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Author(s)	Erdenebat, Temuulen
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Studies on prion propagation in neurons and neurons vulnerable to
prion infection

(神経細胞におけるプリオンの増殖とプリオン感染に脆弱性を示す
神経細胞に関する研究)

Temuulen ERDENEBAT

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ABBREVIATIONS

AP	anteroposterior
Ara-C	cytosine arabinoside
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
Ctx	cortex
CxN	cortical neuron
DAB	3, 3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
dpi	days post inoculation
DMEM	Dulbecco's modified Eagle's medium
DW	distilled water
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin-embedded
GABA	gamma-aminobutyric acid
GABAergic	gamma-aminobutyric acidergic
GdnHCl	guanidine hydrochloride
GFAP	glial fibrillary acidic protein
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
i.c.	intracerebral
IFA	immunofluorescence assay
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IHC	immunohistochemistry

IoU	intersection over union
i.p.	intraperitoneal
mAb	monoclonal antibody
mAP	mean average precision
MAP2	Microtubule-associated protein 2
MEM	minimum essential medium
ML	medial-lateral
min	minute
NEAA	non-essential amino acids
N2a	Neuro2a
pAb	polyclonal antibody
PBS	phosphate buffered saline
PERK	protein kinase RNA-activated-like endoplasmic reticulum kinase
PFA	paraformaldehyde
PK	proteinase K
PrP ^C	cellular isoform of prion protein
PrP ^{Sc}	disease-specific isoform of prion protein
RNAscope	Ribonucleic acid scope
rPrP	recombinant prion protein
PrP-res	protease resistant prion protein
RT	reticular nucleus
r.t.	room temperature
s	second
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
StN	striatal neuron
Str	striatum
Th	thalamus
ThN	thalamic neuron
<i>Vglut1</i>	vesicular glutamate transporter 1

<i>Vglut2</i>	vesicular glutamate transporter 2
<i>Vglut3</i>	vesicular glutamate transporter 3
<i>Vgat</i>	vesicular gamma-aminobutyric acid transporter
VPL	ventral posterolateral nucleus
VPM	ventral posteromedial nucleus

NOTES

This thesis contains one article published as follows.

Chapter I: Excitatory neuron-prone prion propagation and excitatory neuronal loss in prion-infected mice

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PREFACE

Prion diseases are fatal and intractable neurodegenerative disorders affecting both animals and humans, including bovine spongiform encephalopathy, scrapie in sheep and goats, chronic wasting disease in cervids, and Creutzfeldt-Jakob disease (CJD) in humans. These diseases have long incubation periods but become subacutely progressive and inevitably fatal after clinical onset. Neuropathological hallmarks include astrogliosis, microglial activation, neuronal and neuropil vacuolation, neuronal loss, and deposition of a disease-specific prion protein (PrP^{Sc}) in the central nervous systems (CNS) (Collinge, 2001; Prusiner, 1998). PrP^{Sc} is generated from a cellular isoform of prion protein (PrP^C), encoded by the host *Prnp* gene, through post-translational conformational transition. PrP^C is glycosylphosphatidylinositol-anchoring membrane glycoprotein and is most abundantly expressed in neuronal cells, although many other cell types also express it. The first suggested function of PrP^C was immunosuppression (Cashman *et al.*, 1990). After that, PrP^C has been reported to involve various physiological processes such as long-term potentiation in the hippocampus (Collinge *et al.*, 1994; Lledo *et al.*, 1996), circadian rhythm (Tobler *et al.*, 1996), copper ion metabolism (Toni *et al.*, 2005), anti-oxidation (Wong *et al.*, 2001), and stemness of neuronal stem cells (Steele *et al.*, 2006; Santos *et al.*, 2011). Since PrP-deficient mice appear to grow normally and produce offspring (Bueler *et al.*, 1993), PrP is not considered essential for life, and the complete range of its functions has yet to be fully elucidated.

The post-translational misfolding of PrP^C leads to the formation of an abnormal pathogenic isoform, known as PrP^{Sc} (Prusiner, 1998). Upon formation, a new PrP^{Sc} molecule is generated by binding of PrP^C to pre-existing PrP^{Sc}, which follows conformational conversion from α -helix rich PrP^C into β -sheet rich PrP^{Sc} through a process called the seeded conversion mechanism (Caughey *et al.*, 2009). PrP^{Sc}, rich in β -sheets and prone to aggregation, is considered the sole proteinaceous component of prions, the infectious agents of prion diseases,

and prion infectivity is believed to be associated with PrP^{Sc} oligomers/aggregates (Silveira *et al.*, 2005; Wang *et al.*, 2010).

PrP^{Sc} deposition and neurohistopathological lesions, such as neuronal and neuropil vacuolation, are observed in various brain regions of prion-infected mice. However, the distribution varies depending on the prion and mouse strain combinations (Bruce *et al.*, 1994; Bruce, 1993; DeArmond *et al.*, 1993). Intense PrP^{Sc} accumulation in the thalamus and cerebral cortex has been reported in multiple prion strains, including the 22L strain (Carroll *et al.*, 2016; Reis *et al.*, 2015). Some mouse-adapted strains from Gerstmann-Sträussler-Scheinker syndrome and Fatal Familial Insomnia show intense PrP^{Sc} accumulation in the thalamus but mild accumulation in the cerebral cortex (Tateishi *et al.*, 1995; Telling *et al.*, 1996). The prion strain-dependent PrP^{Sc} distribution in the CNS suggests neuronal cell-type tropism or differences in prion propagation efficiency among neuronal cell types, though the underlying mechanism remains largely unknown. Neuronal loss is not solely due to prion propagation in neurons; innate immune responses, such as microglial activation and astrogliosis, are deeply involved in prion disease neuropathology (Carroll *et al.*, 2018; Gómez-Nicola *et al.*, 2013; Makarava *et al.*, 2020; Manuelidis *et al.*, 1987; Mukherjee *et al.*, 2010). Prion propagation in neurons is believed to trigger innate immune responses, similar to the early microglial response to Amyloid β deposition (Wang *et al.*, 2015), and is essential for neuronal degeneration (Chesebro *et al.*, 2005; Mallucci *et al.*, 2003). Therefore, understanding the mechanism behind brain region-dependent prion propagation is crucial for understanding the neuropathophysiology of prion diseases.

In mammals, two transmitters, glutamate and gamma-aminobutyric acid (GABA), are predominantly adjusting neuronal activity transmission in neural circuits in the brain. Glutamate is synthesized in excitatory glutamatergic neurons and astrocytes, while GABA is synthesized in GABAergic inhibitory neurons (Freneau *et al.*, 2004; Tepper *et al.*, 2018). The distribution of glutamatergic and GABAergic neurons varies across brain regions. In the

cerebral cortex, glutamatergic and GABAergic neurons are present in about 8:2 ratio. In the striatum, GABAergic activity accounts for more than 90%. In the thalamic nucleus conversely, the major neurons are known to be glutamatergic, except for most lateral nuclei (Sahara *et al.*, 2012; Tepper *et al.*, 2018; Arcelli *et al.*, 1997). Nowadays, specific marker genes for those neuronal types have been identified and are used to distinguish neuronal cell types and to analyze the localizations of those neurons histologically. Glutamatergic neurons are characterized by the gene expression of vesicular glutamate transporters, Vglut1, Vglut2, and Vglut3 (Vgluts), which specifically and actively fill synaptic vesicles with glutamate (Bellocchio *et al.*, 2000; Takamori *et al.*, 2000; Herzog *et al.*, 2001). Similarly, GABAergic neurons are identified by the presence of a vesicular GABA transporter (Vgat) (Chaudhry *et al.*, 1998). Excitatory and inhibitory neurons have different developmental lineages and numerous molecular and morphological differences (Zhang *et al.*, 2021), which may affect their efficiency in prion propagation and vulnerability to prion infection. The selective loss of parvalbumin-positive GABAergic inhibitory neurons in the brains of CJD patients, which may partly explain extrapyramidal symptoms such as movement disorders, and prion-infected mice suggests that GABAergic neurons are vulnerable to prion infection (Belichenko *et al.*, 1999; Guentchev *et al.*, 1997; Guentchev *et al.*, 1999; Franklin *et al.*, 2008). However, these observations should carefully be re-visited because degeneration of GABAergic neurons was observed in the same degenerative sequence as non-GABAergic neurons in prion-infected hamsters (Bouzamondo *et al.*, 2000; Bouzamondo-Bernstein *et al.*, 2004). Additionally, selective loss of Vglut1-positive synapses in the cerebellum and pontine nuclei was observed in BSE-infected guinea pigs (Sakaguchi *et al.*, 2020). It is expected that the neuropathogenesis of prion diseases involves a complex balance between excitatory and inhibitory neurons, brain regions, and a combination of prion strains and host animals. Thus, detailed analyses targeting brain regions and neuronal cell types where PrP^{Sc} accumulation and neuronal loss are observed will be required to clarify the pathological events that occur in the neuropathological lesions.

Immortalized neuronal and neural cells such as Neuro2a neuroblastoma cells and GT1-7 hypothalamic neuronal cell lines which are permissive to prion propagation have extensively contributed to elucidating the cellular mechanism of prion propagation. Earlier research suggested that PrP^{Sc} generation occurs either on the cell surface or within the endocytic pathway (Caughey and Raymond, 1991; Taraboulos *et al.*, 1992; Borchelt *et al.*, 1992). After the uptake of PrP^{Sc} by the cell, PrP^{Sc} is transported through the endo-lysosomal pathway to late endosomes and lysosomes, (Magalhaes *et al.*, 2005; Jen *et al.*, 2010; Yamasaki *et al.*, 2014). PrP^{Sc} localizes throughout various endocytic compartments, including the plasma membrane, early and recycling endosomes, late endosomes, lysosomes, and the perinuclear Golgi region (Taraboulos *et al.*, 1990; McKinley *et al.*, 1991; Pimpinelli *et al.*, 2005; Veith *et al.*, 2009; Yamasaki *et al.*, 2012; Marijanovic *et al.*, 2009). Endocytic recycling compartment (ERC) or plasma membrane, or possibly both, are now considered potential sites for PrP^{Sc} formation (Marijanovic *et al.*, 2009; Goold *et al.*, 2011; Yamasaki *et al.*, 2014).

The mechanism for prion propagation in cells appears to be well elucidated; however, recent reports showed the existence of PrP^{Sc} on the surface of neuronal cells (Rouvinski *et al.*, 2014), and studies in my laboratory have demonstrated the predominant existence of PrP^{Sc} aggregates as string-like stains on the surface of primary neuronal cells (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020). These results suggest that the dynamics of PrP^{Sc} formation in primary neurons differ significantly from those in immortalized prion-permissive cell lines. The limitation of immortalized neuronal and neural cells is that they do not exhibit any alteration associated with prion propagation. A recent report from my laboratory showed that enhanced phosphorylation of protein kinase RNA-activated-like endoplasmic reticulum kinase (PERK), an early cellular response of the PERK-eukaryotic initiation factor 2-ATF4 unfolded protein response pathway, was induced in prion-infected primary neurons (Tanaka *et al.*, 2020). This suggests the potential of primary neuronal cultures for analyzing the molecular mechanisms of

neurodegeneration caused by prion infection, and thus the mechanism of prion propagation in neurons should also be re-visited.

In addition to neurons, astrocytes also play a significant role in prion disease. Earlier studies identified astrocytes as one of the first sites of PrP^{Sc} accumulation in mice (Diedrich *et al.*, 1991). Immunohistochemical analysis of prion-infected sheep brains demonstrated PrP^{Sc} accumulation with GFAP-positive areas (Hernandez *et al.*, 2014). Primary cerebellar astrocyte cultures from transgenic mice expressing hamster PrP^C were shown to support prion propagation (Cronier *et al.*, 2004), and transgenic mice expressing hamster PrP^C only in astrocytes were still susceptible to prion infection (Raeber *et al.*, 1997). Prions propagated in astrocytes could spread to neurons via cell-to-cell transfer (Victoria *et al.*, 2016). Recently, it has been reported that unfolding protein response in astrocytes is harmful to neurons in prion disease (Smith *et al.*, 2020). Therefore, in addition to the primary neuronal cells, primary astrocytic cultures will also be useful for analyzing the mechanism of prion propagation and the involvement of astrocytes in prion disease neuropathobiology.

In my doctoral dissertation, in Chapter I, I studied the excitatory neuron-prone prion propagation and excitatory neuronal loss using prion-infected mice and prion-infected primary neuronal cultures as *in vivo* and *ex vivo* models. In Chapter II, I describe the utility of primary neuronal and astrocytic cultures for analyzing the mechanism of prion propagation as an *ex vivo* model closer to the brain. The results of my dissertation will provide new insights into future research addressing the mechanism of neuropathobiology of prion diseases and prion-related neurodegenerative disorders.

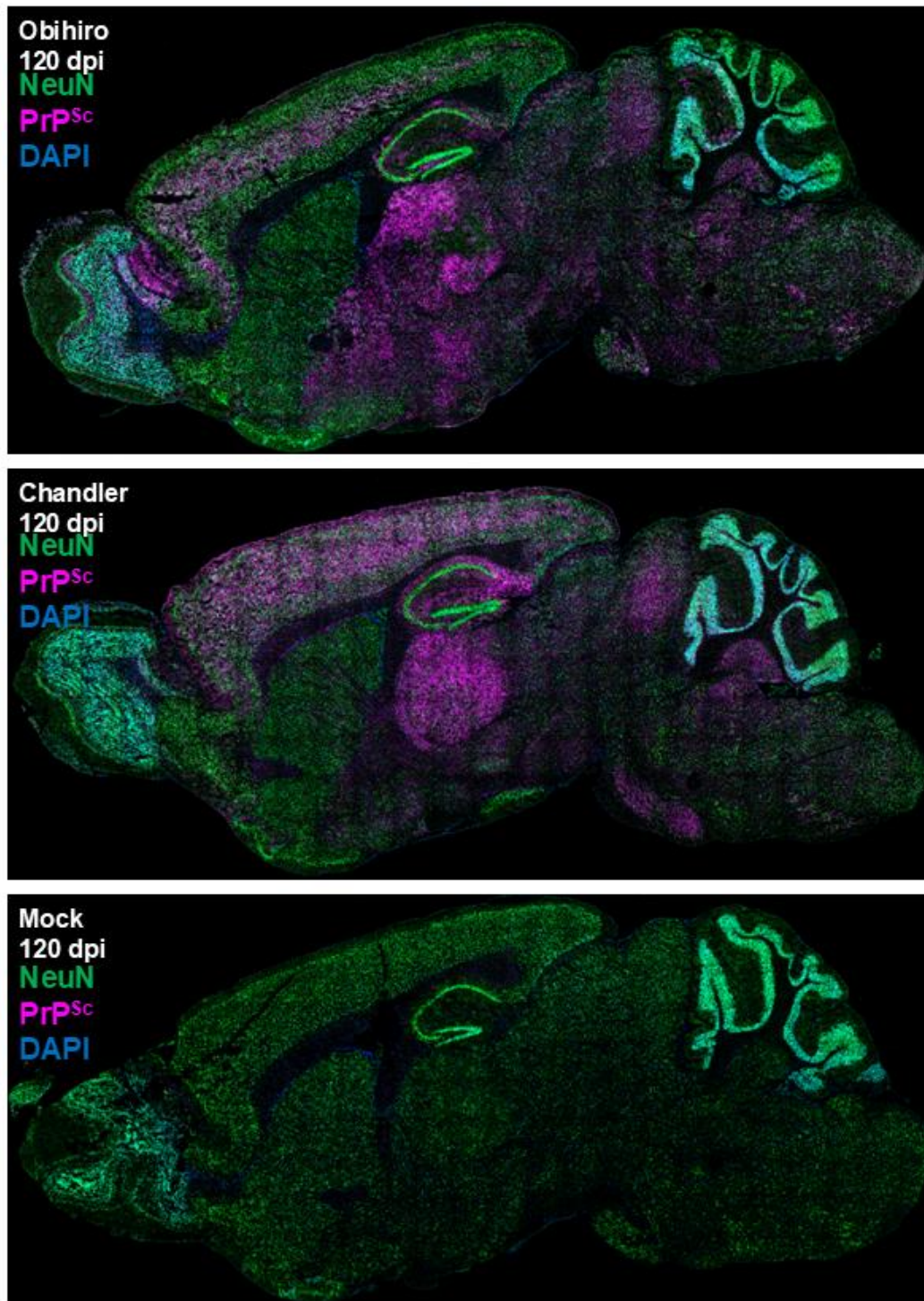
CHAPTER I

Excitatory neuron-prone prion propagation and excitatory neuronal loss
in prion-infected mice

INTRODUCTION

Excitatory and inhibitory neurons have different developmental lineages and numerous molecular and morphological differences (Zhang *et al.*, 2021), which may affect their efficiency in prion propagation and vulnerability to prion infection. The selective loss of parvalbumin-positive GABAergic inhibitory neurons in the brains of CJD patients and prion-infected mice suggests that GABAergic neurons are vulnerable to prion infection (Belichenko *et al.*, 1999; Guentchev *et al.*, 1997; Guentchev *et al.*, 1999). Recent single-cell transcriptome analysis indicated that inhibitory neurons in the hippocampus of RML prion-infected mice are more vulnerable to prion infection (Slota *et al.*, 2022). However, non-GABAergic neurons degenerated earlier than GABAergic neurons and the degeneration of GABA-negative synapses was observed before the degeneration of GABAergic neurons in prion-infected hamsters (Bouzamondo *et al.*, 2000; Bouzamondo-Bernstein *et al.*, 2004). Additionally, selective loss of Vglut1-positive synapses in the cerebellum and pontine nuclei was observed in BSE-infected guinea pigs (Sakaguchi *et al.*, 2020).

Preliminary results in my laboratory showed weaker PrP^{Sc} accumulation in the striatum of prion-infected mice compared to the cerebral cortex and thalamus (Figure I-1). This brain region-dependent PrP^{Sc} accumulation may be related to the striatum composition, which is largely GABAergic neurons (Tepper *et al.*, 2004). I hypothesized that a deeper analysis of this phenomenon would shed light on the neuropathological mechanisms related to neuronal cell-type-dependent prion propagation and cell death in prion diseases. Therefore, in Chapter I, I analyzed brain region-dependent PrP^{Sc} accumulation in prion-infected mice using *in vivo* and primary neuronal cultures. I found that 22L prions propagate more efficiently in glutamatergic excitatory neurons than in GABAergic inhibitory neurons, consistent with the lower PrP^{Sc} accumulation in the striatum. This neuronal cell-type-prone prion propagation will enhance the understanding of prion disease neuropathology.



