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Caroline McLaughlin March 24, 2020

Cannabinoid type 2 receptors regulate striatal inflammation following alpha synuclein-induced nigral degeneration in rat model of Parkinson's disease

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Neuroscience and Behavioral Biology

2020

Abstract

Cannabinoid type 2 receptors regulate striatal inflammation following alpha synuclein-induced nigral degeneration in rat model of Parkinson's disease

By Caroline McLaughlin

Parkinson's disease (PD) is characterized by a loss of dopamine neurons in the nigrostriatal pathway of the brain and by the formation of intracellular inclusions or Lewy bodies composed largely of alpha-synuclein (Asyn). Neuroinflammation accompanies the progression of the disease and plays a key role in Asyn-induced degeneration. There is a growing interest in new therapeutic approaches that aim to reduce these inflammatory responses in an effort to slow the progression of the disease. Specifically, targeting cannabinoid type 2 (CB2) receptors has shown to modulate cytokine release and diminish microglia neurotoxicity. However, it is still not fully understood how pharmacologically targeting CB2 will alter Asyn aggregation and the Asyn-induced inflammatory state. In order to better understand the inflammatory mechanisms of CB2 selective modulation in the striatum, we evaluated the striatal hemispheres of rats unilaterally injected into the substantia nigra with AAV2/5-human wild-type Asyn (2.9E+11 vg/mL) and treated with either systemic SMM-189 (6 mg/kg, ip) or vehicle. SMM-189 is a novel CB2 inverse agonist that has been shown to reduce levels of cytokine and chemokine expression associated with pro-inflammatory microglia. Western blot analyses were conducted on protein extracted from both striatal hemispheres and stained for neuronal markers such as tyrosine hydroxylase (TH), phosphorylated TH, dopamine transporter (DAT), Asyn, and phosphorylated Asyn at serine 129. Additional western blots were performed to evaluate inflammatory markers including GFAP, IBA1, and NfκB. Results show that SMM-189 significantly reduced phosphorylated Asyn and TH expression compared to controls and increased expression of DAT and phosphorylated TH compared to vehicle-treated rats. In addition, inverse and significant correlations were observed between DAT and pSer129 Asyn in both SMM-189 treated rats and control rats; a significant and inverse correlation was seen between TH and Asyn in vehicle treated rats but not in SMM-189 treated rats; and a direct correlation between DAT and Phosphorylated TH, and NFκB and TH was seen in SMM-189-treated rats but not controls. SMM-189 had no effect on microglia IBA1 expression, but does appear to suppress the up-regulation of GFAP expression seen in the ipsilateral side of vehicle-treated subjects. Our findings suggest that CB2 modulation using the inverse agonist SMM-189 decreases striatal toxic forms of Asyn aggregation, but has no effect on inflammatory markers evaluated, and results in enhanced dopaminergic degeneration as indicated by TH, Phospho TH an DAT expression changes.

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Acknowledgments

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Funding

Supported in Part by a Parkinson's Foundation- American Parkinson's Disease Association Summer Student Fellowship, PF-APDA-SFW-1917

I would like to thank the Tansey lab, particularly Valerie Joers and Malu Tansey for training me and supporting me throughout this project. I would also like to thank my mentor Vicky Hertzberg for guiding me under continually changing situations. Lastly, I would like to thank Betty Rodriguez, Tim Sampson, my committee and the NBB department for making sure I had all of the assistance needed.

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Introduction and Background

Parkinson's Disease and neuroinflammation

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder (Cabriera and Masano, 2019). More than 10 million people worldwide live with this disease and around 60,000 Americans are diagnosed with PD every year (Parkinson's Foundation). PD is characterized by a loss of dopamine neurons in the nigrostriatal pathway of the brain and by the formation of intracellular inclusions or Lewy bodies, composed largely of alpha-synuclein (Asyn). Schulz-Schaeffer (2010) suggests that pathological Asyn aggregation result in neurodegeneration or in the case of PD, the loss of dopaminergic neurons, yet the exact mechanism of Asyn toxicity is not clear. There is evidence that phosphorylation on the C-terminal, particularly on the pSer129 site, increases neurotoxicity and contributes to dopamine neuron loss (Okochi et al., 2000). In addition, neuroinflammation accompanies the progression of the disease and plays a key role in Asyn-induced degeneration (Concannon et al., 2016). Microglia, or immune cells of the central nervous system (CNS), are normally abundant in the substantia nigra (SN) and the basal ganglia (Lazdon et al., 2020). Dopaminergic neurons in these regions are susceptible to microglial activity and inflammatory mediators. Microglial cells produce reactive agents like nitric oxide, which can result in cellular stress and in turn neuronal loss (Block et al. 2007).

McGeer et al. (1988), observed high microglial expression in the SN of postmortem brain tissue from patients with PD. Histological staining of the SN of PD patients, show a high number of HLA-DR-positive reactive microglia (McGeer et al., 2008). HLA-DR is a marker of immunocompetent cells, up-regulated on activated microglia (McGeer et al.,

1988). Hence, the first evidence of neuroinflammatory mechanisms associated with PD was reported as early as 1988.

In vivo data including positron emission tomography (PET) scans of patients with PD, confirms that microglia activation is closely linked with the development of the disease. Gerhard et al., 2006 showed that PD patients have a higher mean of [11C](*R*)-PK11195 binding, a PET marker selectively expressed by activated microglia, in the basal ganglia, pons, frontal and temporal cortex. Longitudinal findings suggest that microglia are mostly activated at early stages of PD, and that activation levels off at later stages (Gerhard et al., 2006). Ouchi et al., 2005 also examined [11C](*R*)-PK11195 binding and found higher levels in the midbrain of patients with PD than in healthy control subjects. [11C](*R*)-PK11195 measured values were directly correlated to motor severity measured by the Unified Parkinson's Disease Rating Scale in PD (Ouchi et al., 2005).

Following studies confirmed that neuroinflammation is as a core pathophysiological feature of PD, yet it is not clear if inflammation results in neurodegenerative processes or is the consequence. A study conducted by Hirsch and Hunot (2009) for example, observed the cellular and molecular events characteristic of neuroinflammation associated with loss of dopaminergic neurons, in animal models of PD. They demonstrated that glial cell activation resulted in oxidative stress and cytokine- receptor-mediated apoptosis, likely leading to the progression of the disease. In a recent study, Duffy et al. (2018) suggested that toxic Asyn inclusions trigger neuroinflammation before the onset of neurodegeneration, suggesting that inflammation is a contributing factor to the disease etiology.

Additionally, genome-wide association studies (GWAS) demonstrated that single nucleotide polymorphisms found in inflammatory genes increase risk for PD, and

involvement of susceptibility loci in PD (Satake et al., 2009; Simon- Sanchez et al., 2009; Dzambo et al., 2015). Even though there is extensive evidence indicating the links between inflammatory responses and PD pathology, further research is needed to translate this knowledge into novel therapeutic interventions for this neurodegenerative disease (Hirsch and Hunot, 2009).

Peripheral Inflammation in PD

Systemic inflammation is linked to intracerebral inflammation and neurodegenerative processes in PD patients and animal models (Ferrari and Taerlli, 2011). Microglia activation produces signals that engage peripheral immune cells to enter into the brain. Harms et al. (2018) demonstrated that Asyn overexpressing animal models of PD have higher levels of pro-inflammatory CCR2 + peripheral monocytes in the central nervous system (CNS), suggesting increased infiltration of peripheral immune cells. Brochard et al. (2009) also show that CD4+ and CD8+ T cells present in postmortem SN of human patients with PD and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and are destructive to neurons. Huang et al. (2009) observed a high number of activated T lymphocytes present in the blood of neurodegenerative patients. It is not fully understood whether these T lymphocytes migrate from the brain or are destined to infiltrate the CNS. MPTP-induced dopaminergic degeneration was mitigated in mouse strains lacking mature T lymphocytes, suggesting that reduction in peripheral inflammation, in this case lymphocytes, benefits PD pathologies (Brochard et al., 2009).

Patients with PD undergo changes in BBB function, resulting in CNS damage (Farkas et al., 2000). Blocking the CCR2 receptor on peripheral cells and restricting their

entry past the blood brain barrier (BBB) mitigates Asyn mediated inflammation and neurodegeneration. The known mechanisms where peripheral inflammation is involved with CNS degeneration include 1) T and B-lymphocytes in the central ducts infiltrate and attack central neurons, 2) pro-inflammatory cytokines migrate into the brain activating microglia and 3) macrophages cross the BBB, convert into microglia cells and play a role in degeneration (Odoardi et al., 2012; Wang et al., 2013; Marinova-Mutafchieva et al., 2009). These peripheral mechanisms involved in chronic inflammation may result in degeneration of dopaminergic neurons in the SN and in turn PD pathology (Guan et al., 2019).

Elevated levels of pro-inflammatory cytokines have been observed in the brains of patients with PD (Whitton, 2009). When pro-inflammatory cells are upregulated, T cells and monocytes are recruited and pass through the BBB (Neumann and Wekerle, 1998). High levels of inflammatory cytokines in the brain, plasma and CSF of patients with PD, suggest that peripheral immune responses are associated with neurodegeneration of the disease (Baba et al., 2005; Selikhova et al., 2002; Reale et al., (2000). Reale et al. (2009), for instance showed that patients with PD have higher levels of IL-1b, IFNγ and TNFα, produced by unstimulated peripheral blood mononuclear cells (PBMCs), compared to healthy patients.

Therapeutic Approaches

In addition to the extensive evidence linking PD pathology to neuroinflammation, existing treatments for PD have also shown to have anti-inflammatory effects. For instance, monoamine oxidase-B inhibitors such as Rasagiline, commonly used to increase levels of dopamine in the brain and treat PD, have been shown to reduce active microglia expression

(Stocchi et al., 2015). Furthermore, a meta-analysis conducted by Gagne and Power (2010) observed that use of classical anti-inflammatory drugs like non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) decreased incidence of PD by 15%, suggesting that anti-inflammatory therapies may be beneficial for PD.

Hence, there is a growing interest in new therapeutic approaches that aim to reduce the inflammatory responses and the progression of the PD (Cancanon et al., 2016). Recent evidence regarding the regulatory functions of microglia, suggests that treatments modulating rather than inhibiting microglia activity, could be an effective therapeutic strategy (Tansey and Goldberg, 2010). The challenges associated with testing for anti-inflammatory neuroprotective therapies, is that they require sensitive methods able to detect shifts in microglial phenotypes. This is particularly challenging when taking into account the progressive nature of PD (Joers et al., 2017).

