Blood Neurofilament Light Chain as a Potential Biomarker for Central and Peripheral Nervous Toxicity in Rats

Tomoya Sano,*1 Yasushi Masuda,† Hironobu Yasuno,* Tadahiro Shinozawa,* Takeshi Watanabe,* and Masaaki Kakehi†

*Drug Safety Research and Evaluation, Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan; and †Drug Metabolism and Pharmacokinetics Research Laboratories, Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan

1To whom correspondence should be addressed at Drug Safety Research and Evaluation, Takeda Pharmaceutical Company Limited, 26-1 Muraoka-Higashi 2-Chome, Fujisawa, Kanagawa 251-8555, Japan. E-mail: tomoya.sano@takeda.com.

ABSTRACT

Neurotoxicity is a principal concern in nonclinical drug development. However, standardized and universally accepted fluid biomarkers for evaluating neurotoxicity are lacking. Increasing clinical evidence supports the potential use of neurofilament light (NFL) chain as a biomarker of several neurodegenerative diseases; therefore, we investigated changes in the cerebrospinal fluid (CSF) and serum levels of NFL in Sprague Dawley rats treated with central nervous system (CNS) toxicants (trimethyltin [TMT, 10 mg/kg po, single dose], kainic acid [KA, 12 mg/kg sc, single dose], MK-801 [1 mg/kg sc, single dose]), and a peripheral nervous system (PNS) toxicant (pyridoxine, 1200 mg/kg/day for 3 days). Animals were euthanized 1 (day 2), 3 (day 4), or 7 days after administration (day 8). Increased serum NFL was observed in TMT- and KA-treated animals, which indicated neuronal cell death in the brain on days 2, 4, and/or 8. MK-801-treated animals exhibited no changes in the serum and CSF levels of NFL and no histopathological changes in the brain at any time point. Pyridoxine-induced chromatolysis of the dorsal root ganglion on day 2 and degeneration of peripheral nerve fiber on day 4; additionally, serum NFL was increased. A strong correlation was observed between the serum and CSF levels of NFL and brain lesions caused by TMT and KA, indicating that NFL could be a useful biomarker for detecting CNS toxicity. Additionally, PNS changes were correlated with serum NFL levels. Therefore, serum NFL could serve as a useful peripheral biomarker for detecting both CNS and PNS toxicity in rats.

Key words: neurofilament light chain; neurotoxicity; rat.
Neurofilaments are the major component of the axonal cytoskeleton and consist of 3 types of proteins: neurofilament light (NFL), neurofilament medium, and neurofilament heavy chains (Khalil et al., 2018). Neurofilaments are released into the extracellular space when an axon is damaged and subsequently into the cerebrospinal fluid (CSF) and blood (Khalil et al., 2018).

Recently, the development of an extremely sensitive and reliable assay based on single-molecule array technology (Simoa) has enabled reliable measurement of blood NFL levels (Khalil et al., 2018; Kuhl et al., 2016). NFL is a novel biomarker, as well as a clinical marker, reflecting disease severity, progression, or therapy response in several neurological disorders such as multiple sclerosis, amyotrophic lateral sclerosis, Huntington’s disease, and Alzheimer’s disease (Disanto et al., 2017; Fyfe, 2019; Gaetani et al., 2019; Kuhl et al., 2019; Olsson et al., 2019; Rodrigues et al., 2020). NFL was recently reported to be a useful biomarker of axonal degeneration in chemotherapy-induced peripheral neurotoxicity in both clinical and nonclinical studies (Kim et al., 2020; Meregalli et al., 2018, 2020), including in a rat model of vincristine/cisplatin-paclitaxel-induced peripheral neurotoxicity (Meregalli et al., 2018, 2020). Additionally, researchers have reported that NFL is elevated in mouse models of Huntington’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, Alzheimer’s disease, and Gaucher disease and that both CSF and blood levels of NFL are convincing and useful markers for monitoring disease progression and severity (Baciglou et al., 2016; Loeffler et al., 2020; Soylu-Kucharz et al., 2017). Additionally, elevation of NFL levels in the CSF and serum was observed in experimental pneumococcal meningitis in rats (Le et al., 2020). However, little is known regarding the usefulness of NFL as a nonclinical biomarker of CNS toxicity, especially neuronal cell death.

In this study, we employed the Simoa assay to measure serum and CSF NFL levels, evaluated the potential of NFL as a peripheral biomarker of CNS toxicity, and compared the sensitivities of typical neurotoxicity endpoints such as nervous symptoms and histopathology in rat neurotoxicity models induced by trimethyltin (TMT), kainic acid (KA), and MK-801. TMT induces injury in CNS neurons and several nervous symptoms (Balaban et al., 1988; Bushnell and Evans, 1985; Ceccariglia et al., 2020; Crofton et al., 1990; Ogata et al., 2015). KA, an agonist of kainate glutamate receptor subtypes, causes glutamate excitotoxicity in rodents (Zheng et al., 2011), and KA treatment is one of the most reliable models for temporal lobe epilepsy (Bertoglio et al., 2017; Levesque and Avoli, 2013; Zheng et al., 2011). MK-801, an NMDA receptor antagonist (Olney et al., 1989), has been reported to induce temporal neuronal cell vacuolation and neuronal cell death in rats (Fix et al., 1993, 1995; Kuroda et al., 2015). In addition to these CNS toxicants, we measured NFL levels in a pyridoxine-induced peripheral neuropathy model (Jortner, 2000; Krinke and Fitzgerald, 1988).

