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Short Communication

Identifying the dataset to define the optimal timing of histopathological examination for central nervous system toxicity in MPTP-induced Parkinson's disease monkey model

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Abstract: Determining the optimal timing for histopathological examination following exposure to a test article is crucial for assessing neurotoxicity. However, no study has focused on identifying an ideal dataset to define the optimal timing for histopathological examination of central nervous system (CNS) toxicity in monkeys. Therefore, this study aimed to define a predictive endpoint that would guide us in selecting the optimal timing for histopathological examination of CNS toxicity in monkeys. Four cynomolgus monkeys were administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intravenously at a dosage of 0.6 mg/kg twice at 1-week intervals. Necropsies were performed 1 week after the final dose. The Parkinsonian rating (PR) score and temporal changes in neurofilament light chain and glial fibrillary acidic protein concentrations in the cerebrospinal fluid (CSF) and serum were evaluated and compared with the histopathological findings in the brain. The PR score of all animals administered MPTP increased from days 10 to 11, with some degree of individual variability. Microscopically, all animals showed axonal swelling and vacuolation, with or without microgliosis in the nigrostriatal bundle. However, substantial neurodegenerative findings were observed only in animals with high PR scores at necropsy. A slight increase in CSF biomarker levels at necropsy was also observed in animals with high PR scores. However, their correlation with microscopic findings in these animals was unclear. These data suggest that comprehensive clinical observations, such as PR score alone or combined with other CSF biomarkers, could be further evaluated as potential indicators for triggering anatomic CNS evaluations in monkeys following toxic insults. (DOI: 10.1293/tox.2023-0010; J Toxicol Pathol 2023; 36: 199–204)

Key words: timing of histopathological examination, neurotoxicity, monkey, neurofilament light chain, glial fibrillary acidic protein

To precisely assess the neurotoxicity of the central nervous system (CNS), an understanding of the meaningful and predictive study endpoints; optimal timing of clinical, clinical pathological, or histopathological observations; the scope of impacted anatomical locations within the CNS; and effective histological staining methods are required. Behavioral and pathological evaluations (via cage-side and detailed observations, neurologic examinations, and histopathology assessments) are generally conducted in toxicity studies and are considered the most critical endpoints for neurotoxicity assessment and are sometimes correlated with each other for an individual study. Determining the optimal timing for visible histopathology indicative of nervous tissue injuries following exposure to a test article is crucial in assessing neurotoxicity because the brain requires time to react to the insult and develop observable microscopic pathology. The pathology gradually dissipates as the brain clears away debris and removes evidence of the insult[1]. However, to date, no study has focused on identifying an ideal dataset to define the optimal timing for histopathological examination of CNS toxicity in monkeys. The classic toxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces Parkinson's disease (PD) symptoms in monkeys following the degeneration of dopaminergic neurons[2]. Accelerated decrease in tyrosine hydroxylase (TH, a marker of dopaminergic neurons) immunoreactive neurons after the onset of PD symptoms has been reported[3]. In this study, the correlation between PD symptoms at necropsy and histopathological findings was investigated using an MPTP-induced monkey model of acute neurotoxicity. The early onset of MPTP-related PD symptoms, including loss of threat behavior, decreased locomotor activity, bradykinesia, abnormal posture, freezing, tremor, muscle rigidity, and ataxic gait, was evaluated and compiled as a Parkinsonian rating (PR) score as

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a possible clinical endpoint for correlation with histologic findings. Additionally, serum and cerebrospinal fluid (CSF) neurofilament light chain (NfL), a cytoskeleton protein selectively expressed in neurons and nerves, and glial fibrillary acidic protein (GFAP), a type III intermediate filament protein, both recently reported as valuable biomarkers for detecting neurodegenerative disease or chemically induced neuronal damage[4–7], were evaluated as potential correlative fluid biomarkers.

This study was approved by the Institutional Animal Care and Use Committee of BoZo Research Center, Inc. (Shizuoka, Japan) and was conducted in accordance with the following guidelines: "Act on Welfare and Management of Animals", Act No. 105, October 01, 1973; "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain", Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006; and "Guidelines for Proper Conduct of Animal Experiments", Science Council of Japan, June 01, 2006.