Recent studies have investigated the effects of anti-inflammatory drugs such as glucagon-like peptide 1 (GLP-1), minocycline, TNF inhibitors and cannabinoid type 2 receptors in treating PD-like pathologies (Holst, 2007; Olmos, 2014; Bertilsson et al., 2008). GLP-1 is a peptide hormone that modulates insulin and glucagon secretion (Holst, 2007). GLP-1 agonists, such as exenatide are used to treat type 2 diabetes, and have shown to have neuroprotective qualities in clinical models of PD (Aviles- Olmos et al., 2013). Bertilsson et al. (2008) demonstrated that exenatide promotes neurogenesis and dopaminergic neuron expression in the SN of animal models of PD. The semisynthetic tetracycline mycocline, has shown to diminish nigrostratial dopaminergic degeneration in mouse models of PD, specifically associated with reductions in NO synthase (iNOS) (Du et al., 2001). TNF inhibitors such as XPro1595 have been shown to significantly reduce

microglia and astrocyte expression in the SN and mitigate dopaminergic loss in an animal model of PD (Barnum et al., 2014). Lastly, cannabinoid type 2 (CB2) receptor modulation shows promising results in transforming the innate immune system towards a wound healing phenotype (Reiner et al, 2014). This wound-healing response helps to balance out and avoid detrimental impacts on neuronal populations, associated with extended and unregulated neuroinflammation and oxidative stress (Tansey and Goldberg, 2010). The current study surveys how targeting CB2 receptors modulates neuroinflammation and affects PD-like pathologies.

Cannabinoid Type 2 Receptors

CB2 receptors are peripheral cannabinoid G-protein coupled receptors largely localized on immune cells, including monocytes, macrophages and activated microglia (Nent et al., 2019). CB2 receptors are abundantly expressed on activated microglia, yet otherwise scarce in the overall central nervous system, presumably making CB2 selective drugs attractive for tackling inflammation while avoiding psychoactive effects of cannabis mediated by CB1 receptors (Atwood and Mackie, 2010). There is evidence of up-regulation of CB2 receptors on microglial cells in postmortem tissue of patients with PD, and in the striatum of inflammatory models of PD. (Gómez-Gálvez et al., 2016). Several studies have identified the importance of CB2 in regulating inflammation and supporting a potential neuroprotective role in PD models. CB2 knock-out mice challenged with the neurotoxin MPTP have an exacerbated toxicity (Prince 2009), yet when challenged with LPS have demonstrated increased levels of CD68-immunoreactivity, indicating higher levels of activated microglia and infiltrated peripheral macrophages in the nigra compared to wild-

type mice (Gómez-Gálvez et al., 2016). Pharmacologic selective activation of CB2 receptors resulted in reduced levels of pro-inflammatory markers such as TNFα, IL-1 β, IL-6, and IL-10. Another study conducted by Ehrhart et al. (2005) showed that selective stimulation of CB2 receptors, suppressed cultured microglia TNF-α, and nitric oxide production, both critical signaling molecules implicated in neurodegeneration.

Both cannabinoid receptor agonist and antagonists have been shown to have neuroprotective effects. Cannabinoid receptor agonists are immunosuppressive and neuroprotective in multiple sclerosis (Croxford et al., 2007). Croxford et al., found reduced levels of T cells and CNS infiltration in models of multiple sclerosis administered with doses of a cannabinoid agonist. CB2 selective agonists such as HU-308 have been shown to have neuroprotective roles in Huntington disease. Palazuelos et al., (2009) demonstrated that selective CB2 agonists were effective at reducing striatal neurodegeneration, GABA deficiency and glial activation. In addition, CB2 antagonists show neuroprotective actions such as by attenuating traumatic brain injury (TBI) symptoms (Lopez- Rodriguez et al., 2015). Specifically, cannabinoid antagonism helped reduce neurological deficits, axonal injuries and microglia activation.

CB2 modulation mitigates microglial production of neurotoxic factors (Fernández-Ruiz et al., 2010). As these studies show, CB2 activation inhibits NFκB, a transcription factor of pro-inflammatory mechanisms, and in turn TNF-α (Oh et al., 2010). These findings show the potential of CB2 receptors in modulating cytokine release and diminishing microglia neurotoxicity. In the efforts to investigate CB2 therapeutic approaches, a number of CB2 selective agonist and antagonists have been synthesized.

SMM-189

Out of these novel treatments, a selective CB2 inverse agonist, SMM-189 dampens pro-inflammatory cytokine release such as IL-6, IFN-γ, and MCP-1 from human primary microglia and increases pro-healing phenotype with increased expression of CD206, suggesting SMM-189 can shift the balance of microglia phenotypes (Reiner et al., 2014). Direct comparison between SMM-189 and CB2 agonists demonstrates SMM-189 is superior to other compounds because of the beneficial anti-inflammatory effects (Presley et al., 2015). CB2 inverse agonists like SMM-189 lock active CB2 receptors into an inactive state producing the opposite downstream signaling as an agonist. Since CB2 receptors are negatively coupled to adenylyl cyclase, this inactivation leads to a reduction of adenylyl cyclase inhibition in turn increasing cyclic adenosine monophosphate (cAMP) production. Consequently, the downstream activation of protein kinase A (PKA) phosphorylates cAMP response element binding protein (CREB) leads to decreased pro-inflammatory responses and increased anti-inflammatory effects. Several studies have evaluated the potential of SMM-189 in triggering these pro-healing mechanisms and mitigating the deficits of mild traumatic brain injury (TBI) (Reiner 2014, Bu 2016, Guley 2019). Reiner et al. 2014 found that focal air blast mice models treated with SMM-189 demonstrated improvements in motor, visual and emotional tasks compared to control mice. Guley et al. (2019) found that SMM-189 treatment attenuated functional deficits of TBI, particularly associated with the visual system. Guley et al., suggest that SMM-189 administration biases microglia from a pro-inflammatory state to a wound-healing state. Although there is evidence that SMM-189 may help mitigate the inflammatory responses associated with TBI, its potential in modulating the inflammatory responses associated with PD needs further research.

AAV2/5 Mediated Overexpression of Alpha Synuclein

In order to study the inflammatory mechanisms of SMM-189 in PD, our lab uses an AAV2/5-mediated overexpression of human wild-type Asyn model of PD in rats. Recombinant adeno-associated viral vectors (rAVV) are generated to overexpress levels of human Asyn and mimic the pathological hallmarks of PD seen in humans (Gombash et al., 2013; Gorbatyuk et al., 2008). Previous studies have shown that rAVV2/5 animal models show transfection of neurons leading to progressive neuronal death, where the magnitude and time of neurodegeneration is associated with the amount of Asyn overexpression (Sanchez-Guajardo et al., 2010 and Gorbatyuk et al., 2008). The virus titer leads to dose-dependent increases in inflammation and dopamine neuron degeneration when injected into the SN (Gombash et al., 2013).

Gombash et al. (2013) measured Asyn expression in the striatum and SN following intranigral injection of rAAV2/5, neurodegeneration, and deficits in motor behavior. At 4, 8 and 12 weeks after the virus injection, they found high levels of human wild-type Asyn in the injected SN and adjacent areas of the midbrain. At 8 weeks, there was an increase of terminal dysfunction resulting in motor deficits. In addition, there was a loss of 40-70% of dopaminergic neurons in the nigra over a period of 8-27 weeks (Kirik et al., 2002). There was a loss of more than 50% of nigral TH-immunoreactive neurons at 8 weeks at a dose of 1.0E+13 (Gombash et al., 2013). Immunofluorescent stains showed co-localized expression of Asyn and TH on dopamine neurons. Also, a presence of Asyn expression in the ipsilateral striatum following the intranigral injection, showed that Asyn is anterogradely transported to striatal nuclei.

Immunomodulation of SMM-189

Unpublished preliminary evidence in our lab has shown that daily treatment of SMM-189 for 7 weeks decreased gene expression of TNF while increasing expression of TGF-β in peripheral blood mononuclear cells (PBMCs). Similar trends were seen with increases in anti-inflammatory markers, including YM1 and CD206, in the frontal cortex of rats treated with SMM-189 compared to vehicle, suggesting a role for CB2 to regulate inflammation (Figure 1). Additionally, we found a trend for increased CD68-immunoreactivity in these animals, suggesting that the immunomodulatory effects of SMM-189 may lead to increased phagocytosis. A parallel ongoing study to this one is evaluating the immunomodulatory and neuroprotective effect of SMM-189 in the nigra, the region directly injected with the virus. However, it is still not fully understood how pharmacologically targeting CB2 will alter Asyn aggregation, inflammation and dopaminergic neuron health in the nigral projections within the striatum following Asyn overexpression in the nigra. This project aims to understand how pharmacologically targeting CB2 would specifically alter striatal Asyn aggregation, neuronal integrity and inflammation following virus mediated Asyn overexpression.

Hypothesis

We hypothesized that the selective CB2 inverse agonist treatment, SMM-189, would alter neuroinflammation in the striatum and reduce Asyn toxicity in a rat model of PD. Particularly, we expected that SMM-189 would suppress pro-inflammatory markers, reduce striatal toxic Asyn overexpression and decrease dopaminergic degeneration in the striatum.

Objective

To understand how pharmacologically targeting CB2 would specifically alter striatal Asyn aggregation, neuronal integrity and inflammation following Asyn overexpression.

Materials and Methods

Subjects

We evaluated both striatal hemispheres of male Sprague Dawley rats (Envigo, 3-4mo old) unilaterally injected into the nigra with AAV2/5-human wild-type Asyn (2.9E+11 vg/mL) and treated daily with either systemic SMM-189 (6 mg/kg, ip, n=13) or vehicle (n=8) for 7 weeks starting 1 week after intracerebral injections (Gombash et al., 2013; Yamanda et al., 2004; Reiner et al., 2014). AAV2/5-human wild-type Asyn expression is driven on the chicken beta actin or cytomegalovirus promoter, which promotes neural gene expression. A successful surgery was identified postmortem using immunofluorescent methods to label the rostral to caudal spread of Asyn within the SN. Only animals that were successfully targeted were included in this study (Figure 2). After 7 weeks of treatment, animals were sedated with euthasol (150 mg/kg) and perfused through the left ventricle with ice-cold saline. Brains were extracted and placed in a brain matrix and a coronal cut made rostral to the start of the nigra. Striatum from each hemisphere was dissected and flash frozen in liquid nitrogen. [This section of the procedure was not included in the thesis project timeline. I conducted the following methods as part of the project].

Protein Extraction and Sample Preparation

Protein was extracted from the striatum tissues of those successfully targeted animals. The tissues were lysed with cold TriZOL and lyser beads and incubated with chloroform and methanol. Protein pellets were resuspended in 1% SDS and heated to 50 degrees Celsius before storage. Total protein concentrations of the samples were measured using Pierce BCA Protein Assay Kit. Protein samples were homogenized and prepared at a

1:20 dilution in 1% SDS before BCA assays. Absorbance of BCA standards and diluted samples were measured at 562nm on a spectrophotometer or plate reader. Linear standards were calculated and used to predict sample concentrations. Final stable protein lysates were prepared at 2ug/uL diluted with 4x Lamaelii and 1%SDS buffers. The Lamaelii protein solutions were heated at 80 degrees Celsius for 5 minutes and stored at -20 degrees Celsius.

