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PRINCIPAL INVESTIGATOR:
Serge Przedborski, MD, PhD

CONTRACTING ORGANIZATION:
Columbia University
New York, NY 10032

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Interaction of Synuclein and Inflammation in Dopaminergic Neurodegeneration

Serge Przedborski, MD, PhD

Columbia University
New York, NY 10032

Parkinson’s Disease (PD) is a progressive neurodegenerative disorder characterized by the loss of the dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) of the brain. How the inflammatory response in PD is initiated is unknown. Since synuclein is expressed within the DA neuron, it is possible that synuclein may play a role in the initiation of the inflammatory response. To test this possibility, we injected WT α-synuclein into the SNpc of rats and sacrificed these animals at 2 days (2D), 4D and 7D after injection. At 2D after injection, using MAC-1 immunostaining for microglia, a full-blown inflammatory response was noted in the synuclein-injected SNpc compared to the saline-injected SNpc. This response was still strong at the 7D timepoint. There was also a mild astrocyte response on glial fibrillary acidic protein (GFAP) immunostaining Also noted was a small but non-significant decrease in tyrosine hydroxylase positive neuron numbers in the synuclein-injected rats. These data demonstrate a synuclein-initiated neuroinflammation in an in vivo setting.
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Interaction of Synuclein and Inflammation in Dopaminergic Neurodegeneration

Principle Investigator: Serge Przedborski, MD, PhD

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Prepared By:
Vernice Jackson-Lewis, PhD
Introduction

Parkinson’s Disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s Disease (AD) (Fahn and Przedborski, 2005). It is characterized clinically by resting tremor, slowness of movement, rigidity and postural instability (Fahn and Przedborski, 2005), all attributed to, mainly though not exclusively, the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their dopaminergic terminals in the corpus striatum of the nigrostriatal pathway in the brain ( Hornykiewicz and Kisk, 1987; Fahn and Przedborski, 2005). Although current therapy includes dopaminergic agonists and cholinergic antagonists, the most reliable and most common therapy remains Levodopa (L-DOPA), a precursor for dopamine (DA) (Fahn and Przedborski, 2005). Pathologically, aside from the loss of the DA neurons in the SNpc, PD is characterized by the presence of intraneuronal inclusions called Lewy bodies (Schults, 2006). These inclusions are eosinophilic cytoplasmic aggregates composed of a variety of proteins, such as α-synuclein, ubiquitin, parkin, and neurofilaments and are necessary for the definitive diagnosis of PD (Shults, 2006). Lewy bodies are also found within DA neurons in the SNpc. One of the proteins in the Lewy body, α-synuclein, is a well-conserved protein across mammalian species (Maroteaux et al, 1988; Ueda et al, 1993; George et al, 1995) that is conspicuously expressed throughout the brain and that has been identified in close proximity to synaptic vesicles in presynaptic terminals (Maroteaux et al, 1988; Iwai et al, 1995). Mutations in the α-synuclein protein as well as its overexpression have been linked to the familial form of PD. To date, three missense mutations in α-synuclein (A53T, A30P and E46K) have been noted in the familial form of PD (Polymeropoulos et al, 1997; Kruger et al, 1998; Zarranz et al, 2004). And, in animal studies, overexpression of wild-type (WT) α-synuclein in transgenic mice can lead to a pathology reminiscent of PD (Masliah et al, 2000). Both the A53T and the A30P mutants increase the rate of α-synuclein fibrillation (Sung et al, 2005) and the formation of β-sheets (Narhi et al, 1999) when in solution, and, the overexpression of WT α-synuclein in a number of cell types can result in aggregate formation (Lee and Lee, 2002). Post-translational modification of WT α-synuclein, such as nitrification (Przedborski et al, 2001) and oxidation (Hashimoto et al, 1999), can lead to structural changes in the cells, and if these are not properly cleared, they may give rise to a neurotoxic event within the cells. These findings support the idea that this protein may operate through a toxic gain-of-function mechanism Thus, although none of the α-synuclein genetic aberrations have been found in sporadic PD, since α-synuclein is a major component of Lewy bodies, these data support the possibility that α-synuclein may also play a pathogenic role in sporadic PD.