I-1 Distribution of PrP^{Sc} in brains of prion Chandler and Obihiro strain-infected mice at 120 dpi.

Mice were intracerebrally (i.c.) inoculated with brain homogenates from mock-, Obihiro-, or Chandler strain-infected mice. At 120 dpi, mice were sacrificed, and their brains were subjected to cryosection. Merged images show anti-NeuN mAb (green), PrP^{Sc} (magenta), and DAPI (blue). Images were acquired using a 20× objective lens and are tile scans of sagittal sections generated with ZEN2009 software (black edition). Sagittal brain sections around Plates 111–113 (Paxinos and Franklin, 2013) were used.

MATERIALS AND METHODS

Ethics statement and animals

All mice were housed at the Faculty of Veterinary Medicine, Hokkaido University, in an AAALAC-accredited facility, following guidelines from the Institutional Animal Care and Use Committee (approval No. 23-0041). Jcl:ICR mice were purchased from CLEA Japan (Tokyo, Japan) and acclimated for a week. They had unrestricted access to food and water under a 12-hour light/dark cycle.

Prion strains

Mouse-adapted Prion 22L, Chandler, and Obihiro strains were used. Brains from terminal-stage diseased and age-matched mock-infected mice were used to prepare 10% (w/v) brain homogenates in phosphate-buffered saline (PBS) as stock solutions.

Intracerebral, intraperitoneal, and stereotaxic inoculation

Four-week-old female Jcl:ICR mice were anesthetized with sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) and inoculated either intracerebrally (i.c.) with 20 μ L of 2.5% brain homogenates or intraperitoneally (i.p.) with 200 μ L of 1.0% brain homogenates from prion-infected or age-matched mock-infected mice. In intracerebral inoculation, brain homogenates (20 μ L) were inoculated into the left hemisphere with a two-stage needle of 27-gauge under anesthesia with sevoflurane.

For stereotaxic inoculation, eight-week-old female ICR mice were anesthetized with an intramuscular injection of xylazine (10 mg/kg) and ketamine (100 mg/kg), then placed on a stereotaxic apparatus (Narishige, Tokyo, Japan). A 1 cm midline incision was made on the dorsal skull surface to expose the skull and position the drill over the Bregma point. Coordinates for targeting the right thalamus were anteroposterior (AP) -1.7 mm, medial-lateral (ML), -1.5

mm from Bregma, and 4 mm depth; for the left striatum, AP 1 mm, ML 1.6 mm, and 4 mm depth. Coronal sections for the thalamus and striatum were at Plates 45–46 (Bregma between –1.67 and –1.79 mm) and 22–23 (Bregma between 0.97 and 1.09 mm), respectively (Paxinos and Franklin, 2013). These coordinates avoided ventricles. Brain homogenates (0.5 μ L, 0.1% in PBS) from prion 22L strain-infected or age-matched mock-infected mice were stereotactically inoculated into the thalamus or striatum. Injections were performed with Hamilton syringes (#80301, Hamilton Company, Reno, NV, USA) and 31-gauge steel bevel needles (#7750-22 Hamilton Company) at 0.062 μ L/min using a microinjection pump (#78-8130, KD Scientific, Holliston, MA, USA). The needle remained in place for 2 min post-injection to prevent backflow. The skin incision was closed with a synthetic absorbable suture (#D6284, Ethicon, Raritan, NJ, USA). Needle patency was confirmed before and after injections.

Tissue processing and histology

Mice were euthanized under anesthesia with sevoflurane and their brains were taken and rinsed with PBS, embedded in Tissue-Tek O.C.T. compound (#4583, Sakura Finetek, Torrance, CA, USA) for frozen sections, and stored at –80°C. Ten μ m sagittal plane frozen sections were cut using a cryostat (#Leica CM1950, Leica, Heidelberger Straße, Germany) and mounted on slide glasses (#APS-02, Matsunami, Osaka, Japan). For formalin-fixed paraffin-embedded (FFPE) sections, brains were fixed in 10% neutral buffered formalin (#062-01661, Wako, Osaka, Japan) for 5 days. Whole brains were then cut at the coronal plane to include the entire striatum or thalamus, dehydrated, and embedded in paraffin. FFPE sections were cut into 4 μ m slices using a microtome and mounted on slide glasses (#APS-02, Matsunami). Sections were stained with hematoxylin (#30022, Carazzi's Hematoxylin, Muto Pure Chemicals, Tokyo, Japan) and eosin (#32002, 1% Eosin Y Solution, Muto Pure Chemicals) or subjected to immunohistochemistry (IHC) for PrP^{Sc} detection.

Immunohistochemistry (IHC)

The sections were deparaffinized, rehydrated, and autoclaved at 135°C for 30 min for PrP^{Sc} detection (Song *et al.*, 2008). They were then treated with 3% H₂O₂ in methanol for 5 min, blocked with 5% fetal bovine serum (FBS) in PBS for 30 min at room temperature (r.t.), and incubated overnight at 4°C with anti-PrP monoclonal antibody (mAb) 132 (1 µg/mL). After washing with PBS, the sections were incubated for 1 h at r.t. with anti-mouse IgG F(ab)₂ - Peroxidase antibody (1:2,000, #A3682, Sigma-Aldrich, St. Louis, MS, USA). The sections were washed with PBS and developed using the DAB Substrate Kit, Peroxidase (HRP), with Nickel (#SK-4100, Vector Laboratories, Newark, CA, USA), followed by counterstaining with hematoxylin. Finally, the sections were mounted with Mount-Quick (#Mount-Quick, Daido Sangyo, Tokyo, Japan). Observations and image acquisition were performed using the Keyence BZ-X810 Fluorescence Microscope and BZ-X800 Viewer software (Keyence, Itasca, IL, USA).

Immunofluorescence assay (IFA) for mouse brain

The frozen brain sections were fixed with 4% paraformaldehyde (PFA) (#163-20145, Wako) for 10 min at r.t. After washing with PBS containing 0.01% Tween 20, the sections were permeabilized with 0.1 M glycine and 0.1% Triton X-100 for 20 min at r.t., followed by incubation with 2.5 M guanidine hydrochloride (GdnHCl) for 15 min at r.t. The sections were then blocked with 5% FBS in PBS for 1 h at r.t. and incubated overnight at 4°C with Alexa Fluor 488-conjugated anti-PrP mAb 132 and anti-NeuN rabbit monoclonal antibody (1:2,000, #ab177487, Abcam, Cambridge, UK) in 1% FBS in PBS. For the secondary antibody reaction, the sections were incubated with goat anti-rabbit IgG F(ab')₂ Alexa Fluor 555 antibody (1:1,000, #A-21430, Thermo Fisher Scientific, Waltham, MA, USA) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (#D3571, Thermo Fisher Scientific, 5 µg/mL in 1% FBS in PBS) for nuclei at r.t. for 1 h. Finally, the sections were mounted with Prolong Gold antifade reagent (#P36934, Thermo Fisher Scientific). Tile scan fluorescent images were acquired using

a laser scanning confocal microscope LSM700 (Zeiss, Oberkochen, Germany) with a 20× objective lens.

Primary neuronal cell cultures

In this study, thalamic (ThN), striatal (StN), and cerebral cortex neuronal cultures (CxN) were prepared from ICR mouse embryos at embryonic day 14. Pregnant mice were euthanized with sevoflurane, and the uterus containing the fetuses was removed. The brains of the fetuses were dissected in ice-cold PBS under a stereomicroscope. The medulla oblongata, hippocampi, and meninges were carefully removed, and the thalami, striata, and cerebral cortices were separately collected. Tissue dissociation, cell seeding, and inhibition of astrocyte proliferation were performed as previously described (Tanaka *et al.*, 2016). At 7 days *in vitro* (div), primary neuronal cultures were exposed to microsomal fractions from prion 22L-infected mouse brains, containing 5 ng rPrP equivalent proteinase K (PK) resistant PrP (PrP-res) per 1.0×10^5 cells, by replacing half the medium in each well. At 4 days post inoculation (dpi), the medium was completely replaced with fresh neuronal medium.

SDS-PAGE and immunoblotting

Neurons cultured on 6-well plastic plates were lysed with 300 µL/well of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl [pH 7.5]). The cells underwent one freeze-thaw cycle followed by pipetting. After centrifugation, protein concentrations of the lysates were measured using a DC protein assay kit (#5000116, Bio-Rad-Life Science, Hercules, CA, USA) and adjusted to 0.5-0.8 mg/mL. SDS-PAGE and immunoblotting for PrP-res detection were performed as previously described (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020).

IFA for primary neurons

PrP^{Sc}-specific immunofluorescence staining of prion-infected primary neuronal cultures using anti-PrP mAb 8D5 was performed as previously described (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020). For staining Vglut1, Vglut2, and Vgat, cells were fixed with 4% PFA in PBS at r.t. for 10 min and permeabilized with PBS containing 0.1 M glycine and 0.1% Triton X-100 at r.t. for 10 min. After blocking with 5% FBS in PBS, the cells were incubated with primary antibodies: anti-Vgat rabbit polyclonal antibody (1:4,000, #131002, Synaptic System, Göttingen, Germany), anti-Vglut1 guinea pig polyclonal antibody (1:4,000, #135304, Synaptic System), and anti-Vglut2 guinea pig polyclonal antibody (1:2,000, #135404, Synaptic System). Anti-MAP2 chicken polyclonal antibody (1:2,000, #ab5392, Abcam) was used for staining neurons. Secondary antibodies used were goat anti-chicken IgY conjugated with Alexa Fluor 647 (1:4,000, #ab150173, Abcam) for detecting MAP2, goat anti-rabbit IgG Alexa Fluor 555 (1:4,000, #A21245, Thermo Fisher Scientific) for Vgat, and goat anti-guinea pig IgG Alexa Fluor 488 (1:4,000, #A11073, Thermo Fisher Scientific) for Vglut1 and Vglut2. Nuclei were counterstained with DAPI.

The STAIN Perfect Immunostaining Kit A (#SP-A-1000, ImmuSmol, Bordeaux, France) was used to stain L-glutamate and GABA in glutamatergic and GABAergic neurons in primary neuronal cultures. Cells were fixed, permeabilized, stabilized, and saturated with reagents from the kit according to the manufacturer's instructions. They were then incubated overnight at 4°C with primary antibodies: anti-L-glutamate antibody (1:1,000, #IS1001, ImmuSmol), anti-GABA antibody (1:1,000, #IS1036, ImmuSmol), and mAb 8D5 (1 µg/mL) in the antibody diluent. After three washes with Wash 2 solution, cells were incubated for 60 min at r.t. with secondary antibodies: goat anti-rabbit IgG conjugated with Alexa Fluor 555 antibodies (1:4,000, #A21245, Thermo Fisher Scientific) for anti-L-glutamate, goat anti-chicken IgY conjugated with Alexa Fluor 647 antibodies (1:4,000, #ab150173, Abcam) for anti-

GABA, and F(ab')₂-goat anti-mouse IgG conjugated with Alexa Fluor 488 antibodies (1:4,000, #A-11017, Invitrogen) for PrP^{Sc} in 1% FBS-PBS. Nuclei were counterstained with DAPI.

Stained cells were observed using a Zeiss LSM800 laser scanning microscope (Zeiss) with a 20x objective lens. For higher resolution, Airyscan uses highly sensitive GaAsP detectors on the Zeiss LSM800 with a 63x objective lens. Acquired images were analyzed with ZEN 2.6 software (blue edition).

RNAscope *in situ* hybridization

The RNAscope® 2.5 HD Detection Reagent-RED kit (#322360, ACDbio, Newark, CA, USA) was used for RNAscope *in situ* hybridization following the manufacturer's instructions. FFPE tissue sections were baked at 60°C for 1 h and then deparaffinized. The sections were treated with Hydrogen Peroxide (#322381, ACDbio) for 10 min at r.t. After washing twice with deionized water (DW) for 30 s at r.t., target retrieval was performed by heating the sections at 94°C for 15 min in Target Retrieval Reagent (#322000, ACDbio). After another 30 s wash with DW at r.t., the sections were dehydrated in 100% ethanol for 2 min at r.t. and dried at 60°C for 5 min. Next, the sections were treated with Protease Plus (#322381, ACDbio) for 30 min at 40°C in a moisture chamber within a hybridization incubator and washed twice with DW for 1 min at r.t. The RNAscope probes, including positive control (*Ppib*, #313911), negative control (*Dapb*, #310043), *Vglut1* (*Slc17a7*, #416631), *Vglut2* (*Slc17a6*, #319171), and *Vgat* (*Slc32a1*, #319171) (ACDbio), were pre-warmed at 40°C for 10 min and then hybridized to the tissue sections for 2 h at 40°C in the moisture chamber. After hybridization, the sections were washed with Wash Buffer (#310091, ACDbio) for 2 min at r.t. and then proceeded to signal amplification. For this step, the sections were sequentially incubated with AMP1 for 30 min, AMP2 for 15 min, AMP3 for 30 min, AMP4 for 15 min, AMP5 (containing preamplifiers and amplifiers) for 30 min, and AMP6 for 15 min at 40°C in a moisture chamber, following the manufacturer's instructions. Color development was performed using a mixture of RED-A and

RED-B solutions at a 60:1 ratio for 10 min at r.t. in the dark. The development was stopped by washing with tap water for 2 min. For counterstaining, the sections were stained with hematoxylin. To enhance the contrast between the reddish color developed by the kit and the reddish color of hematoxylin staining, the color of hematoxylin was converted to blue by soaking the sections in 0.02% ammonia for 10 s at r.t. Finally, the tissue sections were dehydrated at 60°C for 15 min, soaked twice in xylene for 3 s each, and mounted with Mount-Quick.

Quantitative analysis of RNAscope staining with deep learning

RNAscope staining bright field images were captured using a Nano Zoomer 2.0RS digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). The virtual slide images were read and cropped with QuPath, an open-source image analysis software. Four brain regions were used as indicators for cropping thalamus images: the hippocampus (dorsal border), the third ventricle (medial border), the reticular nucleus (RT) (lateral border), and the zona incerta (ventral border). The cropped images were further divided into 500×500 pixel ($112.7 \times 112.7 \mu\text{m}$) parcels for annotation and analysis. The number of parcels covered the entire thalamus: 15–18 parcels for the mediolateral axis and 17 parcels for the dorsoventral axis.

To develop a deep learning model for classifying RNAscope-positive and negative cells, Microsoft VoTT (Visual Object Tagging Tool, Microsoft, Redmond, WA, USA) was used for annotation. Sections counterstained with hematoxylin allowed the identification of RNAscope-positive cells by red punctate signals around hematoxylin-labeled nuclei. A total of 366 images were annotated, identifying 2,929 RNAscope-positive cells and 14,115 negative cells, resulting in 17,044 cells. The annotated dataset was exported to Roboflow (ver1.1.44, Roboflow Inc., Iowa, USA) to create training, validation, and test splits: 70% for training, 20% for validation, and 10% for testing.

The computer-assisted object detection and classification algorithm, You Only Look Once version 5 (YOLOv5) (Jocher *et al.*, 2021; Redmon *et al.*, 2016) was used for the precise classification of stained cells. YOLOv5x served as the trained model. The optimal model was selected with a learning rate between 0.01 and 0.1. Model fitness was evaluated using mean average precision (mAP), calculated by averaging the precision over two classes and/or various intersections over union (IoU) thresholds. IoU thresholds were determined by dividing the area of overlap by the area of union. Two mAP values, using IoU thresholds of 0.5 and 0.5-0.9, were employed and weighted at 0.1 and 0.9, respectively.

Statistical analysis.

Statistical analyses were performed using JMP Pro version 17 (JMP, Cary, NC, USA).

RESULTS

Distribution of PrP^{Sc} in brains of prion-infected mice

To investigate PrP^{Sc} distribution in prion 22L strain-infected mice brains, I analyzed PrP^{Sc} deposition using sagittal cryosections from mice inoculated i.c. or i.p. with prions, at 120 and 186 dpi, respectively, around the clinical onset (Figure I-2). PrP^{Sc} signals were absent in age-matched mock-infected mice (Figures I-2A and I-2E), while intense PrP^{Sc} signals were observed in the cerebral cortex and thalamus of i.c. inoculated mice (Figure I-2I). Conversely, PrP^{Sc} signals in the striatum were notably weaker than in the cerebral cortex and thalamus (Figure I-2I). The PrP^{Sc} distribution in i.p. inoculated mice was similar to that in i.c. inoculated mice; intense signals were seen in the cerebral cortex and thalamus, but weaker in the striatum (Figure I-2M). This weak PrP^{Sc} staining in the striatum, regardless of the inoculation route, suggests inefficient 22L prion propagation in the striatum. A similar weaker PrP^{Sc} signal in the striatum compared to the cerebral cortex and thalamus was also observed in mice i.c. inoculated with Chandler and Obihiro strains (Figure I-1).