Four healthy female cynomolgus monkeys of Vietnamese origin, aged 4 years and 1 month to 4 years and 6 months (body weight: 2.75–3.56 kg) at the beginning of the treatment, were individually housed in stainless-steel cages (W 750 × D 750 × H 765 mm; Tokyo Giken Co., Ltd., Tokyo, Japan) with the following environmental conditions: temperature controlled between 18°C and 28°C, relative humidity between 30% and 80%, air exchanges at 9 to 15 times/h, and a 12-h light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m., at an intensity of 150 to 350 lux at 70 cm above the floor level). The dosing regimen for MPTP was modified from the advanced Parkinsonism model in monkeys, which can induce stable PD symptoms[8]. MPTP hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). It was administered to four animals at a dosage of 0.6 mg/kg (0.12 mL/kg) by intravenous bolus injection on days 1 and 8 under anesthesia following intramuscular administration of a 4:1 mixture of ketamine hydrochloride solution (500 mg, Ketalar® for intramuscular injection; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and xylazine hydrochloride solution (0.2 mL/kg, Celactal® 2% injection; Bayer Yakuhin, Ltd., Osaka, Japan). All animals were observed for clinical signs, including PD symptoms twice a day on the day of dosing (at pre-dosing and 4 h post-dosing), once on other days between 7:00 a.m. and 12:30 p.m., and once on the necropsy day. We modified the PR scale used in previous studies[8–10]. The PD symptoms were graded from 0 to 2 daily for each finding (Table 1). The total number of points each day was calculated as the PR score, with a maximum score of 16. Cerebrospinal fluid (CSF) was collected at approximately 0.5 mL/animal/day on days −21 and −8, as well as prior to necropsy on day 15 by suboccipital puncture under anesthesia. Blood samples were drawn from the femoral vein of each animal at approximately 4 mL/day on days −21, −18, −14, −11, −8, −4, 4, 8 (before dosing), 11 and 15 (prior to necropsy). Blood samples were processed into serum. Concentrations of NfL and GFAP in the CSF and serum were measured using a Simoa™ SR-X analyzer (Quanterix Corporation, Billerica, MA, USA) with the Simoa™ NF-light Advantage (SR-X) Kit (Quanterix 103400) and Simoa™ GFAP Discovery (SR-X) Kit (Quanterix102336), according to the manufacturer's instructions. An accelerated decrease in TH-immunoreactive neurons over the days after the onset of PD symptoms has been reported[3]. In this study, since the onset of PD symptoms was observed in all animals after the second dosing on day 8, and a high PD score (8 points) was confirmed in 3 out of 4 animals between days 11 and 15, all animals were exsanguinated from the axillary artery and vein under same anesthesia method described above and necropsied on day 15. The brain, spinal cord (cervical, thoracic, and lumbar), and sciatic nerves of all the animals were fixed in 10% phosphate-buffered formalin. In accordance with the Society of Toxicological Pathology (STP) position paper[11], the organs and tissues were trimmed (spinal cord: transverse only), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The MPTP target

Table 1. Grading for Parkinson's Disease Symptoms

Loss of threat behavior	Bradykinesia
0 threatening to observers in front of the cage	0 normal speed and facility of movement
1 threatening to cage-opening	1 mild slowing of movement
2 threatening or not to touch-stimulation	2 severe slowing of movement
Decreased locomotor activity	Tremor
0 normal general activity	0 no visible tremor
1 small general activity to touch-stimulation	1 rhythmical shaking of the extremities
2 no general activity to touch-stimulation	2 rhythmical shaking of the whole body
Abnormal posture	Muscle rigidity
0 normal rising posture	0 no rigidity
1 flexed posture	1 mild increased resistance to the passive movement of hind limbs
2 sitting	2 severe increased resistance to the passive movement of hind limbs
Freezing	Ataxic gait
0 normal ability to move without interruption	0 normal balance
1 a total continuous duration of freeze for 5–29 seconds	1 mild loss of balance, however, preventing falling
2 a total continuous duration of freeze for 30 seconds or more	2 severe loss of balance and falling

0: no change, 1: slight, 2: severe (scores for the individual findings).