Immuoblot Assays

Samples at a concentration of 10ug/uL were loaded onto a 4-15% gel for the analysis of neuronal markers and 20 ug/uL for inflammatory markers. The neuronal markers tested included tyrosine hydroxylase (TH), phosphorylated TH, dopamine transporter (DAT), alpha-synuclein (Asyn), and phosphoserine 129-Asyn (pSer129). The inflammatory markers examined included glial fibrillary acid protein (GFAP, astrocyte marker), ionized calcium binding adaptor molecule 1 (IBA1, microglia and macrophage marker), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB, transcription factor of inflammatory responses) (Table 1). Following transfer of proteins to a PVDF membrane using a Trans Blot Turbo Transfer System, the immunoblots are immediately fixed with 0.4% paraformaldehyde (PFA) and stained for total protein using Revert 700 (Licor) for reliable normalization. Revert staining was captured and measured on the Licor Odyssey at 700nm for 30 sec.

Membranes were blocked with 5% milk in TBS-Tween for 1 hour at room temperature, and probed with primary antibody overnight at 4 degrees Celsius. The blots were then incubated with appropriate horseradish peroxidase secondary antibodies at room temperature for one hour and developed with SuperSignal West Femto Enhancer or Pico

Peroxide solutions under a chemiluminescent detection imaging system (Azure). To ensure the most accurate signal intensity, we optimized each antibody on test immunoblot assays.

Stripping

Previously stained immunoblot assays were stripped and reprobed before staining for Asyn and phosphorylated TH. The membranes were washed with tris-buffered saline tween (TBST) and incubated at 50 degrees Celcius for 30 minutes with stripping buffer (62.5mM Tris-HCl, pH 6.8; 2% SDS; 100mM 2-mercaptoethanol). The membranes were then washed again with TBST before blocking with 5% milk in TBS-Tween and reprobing with a primary antibody overnight at 4 degrees Celsius.

Quantification and Statistical Analysis

Signal intensities were quantified using ImageStudioLite (Licor) and normalized to total protein values (Figure 3). Each blot had representative treatment groups and were used to normalize between blots. Ipsilateral measurements were further normalized to contralateral signals (±SEM) with the exception of Asyn and pSer129 that were only evaluated in the hemisphere ipsilateral to the virus injection. Two-tailed unpaired t-tests between vehicle and SMM-189 treated groups were conducted using GraphPadPrism (version 8). Two-tailed paired t-tests between ipsilateral and contralateral sides for each marker and treatment group were also conducted. A Pearson correlation matrix was performed to determine relationships between markers of interest. Lastly, a grubs test for outliers with significance level at 0.05 was conducted, and all outliers were excluded.

Results

Asyn Aggregation

Dopaminergic neurons in the SN project to the dorsal striatum, consisting of the caudate nucleus and putamen (Lynd-Balta and Haber, 1994). Gombash et al. (2013) showed that through anterograde transport, Asyn travels from the AAV2/5 injected nigra to the ipsilateral striatum. Hence, we evaluated total Asyn and phosphorylated Asyn expression in the ipsilateral striatum. Our PD model was a virus- mediated Asyn overexpressing rat. A significant difference between ipsilateral and contralateral total Asyn expression for SMM-189 (Figure 7A) and vehicle-treated rats (Figure 7B) served as our positive control, and indicated that our model resembled Asyn-overexpressing PD pathology.

The results show that a systemic SMM-189 treatment following viral mediated human Asyn-overexpression alters total human phosphorylated Asyn compared to controls. Phosphoserine129 Asyn (pSer129) expression normalized to total protein (mean ± std. error) was 632,704± 136,730 densitometric units for controls (n=7) and 122,445± 18,848 densitometric units (n=9) for SMM-189 treated rats where $t(14) = 4.207$, $p = 0.0009$ (Figure 4B). In addition, pSer129 expression normalized to total Asyn values, were close to significantly different between vehicle (0.8752± 3.005 densitometric units) and SMM-189 animals (0.2840± 0.08691 densitometric units), where $t(14) = 2.108$, $p=0.0535$ (Figure 4C). Four animals were excluded for the pSer129 calculations due to high background and unclear bands on one of the blots, along with one significant outlier. However, there was no significant difference in Asyn expression between SMM-189-treated rats and vehicle-treated rats. Asyn ipsilateral expression normalized to total protein was 1.040 x 10E+6 ±

197,937 densitometric units for control animals (n=8) and 716,749 ± 154478 densitometric units for SMM-189-treated animals (n=13), where t (19)=1.288, p= 0.2133 (Figure 4A).

Dopaminergic Markers

Degeneration of dopamine neurons in the SN leads to dysfunction of the dorsal striatum (MacDonald and Monchi, 2011). Thus, we evaluated the effects of SMM-189 on dopaminergic degeneration in the striatum. We observed significant changes in dopaminergic marker expression following Asyn-induced nigral overexpression (Figure 5). TH ipsilateral to contralateral relative expression normalized to total protein was 1.248± 0.01073 densitometric units for control animals (n=8) and 0.6115± 0.07167 densitometric units for SMM-189-treated rats (n=13), where t (19)= 5.132 and p< 0.0001. At the 95% confidence level, TH relative expression was significantly lower in SMM-189-treated rats (Figure 5A). However, enzymatically active TH was significantly higher in SMM-189-treated rats, with a relative expression of 0.8044 ± 0.09207 densitometric units in control animals (n=8) and 1.110± 0.08999 densitometric units in SMM-189-treated animals (n=13); t (19)= 2.245, p= 0.0368 (Figure 5B). Phospho TH normalized to TH levels was also significantly greater for SMM-189 animals (2.091±0.2328 densitometric units) compared to vehicle-treated animals (0.6865± 0.1159 densitometric units) where t (19)= 4.490 and p=0.0003 (Figure 5D).

DAT expression was significantly higher in SMM-189-treated animals (n=12) (1.719± 0.1444 densitometric units) compared to controls (n=8) (0.8567 ± 0.08128 densitometric units), where t (18)= 4.535, p= 0.0003 (Figure 5C). To evaluate the effect of human Asyn expression on dopaminergic markers, comparisons were made between

ipsilateral and contralateral hemispheres within treatment group. A significant increase in ipsilateral DAT expression was found for SMM-189-treated rats, at p=0.0019, t (12)= 3.955 (Figure 7C). Ipsilateral DAT expression was measured at 9.4E+6± 1,264,002 densitometric units and contralateral expression at 6.4E6± 1,164,670 densitometric units. The ipsilateral striatum of vehicle-treated rats did not show any significant differences in the levels of DAT expression compared to the uninjected hemisphere.

Inflammation

There was no significant difference in inflammatory marker expression between the SMM-189-treated rats and vehicle-treated rats. The ipsilateral to contralateral relative expression, normalized to total protein for NFκB, was at 0.7789± 0.05423 densitometric units for control animals (n=7) and 0.7438± 0.04984 densitometric units (n=13) for SMM-189-treated animals, where t (19)= 1.235, p= 0.2320 (Figure 6A). A significant outlier was excluded using grubs test for outliers with significance level at 0.05. Control animals (n=8) showed an IBA1 expression of 0.9657± 0.3567 densitometric units compared to 2.217± 0.7892 densitometric units for SMM-189 animal (n=12) expression, where t (18)= 1.230 and p=0.2345 (Figure 6B). Ipsilateral to contralateral relative expression, normalized to total protein for GFAP was at 1.179± 0.04550 densitometric units for control animals (n=8) and 1.280±0.1168 densitometric units for SMM-189-treated animals (Figure 6C). Although no significant differences were evident across treatment groups, GFAP normalized expression was significantly higher on the ipsilateral side compared to the contralateral side for vehicle-treated rats. A two-tailed paired t-test showed a significant difference at t (7)=

3.986, p=0.0053 (Figure 7D) with an expression of 23.55±1.250 densitometric units for the ipsilateral side compared to a contralateral expression of 20.09±1.121densitometric units.

Correlations

A Pearson's correlational matrix was conducted in order to evaluate whether CB2 modulations changes the relationship between different marker expressions and their communication. Such correlations allow us to examine whether there are any interactions between the changes in neuroinflammation, the dopamine system and Asyn aggregation that we hypothesized. The correlational matrix demonstrates a significant inverse correlation between DAT and pSer129 expression in SMM-189 (r= -0.7963, p= 0.0321) and vehicle-treated rats (r=-0.6830, p=0.0436) (Figure 8A-B). Asyn and TH expression are significantly inversely correlated for vehicle-treated rats (r=-0.690, p=0.058) but not for SMM-189-treated rats (r=-0.2491, p=0.4119) (Figure 8C-D). In addition, DAT and phosphorylated TH are directly and significantly correlated for SMM-189 subjects (r=0.5551, p=0.049), but not for control animals (r= -0.1596, p= 0.7058) (Figure 9A-B). SMM-189-treated rats also showed a direct and close to significant correlation between NFκB and TH (r=0.5349, p=0.0596), whereas vehicle-treated rats showed no such trends (r=0.3149, p=0.4915) (Figure 9C-D).

Discussion

Asyn Aggregation

Our results show that SMM-189 rats had significantly lower levels of total pSer129 expression compared to controls. In addition, the ratio of pSer129 to total Asyn in SMM-189-treated rats was close to significantly lower than vehicle-treated rats. Hence, following an artificial increase in human Asyn levels with AVV2/5 technology, SMM-189 is able to reduce the pathological phosphorylation of Asyn, while eliciting less of an effect on total human Asyn expression. A close to significant reduction in pSer12: total Asyn and a significant reduction in total pSer129 in our experimental group suggest that CB2 modulation is decreasing toxic forms of Asyn. Reduction in Asyn aggregation, as suggested by different studies, could help reduce inflammatory responses and neurodegeneration associated with PD pathology (George and Brundin 2015, Schulz-Schaeffer 2010). In PD, 90% of total Asyn is phosphorylated at the pSer129 residue compared to only 4% in healthy human brains (Arawaka et al., 2017). Targeting pSer129- phosphorylation could thus be an effective approach for reducing Asyn aggregation in PD. Asyn aggregation may be an initial response leading to neurodegeneration (Zhang et al., 2005). Zhang et al., 2005 show how Asyn aggregation leads to a greater number of activated microglia and dopaminergic neurotoxicity, linked to activation of NADPH oxidase, a membrane bound enzyme localized on microglia. Zhang et al. (2005) hypothesized that Asyn aggregates form soluble oligomers that are damaging to proteolysis, which in turn leads to cell death. The fact that we observed reductions in pSer129 and not total Asyn expression indicates that CB2 modulation is specifically targeting pathological symptoms of interest in treating PD.

In developing new therapeutic approaches for PD, it is key to examine microglial ability to clear away toxic forms of Asyn (Zhang et al., 2005).

Dopaminergic Markers

Our animal model uses a lower titer dose of the virus that does not produce significant levels of degeneration, as demonstrated by the non-significant results of dopaminergic markers between the injected and uninjected hemispheres in the vehicle-treated rats. However, TH levels were significantly lower in SMM-189-treated rats compared to controls, indicating an enhanced neurodegeneration in SMM-189 subjects. SMM-189 treatment could be having a direct effect on neurons, even though CB2 receptors are not thought to be localized on neurons. Alternatively, SMM-189 treatment could be targeting CB1 receptors in the striatum, where they are synthesized and highly expressed (Julian et al., 2003). Striatal CB1 receptors are particularly localized on presynaptic GABAergic terminals, playing a pivotal role in neurotransmission and influencing dopaminergic neuron activity (Julian et al., 2003). Hence, SMM-189 may alter dopaminergic neurotransmission through CB1 receptors and not necessarily destroying dopamine-producing neurons. In such a case, this could suggest that SMM-189's very low affinity for CB1 receptor binding may have very significant effects (Presley et al., 2015). Therapeutic approaches that target CB1 receptors have lost interest due to adverse side effects such as depression and anxiety (Pertwee, 2012). Thus, the potential effects that SMM-189 has on CB1 receptors needs to be further investigated. In order to assess whether SMM-189 is resulting in a loss of neurons, neuronal markers such as Neun and Map2 must be evaluated.