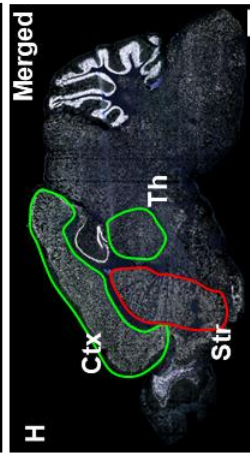
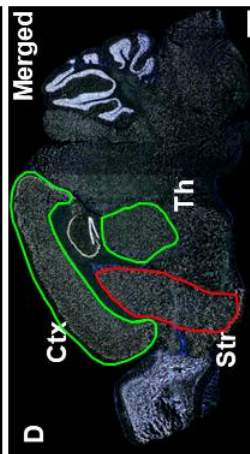
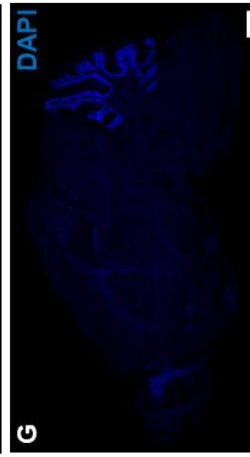
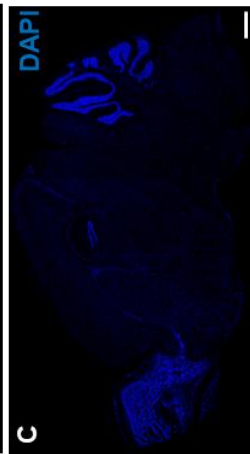
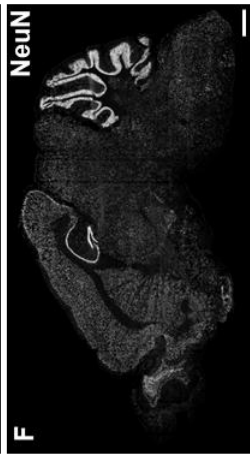
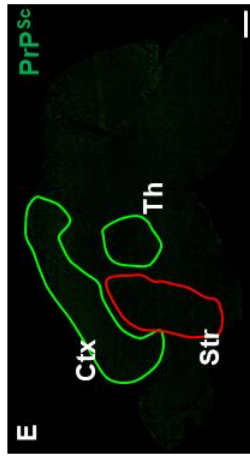
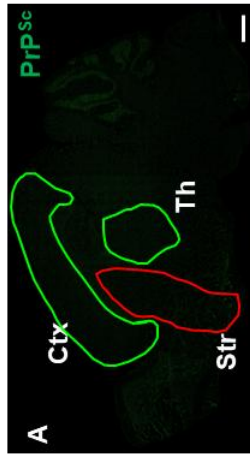
The weak PrP^{Sc} deposition in the striatum suggests that prions propagate more efficiently in the cerebral cortex and thalamus. To further analyze this, I stereotactically injected 22L prions into either the thalamus or striatum and analyzed PrP^{Sc} deposition by IHC (Figure I-3). No visible PrP^{Sc}-positive stains were observed in the thalamus and striatum after inoculating age-matched mock-infected brain homogenates (Figures I-3A to I-3C, I-3G to I-3I). In the thalamus, positive signals were observed along the needle track at 1 dpi, likely from the PrP^{Sc} in the inoculum (Figure I-3D). By 14 dpi, diffuse PrP^{Sc} stains were observed both along and away from the needle track (Figure I-3F). Conversely, in the striatum, PrP^{Sc} stains were observed mainly along the needle tracks up to 14 dpi (Figures I-3J to I-3L). These results suggest that

Mock

22L

IC

IP



IC

IP

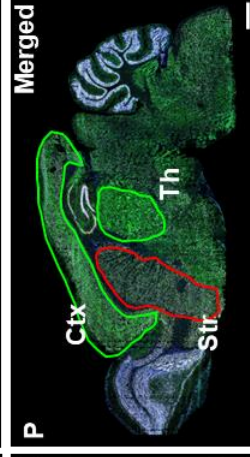
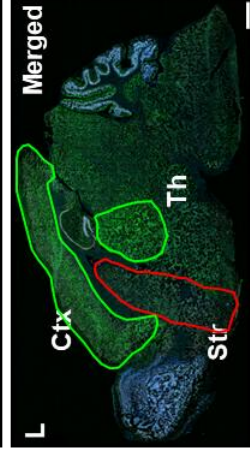
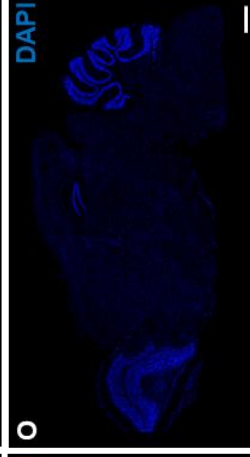
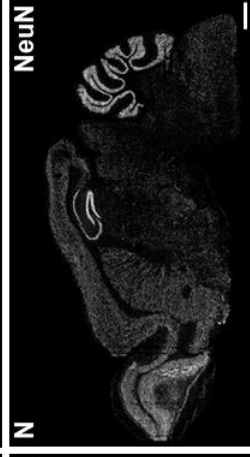
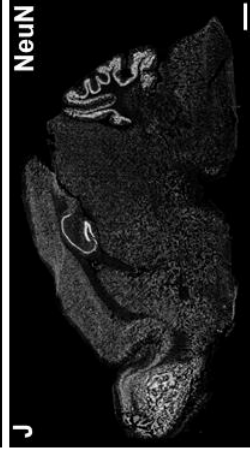
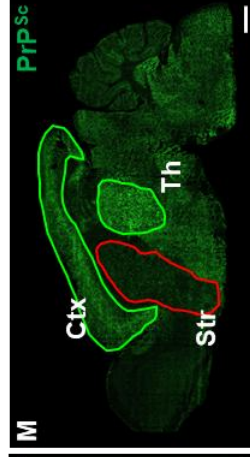
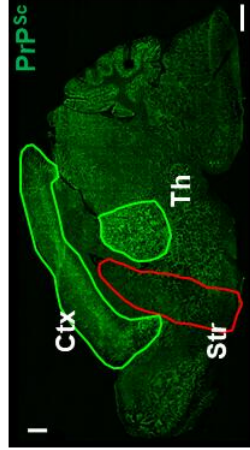


Figure I-2 Distribution of PrP^{Sc} in the brains of prion-infected mice.

Mice were inoculated intracerebrally (i.c.) or intraperitoneally (i.p.) with 20 µL of 2.5% or 200 µL of 1.0% brain homogenates, respectively, from either mock-infected (A-D, E-H) or prion 22L strain-infected mice (I-L, M-P). Mice were sacrificed at 120 dpi (i.c.) or 186 dpi (i.p.), and their brains were subjected to cryosection. Sagittal sections were cut using a cryostat and stained with anti-PrP mAb 132 to detect PrP^{Sc} (green, A, E, I, M) and anti-NeuN mAb for neuronal cells (white, B, F, J, N). Nuclei were counterstained with DAPI (blue, C, G, K, O). Merged images are shown (D, H, L, P). Images were acquired using a 20× objective lens and are tile scans of sagittal sections generated with ZEN2009 software [black edition]. The cortex (Ctx) and thalamus (Th) regions are outlined in green, while the striatum (Str) is outlined in red. Scale bars: 1 mm. Sagittal brain sections around Plates 111 to 113 (Paxinos and Franklin, 2013) were used.

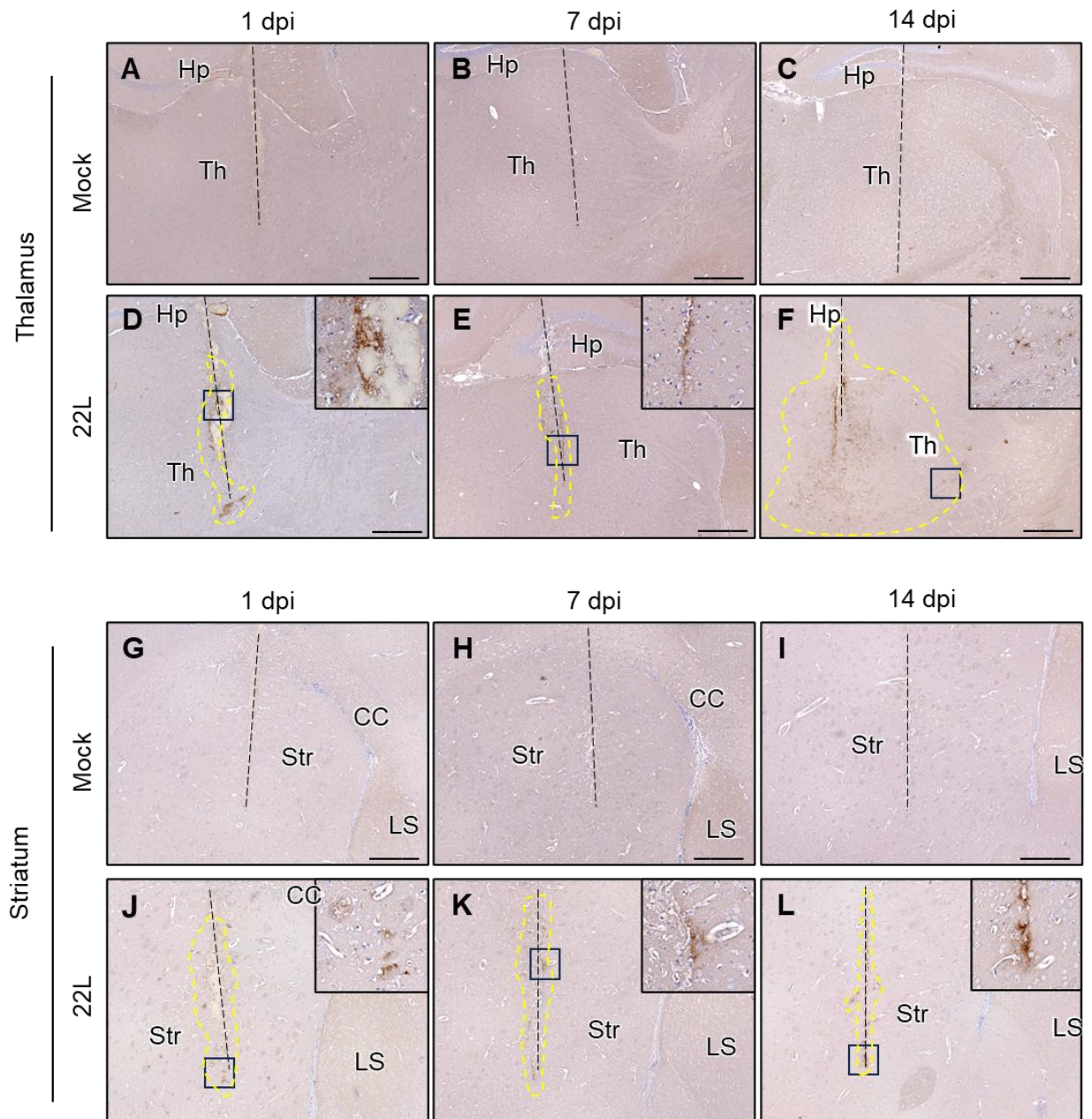


Figure I-3 PrP^{Sc} detection after stereotaxic injection of prions.

Brain homogenates from mock- or 22L prion-infected mice (0.1%, 0.5 μ L) were injected into the thalamus (A-F) or striatum (G-L) using a stereotaxic apparatus. Brains were collected at 1 (A, D, G, J), 7 (B, E, H, K), and 14 (C, F, I, L) days post-injection and fixed with 10% neutral buffered formalin. Coronal sections corresponding to Plates 45–46 (thalamus) and 22–23 (striatum) (Paxinos and Franklin, 2013) were cut for immunohistochemical detection of PrP^{Sc} using anti-PrP mAb 132. FFPE blocks containing either the thalamus or striatum were serially cut into 4 μ m sections at the coronal plane using a microtome. Sections in which needle tracks were observed were used for PrP^{Sc} staining with IHC. Dashed lines indicate needle tracks. Higher magnifications of boxed regions in the 22L strain-injected thalamus and striatum are shown in the upper-right corners of each lower magnification image. The region surrounded by dotted lines in the 22L strain-injected thalamus (F) indicates the spread of PrP^{Sc}-positive regions from the needle track. Th: thalamus, Hp: hippocampus, Str: striatum, CC: corpus callosum, LS: lateral septum. Scale bars: 300 μ m. Dotted lines indicate regions with positive signals determined using Image J (ver 1.8.0).

22L prions propagate more in the thalamus than in the striatum. We did not analyze prion propagation in the cerebral cortex due to its insufficient thickness for stereotaxic injection.

Distribution of glutamatergic excitatory and GABAergic inhibitory neurons in mouse brain

Reports indicate that the cerebral cortex has a high proportion of Vglut1-positive excitatory neurons, while Vglut1 or Vglut2-positive neurons are present in the thalamus, and Vgat-positive inhibitory neurons predominate in the striatum (Fremeau *et al.*, 2004; Rudy *et al.*, 2011). Given the inverse relationship between GABAergic neuron distribution and PrP^{Sc} deposition in the striatum (Figure I-2), I first confirmed the gene expression of *Vgat* (a marker for GABAergic inhibitory neurons) and *Vglut1* and *Vglut2* (markers for glutamatergic excitatory neurons) in mouse brains using RNAscope *in situ* hybridization (Figure I-4). *Vglut1*-positive cells were mainly observed in the cerebral cortex, pyramidal cell layers, dentate gyrus of the hippocampus, thalamus, and granular cell layer of the cerebellum. *Vglut2*-positive cells were primarily found in the thalamus and midbrain. On the contrary, *Vgat*-positive cells were predominantly located in the striatum and reticular nucleus of the thalamus, with an additional presence in the midbrain and sparse distribution in the cerebral cortex (Figure I-4).

Prion propagation in primary neuronal cultures from cortex, thalamus, and striatum

The predominance of GABAergic neurons (Figure I-4) and the inefficient 22L prion propagation in the striatum (Figures I-2 and I-3) suggest a neuronal cell-type-dependent propagation efficiency of the 22L strain. To investigate this, I prepared primary neuronal cultures from cortices (CxN), thalami (ThN), and striata (StN) to analyze prion propagation *in vitro* based on neuronal cell type.

First, I examined the expression of Vglut1 and Vglut2, synaptic vesicle membrane transporters specific to glutamatergic excitatory neurons, and Vgat, a synaptic GABA

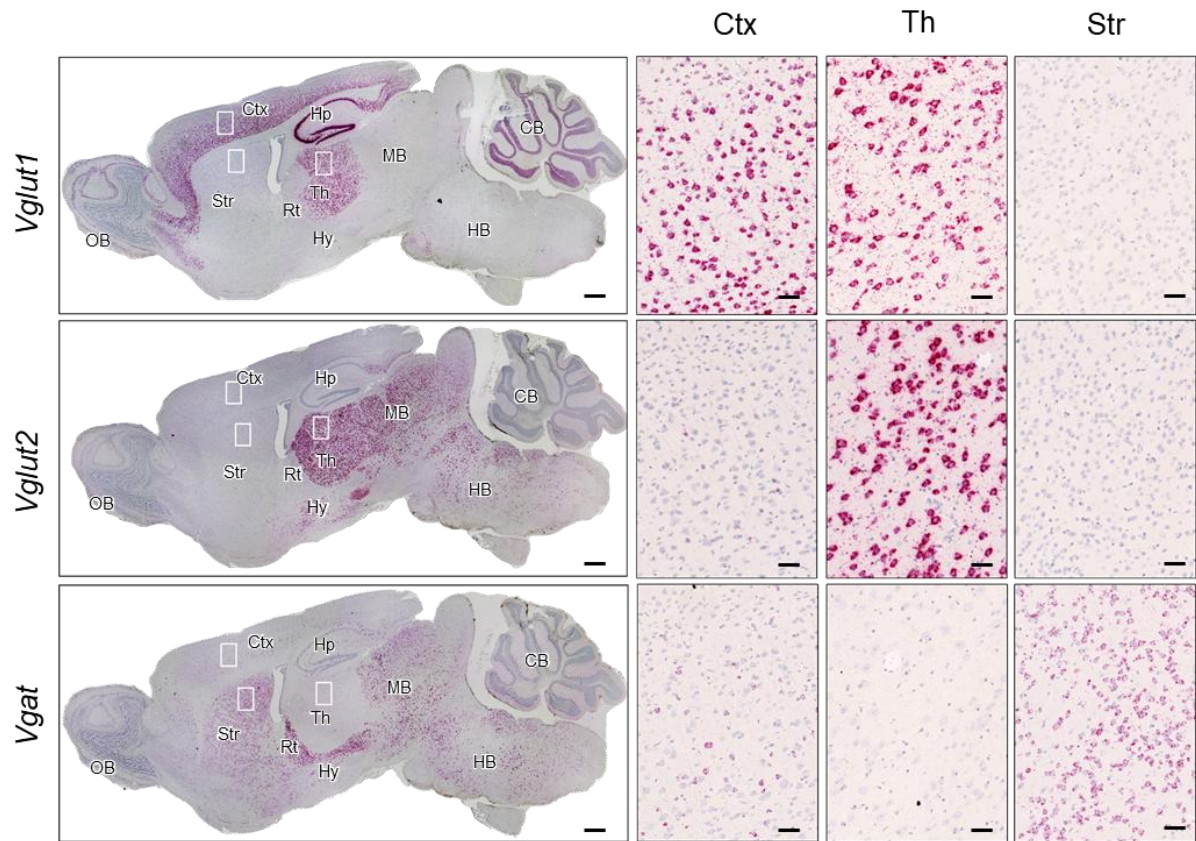


Figure I-4 Expression of *Vglut1*, *Vglut2*, and *Vgat* mRNA in mouse brain.

RNAscope *in situ* hybridization was used to detect the gene expression of *Vglut1* and *Vglut2*, markers for glutamatergic neurons, and *Vgat*, a marker for GABAergic neurons, in sagittal brain sections from an uninfected adult ICR mouse. The leftmost column displays a whole sagittal section, while the three images on the right show magnified views of the boxed regions in the cortex (Ctx), thalamus (Th), and striatum (Str). Positive signals for the target RNA appear reddish, developed with RNAscope® 2.5 HD Detection Reagent (RED), while counterstaining with hematoxylin produces light blue staining. Images were captured with a NanoZoomer 2.0RS (Hamamatsu Photonic K.K., Hamamatsu, Japan) using a 40× objective and stitched with NanoZoomer 2.0RS software (Hamamatsu Photonic K.K., Hamamatsu, Japan). Scale bars are 1 mm in the sagittal plane images and 0.1 μ m in the magnified images. OB: olfactory bulb, Hp: hippocampus, Rt: reticular nucleus, Hy: hypothalamus, MB: midbrain, HB: hindbrain, CB: cerebellum.

transporter of GABAergic and glycinergic neurons, in each primary neuronal culture, using immunofluorescence assay to assess if the proportion of Vglut1-, Vglut2-, and Vgat-positive neurons in the cultures reflect their proportion in the brain regions from which the tissues were collected (Figure I-5). Vglut1-positive neurons were predominantly observed in CxN, with a few Vglut2- and Vgat-positive neurons also present. In ThN, Vglut2-positive neurons were mainly observed, with some Vgat-positive neurons. Conversely, Vgat-positive neurons were the most abundant in StN (Figures I-5A and I-5B). Since the proportion of Vglut1-, Vglut2-, and Vgat-positive neurons in each neuronal culture from mouse embryos appears roughly consistent with their proportions in adult mouse brains (as shown by RNAscope analysis in Figure I-4), I used CtN, ThN, and StN to analyze whether prion propagation differs depending on neuronal cell type.