It is now recognized that neuroinflammation is very much a part of the PD morphological picture (McGeer and McGeer, 2008) and that glial activation is thought to be a contributor to DA neurodegeneration (Fahn and Przedborski, 2009). However, how glial cells and neuroinflammation are involved in PD is not known. It is theorized that the observed activation of glial cells in neuroinflammation may be the cause of the progressive nature of the disease, but this remains to be clarified. We do know that, on autopsy, activated glial cells are present in the SNpc of PD brains and there seems to be evidence of an on-going active degenerative process in the SNpc (Langston et al, 1999). We also know that pro-inflammatory components, such as interleukin 1β (IL-1β) and prostaglandin PGE2, are present in increased levels in the SNpc and in the CSF from PD patients (Mogi et al, 1994, 1996, 2000). In PD models, not only are activated glia present in the SNpc, also present are markers of inflammation, such as the
elevation of NADPH oxidase (Wu et al, 2002), inducible nitric oxide synthase (iNOS) (Liberatore et al, 1999) and PGE2 (Teismann et al, 2003). One interesting finding is the presence of post-translational modified α-synuclein (Przedborski et al, 2001) within the DA neurons of the SNpc. Since this is the case, it is possible that there is some connection between this finding, oxidative stress and neuroinflammation. While we do not know the exact mechanism here, previous work with an injection of neuromelanin into the SNpc of rats resulted in an inflammatory response along with synuclein accumulation within the DA neurons of the SNpc (Jackson-Lewis et al, 2008). This suggests that if enough of an accumulation of synuclein occurs within the DA neuron, this can cause the cell to rupture, spewing its contents including synucleins into the extracellular space. Thus, the released accumulated synuclein may initiate the neuroinflammation seen in PD.

Body of Research

Our overall goal here is to examine the relationship between neuroinflammation and the synucleins. Recent evidence, both in vitro and in vivo, suggest that synuclein can initiate inflammation and DA neuronal degeneration. For instance, in the MPTP mouse model of PD, not only is there an inflammatory response, there is also an oxidative and nitrative modification of α-synuclein (Przedborski et al, 2001). It has also been shown that α-synuclein as well as its mutants can induce the up-regulation of pro-inflammatory markers such as the inflammatory mediator intercellular adhesion molecule-1 (I-CAM) and interleukin-6 (IL-6) in human astrocytes (Klegeris et al, 2006). Furthermore, Gao et al (2008) have shown that there is a link between DA neurodegeneration, neuroinflammation and the oxidation and nitration of α-synuclein. Thus, to investigate the possible link between neuroinflammation and the synucleins in terms of PD, we have proposed the following specific aims.

Specific aim-I. Assess the effects of extracellular α-synuclein on glial mobility and proliferation.

**Hypothesis:** Post-translationally modified and mutated α-synuclein species provoke a greater clustering of glial cells around the site of intracerebral injection than wild-type soluble α-synuclein. In magnitude, this inflammatory response parallels the known pathogenicity of a range of different α-synuclein species, and is due to a combination of key features of the glial response, namely proliferation and chemotaxis.

**Plan:** (A) Rats will receive into the substantia nigra a single injection of modified (fibrillar, nitrated, or oxidized) or unmodified wild-type or PD-linked mutant α-synucleins. Brains will then be harvested and glial cell morphology, topography and density will be compared among each different group. (B) Glial cultures will be exposed to the same set of α-synuclein species and their effects on glia chemotaxis and proliferation will be compared.

Specific aim-II. Examine the stability of extracellular α-synuclein and its effect on glial cell activation.

**Hypothesis:** Modified and mutated α-synuclein species are not cleared (or engulfed) by glial cells as efficiently as is WT soluble α-synuclein and are more potent than wild-type soluble α-synuclein in stimulating the production of a variety of factors such as reactive oxygen species (ROS) or nitric oxide (NO) from glial cells.
**Plan:** (A) The stability and phagocytosis of extracellular α-synuclein species will be compared *in vivo* (using the SA-IA model) and *in vitro* (using primary glial cultures) by immunostaining for α-synuclein and glial markers and confocal microscopy, and by an *in vitro* protein fragmentation assay. (B) The effect of the α-synuclein species on the *in vivo* and *in vitro* production of chemokines, cytokines, ROS and NO by glia will also be compared.