This study aimed to investigate whether increased NFL levels in the CSF and serum can be utilized for detecting neuronal damage in TMT-, KA-, MK-801-, and pyridoxine-induced neurotoxicity models and to discuss the feasibility and usability of NFL as a blood biomarker of neurotoxicity.

**MATERIALS AND METHODS**

**Animals and treatments.** This study was approved by the Institutional Animal Care and Use Committee, Shonan Health Innovation Park. We used male Sprague Dawley rats (Charles River Laboratories Japan Inc.) with weights ranging from 207 to 260 g. The rats were selected using standardized normal values calculated from the body weights. These animals were allocated to 12 groups, each comprising 4 males: 5 groups (Control, TMT, KA, MK-801, and pyridoxine) necropsied at 1 day after the first dose (day 2), 5 groups (Control, TMT, KA, MK-801, and pyridoxine) necropsied at 3 days after the first dose (day 4), and 2 groups (Control and TMT) necropsied at 7 days after the first dose (day 8). The animals were individually housed in metal cages with stainless-steel wire mesh bottoms equipped with a stainless-steel resting board and chew toys (i. chews, ASAP and Nylon Bone, Bio Serv., New Jersey) as animal enrichment devices. The conditions of the room were as follows: temperature of control range: 20–26°C, relative humidity of control range: 40–70%, air exchange 10–25 times/h, and a 12-h light/dark cycle. The animals were allowed free access to a pelleted laboratory animal diet (CR-LPF, ORIENTAL YEAST Co. LTD., Tokyo, Japan) and tap water. Animals received a single oral gavage dose of TMT (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at 10 mg/kg, a single subcutaneous injection of KA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 12 mg/kg, single subcutaneous injection of MK-801 (FUJIFILM Wako Pure Chemical Corporation) at 1 mg/kg or pyridoxine (Sigma-Aldrich Japan, Tokyo, Japan) at 1200 mg/kg/day (600 mg/kg BID) for 1 or 3 days; they were sacrificed at various time points (1, 3, or 7 days after administration). The dose level of each compound was set on the basis of doses reported in the literature (Covolan and Mello, 2000; Fix et al., 1995; Jortner, 2000; Kuroda et al., 2015; Ogata et al., 2015) or internal data (data not presented). Clinical signs were observed daily, and body weight was measured on days 2, 4, and 8.

**Tissue collection, blood, and CSF sample collection and preparation.** For collecting the brain tissues, blood, and CSF samples at each necropsied time point, the animals were anesthetized with 4% isoflurane in oxygen as a carrier gas. Blood samples were collected from the abdominal aorta using a syringe without anticoagulant under isoflurane anesthesia prior to necropsy. The blood samples were kept at room temperature for 30 min and subsequently centrifuged at 1700 × g for 10 min at 4°C for obtaining serum. Immediately after euthanasia by exsanguination, the skin and muscles around the back of the neck were removed, and the dura mater of the cisterna magna was exposed. CSF samples were collected via cisterna magna puncture using a syringe and needle. We necropsied the animals; collected the tissues, including the brain, spinal cord (cervical, thoracic, and lumbar spine), sciatic nerve, tibial nerve, dorsal root ganglion (DRG; including the lumbar spine L1 and L2 and the cervical spine), and eyes (including the optic nerve); and fixed them in 10 vol% neutral buffered formalin for subsequent microscopic examination.