My laboratory previously reported that anti-PrP mAb 8D5 detects PrP^{Sc} as string-like stains in prion-infected primary neurons under non-denatured conditions (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020). Figure I-6A shows immunofluorescence detection of PrP^{Sc} using mAb 8D5 in neurons exposed to microsomal fractions from 22L-infected mouse brains. String-like stains were observed in all three neuronal cultures infected with 22L prions, while no PrP^{Sc} stains were seen in mock-infected cultures (Figure I-6A). Immunoblotting revealed time-dependent increases in PrP-res from 7 to 28 dpi in CxN, ThN, and StN exposed to 22L prions, indicating successful prion propagation (Figure I-6). Additionally, PrP-res levels in CxN appeared higher than in ThN and StN (Figure I-6C). Although neurons with string-like PrP^{Sc} stains were present in all three cultures, the frequency of such neurons appeared to vary. Therefore, I classified PrP^{Sc}-positive neurons based on the number of string-like PrP^{Sc} stains (Tanaka *et al.*, 2020) to compare prion propagation efficiency among neuronal cultures.

Figure I-7A illustrates the classification of PrP^{Sc}-positive neurons based on the number of string-like PrP^{Sc} signals on the surface of the soma. Neurons with five or more, fewer than five, and no apparent string-like PrP^{Sc} signals surrounding the soma were categorized as

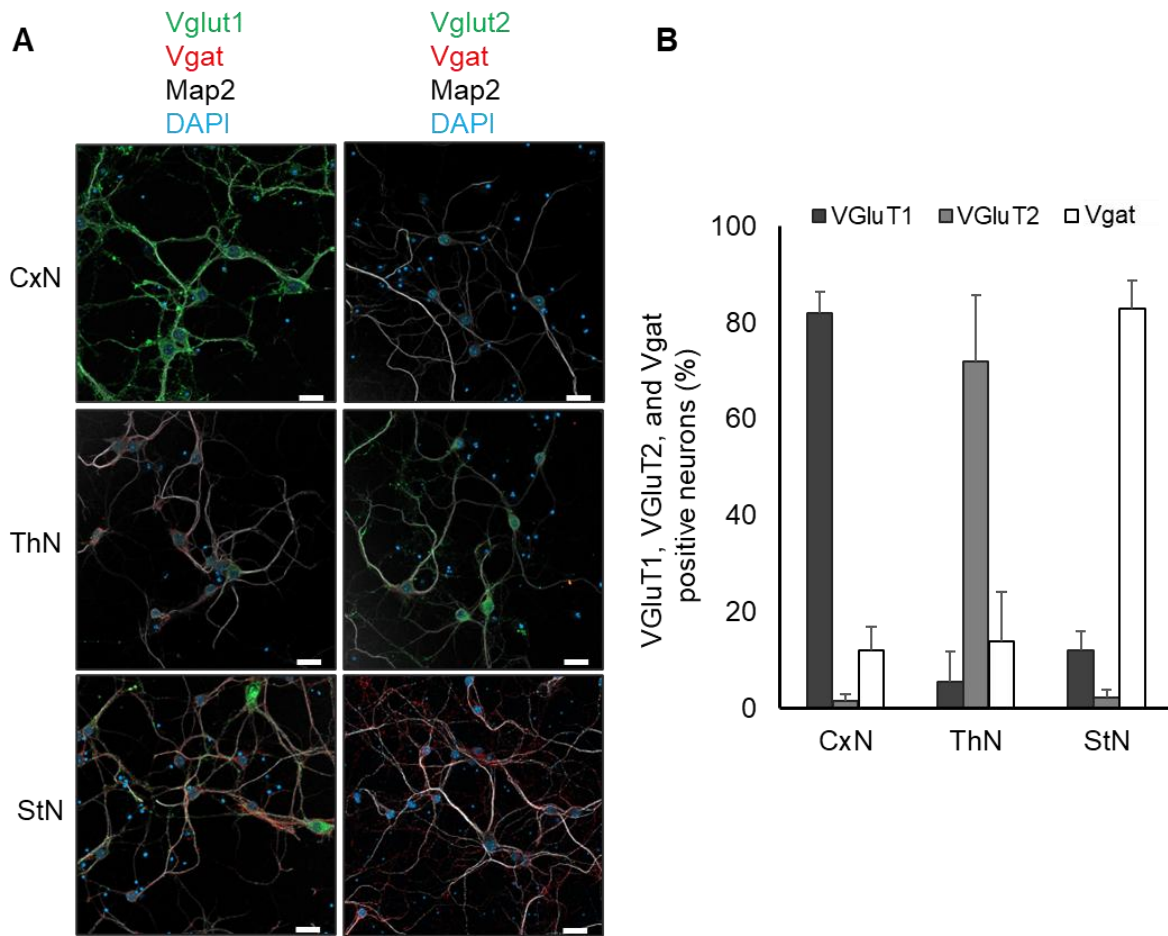


Figure I-5 Expression of Vglut1, Vglut2, and Vgat in primary neuronal cultures.

(A) Expression of markers for glutamatergic excitatory and GABAergic inhibitory synapses in primary neuronal cultures from cortices (CxN, top), thalami (ThN, middle), and striata (StN, bottom). Neuronal cell bodies and dendrites were visualized with anti-MAP2 polyclonal antibody (pAb) (gray). Glutamatergic excitatory synaptic markers were stained with anti-Vglut1 (green, left column) or Vglut2 (green, right column), while GABAergic inhibitory synaptic markers were detected with anti-Vgat (red). Scale bars: 20 μ m. (B) Proportion of Vglut1-, Vglut2-, and Vgat-positive cells in CxN, ThN, and StN. The graph shows the mean $\pm$ SD of six independent experiments. A total of 277, 225, and 398 cells from CxN, ThN, and StN, respectively, were analyzed. Total cell numbers included cells that appeared negative for Vglut1, Vglut2, and Vgat: 14 in CxN, 20 in ThN, and 11 in StN.

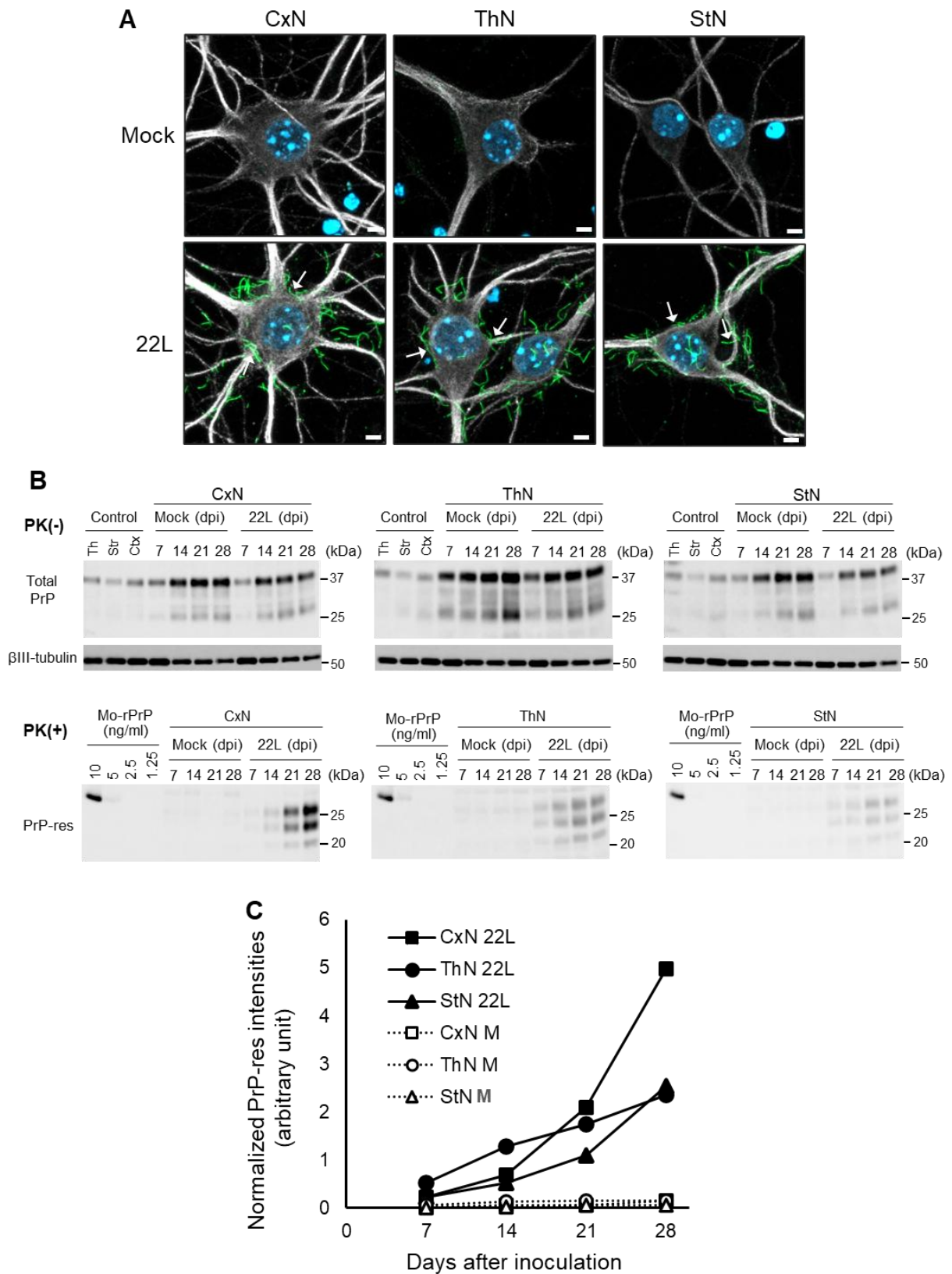
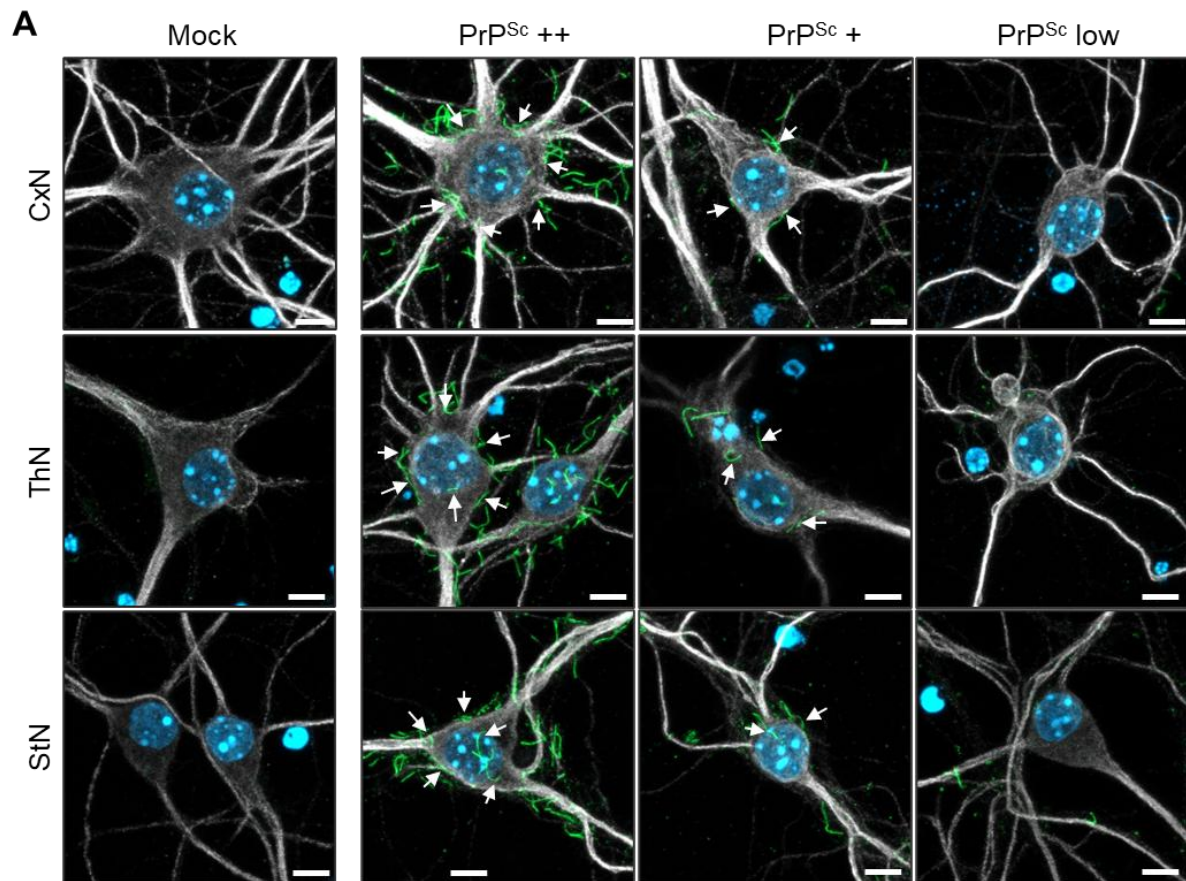


Figure I-6 Detection of PrP^{Sc} in primary neuronal cultures infected with prions.

(A) PrP^{Sc} detection in 22L prion-infected neuronal cultures using PrP^{Sc}-specific immunostaining with mAb 8D5 (green) at 14 dpi. Upper and lower panels show mock-infected and 22L prion-infected primary neuronal cultures from cortices (CxN), thalami (ThN), and striata (StN), respectively. Arrows indicate string-like PrP^{Sc} strains. Neuronal dendrites were visualized with anti-MAP2 pAb (#ab18207, Abcam) staining (gray), and nuclei were counterstained with DAPI (blue). Scale bars: 5 μ m. (B) Representative immunoblot images of total PrP, β III-tubulin, and PrP-res in mock- and prion 22L strain-infected primary neuronal cultures at 7, 14, 21, and 28 dpi. Cell lysates without PK treatment (PK (-)) were used to detect total PrP (mAb 31C6) and β III-tubulin, while PK-treated cell lysates (PK (+)) were used to detect PrP-res (mAb 31C6). Recombinant PrP (rPrP) was used for quantification of PrP-res and standardization among different blots. Controls represent lysates of each neuronal culture at 21 days in vitro. Molecular markers on the right are in kDa. (C) Quantification of PrP-res in (B). The amount of PrP-res in different blots was standardized using rPrP intensities, and PrP-res levels were normalized with corresponding β III-tubulin signal intensities. Results from two independent experiments are shown.



B

	PrP ^{Sc} ++	PrP ^{Sc} +	PrP ^{Sc} low
CxN	99**	24(*)	8(*)
ThN	133	52	20
StN	97(**)	52	27*

$p < 0.01$, χ^2 test

Figure I-7 Classification of prion-infected neurons by degree of string-like PrP^{Sc} signals.

(A) Immunofluorescence detection of PrP^{Sc}: Primary cortical (CxN), thalamic (ThN), and striatal (StN) neuronal cultures were infected with the prion 22L strain. At 14 dpi, cultures were stained for PrP^{Sc} using anti-PrP mAb 8D5 (green) and for neuronal bodies and dendrites using anti-MAP2 pAb (gray). Nuclei were counterstained with DAPI (blue). Neurons with five or more, fewer than five, and no apparent string-like PrP^{Sc} signals around the soma were classified as PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low neurons, respectively. Mock indicates mock-infected cultures. Arrows highlight string-like PrP^{Sc} stains. Scale bars: 5 μ m. (B) Classification of PrP^{Sc}-positive neurons: A total of 131 neurons from CxN (9, 54, 68: cell numbers per experiment), 205 neurons from ThN (39, 87, 79), and 176 neurons from StN (19, 81, 76) were classified into PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low categories across three experiments. Statistical analysis was performed using the χ^2 test, followed by residual analysis. ** and (**) indicate items with higher and lower than expected counts, respectively ($p < 0.01$), while * and (*) indicate items with higher and lower expected counts, respectively ($p < 0.05$), by residual analysis.

PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low neurons, respectively (Figure I-7A). I conducted three independent experiments, classifying a total of 131, 205, and 176 neurons from CxN, ThN, and StN, respectively, into these categories (Figure I-7B). The frequency of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low neurons varied significantly among CxN, ThN, and StN (χ^2 test, $p < 0.05$). The frequency of PrP^{Sc} ++ neurons was significantly higher than the expected count in CxN, while PrP^{Sc} + and PrP^{Sc} low neurons were significantly lower than the expected count. In contrast, StN had significantly lower frequencies of PrP^{Sc} ++ and higher frequencies of PrP^{Sc} low neurons (residual analysis following χ^2 test). These results suggest that the 22L strain propagates more efficiently in CxN than in StN.

