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Stereotaxic Targeting of Alpha-Synuclein Pathology in Mouse Brain Using Preformed Fibrils

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Abstract

The accumulation of intraneuronal inclusions containing misfolded alpha-synuclein (aSyn) within the central nervous system (CNS) is a common feature found in several neurodegenerative disorders including Parkinson's disease (PD). Emerging evidence indicates that aSyn amyloid fibrils, a configuration that is present within these characteristic inclusions, are capable of self-replicating by templating the conversion of endogenously expressed aSyn in neurons. Stereotaxic administration of synthetic α -synuclein preformed fibrils (PFFs) into the mouse brain has been shown to seed the formation of intracellular aSyn pathology reminiscent of Lewy body (LB) inclusions present in human PD and related synucleinopathies. Moreover, pathology can be targeted to specific CNS regions. This experimental approach provides a versatile platform for investigating PD-like LB pathology in vivo. We focus here on procedures for initiating aSyn inclusion formation at various regions of the mouse brain using computer-assisted motorized stereotaxic microinjection of aSyn PFFs and discuss appropriate strategies for controls and analysis.

Keywords

α -Synuclein; Transmission study; Stereotaxic; Microinjection; in vivo model; α -Synuclein preformed fibrils (PFFs)

1 Introduction

Alpha-synuclein (aSyn) is a major component of Lewy bodies (LBs) and Lewy neurites (LNs), the intracellular inclusions characteristic of Parkinson's disease (PD) and dementia with LBs (DLB). LBs/LNs are also present in the brains of a sizeable portion of Alzheimer's disease patients in addition to nearly all sporadic and familial PD patients [1, 2]. Since point mutations or amplification of the aSyn gene (SNCA) causes autosomal dominant forms of familial PD [3–6], this implies a central pathogenetic role for this protein in sporadic as well as familial PD.

A number of important observations have emerged from extensive postmortem studies on the neuroanatomical distribution of LBs/LNs at various stages of PD. Firstly, LBs/LNs affect multiple CNS regions in PD, DLB, and AD patients and although the patterns of distribution may be heterogeneous, they overlap considerably between individuals [7, 8]. Secondly, motor and non-motor symptoms strongly correlate with the extent of aSyn pathology and the function associated with the affected areas [9, 10]. Thirdly, aSyn pathology accumulates progressively, affecting new CNS regions over time, while pathology in previously affected areas increases in severity [10]. Most commonly, LB/LN pathology first develops in lower brainstem nuclei, olfactory nuclei, and peripheral neurons, affecting the SNpc in midcourse of disease, while neocortical involvement typically occurs last. Although some patients deviate from this pattern [8], the majority of patients appear to exhibit this stereotypic progression of aSyn pathology described by Braak and colleagues [7, 8, 11].

The progressive and sequential spread of LBs/LNs from affected to unaffected CNS regions over time is consistent with the transmission of a pathogenic process from diseased to healthy neurons [10, 12]. In fact, LBs/LNs are frequently detected in gastrointestinal, cardiac, as well as olfactory neurons during early stages of PD [13, 14], suggesting that spread can occur over long distances [12]. One of the first clues that the transmissible agent in PD might be misfolded aSyn itself comes from postmortem studies showing the time-dependent formation of LBs in mesencephalic neurons grafted into PD patients [15, 16].

In contrast to its highly soluble state in healthy brains [17], aSyn in LBs and LNs exist as β -sheet-rich amyloid fibrils, an ultra-structural arrangement shared with other misfolded proteins that form the signature aggregates found in other neurodegenerative diseases [18]. Recombinant aSyn, which has no native secondary structure [19], also assembles into fibrils at micromolar concentrations [20, 21]. A common property of amyloids is their ability to catalyze and template the assembly-soluble monomers into fibrils [22]. For aSyn, this activity is apparent in both cell-free and cell-based systems and we and others have previously demonstrated that the internalization of purified recombinant aSyn PFFs to a variety of cells expressing aSyn, including mouse primary neurons, results in the seeded formation of insoluble inclusions that share many of the same morphological features and biochemical markers as human LBs, such as aSyn phosphorylation at Ser129 (pSyn) and recruitment of ubiquitin and heat-shock proteins [23, 24].