**Histopathological examination.** In accordance with the STP position paper (Bolon et al., 2013), the brain and spinal cord (cervical, thoracic, and lumbar, transverse only) were trimmed, embedded in paraffin, and sectioned. The 4-μm thick sections were stained with hematoxylin and eosin (HE). Additionally, the HE-stained slides of the sciatic nerve, tibial nerve, DRG (including the lumbar spine L1 and L2 and the cervical spine), and eyes (including the optic nerve) were prepared for microscopic examination. Staining with Fluoro-Jade C (FJ-C), a neuronal cell death marker, was additionally performed for the brains of representative animals.
necropsied on days 4 and 8 in the TMT groups and on days 2 and 4 in the KA groups and for the brains of all animals in which neuronal cell necrosis was not identified in HE sections from the other groups. For immunohistochemistry, brain sections of 4 control animals necropsied on day 2 and that of all animals from the TMT and KA groups were prepared for characterizing gliosis observed in the HE sections. After deparaffinization, immunohistochemistry using anti-glia fibrillary acidic protein (GFAP) antibody and anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody was performed using Leica Bond-Rx autostainers (Leica Biosystems K.K., Tokyo, Japan) in accordance with the manufacturer’s instructions. Additionally, we performed heat-induced epitope retrieval in BOND Epitope Retrieval Solution 1 (AR9961, Leica Biosystems K.K.) for both antibodies, Bond Polymer Refine Detection (DS-9800, Leica Biosystems K.K.), and blocking using Protein Block (X0909, DAKO, Agilent Technologies Japan Ltd., Tokyo, Japan) in accordance with standard procedures. The sections were incubated with anti-GFAP rabbit polyclonal antibody (1:2 dilution, IR524, DAKO) or anti-Iba1 rabbit polyclonal antibody (1:5000 dilution, ab178847, Abcam, Cambridge, UK) for 30 min at room temperature. Immunoreactivity was detected and visualized using Bond DAB Enhancer (AR9432, Leica Biosystems K.K.) before the sections were counterstained with hematoxylin.

Measurement of NfL levels in CSF and serum. NfL levels in CSF and serum were measured using a Simoa SR-X analyzer (Quanterix Corporation, Massachusetts) with a Simoa NF-light Advantage (SR-X) Kit (Quanterix, No. 103400) according to the manufacturer’s instructions.

Statistical analysis. The data on body weights and organ weights were tested by the F test for homogeneity of variance between the control group and each test article group. When the variances were homogeneous, Student’s t test was used; when the variances were heterogeneous, the Aspin and Welch t test was performed to compare the mean in the control group with that in the test article group. Correlations between CSF and serum NfL levels were determined using Pearson’s correlation coefficient.

The F test was conducted at a significance level of 0.20, and the other tests were conducted at 2-tailed significance levels of 0.05 and 0.01. Analyses were performed using SAS version 9.3 (SAS Institute Inc.).

Table 1. Effects of TMT, KA, MK-801, and Pyridoxine Treatment on Body Weights, Brain Weights, and Nervous Symptoms

| Test Article | Dose | Trimethyltin 10 mg/kg, po, single | Kainic Acid 12 mg/kg, sc, single | MK-801 1 mg/kg, sc, single | Pyridoxine 1200 mg/kg/day, ip, 3 days |
|--------------|------|-----------------------------------|---------------------------------|----------------------------|----------------------------------|
| Necropsy timing (day 1 is the day of first dosing) | | Day 2 | Day 4 | Day 8 | Day 2 | Day 4 | Day 2 | Day 4 | Day 2 | Day 4 |
| No. of animals | 4 | 4 | 4 | | 4 | 4 | | 4 | 4 | 4 |
| Body weight (% difference from control) | | — | — | — | 10%* | — | 13%* | — | 16%** | — | 16%* |
| Absolute brain weight (% difference from control) | | — | — | — | 6%* | — | — | — | — | — | — |
| Representative clinical signs of potential effects on central or peripheral nerve (incidence and observation timing) | | | | | | | | | | | |
| Decreased locomotor activity | — | — | — | | 1 | (day 2) | — | 4 | (1-h AD) | — | — |
| Prone position | — | — | — | | 1 | (day 2) | — | 4 | (1-h AD) | — | — |
| Hunchback position | — | — | — | | 1 | (day 2) | — | — | — | — | — |
| Bizarre behavior | — | — | — | | — | — | — | 1 | (1-h AD) | — | — |
| Soiled fur | — | — | — | | 2 | (days 6–8) | 3 | 1 | (days 2–4) | — | — |
| Irritability | — | 3 | 4 | (days 4–8) | 4 | 4 | (4-h AD, day 2–4) | 1 | 4 | (1-h AD) | — | — |
| Salivation | — | — | — | | 4 | (1- and 4-h AD) | 3 | (1- and 4-h AD) | 2 | (1-h AD) | — | — |
| Irregular respiration | — | 1 | — | (4-h AD) | — | — | — | — | — | — | — | — |
| Staggering gait | — | — | — | | 1 | (1-h AD) | — | 1 | (4-h AD) | — | — | — | — |
| Twitch | — | 3 | 4 | (days 4–8) | 1 | 4 | (4-h AD) | — | — | — | — | — | — |
| Tremor | — | — | — | | 1 | (days 7 and 8) | — | — | — | — | — | — | — |
| Wet dog shake | — | — | — | | 4 | (1-h AD) | — | — | — | — | — | — | — |
| Convulsion | — | — | — | | 3 | (days 4, 5, and 7) | — | — | — | — | — | — | — |

AD, after the first dose; —, not remarkable finding. *p < .05 versus control, **p < .01 versus control.
RESULTS

Clinical Signs, Body Weight, and Brain Weight
The clinical signs, body weight, and brain weight changes are summarized in Table 1. After a single administration of TMT, convulsions, tremors, twitches, and/or irritability were observed on day 4 and thereafter (day 8). In the KA group, wet dog shakes, twitch, salivation, and/or irritability were noted from the day of dosing (day 1) to day 4. The animals in the MK-801 group showed a severe decrease in locomotor activity on day 1. In the TMT-treatment group, the body weight significantly decreased on days 4 and 8. Additionally, the KA-treated animals showed a reduction in body weight on days 2 and 4. The pyridoxine group showed a decrease in body weight on days 2 and 4, without any neuronal clinical signs. Absolute brain weight did not change in any group on days 2 and 4. On day 8, absolute brain weight was decreased in the TMT group; however, this was possibly related to body weight reduction and animal growth retardation.