Prion propagation in glutamatergic and GABAergic primary neurons

The predominance of Vglut1-positive neurons in CxN aligns with the abundance of Vglut1-positive neurons in the cerebral cortex, while the predominance of Vgat-positive neurons in StN corresponds with the abundance of GABAergic neurons in the striatum. These observations suggest that the propagation efficiency of the 22L strain differs between glutamatergic excitatory and GABAergic inhibitory neurons. To explore this possibility, I first distinguished glutamatergic neurons from GABAergic neurons using the STAIN Perfect Immunostaining Kit A (Figure I-8). The kit identified glutamatergic neurons as L-glutamate-positive but GABA-negative, and GABAergic neurons as both L-glutamate- and GABA-positive. Most neurons in the CNS use L-glutamate as an excitatory neurotransmitter, while GABA is used as an inhibitory neurotransmitter in inhibitory neurons (Amara and Kuhar, 1993). As expected, most neurons in primary cultures were divided into two populations: L-glutamate-positive but GABA-negative neurons (Figure I-8, upper panel) and L-glutamate- and GABA-positive neurons (Figure I-8, lower panel). CxN comprised approximately 70% glutamatergic and 23% GABAergic neurons; ThN had about 62% glutamatergic and 29% GABAergic

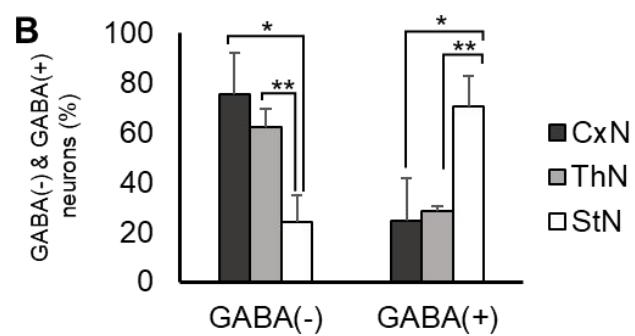
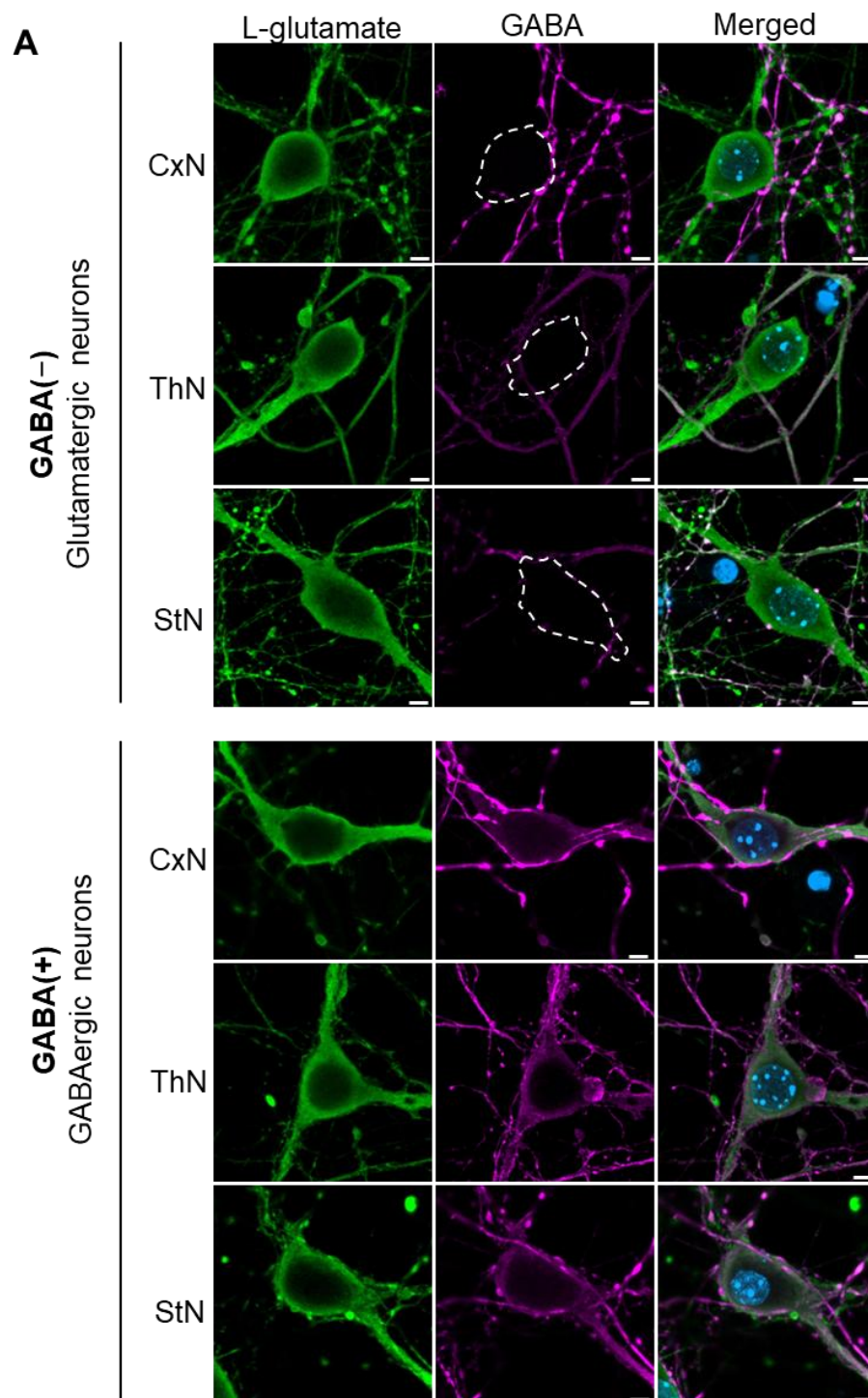
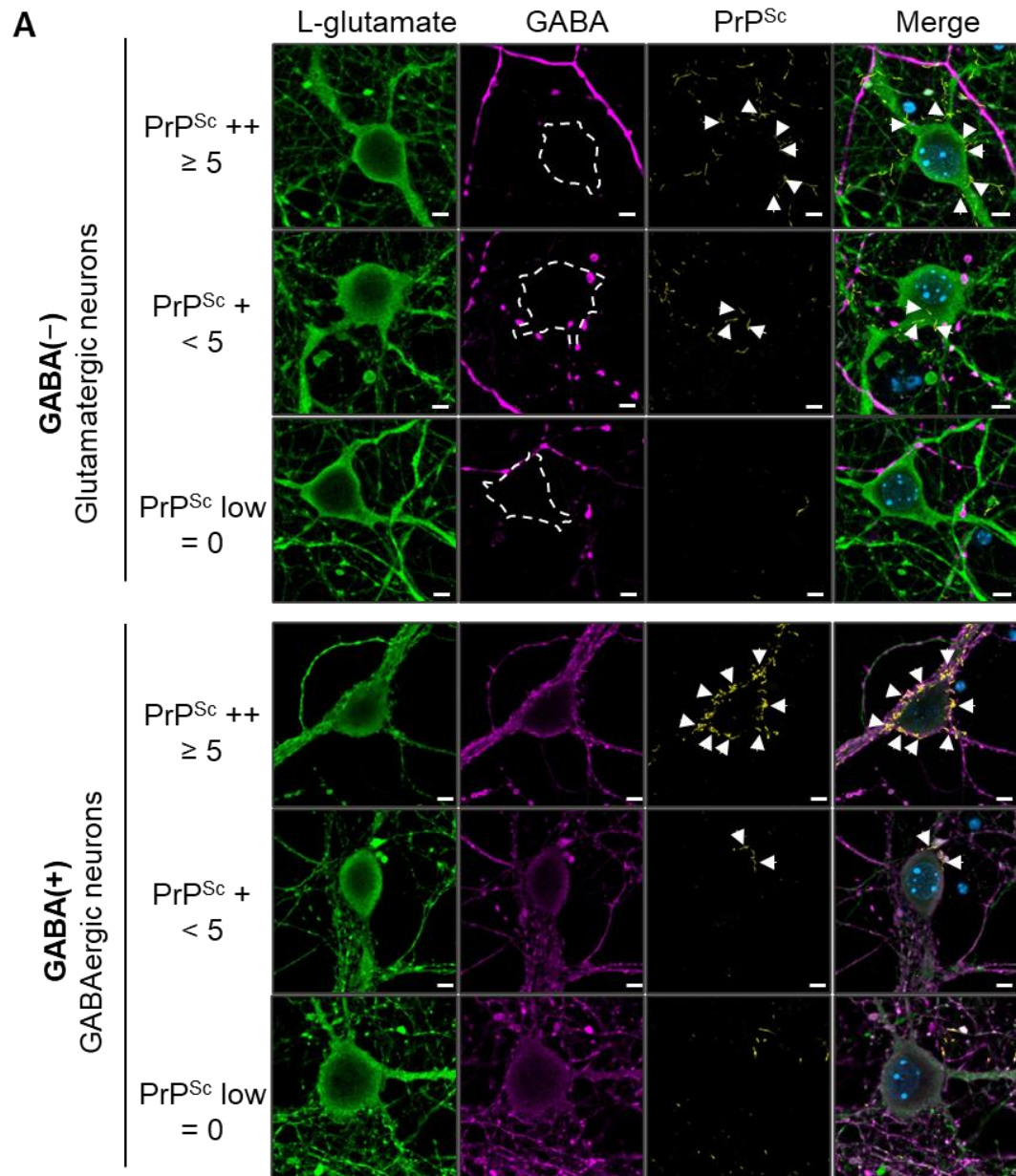


Figure I-8 Distinction between GABA-negative glutamatergic neurons and GABA-positive GABAergic neurons.

(A) Distinction of glutamatergic and GABAergic neurons: Primary cortical (CxN), thalamic (ThN), and striatal (StN) neuronal cultures at 14 dpi were co-stained with anti-L-glutamate (green) and anti-GABA (violet) antibodies using the STAIN Perfect Immunostaining Kit A. Nuclei were counterstained with DAPI (blue) to distinguish GABA-negative glutamatergic neurons from GABA-positive GABAergic neurons. Representative images of GABA-negative glutamatergic neurons (upper panel) and GABA-positive GABAergic neurons (lower panel) from three different neuronal cultures are shown. Cells enclosed by dotted-lines are L-glutamate-positive but GABA-negative. Scale bars: 5 μ m. (B) Proportion of GABA-negative glutamatergic and GABA-positive GABAergic neurons: A total of 184, 234, and 190 cells from CxN, ThN, and StN, respectively, were analyzed. Total cell numbers included cells that appeared negative for both glutamate and GABA: 14 in CxN, 22 in ThN, and 10 in StN. Graphs show mean $\pm$ SD from three independent experiments. Statistical analysis was performed using Student's *: $p < 0.05$, **: $p < 0.01$.

neurons, while StN consisted of roughly 71% GABAergic and 24% glutamatergic neurons (Figure I-8).

I then performed multiple immunofluorescence staining using the STAIN Perfect Immunostaining Kit A to detect L-glutamate and GABA, along with mAb 8D5 for PrP^{Sc}. Figure 9A shows representative images for classifying PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low neurons in either glutamatergic (upper panel) or GABAergic neurons (lower panel). Neurons were categorized based on the string-like PrP^{Sc} stains around the soma, as in Figure I-9. A total of 145, 209, and 185 neurons in CxN, ThN, and StN, respectively, were analyzed. The frequencies of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low neurons in GABA-negative and GABA-positive neurons in CxN differed significantly ($p < 0.05$, Fisher's exact test). Significant differences were also observed in ThN ($p < 0.05$, χ^2 test), with residual analysis showing a significantly lower frequency of PrP^{Sc} low GABA-negative neurons and a significantly higher frequency of PrP^{Sc} low GABA-positive neurons ($p < 0.05$). Significant differences were also observed in StN ($p < 0.01$, χ^2 test); the frequency of PrP^{Sc} ++ GABA-negative neurons was significantly higher, while that of PrP^{Sc} ++ GABA-positive neurons was significantly lower than the corresponding expected counts. Given the varying proportions of GABA-negative and GABA-positive neurons among the three neuronal cultures, I combined the results and compared the frequencies of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low in GABA-negative and GABA-positive neurons. The frequencies of PrP^{Sc} ++ neurons were significantly higher than the expected count in GABA-negative neurons and lower than the expected count in GABA-positive neurons ($p < 0.01$). Conversely, the frequencies of PrP^{Sc} low neurons were significantly lower in GABA-negative neurons and higher in GABA-positive neurons ($p < 0.01$). These findings indicate that the 22L prion strain propagates more efficiently in GABA-negative glutamatergic neurons than in GABA-positive GABAergic neurons in primary neuronal cultures.



B

CtN (145)	PrP ^{Sc}		
	++	+	low
GABA(-)	78	39	12
GABA(+)	6	10	0
$p < 0.05$, Fisher's exact test			

ThN (209)	PrP ^{Sc}		
	++	+	low
GABA(-)	89	50	11(*)
GABA(+)	29	18	12*
$p < 0.05$, χ^2 test			

StN (185)	PrP ^{Sc}		
	++	+	low
GABA(-)	39**	11	3(*)
GABA(+)	66(**)	42	24*
$p < 0.01$, χ^2 test			

Total (539)	PrP ^{Sc}		
	++	+	low
GABA(-)	206**	100	26(**)
GABA(+)	101(**)	70	36**
$p < 0.01$, χ^2 test			

Figure I-9 PrP^{Sc} staining with glutamatergic and GABAergic neurons.

(A) Representative images: At 14 dpi, primary neurons infected with the prion 22L strain were co-stained with anti-L-glutamate Ab (green, leftmost column), anti-GABA Ab (violet, second column from left), and anti-PrP mAb 8D5 (yellow, second column from right). Nuclei were counterstained with DAPI (blue). The rightmost column shows merged images. Representative images of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low glutamatergic neurons (upper panel) and GABA-negative GABAergic neurons are shown. Classification of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low is described in Fig. 6. Arrows indicate string-like PrP^{Sc} stains. Scale bars: 5 μ m. (B) Comparison of PrP^{Sc}-positive neurons: Tables show the numbers of PrP^{Sc} ++, PrP^{Sc} +, and PrP^{Sc} low cells in GABA-negative and GABA-positive neurons in CxN, ThN, and StN. A total of 145 CxN (48, 47, 50: cell numbers per experiment), 209 ThN (41, 107, 61), and 185 StN (57, 77, 51) were analyzed. Statistical analysis was performed using the χ^2 test, followed by residual analysis. ** and (***) indicate items with higher and lower than expected counts, respectively ($p < 0.01$), while * and (*) indicate items with higher and lower than expected counts, respectively ($p < 0.05$) by residual analysis.

Type of neurons that are lost in the thalamic nuclei

My results indicate that the 22L strain propagates more efficiently in glutamatergic neurons than in GABAergic neurons. My laboratory has observed massive neuronal loss in the lateral thalamus of Jcl:ICR mice infected with the 22L, Chandler, and Obihiro strains (unpublished observation), similar to previous reports (Cunningham *et al.*, 2005; Reis *et al.*, 2015). Thus, to identify the types of neurons lost in the lateral thalamus of 22L strain-infected mice, I analyzed *Vglut1*, *Vglut2*, and *Vgat*-expressing cells using RNAscope *in situ* hybridization (Figure I-10). *Vglut1* and *Vglut2* expression was observed in both the lateral and medial thalamus. Compared to age-matched mock-infected mice, the number of *Vglut1*- and *Vglut2*-expressing neurons significantly decreased in the ventral posterolateral nucleus (VPL). In the ventral posteromedial nucleus (VPM), *Vglut1*-expressing neurons tended to decrease but no apparent decrease in *Vglut2*-expressing neurons was observed in 22L strain-infected mice (Figure I-10B). No decrease in *Vgat*-expressing neurons was observed in the reticular nucleus of the thalamus (RT), which is adjacent to VPL and VPM and primarily composed of *Vgat*-expressing interneurons.

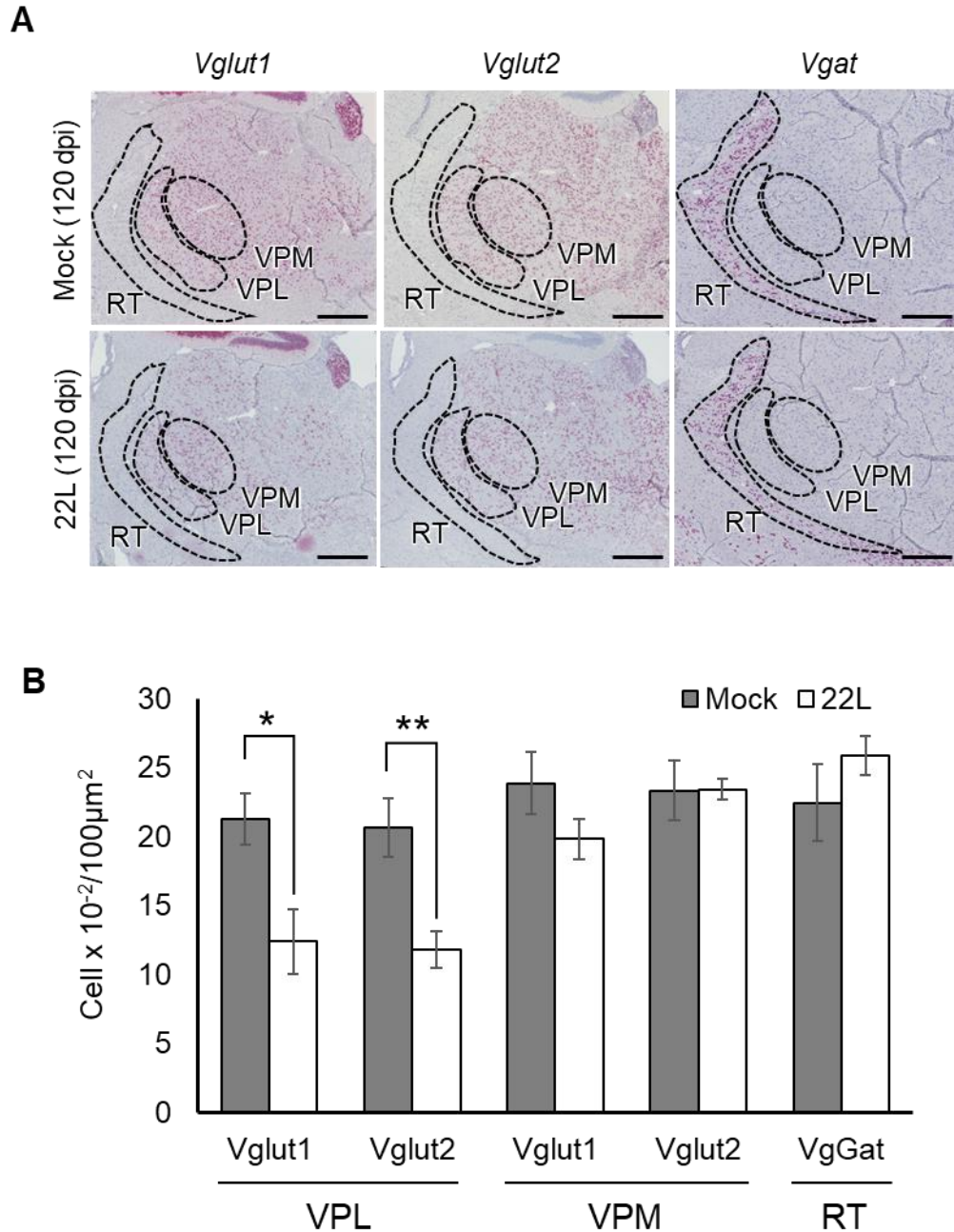


Figure I-11 Type of thalamic neurons lost in prion infection.