Although our results show evidence of enhanced dopaminergic degeneration, phosphorylated TH and DAT expression show that there is compensation mechanism mediated by the remaining neurons, to make up for the loss of dopamine. Phosphorylated TH and DAT expression was significantly higher for SMM-189-treated rats compared to vehicle-treated rats. DAT levels in the ipsilateral hemisphere of SMM-189-treated rats was significantly higher than the contralateral side. These findings suggest that CB2 modulation results in an up-regulation of DAT on the injected side, resulting in more dopamine being transported back into neurons from the synaptic cleft. The amount of enzymatically active TH also increases, in attempt to speed up the rate-determining step of dopamine production. In addition, Phospho TH: TH levels were significantly higher for SMM-189 animals, suggesting that the CB2 treatment increases the ratio of enzymatically active TH to total TH levels. SMM-189 treatment is likely increasing the rate at which TH is phosphorylated. These results support other studies showing how surviving neurons in PD brains increase TH phosphorylation as a compensatory mechanism for dopamine production (Shedhadeh et al., 2019). Masoud et al. (2015), demonstrates that DAT overexpression precedes the loss of dopaminergic neurons, oxidative stress and motor deficits associated with PD. Increased levels of DAT lead to oxidative damage and neurotoxicity susceptibility. Thus, the DAT overexpression in the SMM-189-treated rats suggests dopaminergic neurodegeneration, which will likely result in high levels of oxidative stress.

Inflammation

The inflammatory markers, including NFκB, IBA1 and GFAP, show no significant differences between SMM-189 and control rats. These findings were unexpected, given

what is described in the literature (Reiner et al., 2014; Presley et al., 2015). We had hypothesized that the CB2 inverse agonist would shift the immune system from a pro-inflammatory phenotype to a wound- healing phenotype. We expected to see a decreased expression of IBA1, GFAP and NFκB in our treated rats compared to the controls. CB2 inverse agonists are uniquely characterized by a prolonged downstream mechanism that alters inflammation; distinguishing them from CB2 agonists and antagonists (Reiner 2014, Bu 2016, Guley 2019) Reiner et al., (2014) showed how SMM-189 treatment is associated with CREB activation in microglia and therefore with an inverse agonist action of CB2 receptors. Although previous studies show that SMM-189 activates CB2 receptors, our data does not directly show the inflammatory changes associated with CB2 inverse agonists. Hence, further investigation is needed to demonstrate a direct line of evidence that CB2 signaling has changed. Future studies could look at CREB, PKA expression, CB2 distribution or changes in ligand and cannabinoid concentrations (Barreda-Gómez et al., 2010; Reiner et al., 2014).

Most of all, future work is needed to further understand the inflammatory mechanisms of CB2 modulation. As previously discussed, inflammatory markers in PD models are highly expressed in the brain and in the periphery (Concannon et al., 2016; McGeer et al., 1988; Harms et al., 2018). Hence, SMM-189 could be directly targeting inflammatory responses in the brain or alternatively altering peripheral cells that enter the brain and provide inflammatory cues. In the current study, we attempted to understand the effects of inflammation particularly in the brain, but it is important to further investigate infiltrating cells (Harms et al., 2018). Further studies should look at T cell expression in the brain.

Although the data shows no significant changes in inflammatory expression, we cannot conclude that SMM-189 does not alter inflammatory responses. NFκB is associated with the expression of genes involved in microglial inflammatory and immune responses (Kaltshmidt et al., 2005; Mattson et al., 2005). Inhibition of NFκB has been investigated as a therapeutic approach for targeting microglia-induced inflammation in PD. Even though our results indicate no changes in NFκB expression, this does not designate a lack of changes in all stages of the NFκB pathway. Several components of the pathway play a role in the regulation of inflammation and could be affected. To further determine if there are any alterations in the pathway, IκB kinase expression, a protein involved in NFκB activation, or NFκB nuclear translocation could be examined (Hunot et al., 1997).

IBA1 is a protein expressed on microglia and up-regulated with cell activation. IBA1 staining is a well-established form of labeling microglia activation, often used in western blots and immunohistochemistry stains (Walker et al., 2016; Hu et al., 2009). Hu et al. (2009) showed that IBA1 expression is higher in PD models, associated with neuronal loss. Hence, in order to measure changes in microglia expression, an IBA1 antibody was used. We observed no changes in IBA1 expression. However, immunoblot stains do not directly represent the phenotype of microglia. Thus, a lack of change in striatal IBA1 expression does not necessarily signal a lack of morphological alterations. Previous data in the lab noted that nigral microglia underwent morphological changes following CB2 modulation. Hence, in order to fully assess microglial effects, future experiments should look at immunoflourescent stains and quantify striatal microglia size and shapes following SMM-189 treatment.

Similarly, we observed no changes in GFAP expression between experimental and control subjects. GFAP is a filament protein expressed by astrocytes. Clairembault et al. (2014) demonstrated GFAP overexpression in patients with PD. Therefore, we evaluated whether SMM-189 had any effects on GFAP and astrocyte expression. Although we observed no significant changes in GFAP expression between treated and non-treated animals, we cannot discard any alterations in astrocyte function. Astrocytes are important for metabolic support, regulating synaptic transmission, constructing the BBB, and controlling blood and water transport in the brain. They produce glial-derived neurotrophic factors (GDNF), which are essential to dopamine-producing neurons (Booth et al., 2017). Therefore, we cannot conclude that our PD model shows no disruption of astrocyte function and infiltrating cells. To further assess any changes in astrocyte function, BBB integrity or mature astrocyte expression could be tested, using aldonase C or glutamate transporter-1 (Glt1) markers.

Even though there were no evident changes in GFAP expression between treatment groups, GFAP levels were significantly higher in the ipsilateral side compared to the contralateral side in vehicle-treated rats. This difference between hemispheres was not evident in SMM-189-treated rats. These observations suggest that CB2 modulation is suppressing up-regulation of astrocyte expression in the side of the brain injected with the virus. Glial cells, including astrocytes, help mediate the inflammatory and immune response of endogenous and exogenous cannabinoids (Merrill and Benveniste, 1996). Specifically, activation of cannabinoid receptors on astrocytes, suppresses inflammatory actions. Through RT-PCR and immunohistochemistry, Molina-Holgado et al., 2002 observed expression of CB1 receptors on mice astrocyte cultures. Although further research

is needed to confirm the presence of CB2 receptors on astrocytes, these receptors have been shown to play a functional role in glial inflammatory responses. CB2 receptor antagonists stop inhibitory cannabinoid effects on LPS animal models (Molina-Holgado, 2002).

Correlations

A negative and significant correlation between DAT and pSer129 was evident in both SMM-189 and control rats. This trend suggests that healthier neurons with high DAT levels have less Asyn pathology and aggregation. The neurons overcompensating for dopamine loss in the SMM-189 animals and with higher DAT expression are successfully suppressing increased levels of phosphorylated Asyn. Concurrently, neurons dying off may be a result of high expression of toxic Asyn, such as in vehicle animals with high pSer129 and low DAT expression.

A similar trend is evident only in vehicle-treated rats, where Asyn and TH are negatively correlated at a close to significant level. Again, in vehicle rats, healthier neurons with higher levels of TH are associated with lower levels of total Asyn expression. This trend is not evident in SMM-189 rats, likely revealing that the alterations in TH expression are not driving changes in total Asyn levels.

In SMM-189-treated rats, the correlational data showed a direct positive and significant correlation between phosphorylated TH and DAT. This trend was not seen in control subjects. A positive correlation between phosphorylated TH and DAT signals that there are more active dopamine neurons in the SMM-189 group likely as a result of a degenerating process, and that the remaining neurons are overcompensating by producing more Phospho TH and DAT. This overcompensating mechanism is not present in the

control group, which has lower expression of Phospho TH and DAT compared to the SMM-189 group.

Lastly, there was a positive and close to significant correlation between TH and NFκB in the SMM-189 group, and not in the control group. SMM-189 seems to be altering inflammation with aversive effects on TH levels. Total TH expression is lower in SMM-189 treated rats and closely linked with NFκB and inflammatory responses. CB2 modulation could be disrupting neuronal- immune communication. Diseases characterized by peripheral immune deregulation and inflammation, such as PD, tend to affect neuro-immune communication (Pavlov et al., 2018). Cells in the brain boundaries such as the blood- cerebrospinal fluid (BCSF) and the BBB act as communication bridges across the brain. Regulation of the crosstalk and passage of cells helps control infiltration of noxious cells leading to neuroinflammation (Fuzzati-Armentero et al., 2019). Hence, complications in neuro-immune communication following CB2 targeting could worsen aggregation of blood- derived proteins in the striatum, observed in post-mortem brain tissue of PD patients (Fuzzati-Armentero et al., 2019). Evaluating the presence of infiltrating leukoctyes and markers of tight junctions in the striatum would help to further understand the effect of CB2 targeting on BBB permeability and migration of the innate or adaptive immune system in our model. Some markers that could be stained in the brain in order to examine BBB integrity include S100β and neuron-specific enolase (NSE) (Marchi et al., 2004). In control subjects, NSE is mostly concentrated in the plasma and S100β in the CNS. Thus, staining for these markers before and after opening up the BBB could give insights on BBB integrity, whereas increases in plasma S100β expression and no changes in NSE would be indicative of no neuronal damage (Marchi et al., 2004).

Limitations

A limitation of the current study is that all of the results are generated from a single methodological approach with possible variance. Due to time limits, the findings could not be backed up through other protein measuring techniques such as immunohistochemistry staining. If studies are repeated in the future, evaluating a cohort of animals using immunohistochemistry for similar markers would provide secondary evidence for the changes we observed from the western analysis. Other techniques could have helped to overcome any inconsistencies associated with protein preparations, immunoblot normalization values, quantification of weak signal intensities and western transfers. However, most importantly there could have been some deviation due to inconsistent Asyn transport from the nigra depending on the location of nigral injections. Asyn in anterogradely transported to the ipsilateral striatum following AAV2/5 nigral injection. Hence, Asyn levels in the striatum could have been inconsistent across animals if not targeted exactly across animals, despite an equal titer injection of the virus.

Lastly, our negative control for the AAV2/5 injection included the contralateral brain values. However, peripheral-central neuro-immune crosstalk could have had an effect on the intactness of the contralateral side. Inflammatory molecules produced both in the periphery and the brain, can alter tight junctions and in turn BBB permeability (Fuzzati-Armentero et al., 2019). Hence, the effects of Asyn-induced inflammation could reach the contralateral hemisphere. Future studies should include a separate control animal injected with saline, in order to avoid any inflammatory or PD-pathology cross-talk.