region, substantia nigra, and striatum were included in the brain levels of sections 1–3 (striatum) and 3B–4 (substantia nigra) of the STP position paper. Additionally, the brain level between sections 2 and 3B was processed similarly to evaluate the MPTP target region, broadly. In addition, Fluoro-Jade (FJ) staining using Fluoro-Jade C Ready-to-Dilute Staining Kit (TR-100; Biosensis, Thebarton, SA, Australia), Luxol fast blue (LFB)-HE, and immunohistochemical staining using anti-TH, NfL, ionized calcium-binding adaptor molecule 1 (Iba-1), a marker of microglia, and GFAP antibodies were performed on the brain sections. The staining conditions for each primary antibody are summarized in Table 2. As a control to compare the staining property for IHC staining, paraffin blocks of the normal brain from a female of Vietnamese origin aged 3 years 2 months that were retained in the archives at the Gotemba Laboratory in BoZo Research Center Inc. were provided, sectioned, and stained similarly.

Microscopically, decreased TH stainability in the caudate nucleus and putamen and axonal swelling, vacuolation, and/or microgliosis (via increased Iba-1 expression) in the nigrostriatal bundle were confirmed in all animals treated with MPTP (Table 3, Fig. 1A–1F). No MPTP-related findings were observed in the spinal cord or sciatic nerve of any of the monkeys. The severity of axonal swelling and vacuolation in HE sections was minimal to mild, the findings were limited to the nigrostriatal bundle, and neuronal cell body changes were not confirmed in any brain region, including FJ-stained sections. An acute MPTP administration model in monkeys reported that marked reductions in dopaminergic axons before the loss of nigral cell bodies indicate that nigral neurons degenerate through "dying-back" axonopathy[12]. Morphologically, swelling of nigrostriatal axons has been reported as an early abnormality[13]. Therefore, considering minimal to mild changes only in axons, our current dosing regimen and necropsy timing demonstrated early or mild histopathology before neuronal necrosis or loss in the substantia nigra.

The PR score of each animal was elevated during the week following the second MPTP dose, starting on either day 10 (Nos. 1101 and 1102) or 11 (Nos. 1103 and 1104), with variation in severity noted across the animals on a given day. Decreased locomotor activity, bradykinesia (all animals), abnormal posture, muscle rigidity (two animals), and freezing were commonly observed in three animals (Nos. 1101, 1102, and 1103), with high PR scores more than 7 (Table 4). Among these animals, two with the highest PR scores at necropsy on day 15 (Nos. 1101 and 1102) also showed an increased severity of microscopic nigrostriatal findings, including axonal swelling, vacuolation, microgliosis, and decreased TH stainability in the caudate nucleus and putamen, compared with the two animals with lower PR scores at necropsy (Table 3). Additionally, decreased NfL stainability in swollen axons was noted in these animals, consistent with the histopathological severity observed in the HE sections (Fig. 2A, 2B). In contrast, one animal (No. 1103)

Table 2. Primary Antibodies and Reaction Conditions for Immunohistochemistry

Antibody	Host	Source	Dilution	Antigen retrieval[a]	Detection system
TH	Mouse	Sigma-Aldrich (IHCR1005-6)	prediluted	0.01M citrate buffer (pH6.0)	ENVISION+/ Single Reagents, HRP. Mouse
NfL	Mouse	Abcam (ab7255)	1: 1000		ENVISION+/ Single Reagents, HRP. Mouse
Iba-1	Rabbit	FUJIFILM Wako Pure Chemical Corporation (019-19741)	1: 500		ENVISION+/ Single Reagents, HRP. Rabbit
GFAP	Rabbit	Abcam (ab7260)	1: 2000		ENVISION+/ Single Reagents, HRP. Rabbit

[a]Microwave at 10 minutes. TH: tyrosine hydroxylase; NfL: neurofilament light chain; Iba-1: ionized calcium-binding; GFAP: glial fibrillary acidic protein; HRP: horseradish peroxidase.