Histopathological Examination
Representative histopathological findings in the brain are summarized in Table 2 and Figures 1 and 2.

TMT group. Neuronal cell necrosis was observed in the olfactory bulb, hippocampus, entorhinal cortex, piriform cortex, septal nucleus, and Purkinje cells in the cerebellum from day 4. On day 8, brain lesions were more severe than on day 4, and they expanded to the retrosplenial cortex and thalamus. The necrotic areas were accompanied by increased Iba-1-positive cells, suggesting the occurrence of microgliosis on day 4; on day 8, mixed gliosis identified by Iba-1- and GFAP-positive cells, suggesting that mixed gliosis occurred on days 1 and 4.

KA group. Neuronal cell necrosis was observed in the hippocampus, hypothalamus, thalamus, amygdala, entorhinal cortex, piriform cortex, septal nucleus, and olfactory bulb on days 1 and 4. The necrotic areas were accompanied by increased Iba-1- and GFAP-positive cells, suggesting that mixed gliosis occurred on days 1 and 4.

MK-801 group. No histopathological findings were observed in any of the CNS or PNS tissues. The brains of all MK-801-treated animals were negative for FJ-C staining, suggesting a lack of neurodegenerative changes in the examined regions.

Pyridoxine group. No pyridoxine-related histopathological findings were observed in the brain or spinal cord; though spontaneous focal necrosis was unilaterally noted in the cerebral cortex of 1 animal on day 2. Neuronal chromatolysis in the DRG was observed on days 2 and 4. Additionally, degeneration of the nerve fibers in the sciatic and tibial nerves was noted in 1 animal on day 4. The brains of all pyridoxine-treated animals were negative for FJ-C staining except for 1 case of spontaneous change, suggesting a lack of pyridoxine-related neurodegenerative changes in the examined regions.

NfL Levels in the CSF and Serum
The NfL levels in the CSF and serum of the treated animals are shown in Table 3 and Figure 3.

Table 2. Results of Histopathology in TMT, KA, MK-801, and Pyridoxine-Treated Animals

| Test article Dose | TMT | KA | MK-801 | Pyridoxine |
| | 10 mg/kg, po, single | 12 mg/kg, sc, single | 1 mg/kg, sc, single | 1200 mg/kg/day, ip, 3 days |
| Necropsy timing (day 1 is the day of first dosing) | Day 2 | Day 4 | Day 8 | Day 2 | Day 4 | Day 2 | Day 4 |
| No. of animals examined | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Representative histopathological findings (incidence and severity) | | | | | | | | |
| Cerebrum/necrosis of nerve cell | | | | | | | | |
| Hippocampus | — | 4: + | 3: +, 1: ++ | 4: + | 3: +, 1: ++ | — | — | — |
| Amygdala | — | — | — | — | — | | — | — |
| Thalamus | — | — | 2: ± | 3: ±, 1: + | 3: ± | — | — | — |
| Hypothalamus | — | — | — | 2: ±, 1: + | 2: ± | — | — | — |
| Entorhinal cortex | — | 3: ± | 4: ++ | 4: ++ | 1: ±, 3: ++ | — | — | — |
| Piriform cortex | — | 3: ±, 1: + | 4: ++ | 4: ++ | 1: ±, 2: ++ | — | — | — |
| Septal nucleus | — | 2: ± | 2: ± | 4: ± | 2: ±, 2: + | — | — | — |
| Retrosplenial cortex | — | — | 4: ± | — | — | — | — | — |
| Olfactory bulb | — | 1: ± | 4: ± | 3: ± | 3: ± | — | — | — |
| Cerebellum/necrosis of Purkinje cell | — | 2: ± | 2: ± | — | — | — | — | — |
| DRG/chromatolysis | — | — | — | — | — | — | 4: ± | 4: ± |
| Dorsal nerve root/degeneration of nerve fiber | — | — | 3: ± | — | — | — | — | — |
| Sciatic and tibial nerve/degeneration of nerve fiber | — | — | 4: ± | — | — | — | — | 1: ± |

AD, after the first dose; —, no change or not remarkable finding; ±, minimal; +, mild; ++, moderate.
The serum/CSF NfL levels and the distribution/severity of histopathological neuronal cell necrosis in the brain were correlated in this study (Supplementary Table 1).