Future Directions

Through our results, it is still not clear how SMM-189 is significantly decreasing pSer129. Further inflammatory markers must be evaluated to determine how Asyn aggregates are being affected. In order to further understand how CB2 attenuates inflammatory responses future studies could evaluate T-cells, tumor necrosis factor alpha (TNF alpha), nitric oxide synthase (iNOS), and cytokine production- through enzyme-linked immunosorbent assay (ELISA).

Different lab techniques and administering different doses of the virus could help further investigate the validity of the trends. Evaluation of lymphocytes such as CD4 or CD8+ t-cells or markers of infiltrating macrophages or monocytes would help determine if CB2 effects may have been due to modifications of the infiltrating cells or the resident central immune cells (ie: microglia). Measuring the downstream signaling of CB2 in the striatum including PKA, ERK1/2 and pCREB would serve to determine if GPCR downstream effects or protein synthesis, following CB2 targeting, changes neuronal expression. Other methods to verify the results observed through immunoblot assays would be to examine inflammatory and neuronal gene expression through qPCR evaluations. Lastly, high titer doses of AAV2/5 human wild type Asyn could shed light on the role of CB2 modulation in later stages of PD and at higher rates of dopaminergic degeneration.

Conclusions

Our results suggest that SMM-189 is targeting toxic forms of Asyn aggregates, pSer129. At the same time, SMM-189 is likely having an effect on cannabinoid receptors on neurons, resulting in an enhanced degeneration and lower levels of TH. Alternatively, SMM-189 is not killing neurons but altering neurotransmission, either through CB1 receptors or possible CB2 receptors on neurons which is still a controversy in the field.

The decreased amount of TH expression is linked to inflammatory pathways and increases in NFκB levels. Remaining neurons are undergoing an overcompensation response, overexpressing DAT and Phospho TH. Increases in DAT levels are matched to decreases in pSer129.

In addition, SMM-189 resulted in no changes in inflammatory marker expression compared to controls, namely astrocyte and microglia markers. However, CB2 modulation may be suppressing the up-regulation of astrocytes seen in the ipsilateral side of vehicle treated subjects.

Although SMM-189 significantly decreases pSer129 aggregates, its aversive effects on neurons must be closely taken into consideration. In addition, inflammatory responses must be further evaluated before considering CB2 inverse agonist modulation as a novel therapeutic approach for treating PD.

Figures and Graphs

Antibody/ Anitgen	Company	Product #	Ab made in	Molecular weight	Optimal concentration	Secondary concentration
TH	Millipore	AB152	Rabbit	62 kDa	1_2000	1_2000
Asyn	BioLegend	103-108 4B12/SYN	Mouse	14 kDa	1_500	1_2000
DAT	Novus Bio	NBP2-22164	Mouse	75-50 kDa	1_2000	1_2000
IBA1	ABCAM	AB5076	Goat	17kDa	1_1500	1_2000
Phospho-TH	Phospho solutions	P1580-40	Rabbit	60 kDa	1_1000	1_2000
NFκB p65	Santa Cruz	SC327	Rabbit	65 kDa	1_500	1_2000
GFAP	Dakocyto-mation	Z0334	Rabbit	50 kDa	1_2500	1_2000
pSer129	ABCAM	AB51253	Rabbit	14 kDa	1_500	1_2000

Table 1: Antibody information for each marker tested. Antibody name, company, product number, animal host, molecular weight, loading protein concentration, optimal primary antibody staining concentration and optimal secondary antibody concentration for each marker stained.

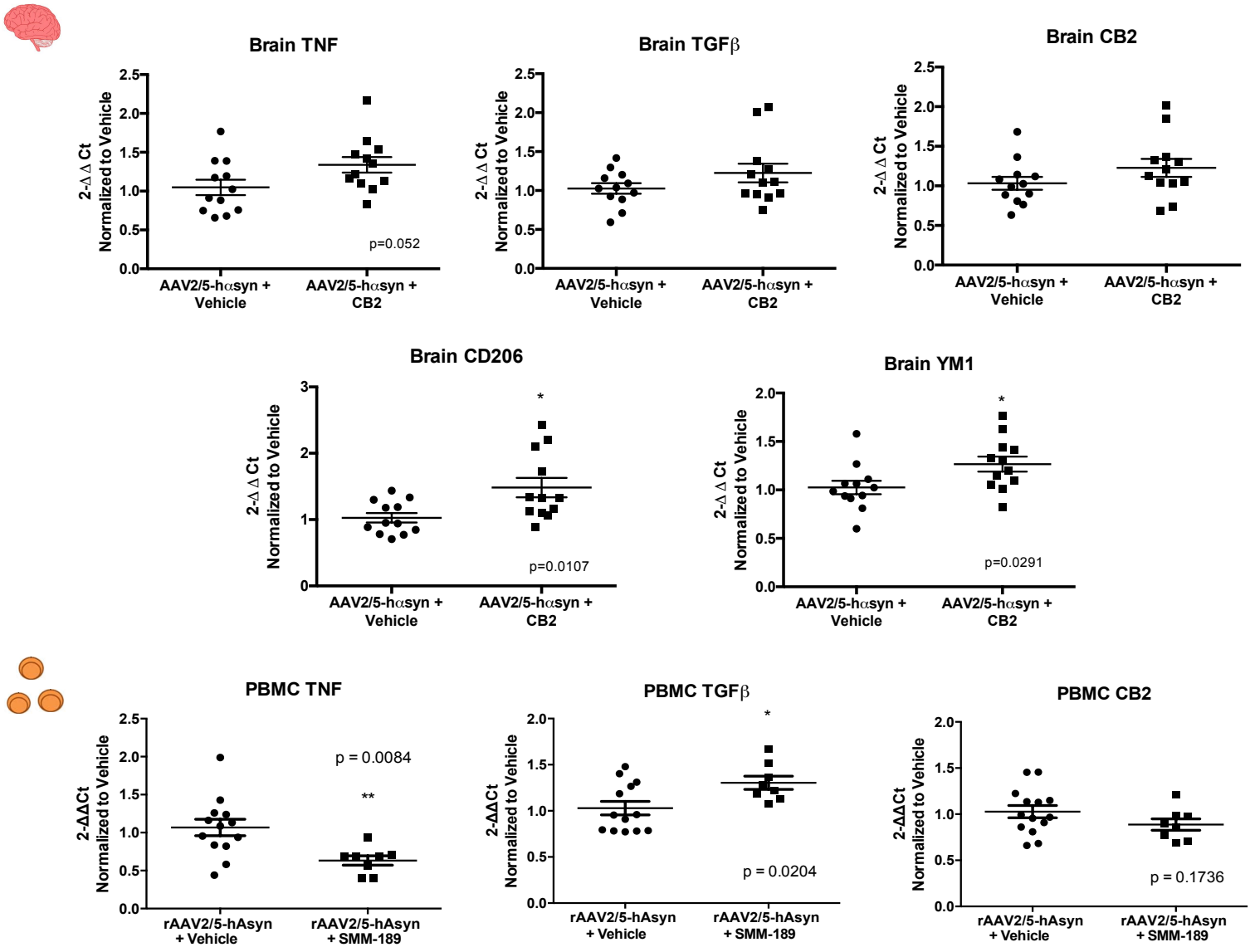


Figure 1: Preliminary data showing how SMM-189 alters gene and marker expression in the frontal cortex and plasma. SMM189 rats showed altered gene expression of anti-inflammatory phenotype markers in the frontal cortex. Subjects administered daily SMM-189 had increased M2 macrophage markers YM1 and CD206 mRNA levels compared to control vehicle-treated animals. Gene expression of endpoint PBMCs show that SMM-189-treated rats had significant increase in wound healing marker TGFβ and decrease in pro-inflammatory marker TNF, indicating changes at the cellular level.

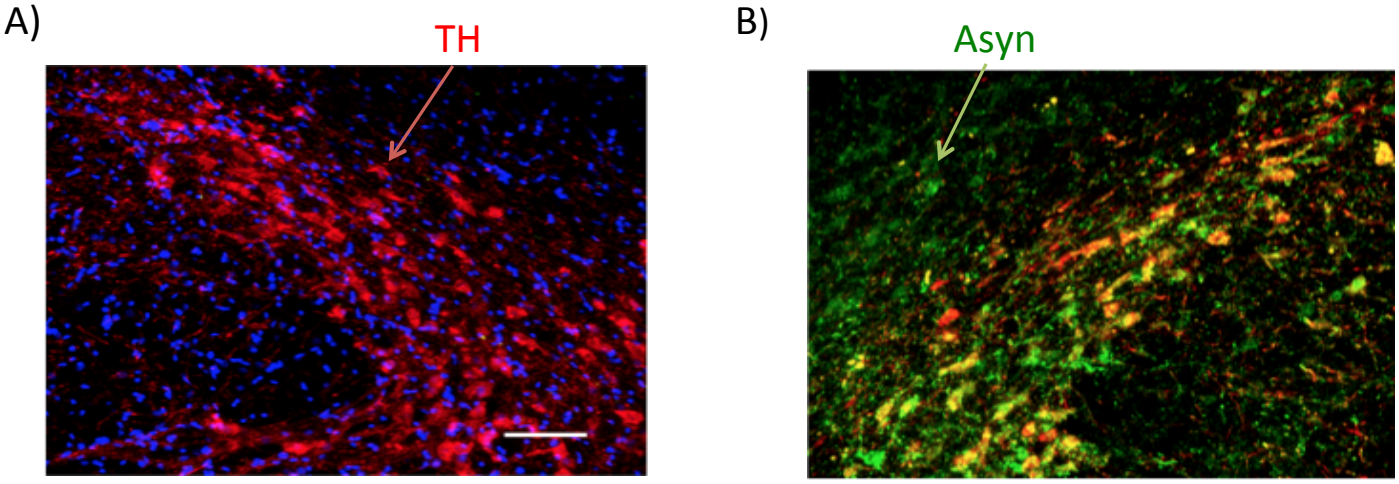


Figure 2: A successful surgery identified postmortem using immunoflourescent staining. A) Unlesioned animal where TH is stained in red and DAPI is counter stained in blue. Overlap of DAPI and TH signal dopamine producing neurons. B) AAVV2/5 human wild type Asyn lesioned animal where green demonstrate a unilateral presence of Asyn in the TH+ nigra neurons. Scale bar= 100um.