**Specific aim-III. Define the mechanism by which glial cells recognize α-synuclein.**

**Hypothesis:** Extracellular unmodified, modified and mutated α-synuclein species interact with glia through specific cell surface receptors, leading to glial cell activation. Moreover, modified and mutated α-synuclein species are more potent in activating immune cells in the brain than wild-type soluble α-synuclein.

**Plan:** (A) Identify the glial receptors involved in the recognition of the α-synuclein species. (B) Characterize the effect of abrogating (knockout mice) and inhibiting (blocking antibodies) glial receptors on endocytosis of and activation by α-synuclein species.

**Key Accomplishments**

**Specific Aim I**

**Production of the synucleins**

The process of making synuclein is a rather long process. While only microgram quantities of the synucleins are needed for the SNpc injections, fairly large quantities are needed for the cell culture experiments. Thus, we have now made about 90 milligrams of WT α-synuclein, part of which we will oxidize or nitrate for the oxidized and nitrated synuclein studies. For the mutated synuclein studies, we have made about 30 milligrams of both the A30P and the A53T mutants which is being used for the *in vivo* studies and to start the *in vitro* studies. We are now in the process of making larger quantities of the oxidized, nitrated and mutated synucleins for the cell culture studies.

**In Vivo Experiments**

We have verified the coordinates for the injection of the synucleins into the SNpc of rats weighing 225-250 grams at the start of the experiments. Following this, in a pilot experiment using 3 rats per group, we injected wild-type α-synuclein, 5.0 micrograms per microliter, total of 4 microliters, into the SNpc. The animals were sacrificed by transcardial perfusion at 2 days (2D), 4D and 7D after injection and brains were processed for immunostaining. After the analyses of these brains, a longer, more in-depth study was performed using the same protocol, 8 rats per group and per timepoint and a total of 8 micrograms of WT α-synuclein. Samples were collected at 1D, 2D, 4D, 7D, 14D and 21D after injection for both immunostaining and western blot analyses. We are now in the process of analyzing these samples. We have also started a pilot for A53T injection into the SNpc using the same protocol as for the WT pilot study.

**In vitro Experiments**

We have succeeded in producing stable microglia and astrocyte cultures from both 1-2 day old rat and mouse pups. We have now made enough WT α-synuclein and mutated synucleins and have begun the cell culture part of these studies.
Specific Aim II
Rats, 8 per group and per time point, received 8 micrograms of WT α-synuclein in 4 microliters of phosphate buffered saline at a rate of 1 microliter per minute. The needle was left in place for an additional 2 minutes to insure diffusion and to prevent splashback. Rats were sacrificed at 1D, 2D, 4D, 7D, 14D and 21D after injection by decapitation; brains were removed and SNpc and striatum were dissected out, frozen and stored at -80°C until analyses. These tissues will be used for western blot analyses for cytokines and chemokines.

Specific Aim III
We are now breeding synuclein KO mice for these studies.

Reportable Outcomes
Specific Aim I
Injection of WT α-synuclein (5.0 µg/µl, total volume 4µl) produced a significant inflammatory response in the SNpc at 2D after the injection as evidenced on MAC-1 immunostaining, an antibody used the staining of microglia. In the synuclein injected animals, microglia in the SN were strongly activated and took on an amoeboid appearance with short thick processes at 2D after WT α-synuclein injection (Fig 1). This effect was still strong at the 7D timepoint (Fig. 1). Clumping of the microglia was noted at the 2D timepoint (Fig 1), which was more intense at 4D and 7D (Figs. 1). Injection of saline in the SNpc of control animals exhibited a very mild reaction to the MAC-1 antibody (Fig 1). The astrocyte response, as evidenced on glial fibrillary acidic protein (GFAP) immunostaining, was quite mild, almost unnoticeable, at 2D after the SNpc injection. At the 7D timepoint, this response, although more pronounced, was still classified as a mild response. Based in the astrocytic response that was obtained with neuromelanin (NM) at 21D after the injection of this substance, it is predicted that the astrocytic response to WT α-synuclein will continue to become stronger and be even more pronounced at the 21D timepoint. Furthermore, the dose of WT α-synuclein used here blew a hole in some of the tissues which indicates that this dose may have been too strong. Thus, we are repeating the study with a less concentrated dose of synuclein (2.0 µg/µl) and more timepoints (1D to 21D after injection).