TMT group. High NfL levels in serum and CSF were observed in all animals that showed brain lesions on days 4 and 8. The apparent and statistically significant elevation of NfL levels in the serum and CSF were confirmed on day 4 ($p < .05$, approximately 15-fold increase in both CSF and serum) and day 8 ($p < .01$ in both CSF and serum, approximately 60- and 26-fold increase in the CSF and serum, respectively). TMT-treated animals showed a strong correlation between CSF and serum NfL levels (Figure 3A, $p < .001$, $r = 0.9001$). In particular, the NfL levels in TMT-treated animals on day 8 were much higher than that on day 4, indicating a correlation between NfL levels and the severity of nervous histopathological findings in the CNS and PNS. Despite the lack of histopathological findings in the nerve tissues and NfL levels in the CSF, a statistically significant increase in serum NfL levels was observed in TMT-treated animals on day 2.

KA group. Apparent increases in NfL levels in CSF and serum were observed on day 2 (approximately 55- and 13-fold increase in the CSF and serum, respectively) and day 4 ($p < .05$ in both CSF and serum, approximately 33- and 11-fold increase in the CSF and serum, respectively) in all KA-treated animals with brain lesions. NfL levels in the CSF and serum were highly correlated in the KA-treated animals (Figure 3B, $p < .001$, $r = 0.9881$).

MK-801 group. No significant differences in NfL levels in the CSF and serum were observed in MK-801-treated animals without any brain lesions.

Pyridoxine group. Apparent increases in NfL levels in the CSF and serum were observed in pyridoxine-treated animals with chromatolysis of the DRG and/or nerve fiber degeneration on day 4 (CSF: approximately 83 folds, serum: approximately 35 folds), but not on day 2, when only chromatolysis was observed in the DRG. In the individual data, 1 animal with DRG lesion on days 2 and 4 showed high NfL levels only in the serum (Figure 3C). The
levels of NfL in both CSF and serum of pyridoxine-treated animals were highly correlated (Figure 3C, \( p < .001, r = 0.9326 \)).

**DISCUSSION**

In this study, we demonstrated that both CSF and serum NfL could serve as potential biomarkers for the detection of toxicity in both the CNS and PNS in TMT-, KA-, and pyridoxine-induced rat models of neurotoxicity.

In this study, NfL levels and brain lesions were correlated at various time points in animals treated with TMT and KA (Supplementary Table 1). Increased NfL levels in the serum and CSF were observed in the KA groups on days 2 and 4 and in the TMT groups on days 4 and 8, where neuronal cell death was present in the brain. The NfL level in the TMT group on day 8 was higher than that on day 4. The increased NfL in the TMT group on day 8 reflected the histopathological progression of the brain lesions by day 8. In the TMT group, increased serum NfL level was also observed on day 2, without any histopathological changes in the brain or alteration of NfL levels in the CSF, suggesting that increased serum NfL levels were not derived from the CNS. However, we cannot deny the possibility that the increase in serum NfL is related to the presence of a very small number of necrotized neuronal cells in the brain. Additionally, peripheral nerve effects such as vacuolation of the spiral ganglion cells in the organ of Corti were reported to be induced 24 h after TMT dosing in rats (Chang and Dyer, 1983). Therefore, further studies involving the quantitative evaluation of neuronal cell death in the brain through stereological analysis or more detailed assessment of peripheral nerve tissues will be needed to clarify not only the precise relationship between the total number of neuronal cell deaths in the whole brain and the NfL values but also the significance of the change in serum NfL in the TMT group on day 2.

In the past decade, multiple fluid biomarker candidates have been proposed for detecting neurotoxicity in nonclinical studies. In KA-treated rats with brain lesions, glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-1 (UCHL-1)
were increased in the CSF and brain tissues 24 h after dosing (Glushakova et al., 2012). Peak elevation of UCHL-1 was observed at 6 h after dosing when no brain lesions were confirmed; it might serve as an early marker of neurotoxicity. Although GFAP and UCHL-1 were not detected in the CSF at 48 and 72 h after KA dosing, neuron-specific αII-spectrin breakdown products in the CSF were increased at the same time point (Glushakova et al., 2012). Thus, the panel use of these markers in the CSF was necessary for the assessment of neuronal damage at each time point. Other studies showed that changes in specific miRNAs and metabolomics data in the CSF, blood, and urine were associated with brain lesions in the TMT-induced rat neurotoxicity model (Imam et al., 2018; Ogata et al., 2015). Ogata et al. (2015) reported that miR9a-3p and miR384-5p could detect neurodegenerative changes in the rat brain; however, histopathological examination was more sensitive for the early detection of brain lesions. Imam et al. (2018) demonstrated that increased acylcarboxylase levels in the CSF, plasma, and urine might act as a predictor of neuronal cell death; however, an increase in acylcarboxylase was observed transiently before the neuronal lesions. In contrast, the CSF and serum levels of NfL in the TMT- and KA groups were consistently increased in animals with neuronal cell necrosis in the brain, potentially representing a robust marker for detecting acute neuronal cell necrosis with high accuracy. Although the change in NfL levels in the CSF was higher than that in the serum in the TMT and KA groups, the change in NfL levels in the serum was sufficient for detecting brain lesions. Collectively, the present results suggest that NfL is released in the CSF and serum in parallel with neuronal cell damage in the brain, confirming the close relationship between the 2 fluid compartments. To the best of our knowledge, this is the first report to demonstrate the value of serum NfL as a peripheral biomarker of neuronal necrosis in the brain. In previous clinical studies, the measurement of serum NfL levels in neurodegenerative diseases enabled the monitoring of ongoing neuronal damage in real time (Disanto et al., 2017; Fyfe, 2019; Gaetani et al., 2019; Kuhle et al., 2019; Olsson et al., 2019). Our data indicate that NfL could be used for detecting drug-induced neuronal cell necrosis in nonclinical studies as well as for monitoring disease progression in patients with neurodegenerative disorder.