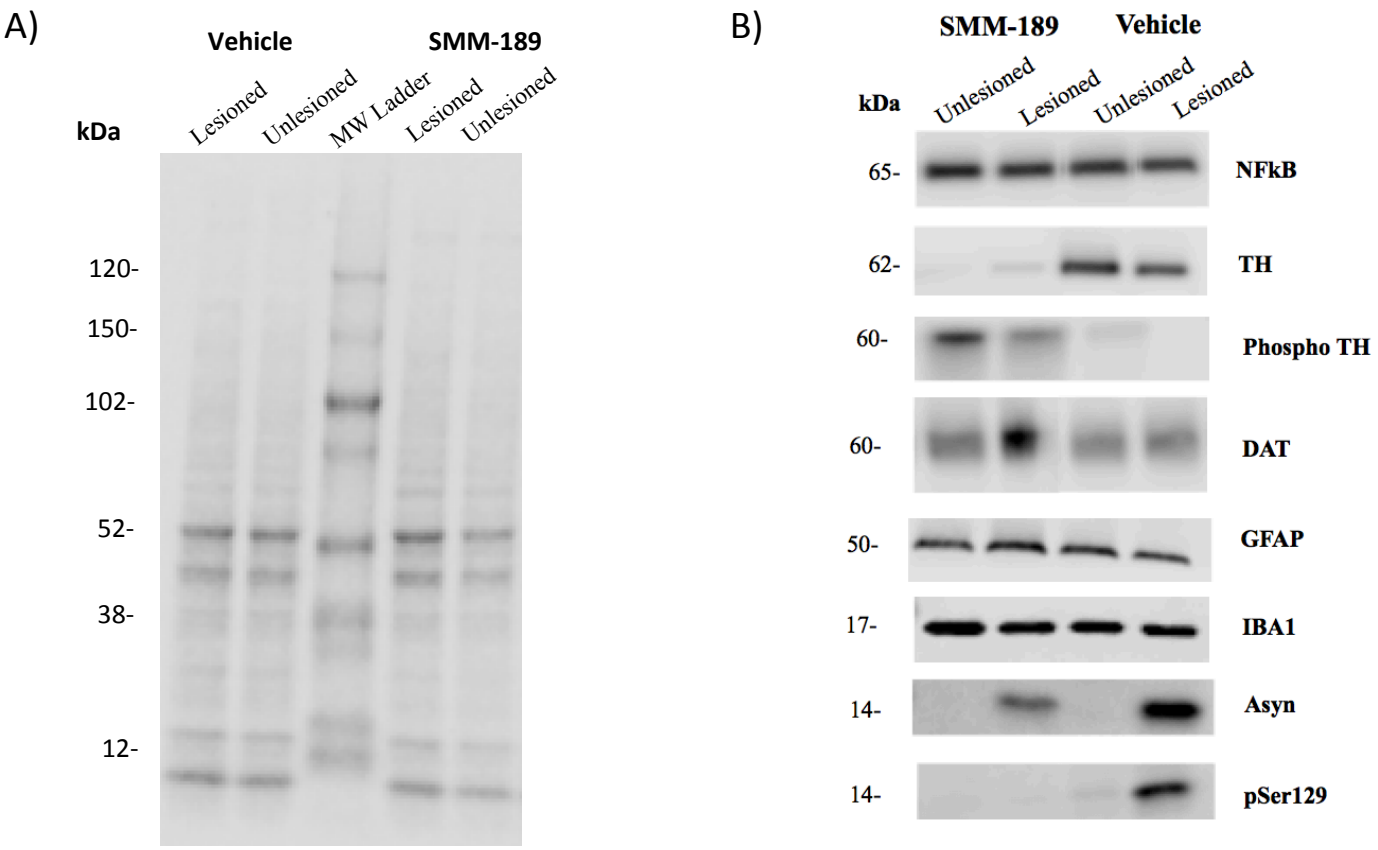
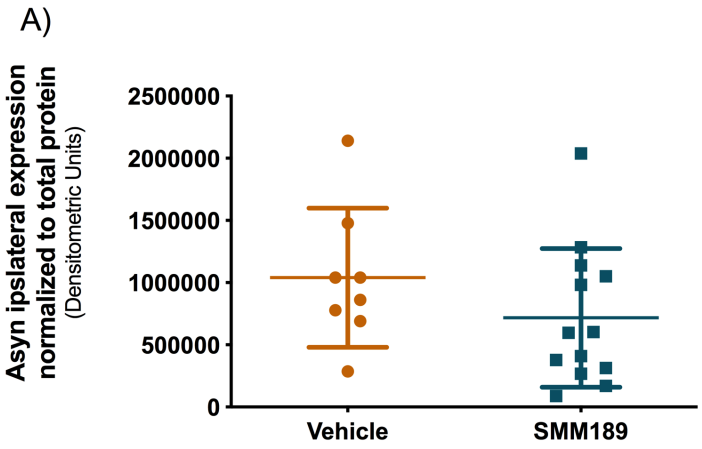
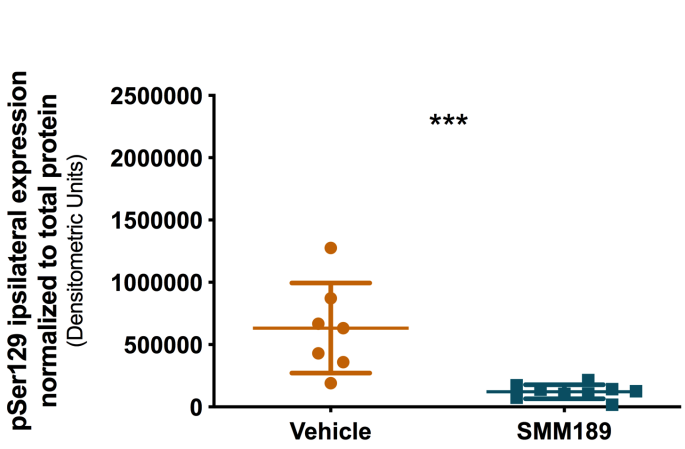


Figure 3: Immunoblot assay signal intensities. A) Total protein of gel loaded with 10-15 ug/uLof sample. Marker signal intensities were normalized to total protein values. B) Representative signal intensities for NFkB, TH, Phosphorylated TH, DAT, GFAP, IBA1 and Asyn, pSer129 from top to bottom.





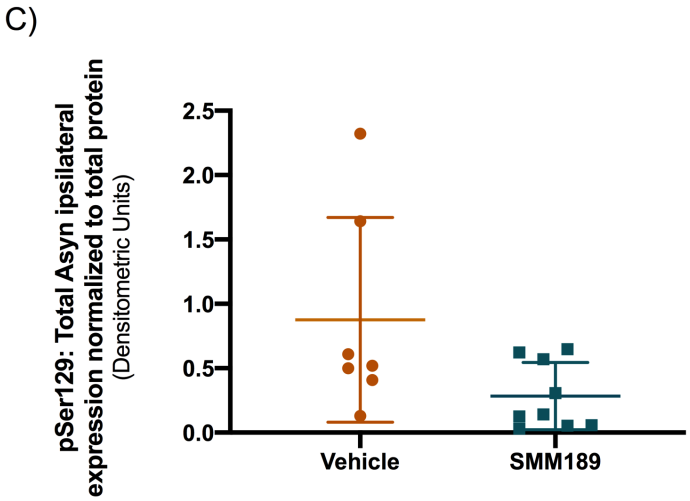


Figure 4: Phosphorylated Alpha-synuclein expression following human Asyn overexpression shows a significant difference between SMM-189 and vehicle-treated rats. A) Systemic SMM-189 treated rats (n=13) (716,749± 154,478) stained with anti-human Asyn antibody (4B12/SYN) had no significant difference in striatum intensity signal compared to vehicle-treated rats (n= 8) (1,039,643± 197,937), where t (19)=1.288, p=0.2133. B) Systemic SMM-189-treated rats (n=9) stained with anti-pSer129 antibody (AB51253) had a significantly lower ipsilateral relative expression, normalized to total protein (122,445± 18,848) compared to vehicle-treated rats (n= 7) (632,704± 136,730), where t (14)= 4.207, ***p= 0.0009. C) pSer129 ipsilateral expression normalized to total Asyn for vehicle animals (0.6865) is close to significantly different to experimental animals (2.091± 0.8394) where t (14)= 2.198, p=0.0535.

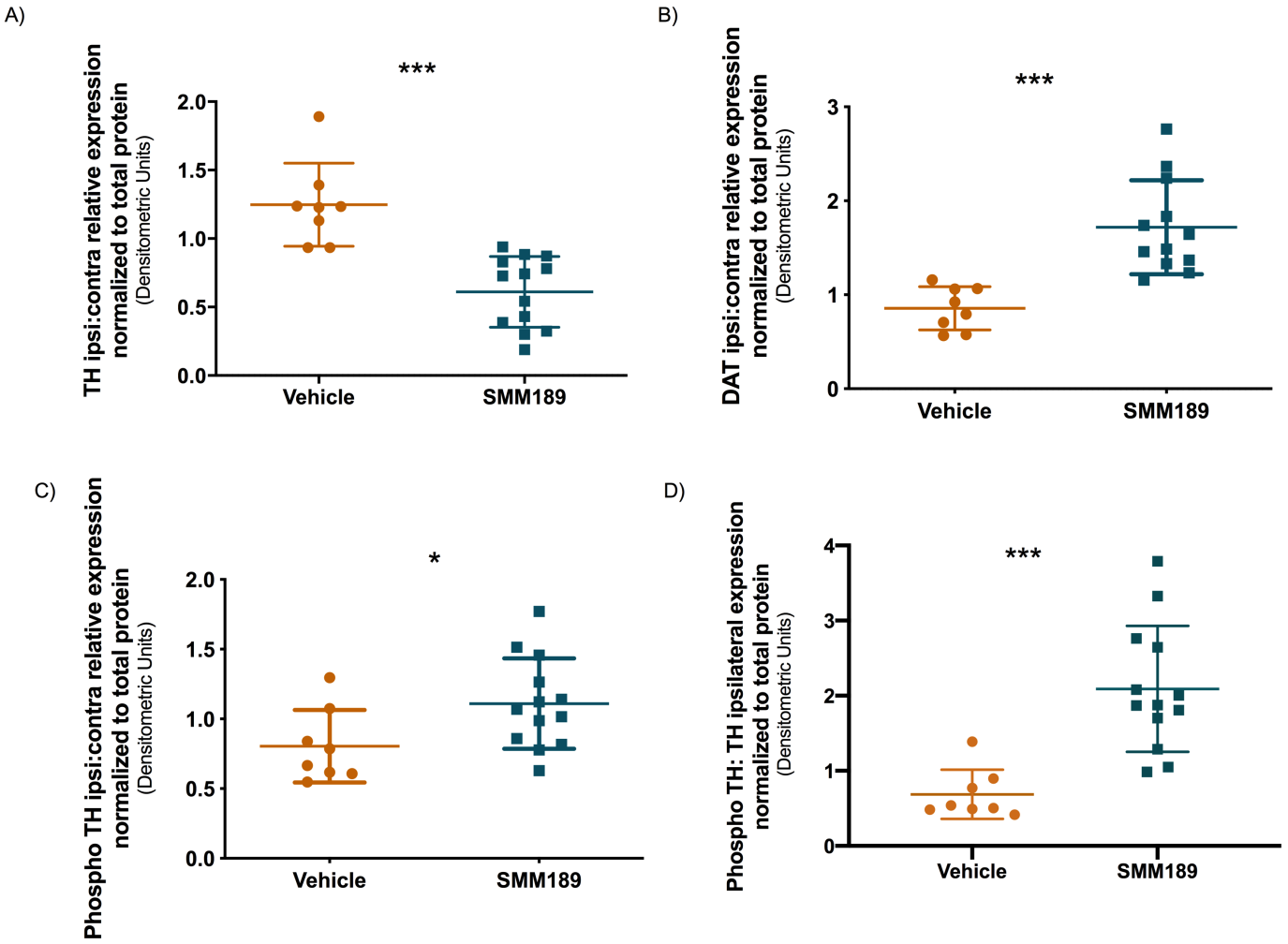
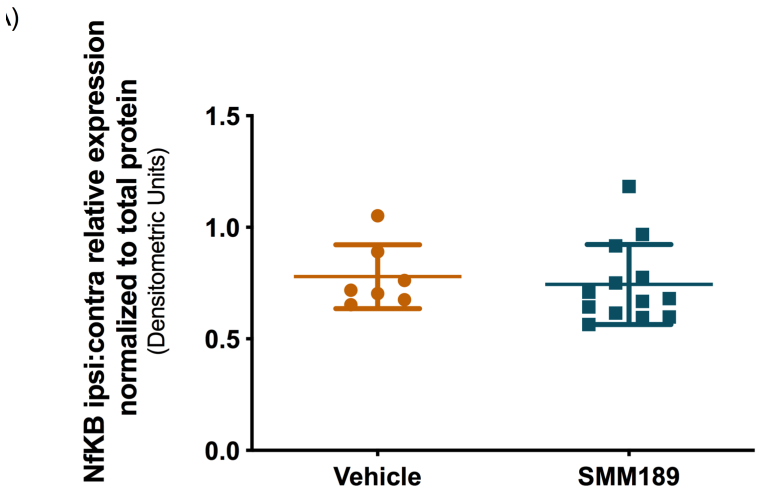
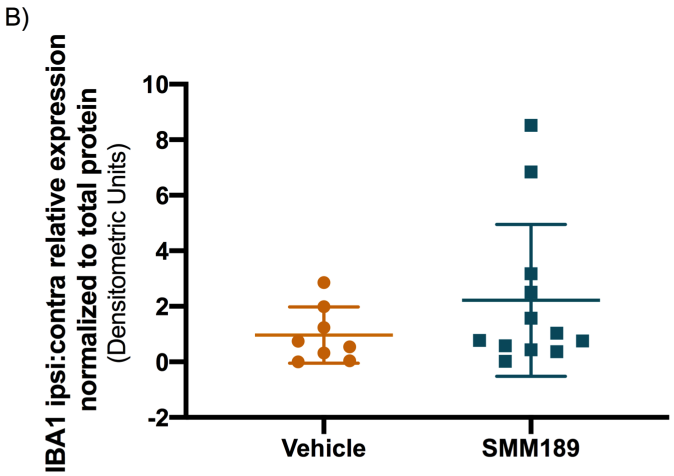