(A) RNAscope analysis: RNAscope *in situ* hybridization was used to detect the gene expression of *Vglut1* and *Vglut2*, markers for glutamatergic neurons, and *Vgat*, a marker for GABAergic neurons, in the thalamus of prion 22L strain-infected mice at 120 dpi. Coronal sections around Plate 45–46 (Paxinos and Franklin, 2013) were used. Positive signals for the target RNA were developed with RNAscope® 2.5 HD Detection Reagent - RED, showing a reddish color, while counterstaining with hematoxylin produced light blue staining. Images were captured with a NanoZoomer 2.0RS using a 40× objective and stitched with NanoZoomer 2.0RS software. The ventral posterolateral nucleus (VPL), ventral posteromedial nucleus (VPM), and reticular nucleus (RT) are enclosed with dashed lines. Scale bar: 0.5 mm. (B) Quantitative analysis: Neurons positive for *Vglut1*, *Vglut2*, or *Vgat* were counted as described in the materials and methods. Graphs show the number of positive cells per 100 μm^2 (mean $\pm$ SD of 3 brains from either mock- or prion-infected mice). Three sections were analyzed from each mouse. Statistical analysis was performed using Student's *t*-test, *: $p < 0.05$, **: $p < 0.01$

DISCUSSION

The distribution of PrP^{Sc} and neuropathological lesions varies depending on prion strains as well as the combination of prion and mouse strains (Bruce *et al.*, 1994; Bruce, 1993; DeArmond *et al.*, 1993). In this study, I attempted to address the underlying mechanisms using *in vivo* and *ex vivo* experiments. I found that the inefficient propagation of the 22L strain in GABAergic inhibitory neurons contributes, if not entirely, to the weaker PrP^{Sc} deposition in the striatum compared to the cerebral cortex and thalamus in 22L strain-infected mice (Figure I-2). After stereotaxic inoculation of the 22L strain into the striatum, which is primarily composed of GABAergic neurons, PrP^{Sc} deposition was observed along the needle tracks. In contrast, diffuse PrP^{Sc} deposits were detected both along and away from the needle track in the thalamus, where excitatory neurons are predominant (Figures I-3 and I-4). The findings indicate that the 22L strain may propagate more efficiently in the thalamus than in the striatum. PrP^{Sc} deposition along the needle tracks after stereotaxic inoculation into the striatum aligns with previous observations (Chesebro *et al.*, 2015; Rangel *et al.*, 2014). Intracerebrally inoculated prions spread through the brain via interstitial fluid flow (Chesebro *et al.*, 2015), and prions associated with neurons are believed to spread through neuroanatomical pathways (Bartz *et al.*, 2002; Fraser, 1982; Reis *et al.*, 2015). Consequently, PrP^{Sc}-positive signals were observed in the striatum, but the PrP^{Sc}-positive areas did not spread efficiently after stereotaxic injection, suggesting that neuronal spread of prions through GABAergic inhibitory neurons or transmission across inhibitory synapses is inefficient. Over 50% of neurons in prion-infected StN were still classified as PrP^{Sc} ++, although the frequency of PrP^{Sc} ++ was significantly lower in prion-infected StN than in CxN (Figure I-7). This suggests that the 22L prion might also propagate efficiently in GABAergic neurons, but the weak PrP^{Sc} deposition in the striatum could be due to the inefficient reach of prions after i.c. and i.p. inoculation. The seemingly contradictory differences might be explained by the experimental condition where many

neuronal cells are exposed to the microsome fraction during prion inoculation, meaning the efficacy of neuron-to-neuron spread may not be fully reflected in the frequencies of PrP^{Sc} ++ neurons. Further studies are needed to precisely address the efficiency of prion propagation in GABAergic neurons and the transmission efficiency between them.

Since the level of PrP^C affects prion propagation (Bueler *et al.*, 1993), I analyzed PrP^C levels in three primary neuronal cultures. The level of PrP^C in the StN was 47% and 62% of that of PrP^C in the CxN and ThN, respectively (Figure I-11C), suggesting that the lower level of PrP^C might be one of the factors in the inefficient prion propagation in StN. However, a similar tendency was not observed in the PrP^C expression in the striatum of mock-infected mice (Figures I-11A and 11B). PrP^C expression and PrP^{Sc} formation are influenced by the cell-to-cell contact (Nakamitsu *et al.*, 2010), and PrP^C expression is essential but not a sole determinant for prion permissiveness of neuronal cells (Slota *et al.*, 2023; Uryu *et al.*, 2007). Thus, because of the complexity of the brain, it is difficult to assess if the PrP^C level of the striatal neurons influences prion propagation efficiency.

Certain subsets of GABAergic inhibitory neurons are reported to be vulnerable in the brains of CJD patients and prion-infected animals (Belichenko *et al.*, 1999; Guentchev *et al.*, 1997; Guentchev *et al.*, 1999). However, these observations are controversial, as some reports indicate that degeneration of GABAergic neurons was observed in the same degenerative sequence of events as non-GABAergic neurons in prion-infected hamsters (Bouzamondo *et al.*, 2000; Bouzamondo-Bernstein *et al.*, 2004). In this study, I analyzed prion propagation and vulnerability in GABA-negative glutamatergic excitatory neurons and GABA-positive GABAergic inhibitory neurons. Using multiple fluorescence staining with L-glutamate, GABA, and PrP^{Sc}, I compared prion propagation efficiency in glutamatergic and GABAergic neurons. The frequency of PrP^{Sc} ++ neurons was significantly higher in glutamatergic neurons than in GABAergic neurons, while PrP^{Sc} low neurons were more frequent in GABAergic neurons than in glutamatergic neurons (Figure I-9). These results suggest that the 22L strain propagates

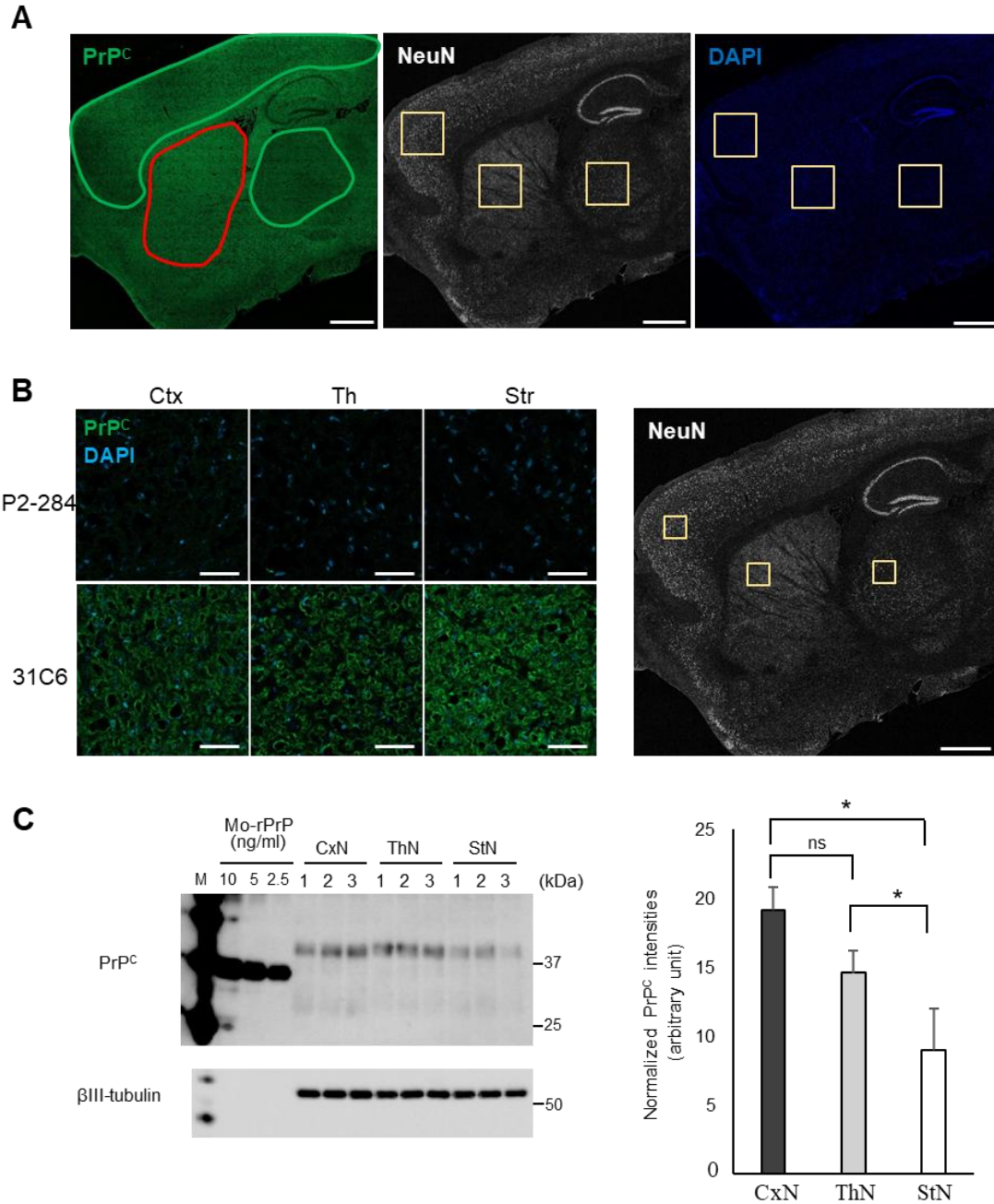


Figure I-11 PrP^C expression in brain and primary neuronal cultures.

(A) Mock-infected mice (i.c.) were sacrificed at 120 dpi and brains were subjected to cryosection. Sagittal sections were cut using a cryostat and stained with anti-PrP mAb 31C6 to detect PrP^C (green) and anti-NeuN mAb for neuronal cells (white). Nuclei were counterstained with DAPI (blue). Images were acquired using a 20× objective lens and tile scan images were generated with ZEN2009 software [black edition]. The cortex (Ctx) and thalamus (Th) regions are outlined in green, while the striatum (Str) is outlined in red. Scale bars: 1 mm. Sagittal brain sections around Plates 111 to 113 (Paxinos and Franklin, 2013) were used. (B) Sagittal brain sections were stained with anti-PrP mAb 31C6 and negative control mAb (anti-feline parvovirus subgroup mAb P2-284) and regions in the cortex (Ctx), thalamus (Th), and striatum (Str) indicated with boxes in the sagittal section on the right are shown. Scale bars are 50 μm and 1 mm, in the left panel images and in the sagittal plane image, respectively. (C) Immunoblot of total PrP^C and βIII-tubulin in primary neuronal cultures from the cortex (CxN), thalamus (ThN), and striatum (StN) at 21 div (in triplicate). The mAb 31C6 was used to detect PrP^C. The graph on the right shows the quantification of PrP^C. Statistical analysis was performed using one-way ANOVA *: $p < 0.05$, ns: not significant.

more efficiently in glutamatergic excitatory neurons than in GABAergic inhibitory neurons. This interpretation aligns with the observation that PrP^{Sc} accumulation is more pronounced in the cerebral cortex and thalamus, regions rich in glutamatergic neurons, compared to the striatum, which is abundant in GABAergic neurons (Figure I-4). To my knowledge, this provides the first direct evidence of excitatory neurons being more permissive to prion propagation.

Massive neuronal losses are observed in the thalamus of mice infected with the 22L strain and other prion strains (Cunningham *et al.*, 2005; Reis *et al.*, 2015). To identify the specific neuronal types affected, RNAscope *in situ* hybridization was used. It revealed a decrease in both *Vglut1*- and *Vglut2*-positive neurons in the VPL at 120 dpi (Figure I-10). However, no decrease in *Vgat*-positive neurons was observed in the RT thalamic nucleus, which is adjacent to the VPL and primarily composed of GABAergic neurons (Pinault, 2004). Sakaguchi *et al.* recently reported a selective decrease in *Vglut1* staining, but not in *Vglut2* and *Vgat* staining along with the loss of granule cells in the cerebellum and decreased *Vglut1* immunoreactivity in the pontine nuclei of BSE-infected guinea pigs (Sakaguchi *et al.*, 2020). Altered *Vglut1* staining has also been noted in the retina of scrapie-infected sheep (Smith *et al.*, 2008). Additionally, no loss of parvalbumin-positive GABAergic neurons was observed in the striatum of prion 139A-infected mice (Gunapala *et al.*, 2010) or in the hippocampus of prion ME7-infected mice (Franklin *et al.*, 2008). These findings suggest the vulnerability of excitatory neurons. Conversely, in addition to the loss of parvalbumin-positive GABAergic neurons in CJD patients and experimental animals (Belichenko *et al.*, 1999; Guentchev *et al.*, 1997; Guentchev *et al.*, 1999), recent single-cell transcriptome analysis indicates the vulnerability of inhibitory neurons in the hippocampus to prion infection (Slota *et al.*, 2022). Human cerebral organoids with the familial CJD mutation (PrP E200K) also showed impairment of GABAergic neurons (Foliaki *et al.*, 2021). Thus, while I observed excitatory neuron-prone prion propagation and loss of excitatory neurons in prion-infected mice, I believe

that the neuropathogenesis of prion diseases involves a complex balance between excitatory and inhibitory neurons. Since synaptic losses and axonal degeneration occur earlier than neuronal losses in prion diseases (Liberski and Budka, 1999; Reis *et al.*, 2015; Sisková *et al.*, 2009), and acute neurotoxicity caused by the induction of PrP^C-dependent neurotoxic signals through PrP^C-PrP^{Sc} interaction appears to be selective to excitatory synapse in the neuronal culture model (Fang *et al.*, 2018; Foliaki *et al.*, 2018), functional analyses of both excitatory and inhibitory neurons are needed to understand the precise neuropathogenesis.

Losses of excitatory neurons and synapses are well-documented in Alzheimer's disease (Kashani *et al.*, 2008; Mi *et al.*, 2023; Rodriguez-Perdigon *et al.*, 2016), tauopathies (Fu *et al.*, 2017), and Parkinson's disease (Zhao *et al.*, 2023). Additionally, the loss and dysfunction of inhibitory neurons are implicated in Alzheimer's disease pathology (Mathys *et al.*, 2023; Palop and Mucke, 2016), though the mechanisms of neuronal dysfunction remain unclear. Understanding the neuropathology of prion diseases, including the mechanisms of neuronal cell-type-prone prion propagation and neuronal vulnerability, will enhance the integrated understanding of neurodegenerative disorders caused by protein misfolding. One advantage of prion disease research is the availability of primary neuronal cultures susceptible to prions. Although apparent neuronal death or synaptic alterations were not observed in prion-infected primary neuronal cultures (Tanaka *et al.*, 2020), enhanced phosphorylation of protein kinase RNA-activated-like endoplasmic reticulum kinase (PERK), an early cellular response of the PERK-eukaryotic initiation factor 2-ATF4 unfolded protein response pathway, was induced in prion-infected primary neurons (Tanaka *et al.*, 2020). This suggests the potential of primary neuronal cultures for analyzing the molecular mechanisms of neurodegeneration caused by prion infection. Further improvement of these cultures as models for prion disease neuropathology will be key to elucidating the molecular mechanisms underlying prion neuropathology.

BRIEF SUMMARY

The accumulation of a disease-specific isoform of prion protein (PrP^{Sc}) and histopathological lesions, such as neuronal loss, are unevenly distributed in the brains of humans and animals affected with prion diseases. This distribution varies depending on the diseases and/or the combinations of prion strain and experimental animal. The brain region-dependent distribution of PrP^{Sc} and neuropathological lesions suggests a neuronal cell-type-dependent prion propagation and vulnerability to prion infection. However, the underlying mechanism is largely unknown. In this study, I provided evidence that the prion 22L strain propagates more efficiently in excitatory neurons than inhibitory neurons and that excitatory neurons in the thalamus are vulnerable to prion infection. PrP^{Sc} accumulation was less intense in the striatum, where GABAergic inhibitory neurons predominate, compared to the cerebral cortex and thalamus, where glutamatergic excitatory neurons are predominant, in mice intracerebrally or intraperitoneally inoculated with the 22L strain. PrP^{Sc} stains were observed along the needle track after stereotaxic injection into the striatum, whereas they were also observed away from the needle track in the thalamus. Consistent with inefficient prion propagation in the striatum, the 22L prion propagated more efficiently in glutamatergic neurons than GABAergic neurons in primary neuronal cultures. RNAscope *in situ* hybridization revealed a decrease in *Vglut1*- and *Vglut2*-expressing neurons in the ventral posterolateral nuclei of the thalamus in 22L strain-infected mice, whereas no decrease in *Vgat*-expressing neurons was observed in the adjacent reticular nucleus, mainly composed of *Vgat*-expressing interneurons. The excitatory neuron-prone prion propagation and excitatory neuronal loss in 22L strain-infected mice shed light on the neuropathological mechanism of prion diseases.

Chapter II

Utility of primary neuronal and astrocytic cell cultures in the analysis of
prion propagation mechanism.

INTRODUCTION

Immortalized neuronal and neural cells such as Neuro2a (N2a) neuroblastoma cells and GT1-7 hypothalamic neuronal cell line which are permissive to prion propagation have extensively been utilized for analyzing the mechanism of prion propagation and screening of anti-prion compounds and have provided much important knowledge on the cellular mechanism of prion propagation (Schatzl *et al.*, 1997; Pimpinelli *et al.*, 2005; Campana *et al.*, 2006; Marijanovic *et al.*, 2009; Veith *et al.*, 2009; Goold *et al.*, 2011; Goold *et al.*, 2013; Marzo *et al.*, 2013). However, there are limitations in investigating neurodegeneration because they do not show apparent degeneration after prion infection. In Chapter I, I showed the utility of primary neuronal cultures for analyzing neuronal cell tropism of prion propagation. In the brains of prion-infected animals, prions propagate not only in neurons but also in astrocytes (Diedrich *et al.*, 1991; van Keulen *et al.*, 2015; Carroll *et al.*, 2016; Yamasaki *et al.*, 2018). Additionally, microglia are also positive for PrP^{Sc} (Yamasaki *et al.*, 2018; Makarava *et al.*, 2024) although prions propagate in microglia remains to be elucidated (Carroll and Chesebro, 2019; Aguzzi and Zhu, 2017). Prions are known to spread via neuroanatomical pathways (Fraser, 1982; Bartz *et al.*, 2002; Reis *et al.*, 2015) the same as in Chapter I; however, in addition to neuron-to-neuron spread, recent studies also have suggested astrocytes are involved in the spread of prions between neurons and astrocytes (Victoria *et al.*, 2016; Hollister *et al.*, 2015). Recently, unfolding protein response in astrocytes is harmful to neurons in prion disease (Smith *et al.*, 2020). Thus, in addition to primary neuronal cultures, primary astrocytic cultures will also be useful as a more physiologically relevant model for analyzing the involvement of astrocytes in the neuropathobiology of prion diseases.