For neurotoxicity evaluation, some of the TMT-treated animals with neuronal cell necrosis in the brain and NfL elevation did not show clinical signs on day 4. On the other hand, all animals in the KA-treated group showed neurological clinical signs such as wet dog shake, twitch, and/or irritability on day 1, whereas neuronal cell necrosis in the brain and NfL elevation were noted on days 2 and 4. Hence, NfL may be a good marker for neuronal cell necrosis in the brain, but in terms of neurotoxicity assessment, abnormal clinical signs may appear earlier, as in the case of KA. In this study, we used severe neurotoxicity models, and further evaluation with milder neurotoxic agents could help to understand the true detectability of neuronal cell necrosis. Additionally, whether NfL can predict neuronal necrosis in the brain or detect biochemical and/or physiological events prior to the obvious neuronal cellular damage was not confirmed in this study, so that further investigations are needed to clarify this point.

NfL levels in both CSF and serum were increased with pyridoxine treatment, a PNS toxicant, on day 4, when nerve fiber degeneration occurred in peripheral nerves. According to previous studies, the primary target of pyridoxine-induced sensory neuropathy in rats is believed to be the cell body of DRG neurons, followed by secondary nerve fiber degeneration (Jortner, 2000;
Krinke and Fitzgerald, 1988). In a previous report of increased blood NfL levels in vincristine-, paclitaxel-, and cisplatin-induced rat models of peripheral nerve injury (Meregalli et al., 2018, 2020), serum NfL was highly sensitive to primary axonal damage, especially in paclitaxel-treated rats. Therefore, our data are consistent with previous reports (Meregalli et al., 2018, 2020), and NfL is a good indicator for monitoring nerve fiber lesions in the PNS. Interestingly, in the present study, an increase in serum NfL was observed on days 2 and 4 in some animals, which showed chromatolysis in the DRG but not nerve fiber lesions. These data suggest that serum NfL levels are associated with chromatolysis in the DRG. Additionally, NfL levels in the CSF were increased in the pyridoxine model only on day 4 in this study. Given the lack of CNS lesions in the pyridoxine-treated animals, NfL might be released from the neuronal cell body or its surrounding nerve fibers into the CSF through direct communication between the DRG region and CSF in the subarachnoid space (Joukal et al., 2016).

In this study, we demonstrated that the NfL levels in the serum and CSF can indicate neuronal cell death in the brain and changes in peripheral nerves with a sensitivity similar to that of histopathological examination. Considering the strong correlation between the CSF and serum in neuronal cell necrosis in the brain, the current results provide conceptual evidence for the use of serum NfL as a promising peripheral biomarker for the evaluation of neuronal cell death in the brain. Utilization of NfL for neurotoxicity evaluation in drug development could facilitate identification of neurotoxicity risk and expedite the drug development process through achieving a balance between efficacy and safety and reducing the time to patient access for innovative medicines.

SUPPLEMENTARY DATA
Supplementary data are available at Toxicological Sciences online.

ACKNOWLEDGMENTS
The authors would like to thank Dr Kazumi Ohuchi of the Drug Metabolism and Pharmacokinetics Research Laboratories, Takeda Pharmaceutical Company Limited for supporting NfL measurements, and Axcelead Drug Discovery Partners Inc. for conducting the animal study and their excellent technical support during this work. We would like to thank Editage (www.editage.com; last accessed October 2021) for English language editing.

DECLARATION OF CONFLICTING INTERESTS
All authors are employees of Takeda Pharmaceutical Company (Osaka, Japan).

REFERENCES
Bacioglu, M., Maia, L. F., Preische, O., Schelle, J., Apel, A., Kaeser, S. A., Schweighauser, M., Eninger, T., Lambert, M., Pilotto, A., et al. (2016). Neurofilament light chain in blood and CSF as marker of disease progression in mouse models and in neurodegenerative diseases. Neuron 91, 494–496.