Administration of synthetic aSyn PFFs in vivo also induces the formation of LB- and LN-like pathology in aSyn-overexpressing transgenic mice (e.g., M83 line), as well as accelerates the neurological phenotype and dramatically reduces their survival [25]. The pathology burden in PFF-injected mice is also significantly increased compared to untreated aged M83 mice and more widely distributed, consistent with the amplification and spread of pathological aSyn. More recently, we have shown that intrastriatal injection of mouse aSyn PFFs into wild-type mice from a variety of genetic backgrounds leads to LB/LN formation in multiple connected regions, including the substantia nigra pars compacta which progressively degenerates, and results in loss of striatal DA and impaired motor function [26]. Biochemical analysis shows that aSyn PFFs trigger the pathological conversion of host-expressed aSyn, whereas PFFs are nontoxic and do not induce aSyn pathology in the absence of endogenous aSyn expression in aSyn knockout mice [24–28].

Subsequent studies indicate that seeding with aSyn PFFs or misfolded aSyn isolated from the brains of patients with a range of synucleinopathies also effectively seeds the formation of intracellular pathology with distinct characteristics [27, 29, 30]. Similarly, conformational strains of PFFs have been generated using recombinant aSyn [31, 32]. Furthermore, this phenomenon has been recapitulated in a variety of laboratory animals including mice, rats, and nonhuman primates (e.g., macaque, marmoset) [27, 33, 34]. These models thus provide a versatile platform for studying the basic mechanisms associated with transmission as well as for evaluating therapeutics directed against PD processes which have been reviewed elsewhere [35–37].

The delivery of PFFs or brain-derived aSyn is typically achieved through stereotaxic methods, although induction of pathology via intramuscular [38] and enteric [39, 40] routes has also been described. Methods for stereotaxic microinjection of suspensions into rodent brain are well established and PFFs have been successfully targeted to many different CNS regions [41–45]. Here, we describe a method for injection of aSyn PFFs into the mouse brain and validation of the resultant seeded pathology using immunohistochemistry, and discuss considerations that can influence seeding efficiency in vivo.

2 Materials

2.1 Sonication of aSyn PFFs

1. Purified monomeric recombinant aSyn: Please see Powers et al., this issue.
2. Recombinant α Syn fibrils: Please see Volpicelli-Daley, this issue.
3. Sonicator: Prior to injection, aSyn PFFs are sonicated using a high-power bath sonicator system such as the Bioruptor Plus (Diagenode; Denville, NJ). Aerosols generated during PFF sonication are contained using these closed systems. Alternatively, a handheld probe can also be used. However, this should be done within the confines of a BSL2 safety cabinet to reduce the risk of exposure to aerosolized PFFs.
4. Sterile PBS without Ca^{2+} and Mg^{2+} (Mediatech, MT21031CV).

2.2 Stereotaxic Injection into Mouse Brain

1. Mouse lines for microinjections: The aSyn PFF injection approach has been successfully employed in wild-type (i.e., non-transgenic) mice from a number of genetic backgrounds. The most commonly used are B6C3 (F1) mice generated from a cross between female C57BL/6 and male C3H lines (Charles River Laboratories). Procedures described here were developed based on this mouse strain (see Note 1). Wild-type animals are suitable for injection from 2 to 3 months of age onwards. Approval from relevant local authorities is necessary for all procedures involving live animals. The procedures described here have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

2. Motorized stereotaxic apparatus: The stereotaxic apparatus used consists of a KOPF Model 902LS Dual small animal stereotaxic instrument equipped with Lazy Susan Base Plate (David Kopf Instruments, Tujunga, CA). Attached to this is a motorized stereotaxic microdrive system (StereoDrive, NeuroStar, Tübingen, Germany) and electronic microinjection system (InjectoMate, NeuroStar). The equipment is housed within a HEPA-grade safety cabinet (see Fig. 1).
3. Injection needles: Microinjections are performed using syringes with cemented needles. The Hamilton Company (Reno, NV) produces a 33-gauge, 10 μ L cemented needle, 0.5–1.0 in. long, point style #4, with 45° bevel (Hamilton catalog #80308) suited for this application. Syringes are flushed a minimum of 20 times with 70% ethanol by alternatively drawing up and expelling into the syringe. Clean syringes should be stored in 70% ethanol for 2 h or overnight before using. In addition, the use of separate syringes for different types/batches of aSyn is highly recommended to avoid crosscontamination since even trace quantities of aSyn PFFs can potentially initiate pathology.
4. Scissors (Fine Science Tools #14088–10).
5. Fine forceps (#5 size).
6. Needle holder (Miltex #17–1020).
7. Surgical gowns.
8. Gauze and cotton applicators.
9. Veterinary surgical drapes.
10. Balance with cage for body weight measurement.
11. Surgical shaver.
12. Meloxicam (5 mg/mL).
13. Ketamine (100 mg/mL).
14. Xylazine (20 mg/mL).
15. Acepromazine (10 mg/mL).
16. Bupivacaine (0.25%)/lidocaine (0.5%) mixture.
17. Povidone-iodine solution.
18. Heating pad.
19. Yohimbine solution (2 mg/mL).
20. Nylon sutures.
21. Warming lamp.
22. 70% Ethanol solution.
23. 1% SDS solution.