Figure 5: Increased striatal expression of phosphorylated tyrosine hydroxylase (Phospho TH) and dopamine transporter (DAT), and lower levels of tyrosine hydroxylase (TH) in SMM-189-treated rats. A) Systemic SMM-189-=treated rats (6 mg/kg, ip)(n=13) stained with anti-TH antibody (AB152) had a significantly lower ipsilateral to contralateral relative expression, normalized to total protein (0.6115± 0.07167), compared to vehicle treated rats (n= 8) (1.248± 0.1073), where t (19)= 5.132, ***p< 0.001. B) Systemic SMM-189 treated rats stained with mouse anti-DAT antibody (NBP2-22164) had a significantly higher contralateral relative expression, normalized to total protein (1.719 ±0.1444) than vehicle treated rats (0.8567± 0.08128). Unpaired t-test shows a significant difference at the 95% confidence level where t (18)=4.535, ***p= 0.0003. C) Systemic SMM-189-treated rats stained with anti-Phospho TH antibody (P1580-40) had a significantly higher contralateral relative expression, normalized to total protein (1.110 ±0.08999) compared to vehicle-treated rats (0.8044±0.09207), where t (19)= 2.245, *p= 0.0368.





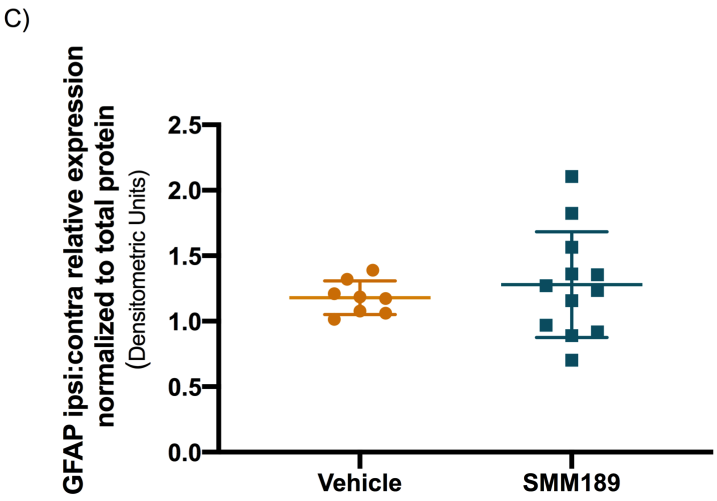


Figure 6: Inflammatory marker expression following human Asyn overexpression show no significant difference between SMM-189 and vehicle-treated rats A) Systemic SMM-189 treated rats (6 mg/kg, ip) (n=13) stained with NFB (SC327) (0.7438± 0.04984) had no significant difference in ratio of ipsilateral to contralateral striatum intensity signal compared to vehicle treated rats (n= 7) (0.7789±0.05423), where t(18)= 0.4446, p=0.6619. B) No significant difference was found for rats stained with anti-IBA1 antibody (AB5076) where vehicle-treated rats (n=8) had an expression of 0.9657± 0.3567 and SMM-189-treated rats (n=12) had an expression of 2.217±0.7892; t (18)=1.230, p=0.2345. C) There was no significant difference in GFAP expression (Z0334) between control animals (n=8) (1.179 ± 0.04550) and SMM-189-treated animals (n=12) (1.280± 0.1168); t (18), p=0.5078.

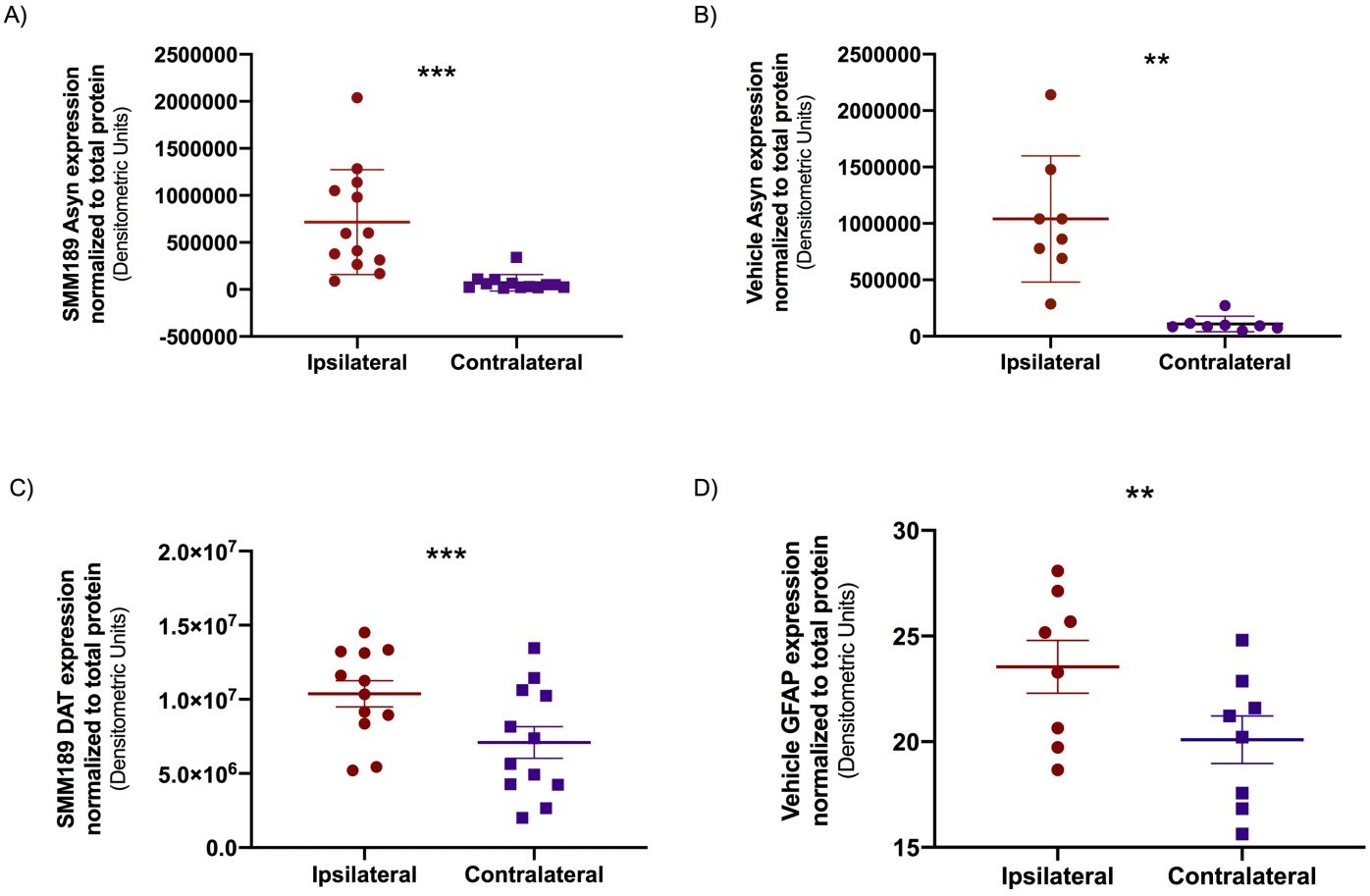


Figure 7: Shows positive control for Asyn overexpressing models & how SMM-189 treated rats have high DAT expression on the ipsilateral side and vehicle rats have high GFAP expression on the ipsilateral side. A) A two-tailed paired t-tests shows that Asyn expression on SMM-189 rats is significantly higher on ipsilateral side (716,749± 154,478) compared to contralateral side (71,006± 24,097), where t (12)= 4.662, p=0.0005. B) Asyn expression of vehicle-treated rats is significantly higher on ipsilateral side (1,039,643± 197,937) compared to contralateral side (108,799± 24,319), where t (7)= 4.755, p=0.0021. C) A two-tailed paired t-test shows that ipsilateral DAT expression (1.03E+7± 881,248) normalized to total protein is significantly higher than contralateral expression (7.08E6± 1,071,050) for SMM-189 subjects, where t (11)= 4.608, ***p=0.0008. D) Ipsilateral GFAP expression for vehicle-treated rats (23.55±1.250) was significantly higher compared to contralateral expression (20.09±1.121) where t (7)=3.986, **p=0.0053.