Table 3. Results of Histopathology Evaluation in the Brain

Animal number	1101	1102	1103	1104
Brain				
Swelling, axon, nigrostriatal	2	2	1	1
Vacuolation, nigrostriatal	1	1	-	-
Microgliosis*	1	1	-	-
TH, Decreased stainability, caudate nucleus	4	3	2	2
TH, Decreased stainability, putamen	3	3	1	2
NfL, Decreased stainability, swelled axon	1	1	-	-

*Diagnosed by immunohistochemistry for ionized calcium-binding adaptor molecule 1 (Iba-1). -: no change, 1 (minimal): Small numbers of lesions (HE and Iba-1 findings)/The staining intensity slightly decreases as compared to the positive control. (NfL and TH findings), 2 (mild): More prominent than that in the minimal. (HE finding)/The decreased staining intensity is clear and more obvious than that of the minimal grade. (TH finding), 3 (moderate): The staining intensity significantly reduces as compared to the minimal grade. (TH finding), 4 (marked): Negative or equivocal stainability. TH: tyrosine hydroxylase; NfL: neurofilament light chain.

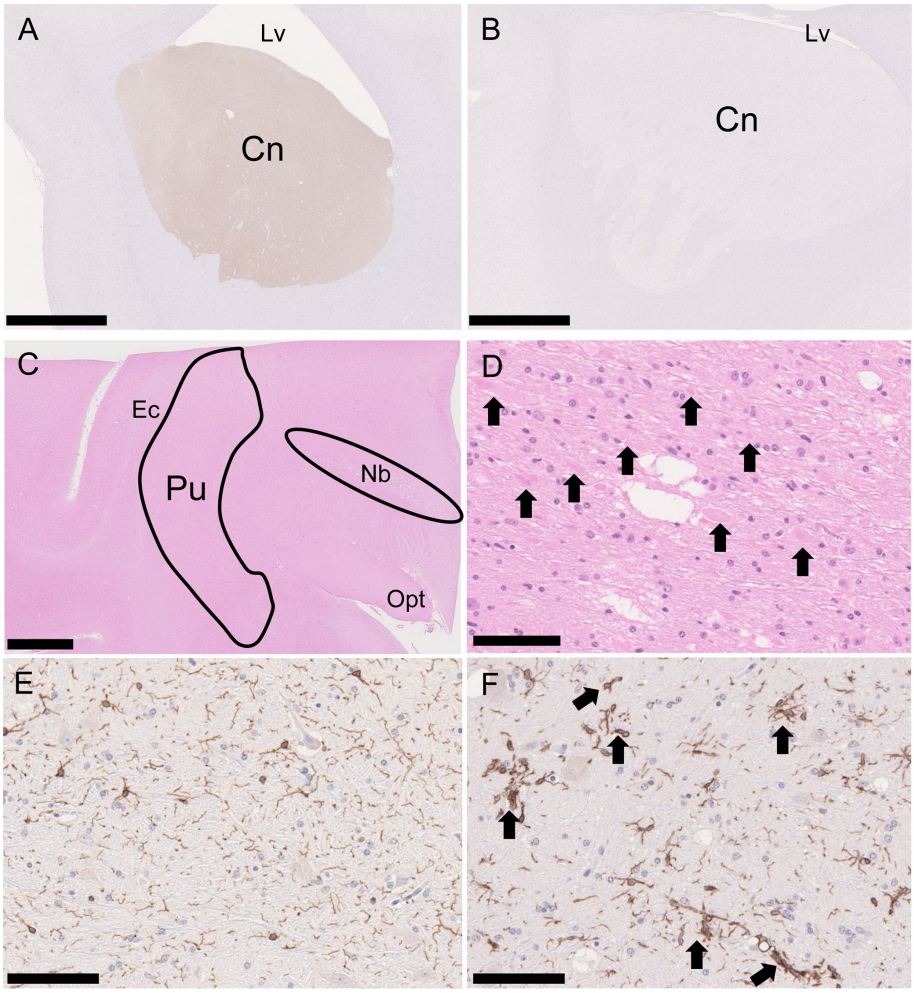

Fig. 1. Microscopic brain lesions induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were analyzed using hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) for tyrosine hydroxylase (TH) and ionized calcium-binding adaptor molecule 1 (Iba-1). A, E: Control; B, C, D, and F: No. 1101. Cn, caudate nucleus; Lv, lateral ventricle; Ec, external capsule; Pu: putamen, Nb, nigrostriatal bundle; opt, optic tract. A, B (IHC for TH): Decreased stainability in the caudate nucleus was observed (B) compared with the control (A). C, D (HE): Low-magnification images of the putamen and nigrostriatal bundles (C). High magnification of nigrostriatal bundles (D). Axonal swelling (black arrows) and vacuolation. E, F (IHC for Iba-1): Nigrostriatal bundles from the control (E). The focal proliferation of microglia (black arrows) was confirmed at nigrostriatal bundle (F) (A–C: bars=2.5 mm; D–F: bars=100 µm).