2.3 Mouse Perfusion and Confirmation of Pathology by Immunohistochemistry

1. Antibodies for immunohistochemistry: Immunohistochemistry using antibodies against aSyn is used to directly visualize aSyn PFF-induced pathology in injected brain samples. A non-exhaustive list of antibodies that have been successfully used is listed (Table 1). Phosphorylation of aSyn at Ser129 is a modification observed in both human synucleinopathy and PFF-injected animals. Antibodies recognizing this epitope (e.g., clone 81A) are sensitive for revealing induced pathology since injected PFFs do not undergo significant levels of phosphorylation. Other suitable antibodies include those that detect aSyn disease-associated conformations (e.g., Syn506) (Fig. 2). Pan-aSyn antibodies can also be used, although injected PFFs may also be detected and results should be interpreted with care. Information provided is based on immunostaining of paraffin-embedded brain sections, although the antibodies listed are also generally compatible with floating sections prepared from freshly fixed or cryopreserved tissue.
2. Ketamine/xylazine/acepromazine mixture.
3. Blunt-tipped 18-gauge butterfly needle, connected to perfusion tubing to syringe and peristaltic pump.
4. Saline solution with heparin (10 U/mL).
5. Fixative solution such as 70% ethanol (in 150 mM NaCl) or 4% paraformaldehyde (in PBS).

3 Methods

3.1 Sonication of aSyn PFFs

If previously frozen, PFFs should be allowed to be thawed in a 37 °C water bath on the day of use. All other procedures can be performed at room temperature unless stated. The aSyn PFF stock is diluted from 5 mg/mL to 2 mg/mL in sterile PBS in a 1.5 mL Eppendorf tube. This preparation is sonicated using the Bioruptor Plus at high power for 10 cycles (30 s on, 30 s off) at a constant temperature of 10 °C. As PFFs can settle over time, gently flick the closed Eppendorf tube to mix contents with a vortex before drawing into the syringe for injection. The sonicated PFF solution should be used on the day of sonication [46].

Extended storage is not recommended as this increases the risk of microbial contamination.

3.2 Stereotaxic Injection into Mouse Brain

1. Prior to entering the surgery/procedure room, ensure that all persons have proper attire and personal protective equipment (PPE), including face mask, hair net, and shoe covers. The surgeon should wash hands extensively with sterile soap before putting on a sterile gown and sterile gloves (*see Note 2*).
2. Weigh the animal to be treated and inject mouse subcutaneously with meloxicam (5 mg/kg).

3. Place mouse under appropriate general anesthesia using a mixture of ketamine/xylazine/acepromazine (60–100 mg/kg; 8–12 mg/kg; 0.5–2 mg/kg) administered as an intraperitoneal (i.p.) injection.
4. After the mouse has reached a surgical plane (i.e., nonresponsive to toe pinch or ear flick), inject a mixture of bupivacaine: lidocaine (3.5 and 4 mg/kg, respectively) subcutaneously along the incision site. For intrastriatal injections, this is along the midline slightly between the eyes and the ears. After allowing this local anesthetic to take effect, remove hair on the middle of the head with shaver.
5. Disinfect the shaved skin area with povidone-iodine solution using a sterile cotton swab, and then wash the area with sterile saline. Repeat this step a total of three times.
6. Place the anesthetized mouse on a heating pad to maintain body temperature at 37 °C during surgery.
7. Cover the mouse with a sterile drape. Maintain a small opening for access to the scalp. All efforts should be made to maintain sterility of this surgical field.
8. Open the scalp using a scalpel blade (#11) to expose the skull.
9. Turn on the StereoDrive computer and open the StereoDrive interface software that controls the motorized stereotaxic system.
10. Calibrate the stereotaxic frame, making sure that the readings on the StereoDrive software match those on the actual stereo-taxic device/frame.
11. Flush the Hamilton syringe 20 times with sterile PBS.
12. Initialize the InjectoMate on the motorized stereotaxic system. Load no more than 9.1 µL of PFF solution into the syringe. Unassembled (monomeric) aSyn has minimal seeding capacity and can also be used as a negative injection control.
13. Ensure that the top surface of the skull is level (i.e., horizontal) using the StereoDrive software. To do this, move the syringe needle to measure the heights at Bregma and Lambda for anterior-posterior levels, and at 2 mm on either side of the midline to ensure that the left and right sides are level. Adjust the heights of these landmarks to the same level as needed. Tolerance should be <0.1 mm between Bregma/Lambda and left/right axes (*see* Note 3).
14. Input the desired target coordinates into StereoDrive (*see* Table 2 for suggested coordinates of common CNS targets). The motorized stereotaxic arms holding the syringe needle will automatically move to the indicated location on the skull and place the needle tip on the surface to skull.
15. Move the stereotaxic arm holding the syringe needle away and make a small V-shaped notch on the surface of the skull with a disposable 30-gauge needle. Then use needle to manually bore a small hole into the skull overlaying the injection site sufficient for the needle to enter (*see* Note 4).