Balaban, C. D., O’Callaghan, J. P., and Billingsley, M. L. (1988). Trimethyltin-induced neuronal damage in the rat-brain - Comparative studies using silver degeneration stains, immunocytochemistry and immunoassay for neurotontypic and gliotypic proteins. Neuroscience 25, 337–361.

Bertoglio, D., Amhaoul, H., Van Eetveld, A., Houbrechts, R., Van De Vijver, S., Ali, I., and Dedeurwaerdere, S. (2017). Kainic acid-induced post-status epilepticus models of temporal lobe epilepsy with diverging seizure phenotype and neuro-pathology. Front. Neurol. 8, 588.

Bolon, B., Garman, R. H., Pardo, I. D., Jensen, K., Sills, R. C., Roulous, A., Radosvky, A., Bradley, A., Andrews-Jones, L., Butt, M., et al. (2013). STP position paper: Recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. Toxicol. Pathol. 41, 1028–1048.

Bushnell, P. J., and Evans, H. L. (1985). Effects of trimethyltin on homeocage behavior of rats. Toxicol. Appl. Pharmacol. 79, 134–142.

Ceccariglia, S., Alvino, A., Del Fa, A., Parolini, O., Michetti, F., and Gangitano, C. (2020). Autophagy is activated in vivo during trimethyltin-induced apoptotic neurodegeneration: A study in the rat hippocampus. Int. J. Mol. Sci. 21, 175.

Chang, L. W., and Dyer, R. S. (1983). Trimethyltin induced pathology in sensory neurons. Neurobehav. Toxicol. Teratol. 5, 673–696.

Covolan, L., and Mello, L. E. (2000). Temporal profile of neuronal injury following pilocarpine or kainic acid-induced status epilepticus. Epilepsia Res 39, 133–152.

Crofton, K. M., Dean, K. F., Menache, M. G., and Janssen, R. (1990). Trimethyltin effects on auditory function and cochlear morphology. Toxicol. Appl. Pharmacol. 105, 123–132.

Disanto, G., Barro, C., Benkert, P., Naegelein, Y., Schadelin, S., Giardiello, A., Zecca, C., Blennow, K., Zetterberg, H., Leppert, D., et al.; the Swiss Multiple Sclerosis Cohort Study Group. (2017). Serum neurofilament light: A biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81, 857–870.

Fix, A. S., Horn, J. W., Wightman, K. A., Johnson, C. A., Long, G. G., Storts, R. W., Farber, N., Wozniak, D. F., and Olney, J. W. (1992). Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK-801 (dizocilpine maleate): A light and electron microscopic evaluation of the rat retrosplenial cortex. Exp. Neurol. 123, 204–215.

Fix, A. S., Wozniak, D. F., Truex, L. L., McEwen, M., Miller, J. P., and Olney, J. W. (1995). Quantitative analysis of factors influencing neuronal necrosis induced by MK-801 in the rat posterior cingulate/retrosplenial cortex. Brain Res. 696, 194–204.

Fyfe, I. (2019). Neurofilament light chain - New potential for prediction and diagnosis. Nat. Rev. Neurol. 15, 557.

Gaetani, L., Blennow, K., Calabresi, P., Di Filippo, M., Parnetti, L., and Zetterberg, H. (2019). Neurofilament light chain as a biomarker in neurological disorders. J. Neurol. Neurosurg. Psychiatry 90, 870–881.

Glushakova, O. Y., Jeromin, A., Martinez, J., Johnson, D., Denslow, N., Streeter, J., Hayes, R. L., and Mondello, S. (2012). Cerebrospinal fluid protein biomarker panel for assessment of neurotoxicity induced by kainic acid in rats. Toxicol. Sci. 130, 158–167.

Imam, S. Z., He, Z., Cuevas, E., Rosas-Hernandez, H., Lantz, S. M., Sarkar, S., Raymick, J., Robinson, B., Hanig, J. P., Herr, D., et al. (2018). Changes in the metabolome and microRNA levels in biological fluids might represent biomarkers of neurotoxicity: A trimethyltin study. Exp. Biol. Med. (Maywood) 243, 228–236.
Jortner, B. S. (2000). Mechanisms of toxic injury in the peripheral nervous system: Neuropathologic considerations. Toxicol. Pathol. 28, 54–69.

Joukal, M., Klusakova, I., and Dubovy, P. (2016). Direct communication of the spinal subarachnoid space with the rat dorsal root ganglia. Ann. Anat. 205, 9–15.

Khalil, M., Teunissen, C. E., Otto, M., Piel, F., Sormani, M. P., Gatteringer, T., Barro, C., Kappos, L., Comabella, M., Fazekas, F., et al. (2018). Neurofilaments as biomarkers in neurological disorders. Nat. Rev. Neurol. 14, 577–589.

Kim, S. H., Choi, M. K., Park, N. Y., Hyun, J. W., Lee, M. Y., Kim, H. J., Jung, S. K., and Cha, Y. J. (2020). Serum neurofilament light chain levels as a biomarker of neuroaxonal injury and severity of oxaliplatin-induced peripheral neuropathy. Sci. Rep. 10, 7995.