In Chapter II, I analyzed prion propagation in primary astrocytes derived from mixed glial culture and compared that in primary neurons to show the utility of primary astrocytic culture. Cellular localization of PrP^{Sc} in primary astrocytes was completely different from that

in primary neurons, suggesting the difference in the mechanism of PrP^{Sc} formation. However, the block of cell surface PrP^C by anti-PrP antibody affected PrP^{Sc} formation in both primary neurons and astrocytes. Anti-PrP antibodies administrated via intraventricular infusion or peripheral route bind to PrP^C and inhibit PrP^{Sc} formation in the brain (Song *et al.*, 2008; Ohsawa *et al.*, 2013). The fact that the anti-PrP antibody affects the production of PrP^{Sc} in primary neurons and astrocytes is an example of how primary neuronal and neural cultures can be useful models for reproducing the environment in the brains of prion-infected animals.

MATERIALS AND METHODS

Mouse neuroblastoma cell line

Subclones of N2a mouse neuroblastoma cell line N2a-3 (Uryu *et al.*, 2007) and N2a-3 cells persistently infected with prion 22L strain, ScN2a-3-22L (Nakamitsu *et al.*, 2010), were used. The ScN2a-3-22L cells maintain PrP^{Sc} without significant loss of PrP^{Sc} in more than 50 passages. Cells were passaged at a 1:10 dilution ratio every 3–4 days and were cultured in Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals) containing 10% FBS (Gibco), MEM non-essential amino acids (NEAA; Gibco), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Gibco) at 37 °C in 5% CO₂ atmosphere. For PrP^{Sc} detection using IFA and immunoblot analysis, cells were first seeded into 6 well plates (Nunc, Denmark) at a 1:20 split ratio.

Primary astrocytic cell culture

Primary astrocytic cell cultures were prepared from 5-day-old ICR neonatal mice. The mice were euthanized with sevoflurane, and their brains were collected in HEPES (Gibco-Thermo Fisher Scientific) and 10 μ M sodium pyruvate (Gibco-Thermo Fisher Scientific) (HEPES++). The olfactory bulb, brainstem, and cerebellum were removed, and the cerebrum and diencephalon were used for further processing. Tissues were digested using the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, USA) according to the manufacturer's instructions with some modifications: the enzymatic reaction was conducted on ice to minimize cell damage. The digested tissues were gently pipetted to aid dispersion, and the resulting cell suspensions were filtered through a 100 μ m nylon mesh cell strainer (CORNING, USA) to remove undigested tissue clumps. The cells were then collected by centrifugation at $300 \times g$ for 10 minutes at 4°C. Throughout the process, an ice-cold environment was maintained using HEPES++. The harvested cells were seeded at a density of 5×10^6 cells per cm² into T75 flasks

(Thermo Fisher Scientific) containing DMEM supplemented with inactivated FBS, NEAA (Gibco), and PenStrep (Gibco). The following day, non-adherent cells were removed by shaking the flasks, and the entire medium was replaced, subsequently, the cultures were incubated for three additional days with 100 μ M Ara-C in order to suppress the microglial proliferation. For the recovery of astrocytes, the cultures were shaken at 200 rpm at 37°C for 3 h to remove microglia and astrocytes were then detached from flasks by treating the cultures with Accutase (Thermo Fisher Scientific) for 10 minutes at 37°C. Flasks were washed with 10 mL of DMEM, and the cells were collected by centrifugation at $460 \times g$ for 5 min at 4°C. The cells were resuspended with DMEM and seeded at a density of 1×10^4 cells per cm^2 into 8-well chambers or 6-well plates. Half of the culture medium was replenished with fresh DMEM twice a week to maintain optimal cell growth. At 3 div, primary astrocyte cell cultures were exposed to microsomal fractions from prion 22L-infected mouse brains, containing 250 ng rPrP equivalent PrP-res per 1.0×10^4 cells, by replacing half the medium in each well. At 4 dpi, the medium was completely replaced with fresh medium.

IFA

For staining PrP^{Sc}, cells were fixed with 4% PFA in PBS at r.t. for 10 min and permeabilized with PBS containing 0.1 M glycine and 0.1% Triton X-100 at r.t. for 10 min and then treated with 6M GdnHCl at r.t. for 10 min. After blocking with 5% FBS in PBS, the cells were incubated with primary antibodies: anti-PrP mAb 132 (1 mg/mL) anti-PrP mAb 31C6 (1 mg/mL), and anti-GFAP rat mAb (Calbiochem, Germany). Secondary antibodies used were anti-mouse IgG Goat F(ab)'2 Alexa Fluor 488 (1:4,000), anti-rat IgG goat Alexa Fluor 555 (1:4,000), and nuclei were counterstained with DAPI. Cells were observed using a Zeiss LSM800 laser scanning microscope (Zeiss) as described in Chapter I.

SDS-PAGE and immunoblotting

Cells cultured on 6-well plastic plates were lysed with 300 μ L/well of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl [pH 7.5]). The cells underwent one freeze-thaw cycle followed by pipetting. After centrifugation, protein concentrations of the lysates were measured using a DC protein assay kit (Bio-Rad-Life Science, USA) and adjusted to 0.5-0.8 mg/mL. SDS-PAGE and immunoblotting for PrP-res detection were performed as described in Chapter I.

RESULTS

Detection of PrP^{Sc} in immortalized neuroblastoma cells

It is well established that PrP^{Sc} is stained as peri-nuclear granular stains in prion-infected neuroblastoma cells such as N2a cells (Pimpinelli *et al.*, 2005; Campana *et al.*, 2006; Veith *et al.*, 2009; Marijanovic *et al.*, 2009; Nakamitsu *et al.*, 2010; Goold *et al.*, 2011; Goold *et al.*, 2013; Marzo *et al.*, 2013) and that treatment of prion-infected neuroblastoma cells with anti-PrP antibody reacting cell-surface PrP^C reduces the PrP-res level in prion-infected cells (Enari *et al.*, 2001; White *et al.*, 2003; Kim *et al.*, 2004). First, I attempted to confirm this phenomenon (Figure II-1). As expected, PrP^{Sc} was detected in ScN2a-3-22L cells as peri-nuclear granular stains that were not observed in uninfected cells. The intensity of PrP^{Sc} stains was apparently reduced when cells were treated with mAb 31C6, compared to those treated with the negative control antibody, mAb P2-284 (Figure II-1A). In immunoblot analysis, the PrP-res level in mAb 31C6-treated ScN2a-3-22L cells was reduced to 23 % of that in negative control mAb-treated cells with the 3-day antibody treatment (Figure II-1B). This confirms the fact that mAb 31C6 recognizing mouse PrP aa144-149, which binds PrP^C expressed on the cell surface, prevents PrP^{Sc} formation in neuroblastoma cells persistently infected with prions (Kim *et al.*, 2004).

Effect of anti-PrP mAb on the PrP^{Sc} formation in primary neurons

Previous studies have demonstrated that string-like PrP^{Sc} stains are predominantly localized on the surface of primary cerebral neurons (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020), which is completely different from PrP^{Sc} stains in prion-infected neuroblastoma cells as shown in Figure II-1A. Since PrP^{Sc} formation in the cells was mainly elucidated from cellular biological analysis using immortalized cells of neural and neuronal origins (Pimpinelli *et al.*, 2005; Marijanovic *et al.*, 2009; Veith *et al.*, 2009; Yamasaki *et al.*, 2012; Yamasaki *et al.*, 2014),

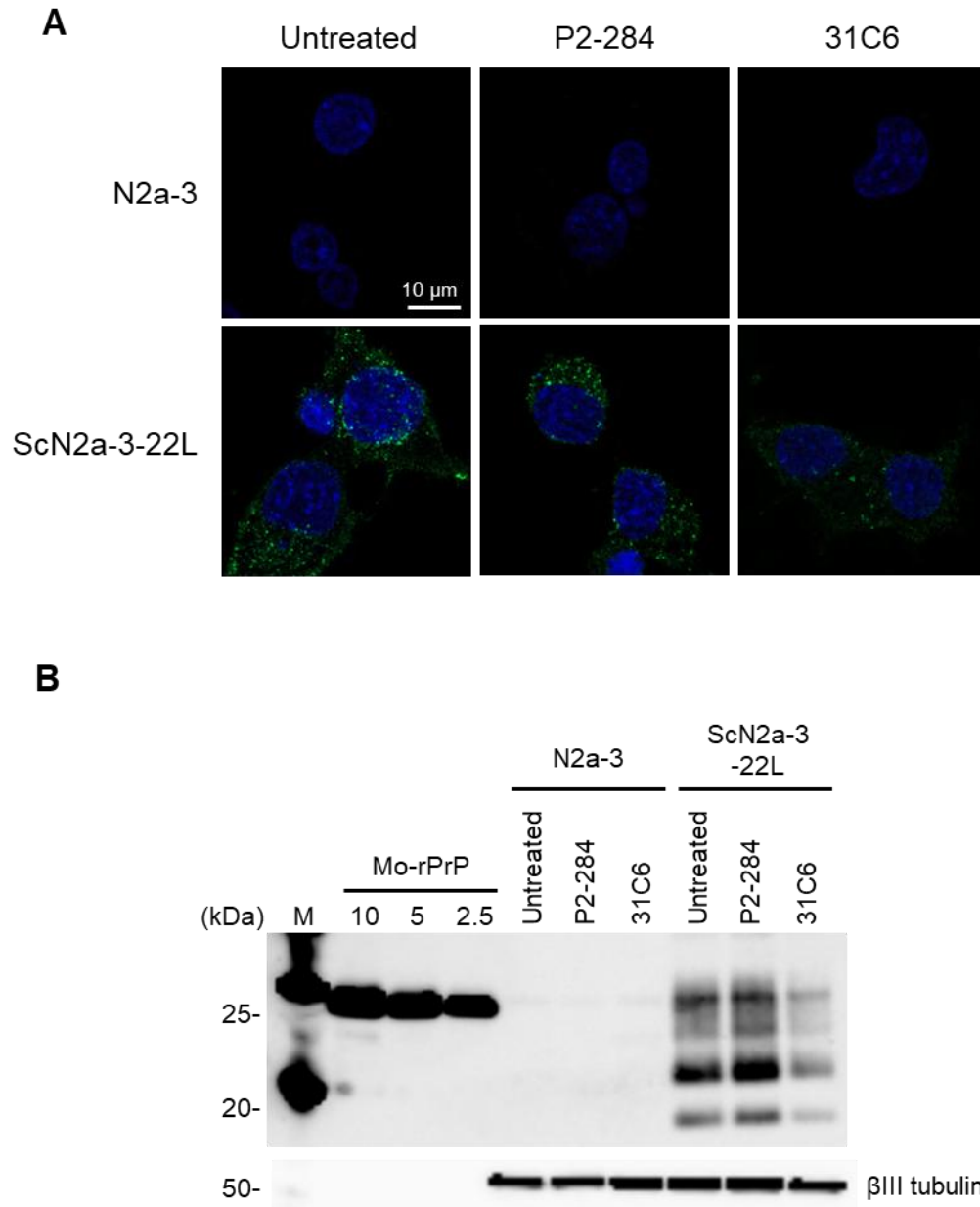


Figure II-1. Detection of PrP^{Sc} in immortalized neuroblastoma cells.

(A) Immunofluorescence detection of PrP^{Sc} using mAb 132. N2a-3 and ScN2a-3-22L cells were cultured for 3 days with 1 μg/mL of mAb P2-284 (negative control) or anti-PrP mAb 31C6 or without antibodies (untreated) and were subjected to PrP^{Sc}-specific staining using anti-PrP mAb 132. Merged images of the PrP^{Sc} signals (green) and nuclear (DAPI, blue) are shown. Scale bars: 10 μm. (B) Immunoblot analysis. For detection of PrP-res, PK treated cell lysates adjusted based on the protein concentration of the corresponding cell lysate were loaded. Immunoblot of βIII tubulin (bottom) is shown as an internal marker for PK-untreated lysates. Molecular mass markers are shown in kDa on the left.

the string-like PrP^{Sc} stains on the surface of primary neurons (Tanaka *et al.*, 2016; Tanaka *et al.*, 2020) (Figures I-6 in Chapter I) suggest that mechanisms underlying PrP^{Sc} formation differ between immortalized cell lines and primary neuronal cells. Primary neuronal cultures are believed to be better *ex vivo* system than immortalized cell lines, I analyzed the effect of anti-PrP mAb 31C6 on the PrP^{Sc} formation in primary neuronal cultures. Since string-like PrP^{Sc} stains were observed on the surface of primary neuronal cultures, mAb 31C6 was added to primary neuronal cultures at 4 dpi, and cells were incubated for 3 to 9 days in the presence of mAb 31C6. Then, PrP^C and PrP^{Sc} on the surface of primary neuronal cells were directly detected with mAb 31C6 conjugated with Alexa Fluor 555 and mAb 8D5 conjugated with Alexa Fluor 488, respectively, by adding those fluorescence dye-labeled antibodies simultaneously into the live cells (Figure II-2). In mock-infected cells without antibody treatment or treated with negative control mAb (P2-284), PrP^C could be detected as diffused stains on the surface of neurons. Diffuse PrP^C reddish stains which were observed in cells without mAb 31C6 treatment (Untreated) and treated with mAb P2-284 were not detected in cells treated with mAb 31C6 regardless of prion infection. This is probably due to the blocking of the epitope on PrP^C by mAb 31C6 added to the culture. String-like PrP^{Sc} stains were detected in 22L-infected primary neurons without antibody treatment or treated with mAb P2-284 at 7 dpi and increased in abundance by 13 dpi (Figure II-2). String-like PrP^{Sc} stains were undetectable in mAb 31C6-treated 22L-infected primary neurons at both 3 and 9 days of treatment, suggesting that mAb 31C6 inhibited the PrP^{Sc} formation in primary neurons infected with prions. Green dotted stains by mAb 8D5 conjugated with Alexa Fluor 488 were observed both in mock-infected and prion-infected primary neurons treated with mAb 31C6. This may be a cross-linking effect by the mAb 31C6 added to neuronal cultures. It is reported that anti-PrP mAb 132 recognizing mouse PrP aa119-127 showed low affinity in monovalent binding but divalent binding confers high binding avidity to mAb 132 (Suzuki *et al.*, 2019). Indeed, mAb 132 did not bind PrP^C on the surface but it binds PrP^C on the cell surface after crosslinking of PrP^C by mAb 31C6, which

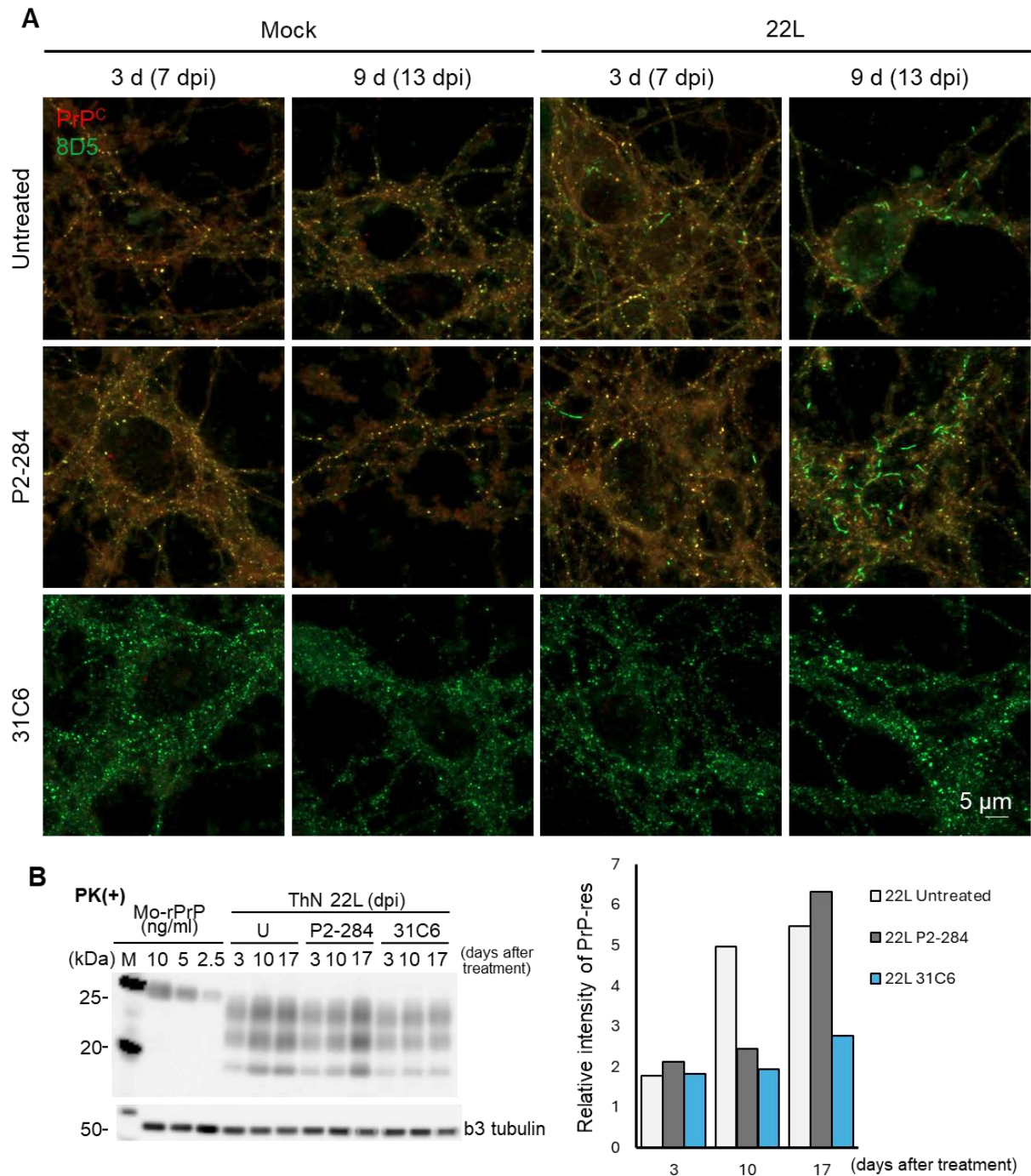


Figure II-2 Effect of anti-PrP mAb on the PrP^{Sc} formation in primary neurons.

