in PD. A gene therapy approach may have the distinct advantage as a therapeutic delivery to only the CNS. This would reduce any significant alteration of the peripheral immune system that may result in undesired effects. It could be further refined with drug regulatable promoters such as the TetR system for greater safety and translation into a human therapy.

## MATERIALS AND METHODS

***Viral vectors.*** Rat fractalkine (GI: 19745168) was cloned from Fisher F344 rat brain mRNA. Native fractalkine was generated from αα 1–393 (nFKN) and soluble from αα 1–334 (sFKN). Membrane bound mutant (mFKN) is the full-length fractalkine with the mutations R335A and R336A. All clones were confirmed by sequence analysis. All FKN clones were tagged on the C-terminus with the flag tag (DYKDHDGDYKDHDIDYKDDDDK). cDNAs were cloned into the pTR2-MCS vector which contains the CBA promoter and SV40 poly A signal. We also constructed a GFAP promoter vector. This vector consisted of AAV2 terminal repeats, the GFAP promoter region (1700bp), multiple cloning site, and a BGH poly A signal. sFKN and GFP were cloned into this vector; these vectors were termed G-sFKN and G-GFP respectively. Human α-synuclein construct was described previously.[11,49] All viruses were AAV serotype 9 with AAV2 TRs and were produced as described previously.[34] Viruses were titered using dot blot method[30] and expressed as vector genomes (vg)/ml.

***Animal husbandry.*** Animal experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use committee of the University of South Florida. Three-month-old male Fisher 344 (F344) rats (Harlan Sprague Dawley, Indianapolis, IN), were pair-housed in environmentally controlled conditions (12:12 hours light:dark cycle at 21 ± 1 °C). Food and water were provided ad libitum. Animals were injected unilaterally with 1 µl of human synuclein virus ($1 \times 10^{13}$ vg/ml) and 1 µl of our control virus (GFP) or FKN virus ($4 \times 10^{12}$ vg/ml) into the right substanti nigra. Viruses were mixed prior to injection and a total of 2 µl was injected. The FKN viruses included the nFKN, mFKN, sFKN, and G-sFKN. Surgery was performed as described previously.[33,49] Eight animals per group were used. Injection coordinates were AP −5.6 mm, LAT −2.4 mm, and DV −8.2 mm from bregma. Virus was injected using the convection enhanced delivery (CED) method described previously.[34] Rotarod behavioral testing of the rats was performed as described previously.[11] The cylinder test was used to test the forelimb activity of the rats when rearing in a confined cylinder. Animals were tested prior to surgery and 2 months after surgery. Animals were placed in a 24 cm high and 16 cm diameter cylinder. Forelimb contacts while rearing are scored with a total of twenty contacts for each animal. The number of impaired or nonimpaired contacts are calculated as a percentage of total contacts. A timeline for injections and testing is shown in **Figure 1k**.

Immunohistochemical analysis was performed as described previously with free floating sections.[11,30] Briefly, every sixth section spanning, the substantia nigra were chosen for each analysis to achieve the best statistical analysis. For each marker, sections from all animals were placed in a multisample staining tray, and endogenous peroxidase was blocked (10% methanol, 10% $H_20_2$ in phosphate-buffered saline (PBS); 30 minutes). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution), and incubated overnight in appropriate primary antibody. Sections were washed in PBS, then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), if necessary. The tissue was again washed after 2 hours, and incubated with Vectastain Elite ABC kit (Vector Laboratories) for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.03% $H_20_2$. Tissue sections were mounted onto slides, dehydrated, and cover slipped. Each immunochemical assay omitted some sections from primary antibody incubation period to evaluate nonspecific reaction of the secondary antibody. Antibodies used

were: mouse anti-TH, Immunostar (Hudson, WI, # 22941); mouse anti-Neu-N, Millipore (Temecula, CA, # 3777), mouse anti-RT1B (OX-6), BD Biosciences (San Jose, CA, # 554926); mouse anti-CD45, BD biosciences (# 550566); mouse anti-flag biotinylated, Genscript (Piscataway, NJ, A01429) and anti-mouse as secondary antibody Vector Labs (# BA-2001). Due to the limited number of sections containing the substantia nigra we were limited in the number of microglia markers that could be tested at this time, so that we could perform reliable statistical estimates of the critical variables tested.

TH and Neu-N staining stereology counts were performed as described in Nash *et al.* [30]. OX-6 stereology was performed as described in Pabon *et al.* [11]. Images were taken using the Zeiss Mirax scanner. Percentage area analysis was performed using IAE-NearCYTE image analysis software (http://www.nearcyte.org) used previously.[30] Statistical analysis was performed using Statview one-way analysis of variance.

## ACKNOWLEDGMENTS

This study was Funded by MJ Fox Foundation. The authors declare no competing financial interests.

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