Krinke, G. J., and Fitzgerald, R. E. (1988). The pattern of pyridoxine-induced lesion - Difference between the high and the low toxic level. Toxicology 49, 171–178.

Kuhle, J., Barro, C., Andreasson, U., Derfuss, T., Lindberg, R., Sandelius, A., Liman, V., Norgren, N., Blennow, K., and Zetterberg, H. (2016). Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and simoa. Clin. Chem. Lab. Med. 54, 1655–1661.

Kuhle, J., Kropshofer, H., Haering, D. A., Kundu, U., Meinert, R., Barro, C., Dahlke, F., Tomic, D., Leppert, D., and Kappos, L. (2019). Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. Neurology 92, E1007–E1015.

Kuroda, K., Suzumura, K., Shirakawa, T., Hiaraishi, T., Nakahara, Y., Fushiki, H., Honda, S., Naraoka, H., Miyoshi, S., and Aoki, Y. (2015). Investigation of mechanisms for MK-801-induced neurotoxicity utilizing metabolomic approach. Toxicol. Sci. 146, 344–353.

Le, N. D., Muri, L., Grandgirard, D., Kuhle, J., Leppert, D., and Leib, S. L. (2020). Evaluation of neurofilament light chain in the cerebrospinal fluid and blood as a biomarker for neuronal damage in experimental pneumococcal meningitis. J. Neuroinflamm. 17, 293.

Levesque, M., and Avoli, M. (2013). The kainic acid model of temporal lobe epilepsy. Neurosci. Biobehav. Rev. 37, 2887–2899.

Loeffler, T., Schilcher, I., Flunkert, S., and Hutter-Paier, B. (2020). Neurofilament-light chain as biomarker of neurodegenerative and rare diseases with high translational value. Front. Neurosci 14, 579.

Meregalli, C., Fumagalli, G., Alberti, P., Canta, A., Carozzi, V. A., Chiorazzi, A., Monza, L., Pozzi, E., Sandelius, A., Blennow, K., et al. (2018). Neurofilament light chain as disease biomarker in a rodent model of chemotherapy induced peripheral neuropathy. Exp. Neurol. 307, 129–132.

Meregalli, C., Fumagalli, G., Alberti, P., Canta, A., Chiorazzi, A., Monza, L., Pozzi, E., Carozzi, V. A., Blennow, K., Zetterberg, H., et al. (2020). Neurofilament light chain: A specific serum biomarker of axonal damage severity in rat models of chemotherapy-induced peripheral neurotoxicity. Arch. Toxicol. 94, 2517–2522.

Ogata, K., Sumida, K., Miyata, K., Kushida, M., Kuwamura, M., and Yamate, J. (2015). Circulating miR-9-9and miR-384-5p as potential indicators for trimethyltin-induced neurotoxicity. Toxicol. Pathol. 43, 198–208.

Olney, J. W., Ikonomidou, C., Mosinger, J. L., and Friedich, G. (1989). MK-801 prevents hypobaric-ischemic neuronal degeneration in infant rat brain. J. Neurosci. 9, 1701–1704.

Olsson, B., Alberg, L., Cullen, N. C., Michael, E., Wahlgren, L., Kroksmark, A. K., Rostasy, K., Blennow, K., Zetterberg, H., and Tulinius, M. (2019). NFL is a marker of treatment response in children with SMA treated with nusinersen. J. Neurol. 266, 2129–2136.

Rogrigues, F. B., Byrne, L. M., Tortelli, R., Johnson, E. B., Wijeratne, P. A., Arridge, M., De Vita, E., Ghazaleh, N., Houghton, R., Furby, H., et al. (2020). Mutant Huntingtonin and neurofilament light have distinct longitudinal dynamics in Huntington’s disease. Sci. Transl. Med. 12, eabc2888.

Sirmashetty, V. B., Nickel, J., Omiecinksi, C., Gohlke, B. O., Drwal, M. N., and Preissner, R. (2016). Withdrawn—a resource for withdrawn and discontinued drugs. Nucleic Acids Res. 44, D1080–D1086.

Soylu-Kucharz, R., Sandelius, A., Sjogren, M., Blennow, K., Wild, E. J., Zetterberg, H., and Bjorkqvist, M. (2017). Neurofilament light protein in csf and blood is associated with neurodegeneration and disease severity in Huntington’s disease R6/2 mice. Sci. Rep. 7, 14114.

Walker, A. L., Imam, S. Z., and Roberts, R. A. (2018). Drug discovery and development: Biomarkers of neurotoxicity and neurodegeneration. Exp. Biol. Med. (Maywood) 243, 1037–1045.

Zheng, X. Y., Zhang, H. L., Luo, Q., and Zhu, J. (2011). Kainic acid-induced neurodegenerative model: Potentials and limitations. J. Biomed. Biotechnol. 2011, 457079.