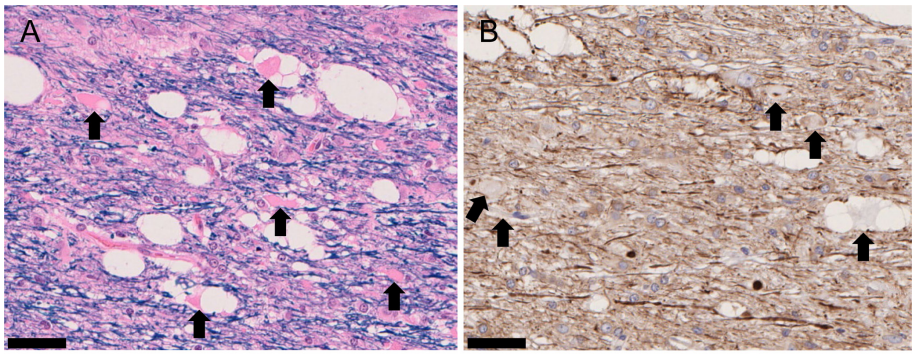

Fig. 2. Microscopic brain lesions induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were analyzed using Luxol fast blue (LFB)-hematoxylin and eosin (HE) and immunohistochemistry (IHC) for neurofilament light chain (NfL). A, B: No. 1101. A (LFB-HE) and B (IHC for NfL): Decreased stainability of NfL in the swollen axons (black arrows) (A, B: bars=50 µm).

with a low PR score (1 point) at necropsy, despite having a higher score earlier in the week (8 points on days 11 to 13), and one animal (No. 1104) that presented with a consistently low PR score (1 point) during this week had only a few axonal swellings without vacuolation, microgliosis, decreased NfL stainability, and a less severe decrease in TH staining in the caudate and putamen than the more severely affected animals (Table 3). These data suggest that the PR score at necropsy was associated with the severity of histopathological findings.

In NfL and GFAP measurements, an apparent increase was not observed in either the CSF or serum; however, the animals with greater histopathology severity showed a slight increase in NfL (5.6-fold from the average pretreatment value of No. 1102) and GFAP (6.0-fold from the average pretreatment value of No. 1101) in the CSF at necropsy, although a slight increase in NfL in the CSF was also noted in another animal with only minimal to mild histopathological findings (11.3-fold from the average pretreatment value of No. 1103) (Fig. 3A–3D). Slight increases in CSF biomark-

Table 4. Results of Parkinson's Disease Symptoms Starting on Day 10

Animal number	1101						1102						1103						1104					
Study day (from Day 10)	10	11	12	13	14	15	10	11	12	13	14	15	10	11	12	13	14	15	10	11	12	13	14	15
Loss of threat behavior	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Decreased locomotor activity	2	2	2	2	2	2	0	0	0	1	2	2	0	2	2	2	1	0	0	0	0	0	0	0
Bradykinesia	1	2	2	2	2	2	0	1	1	1	2	2	0	2	2	2	1	1	0	1	1	1	1	1
Abnormal posture	1	2	2	2	2	2	0	0	0	1	1	1	0	2	2	2	1	0	0	0	0	0	0	0
Freezing	0	2	2	2	2	2	0	0	0	1	2	2	0	2	2	2	0	0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle rigidity	0	0	0	0	1	1	1	1	1	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Ataxic gait	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Parkinsonian rating (PR) score	4	8	8	8	9	9	1	2	2	5	9	9	0	8	8	8	3	1	0	1	1	1	1	1

0: no change, 1: slight, 2: severe (scores for the individual findings).
The total number of points for each day was calculated as the Parkinsonian rating (PR) score, with maximum points being 16.
PD symptoms were absent prior to Day 10.