16. Return the stereotaxic arm with the syringe needle above the borehole and push a small drop (0.1 μ L) of injection material out of needle to prime. Remove drop with a cotton-tipped applicator (*see* Note 5). Insert the needle into brain to desired depth using the software interface. The system is now ready for PFF injection.
17. Using the InjectoMate interface on the motorized stereotaxic system, inject the desired volume of PFF solution. Typical injections contain a total of 5 μ g aSyn PFFs (in 2.5 μ L sterile PBS) or the same volume of monomeric aSyn or PBS alone as a negative control. Injections made into the striatum, hippocampus, and neocortex are done so at a rate of 0.4 μ L/min. For smaller nuclei such as pons or locus coeruleus, a flow rate of 0.1 μ L/min is more suitable. The injection needle should remain in place for at least an additional 1 min after injection is complete before it is gently withdrawn over a period of at least 30 s which minimizes leakage (*see* Note 6).
18. After injection, close the scalp using individual nylon stitches.
19. While the mouse is still anesthetized, provide an injection of yohimbine (xylazine antagonist; 2.0 mg/kg, i.p.) and a subcutaneous injection of buprenorphine SR (1 mg/kg) for analgesia. This should be followed by a subcutaneous injection of warm sterile 0.9% sodium chloride solution (1 mL total).
20. Place animal in a recovery cage under a warming lamp. Temperature and condition of the animal should be checked every 5 min until the mouse can self-ambulate.
21. Flush the used Hamilton syringe 20 times with sterile water followed by 20 times with 70% ethanol solution. Cleaned syringes can be stored loaded with ethanol.
22. Post-surgery, mice should be housed individually for 10 days or until sutures are eliminated from the skin, at which time normal housing arrangements can be resumed.

3.3 Coordinates for Different CNS Sites

LB/LN pathology formation and accumulation following the injection of misfolded aSyn is a highly dynamic process. This should be taken into consideration, together with the goals of the experiment, when selecting the anatomical location for stereotaxic injection. The accumulation of inclusions typically begins in axons in the vicinity of the injection site and propagates in a retrograde fashion over time but anterograde propagation is also observed. Cell-to-cell spread of pathology may also occur in a time-dependent manner, thus expanding the distribution of inclusions well beyond the initial injection site.

A list of injection sites that lead to productive aSyn pathology is given in Table 2. Note that these coordinates are based on established stereotaxic atlases but significant variation may be present between animals, even those that share similar genetic backgrounds. Particular attention should be paid to the age and weight of the mice used as this also affects brain dimensions. Where the surgeon is unfamiliar with a target, it is recommended that pilot

injections using a dye (e.g., Brilliant Blue) be performed as a proxy and the target inspected immediately to confirm the accuracy of the coordinates. Pilot studies are also recommended to assess the distribution of PFFs at and around a particular target site. This is particularly important if using brain homogenates as these specimens are often more viscous than PFF suspensions.

3.4 Mouse Perfusion and Confirmation of Pathology by Immunohistochemistry

Intraneuronal inclusions are expected to develop in the CNS within 30 days after injection with active aSyn PFFs in wild-type mice. Examination of pathology should be confirmed by immunohistochemistry after sacrifice and transcardial perfusion/fixation of the tissue as previously described [25].

1. Anesthetize the mouse using a single combined i.p. injection of ketamine/xylazine/acepromazine (100 mg/kg/10 mg/kg/10 mg/kg). Once the animal is no longer responsive to toe pinch or ear flick, restrain the mouse in a supine position on a dissection block using tape to secure the fore- and hind limbs.
2. Using a pair of clean surgical scissors, cut open the thorax to expose the heart. Avoid damaging major blood vessels close to the heart. Gently introduce a small hole in the right atrium with scissors as an exit port for the blood. Insert a blunt-tipped 18-gauge butterfly needle connected to perfusion tubing, syringe, and pump into the left ventricle.
3. Pump heparinized saline through the mouse to rinse out the blood. This step helps reduce the background staining.
4. Remove brain and spinal cord from the mouse and fix in 70% ethanol (in 150 mM NaCl) or 4% paraformaldehyde (in PBS) overnight at 4 °C. Tissues should then be processed for paraffin embedding, sectioning, and collection on slides. Dispose of carcass according to local and institutional regulations.
5. Tissue sections on the slides are processed using routine immunohistochemical procedures as described in Sanderson et al., this issue.