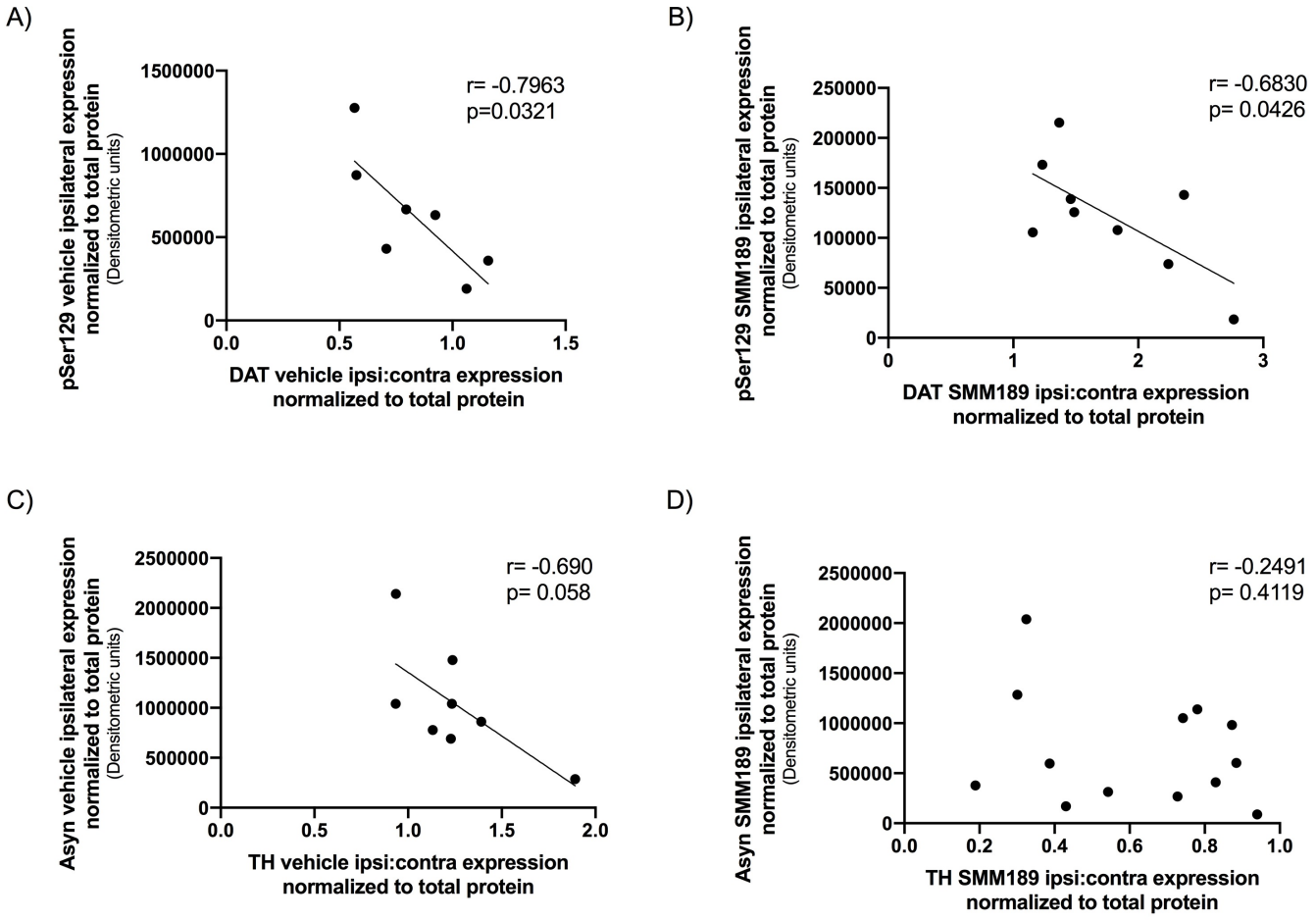


Figure 8: Healthy dopamine producing neurons are associated with reduced Asyn aggregation. A) pSer129 and DAT ipsi:contra expression normalized to total protein are significantly and inversely correlated in vehicle-treated rats, where r=-0.7963 and p=0.0321. B) A similar trend is evident in SMM-189-treated rats, where pSer129 and DAT expression are inversely correlated at r=-0.6830 and p=0.0426. C) Asyn and TH expression are inversely correlated at a close to significant level in vehicle-treated rats (r= -0.690, p= 0.058) D) but not in SMM-189 treated rats (r=-0.2491, p=0.4119).

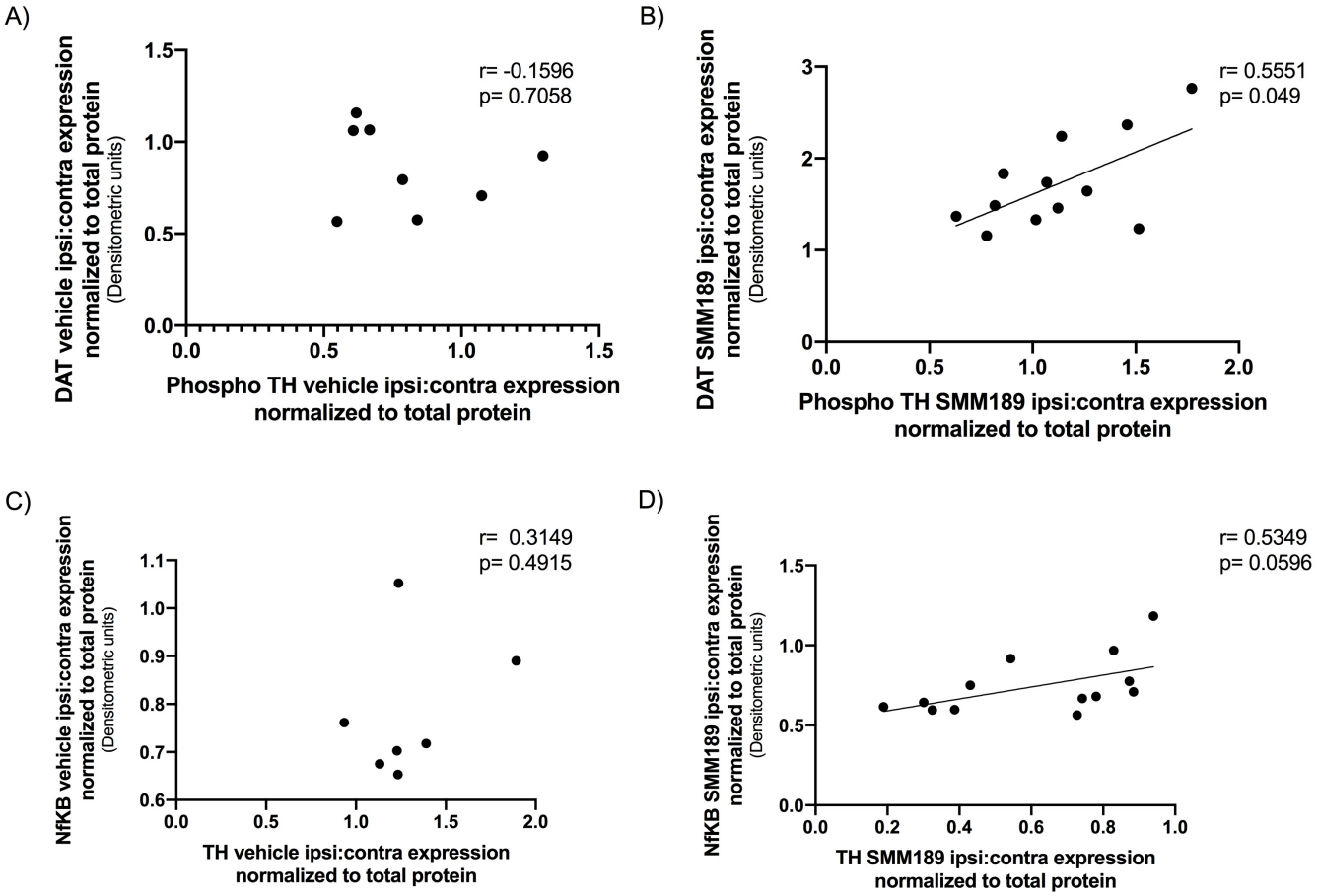


Figure 9: CB2 modulation results in a positive correlation between DAT and Phospho TH and NFκB and TH. A) DAT and Phospho TH ipsi:contra expression normalized to total protein show no significant correlation in vehicle treated rats, where r=-0.1596 and p=0.7058 B) but are significantly and directly correlated in SMM189-treated rats, where r= 0.5551 and p=0.049. C) NFκB and TH expression show no significant trend in vehicle-treated rats (r=0.3149 and p=0.4915) C) but show a direct and close to significant correlation for SMM-189 treated animals (r=0.5349, p=0.0596).

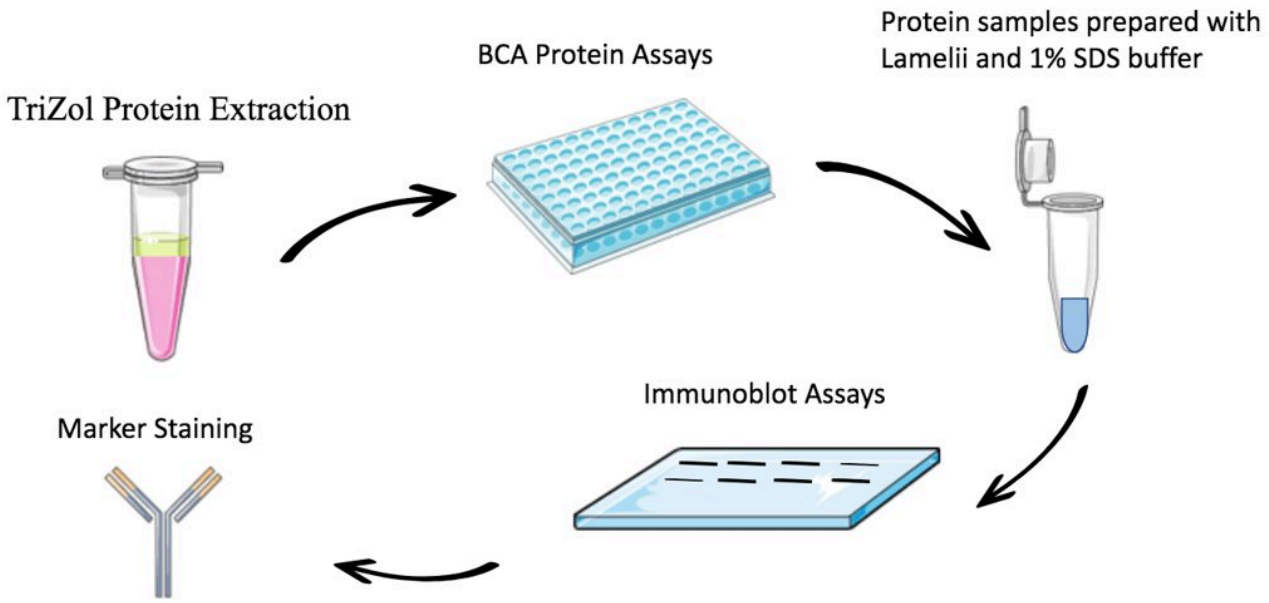


Figure 10: Visual representation of methodology. Striatum protein of successfully targeted animal tissue was extracted through Trizol protein extraction. BCA proteian assays were conducted to measure total protein and to prepare protein samples with Lamelii and 1% SDS buffer. Immunoblot assays were conducted and stained for a number of neuronal and inflammatory markers.

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