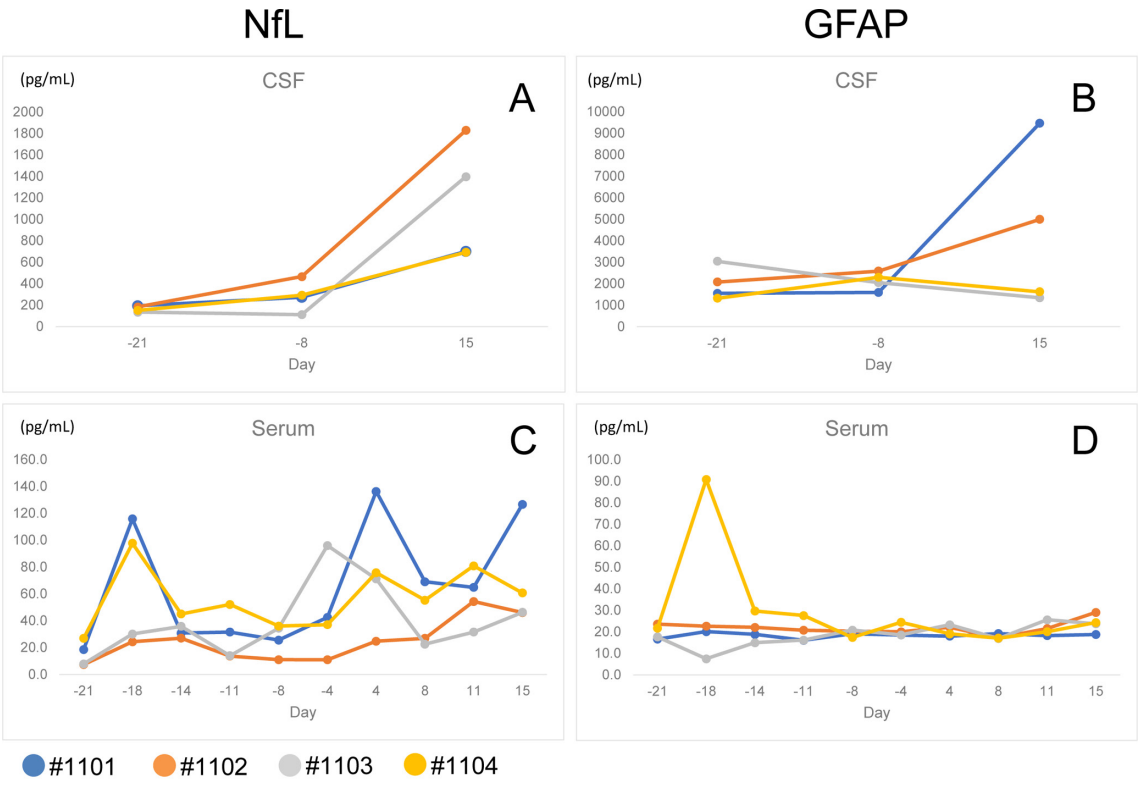

Fig. 3. Neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) levels in the cerebrospinal fluid (CSF) (A, B) and serum (C, D) A, B (CSF): Slight increases in NfL (Nos. 1102 and 1103) and GFAP (No. 1101) levels on day 15. C, D (serum): No increase was observed in either NfL or GFAP.

ers at necropsy were observed in these three animals mentioned above; however, a clear correlation with microscopic findings was not observed in these animals. Since the number of animals and CSF sampling points are limited in this study, the reason for the discrepancy was not apparent.

High PR scores at necropsy were associated with neurodegeneration, including microgliosis, vacuolation, and axonal degeneration, with decreased stainability of TH and NfL. While NfL and GFAP in the CSF may be potential biomarkers to define the timing of histopathological evaluation, further studies are needed to validate the utility of these biomarkers using more animals administered MPTP or different neurotoxic drugs. Therefore, comprehensive clinical observations such as the PR score alone or combined with CSF biomarkers could be further evaluated as potential indicators for triggering anatomical CNS evaluations in monkeys following toxic insults, including those other than the MPTP model.

Disclosure of Potential Conflicts of Interest: The authors declare no conflicts of interest associated with this study.

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