3.5 Decontamination of Instruments and Work Surfaces

Given that small quantities of aSyn PFFs can trigger aggregation in permissive hosts, it is essential that residual PFFs be thoroughly removed from all instruments and work surfaces after procedures. Common detergents such as SDS effectively solubilize recombinant aSyn PFFs [47] and thus greatly reduce their activity. Any spills or aerosol contamination should be neutralized immediately.

1. Clean all exposed work surfaces with 1% SDS after use. Allow solution to sit for 1 min before wiping with paper towels. Dispose of towels and repeat procedure using distilled water to remove any residue (*see Note 7*).
2. Microinjection needles should be rinsed extensively before storage. To further avoid contamination, it is essential that each needle assembly be used for only one type of injection material (e.g., PFF strain, monomer).

4 Notes

1. To date, efficient seeding has also been reported in C57BL/6J [28,43], C3H/HeJ [48], and CD-1 [42] mice. aSyn knockout mice are nonpermissive to seeding with PFFs and can be used as negative controls in many cases. Additional considerations should be made when using transgenic lines (e.g., those over-expressing aSyn) as their neuroanatomy may deviate from wild-type animals and/or develop age-dependent phenotypes. In addition, mice may differ in their response to anesthesia depending on background strain.
2. Surgical tools and materials should be steam sterilized at 120 °C for 30 min prior to surgery.
3. Levelling the skull is critical for precise stereotaxic injections, especially when targeting relatively small regions or nuclei. A tolerance of no more than 0.01–0.03 mm for Bregma/Lambda and left/right should be maintained.
4. To prevent damage to the skull and brain surface during surgery, manually bore a small hole into the skull overlying the injection site with the smallest needle that can get least damage which is better when imaging the brain sections.
5. Priming of the needle by advancing a small drop of injection material from needle tip prior to inserting into brain helps avoid introducing air bubbles.
6. Using a faster infusion rate will lead to increased leakage of the PFF suspension beyond the target site. Slow removal of the needle also reduces leakage into areas surrounding the injection tract due to drawback and is especially critical when targeting smaller nuclei.
7. Dispose unused PFFs and PFF-contaminated waste (e.g., paper towels, scalpels) according to local regulations.

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Fig. 1. Motorized stereotaxic system for PFF injection. Top: Apparatus housed with a HEPA-filtered biological cabinet. Bottom: The setup consists of a Kopf small-animal stereotaxic instrument with Lazy Susan Base Plate fitted with a motorized stereotaxic microdrive system and electronic microinjection system, and is controlled on a laptop computer

Fig. 2. PFF-induced pathology in mouse brain. Top: Mouse aSyn PFFs in the dorsal striatum 3 days after stereotaxic injection into a aSyn knockout mouse, revealed by immunohistochemistry using Syn506. Bottom: Example of aSyn inclusions in the frontal cortex (FC) and amygdala (Amyg) of a B6C3 (F1) mouse sacrificed 90 days after striatal PFF injection. An antibody against pSer129 was used. Scale bars: 60 μ m (top), 10 μ m

Table 1

Antibodies for detecting aSyn pathology in mouse CNS tissue

Antibody	Antigen	Host species	Dilution	Source
Syn ^{pser129} (81A)	Phospho-Ser129 aSyn (human and mouse)	Mouse	1:10,000	Ref. 15
Syn303	aSyn (human and mouse)	Mouse	1:7,000	Ref. 16
Syn506	aSyn (human and mouse)	Mouse	1:20,000	Ref. 16
SNL-4	aSyn (human and mouse)	Rabbit	1:10,000	Ref. 16

Table 2

Coordinates for different CNS targets

Injection site	Distance from Bregma (mm)	Distance from midline (mm)	Depth from brain surface (mm)
Dorsal striatum	+0.2	2.0	−2.6
Ventral striatum	+0.2	2.0	−3.6
Neocortex	+0.2	2.0	−0.8
Hippocampus (CA1)	−2.5	2.0	−1.8
Lateral accumbens (shell)	+1.45	1.75	−4.0