The number of TH+ neurons was not noticeably different between synuclein-injected and saline-injected animals (Fig.2). However, on counting of these neurons using stereology, there was a small but insignificant difference in TH+ neuron counts in the SNpc between the two groups of mice, the difference at the 7D timepoint being less than 10% (data not shown).

Discussion
Chronic neuroinflammation seems to play a significant role in several neurodegenerative diseases, including PD. Both microglia and astrocytes are activated during injury and this activation seems to push the progressive nature of PD, as it is reported that toxic substances such as the superoxide radical, NO, cytokines and chemokines which help to continue the neuronal death process, are present in the PD brain (McGeer and McGeer, 2004). Lewy bodies are one of the hallmarks of PD (Przedborski and Dauer, 2003) and are found within DA neurons in the SNpc. In our study, we injected WT α-synuclein directly into the SNpc of rats. Results showed a
significant inflammatory response around the injection site and in the SN pars compacta and pars reticulata compared to both the uninjected side and the saline-injected animals. This full-blown neuroinflammatory response was noted as early as the 2D time point after injection and was still maintained at the 7D time point. Since the neuroinflammation was still strongly present at 7D after the injection, it is likely that the injected synuclein initiated the microglial and astrocytic response, in part, via its presence in the extracellular space. The fact that we saw an insignificant amount of DA neuron death in the SNpc actually fits with the pattern of DA neuronal death in PD as this process in the PD patient takes decades and symptoms of the disease are not visible until the 5th decade of life. In cell culture studies, it was noted that DA neuron degeneration was dependent on the presence of synuclein following lipopolyssachride (LPS)-induced microglial activation (Gao et al, 2008). These studies show that DA neuron degeneration is far greater in the presence of microglia plus synuclein than with microglia alone. These same authors also demonstrated this same phenomenon in vivo using synuclein transgenic and knockout mice and LPS (Gao et al, 2008). Other studies (Su et al, 2007) not only demonstrated that MN9D-SYN cells released synuclein into the culture media but also that the SYN conditioned media, when exposed to LPS-treated microglia, exhibited a greater number of activated microglia than the LPS-treated microglia that was not conditioned with synuclein. Once initiated, the response is continual due to the release of or the up-regulation of proinflammatory factors such as COX2, iNOS, IL-1βand IL-6 (Su et al, 2007). Further investigations, such as the new timecourse in which tissues were collected for immunostaining and western blots should sort out the entire scenario sort this out of how the presence of WT α-synuclein initiates the neuroinflammatory response in a time dependent manner.

Conclusions
It is concluded from our pilot studies that the injection of WT α-synuclein does cause an inflammatory response in which there is a strong initial response from microglia and a rather weak initial response from astrocytes. There was also a non-significant decrease in TH+ SNpc neuron numbers. Since we noted that the injection WT α-synuclein used in this pilot study (5.0 micrograms.4 microliters) was too strong as it caused holes in some of the tissues, the study needs to be re-done using less WT α-synuclein.

References


Schults CW. Lewy Bodies. PNAS (USA) 103: 1661-1668, 2006


Figure 1. MAC-1 immunostaining following WT- alpha synuclein injection into the SNpc of rats. Rats were injected with wild-type α-synuclein (5.0 µg/µl, total volume=4µl) into the SNpc and sacrificed at 2 days (D), 4D and 7D after injection. A strong microglial response was noted at the 2D timepoint which was still maintained at the 7D timepoint. Note: because of the tissue damage at 7D, we think that the dose of synuclein was too strong.
Figure 2. Tyrosine hydroxylase (TH) immunostaining in rats 7 Days after WT α-synuclein injection into the SNpc. Rats (n=4) were injected with WT α-synuclein (5μg/μl, total volume, 4 μl) or saline in the SNpc and sacrificed 7D after injection. The noted differences between the two groups of animals did not reach significance at this time.