(A) Detection of cell surface PrP^C and PrP^{Sc} on primary neuronal cultures from thalamus (ThN). Mock- and prion-infected ThN treated with mAb P2-284 (negative control mAb, 1 μ g/mL), anti-PrP mAb 31C6 (1 μ g/mL), or without antibody treatment (Untreated) for 3 and 9 days, corresponding 7 and 13 dpi were stained directly with mAb 31C6 conjugated with Alexa Fluor 555 for PrP^C (reddish color) and mAb 8D5 conjugated with Alexa Fluor 488 for PrP^{Sc}, without fixation of cells. Scale bars: 5 μ m. (B) Immunoblot analysis. PK-treated cell lysates were subjected to immunoblot analysis using anti-PrP antibody mAb 31C6 to detect PrP-res. Cell lysates without PK treatment were subjected to detect β III tubulin (bottom) as an internal marker for PK-untreated lysates. Molecular mass markers are shown in kDa on the left. The graph on the right shows quantitative results. Graph on the right shows quantification of the PrP-res.

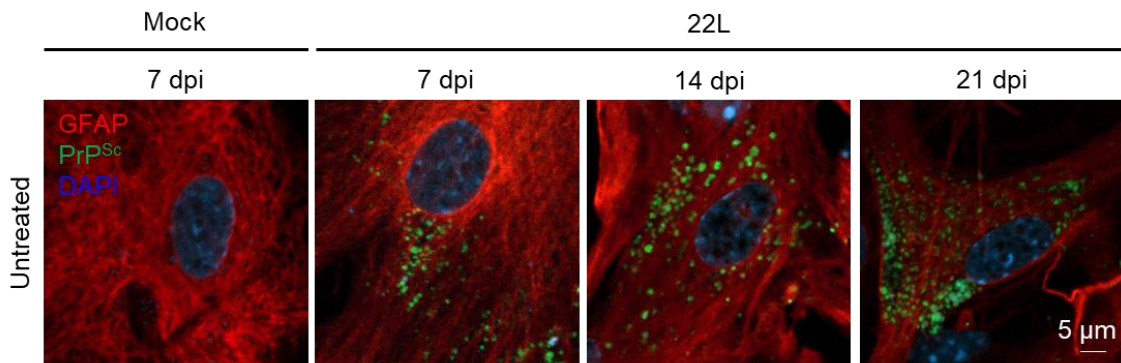
allows mAb 132 divalent binding. However, it remains to be elucidated if this mechanism applies to mAb 8D5.

Figure II-2B shows immunoblot analysis of PrP-res. Although the string-like PrP^{Sc} stains disappeared by the mAb 31C6 treatment, immunoblot detected PrP-res in primary neurons infected with prions up to 17 days of treatment. Quantitative analysis revealed that PrP-res levels in mAb 31C6 treated neurons were 49% and 56% lower than those in antibody-untreated or P2-284-treated neurons at 17 days after antibody treatment. This result also suggests the inhibition of PrP^{Sc} formation by mAb 31C6 in primary neurons. However, considering the kinetics, there is a discrepancy between the inhibition of the formation of string-like PrP^{Sc} formation observed in IFA and PrP-res detected by immunoblotting.

Effect of anti-PrP mAb on the PrP^{Sc} formation in primary astrocytes

Since astrocyte-derived cell line, primary cerebellar granular neuronal cultures with astrocytes and astrocytes derived from human induced pluripotent stem cells infected with prions showed granular PrP^{Sc} stains (Hollister *et al.*, 2015; Victoria *et al.*, 2016; Krejciova *et al.*, 2017). I analyzed PrP^{Sc} formation in primary astrocytes obtained from mixed glial culture. When I analyzed the PrP^{Sc} in prion-infected primary astrocytes, PrP^{Sc} was merely detected with mAb 8D5 (data not shown), thus I used mAb 132 with the pre-treatment of cells with GdnHCl for detecting PrP^{Sc} (Yamasaki *et al.*, 2012). PrP^{Sc} was detected as intracellular granular stains like those detected in prion-infected neuroblastoma cells (Figure II-3A). N-terminal truncated PrP^{Sc} bands, which were the same as PrP-res bands detected after PK-treatment, were observed without PK-treatment (Figure II-3B, PK(-)). This suggests that the majority of PrP^{Sc} in the astrocytes are N-terminally processed, and therefore, PrP^{Sc} was merely detected in prion-infected astrocytes with mAb 8D5 that recognizes the N-terminal part of PrP (aa 31-39). The striking difference in the PrP^{Sc} localization in astrocytes suggests that the mechanism of PrP^{Sc}

A



B

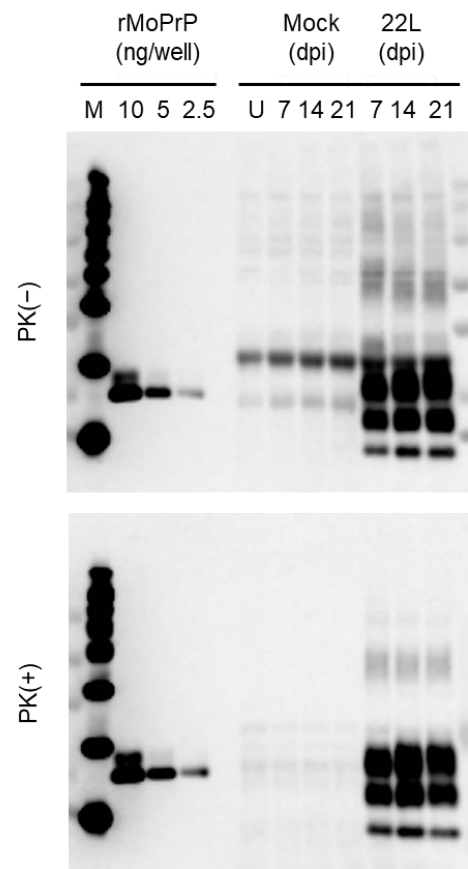


Figure II-3 PrP^{Sc} in prion-infected primary astrocytes.

(A) Detection of PrP^{Sc} in primary astrocytes infected with the 22L prion strain. PrP^{Sc} was stained with PrP^{Sc}-specific immunostaining with mAb 132 (green), while astrocytes were stained with GFAP (red) and nuclei were counterstained with DAPI (blue). The left panel shows mock-infected and the rest shows 22L prion-infected primary astrocytes. PrP^{Sc} was detected at 7, 14, and 21 dpi. Scale bars: 5 μm. (B) Immunoblot analysis. Upper and lower panels show detection of total PrP PK(-) and PrP-res (PK(+)), respectively.

formation, *i.e.*, prion propagation, in primary astrocytes differs from that in primary neurons but exhibits some similarities with Neuro2a cells.

Next, I analyzed PrP^{Sc} and the effect of mAb 31C6 treatment on PrP^{Sc} formation in primary astrocytes (Figure II-4). The granular PrP^{Sc} stains appeared to be increased and larger PrP^{Sc} granular stains in size also appeared to be increased with the days after inoculation in astrocytes treated with negative control antibody (P2-284) (Figure II-4A). In contrast, large granular PrP^{Sc} stains were merely observed in prion-infected astrocytes treated with mAb 31C6. Quantitative analysis showed that difference in ratios of granules with diameter of 0.5-1.0 μm (small), 1.0-2.0 μm (middle), and > 2.0 μm (large) at 17 days antibody treatment: no middle and large PrP^{Sc} granular stains were observed in astrocytes treated with mAb 31C6-treated (Figure II-4B). This result suggests that the binding of mAb 31C6 to PrP^C on the cell surface influences the PrP^{Sc} formation in prion-infected primary astrocytes.

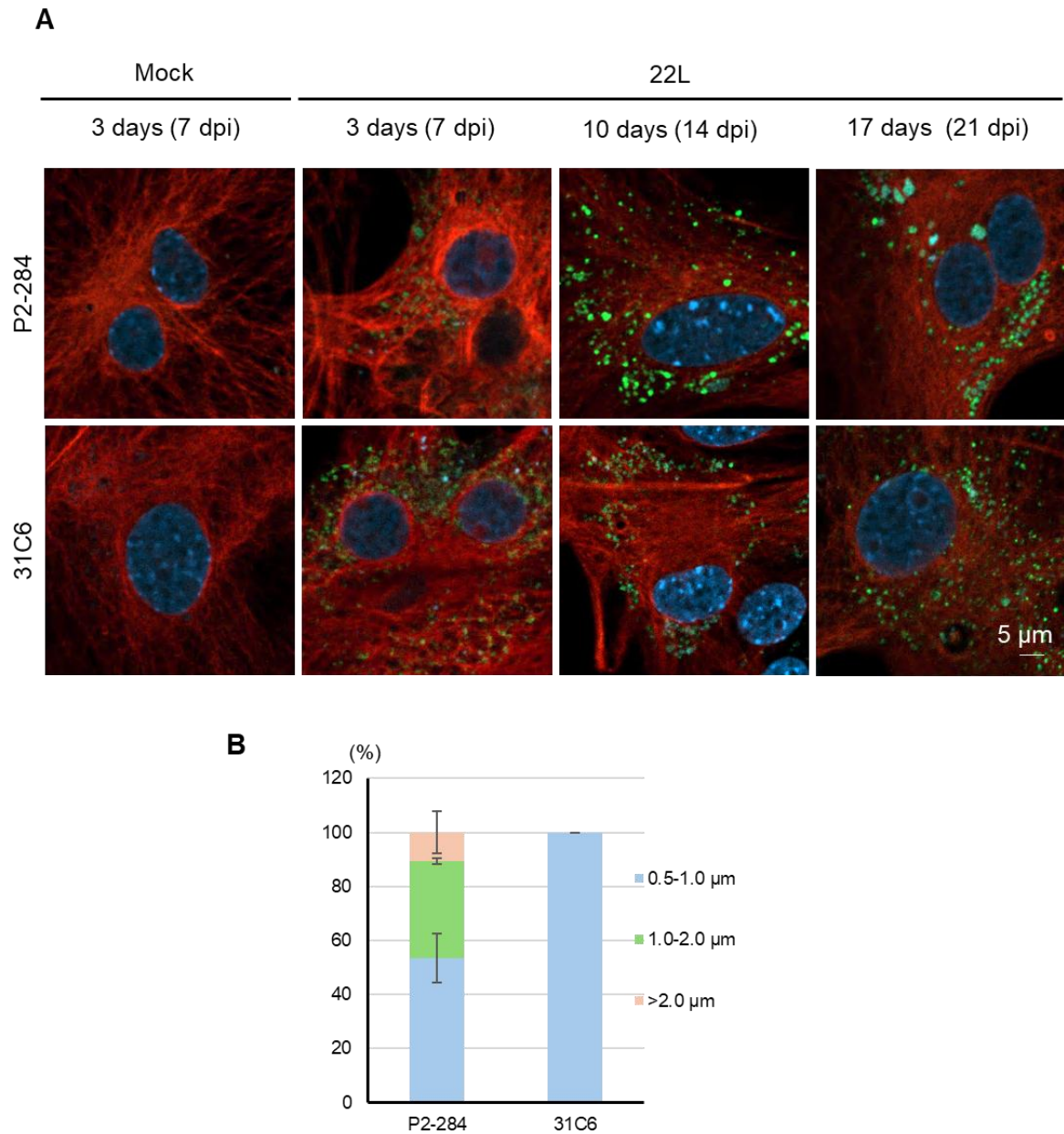


Figure II-4 Effect of anti-PrP mAb on the PrP^{Sc} formation in primary astrocytes.

(A) Effect of mAb 31C6 treatment on PrP^{Sc} in primary astrocytes. PrP^{Sc} was stained with PrP^{Sc}-specific immunostaining with mAb 132 (green), while astrocytes were stained with GFAP (red) and nuclei were counterstained with DAPI (blue). The left panel shows mock-infected and the rest shows 22L prion-infected primary astrocytes. Antibodies were added to the culture medium at 4 dpi at 1 μg/mL and astrocytes were cultured for up to 17 days (21 dpi) with mAb P2-284 (negative control) and anti-PrP mAb 31C6. PrP^{Sc} was detected at 3, 10, and 17 days of treatment. Scale bars: 5 μm. (B) Quantitative analysis. PrP granular stains with diameters of 0.5-1.0 μm (small), 1.0-2.0 μm (middle), and > 2.0 μm (large) were distinguished using Image J (version). The graph shows the average and variations of ratios (%) of small, middle, and large granules at 17 days of treatment. A total of 547, 331, and 532 granules in two astrocytes (one astrocyte per experiment) were assigned.

DISCUSSION

Immortalized neural and neuronal cells such as N2a neuroblastoma cells and GT1-7 hypothalamic neuronal cell line are extensively utilized for analyzing the mechanism of prion propagation and anti-prion compounds (Schatzl *et al.*, 1997; Pimpinelli *et al.*, 2005; Campana *et al.*, 2006; Marijanovic *et al.*, 2009; Veith *et al.*, 2009; Goold *et al.*, 2011; Goold *et al.*, 2013; Marzo *et al.*, 2013; Yamasaki *et al.*, 2014). However, they have limitations in investigating neurodegeneration, and cell culture models closer to the brain are required for re-examination of the mechanism of prion propagation in the brains of prion-infected animals and for elucidating molecular mechanism of neurodegeneration caused by prion infection. For instance, the localization of PrP^{Sc} in neuroblastoma cell lines, peri-nuclear granular stains (Beranger *et al.*, 2002; Marijanovic *et al.*, 2009; Veith *et al.*, 2009) differs from that observed in primary neuronal cultures (Rouvinski *et al.*, 2014; Tanaka, 2016), and N2a cells did not exhibit any alterations caused by prion infection.

Thus, in Chapter II, I explored the potential of primary neuronal and astrocytic cell cultures for analyzing prion propagation mechanisms. As expected, in prion-infected ScN2a-3-22L cells, PrP^{Sc} appeared as perinuclear granular stains, and treatment with the anti-PrP antibody mAb 31C6, which binds to cell-surface PrP^C, reduced PrP-res levels to 23% after three days of treatment (Figure II-1). In prion-infected primary neuronal cells, although cellular localization of PrP^{Sc} is quite different from that observed in ScN2a-3-22L cells, string-like PrP^{Sc} stains on the cell surface disappeared even after the 3-day antibody treatment (Figure II-2). It is well known that the retention of PrP^C on the cell surface by binding of mAb 31C6, which results in changing intracellular trafficking of PrP^C, is the key mechanism of the inhibition of PrP^{Sc} formation in prion-infected neuroblastoma cells (Kim *et al.*, 2004; Yamasaki *et al.*, 2014). It remains to be elucidated if the same mechanism can explain the inhibition of string-like PrP^{Sc} stain formation by mAb 31C6 in primary neurons. Since several reports suggest the binding of

PrP^C and PrP^{Sc} occurs on the cell surface, the binding of mAb 31C6 to PrP^C on the cell surface may directly block the interaction between PrP^C and PrP^{Sc} on the cell surface of primary neurons (Goold *et al.*, 2011; Ribes *et al.*, 2023). In contrast to the almost complete disappearance of string-like PrP^{Sc} stains on the cell surface by 3 to 9 days of treatment with mAb 31C6, PrP-res was about 50% lower than the control even 17 days after antibody treatment (Figure II-2). The results suggest that the inhibition of PrP^{Sc} formation in prion-infected primary neurons by mAb 31C6; however, further studies are needed to clarify mechanisms for inhibition and whether this antibody blocks direct interaction between PrP^C and PrP^{Sc} on the cell surface and PrP^{Sc} degrades after inhibition.

Primary astrocytes have been reported to be susceptible to prion infection and transmit prions to other cells that are permissive to prion infection (Cronier *et al.*, 2004; Victoria *et al.*, 2016). In this study, I showed that granular PrP^{Sc} in astrocytes increased in size over time following prion infection. Furthermore, different from PrP^{Sc} in prion-infected primary neurons, the majority of PrP^{Sc} in prion-infected primary astrocytes was N-terminally processed and thus lacks the epitope for mAb 8D5. The formation of large PrP^{Sc} granular stains (>2.0 μ m) was inhibited by mAb 31C6 (Figure II-4), suggesting that the binding of mAb 31C6 to the PrP^C on the cell surface affects prion propagation in prion-infected primary astrocytes.

Although the cellular localization and staining pattern of PrP^{Sc} differed between primary neurons and astrocytes, results in Chapter II suggest that blocking PrP^C on the cell surface by mAb 31C6 affects PrP^{Sc} formation both in primary neurons and astrocytes. However, compared to ScN2a-3-22L cells in which PrP-res level was reduced to 23 % of that in negative control mAb treated cells with the 3-day treatment, it appears to take longer time to see the effect of mAb 31C6 in primary neurons and astrocytes. This is probably due to the cell division of neuroblastoma cells. The PrP^{Sc} levels in prion-infected were fluctuated and indeed, PrP^{Sc} level decreased immediately after passage and recovered with the increase of cell density (Ghaemmghami *et al.*, 2007; Nakamitsu *et al.*, 2010). The mAb was added to ScN2a-3-22L

24 h after passage in the current experiments, the timing when the PrP^{Sc} level has not recovered. Therefore the impact of the inhibitory effect is pronounced. On the contrary, primary neuronal neurons and astrocytes are not divided, the effect of antibodies on PrP^{Sc} may reflect a true inhibitory effect. Therefore, primary neuronal and neural cultures are suitable for the purpose of screening drugs that truly inhibit PrP^{Sc} production.

Prion-permissive neuronal and neural cells such as N2a neuroblastoma cells and GT1-7 hypothalamic neuronal cell line greatly contributed to the elucidation of the cellular mechanism of prion propagation. However, those cells are not useful for analyzing the molecular mechanism of neurodegeneration because they do not show any degeneration or deficit after prion infection. One of the obstacles to elucidating the neuropathobiological mechanism of prion diseases is the limitation of cell culture models. Neurospheres (Giri *et al.*, 2006; Milhavet *et al.*, 2006), organotypic slice culture (Falsig and Aguzzi, 2008), and cerebral organoid (Groveman *et al.*, 2019; Groveman *et al.*, 2021) have been proposed and used as *ex vivo* tools for the elucidating mechanism of prion propagation and neuropathobiology. Particularly recent advances in cerebral organoids are intriguing (Groveman *et al.*, 2023; Foliaki and Haigh, 2023) as three-dimensional *ex vivo* systems include neurons, astrocytes, and oligodendrocytes and thus may mimic partly brain tissues. The advantage of primary neuronal and neural cells of each of the cell types that constitute the brain is to allow detailed cell-by-cell analyses of alteration caused by prion propagation, the functions of cells derived from specific brain regions and individual cell types, and therefore, they also seem to be a valuable *ex vivo* tools for studying cellular prion propagation, neurodegeneration, and evaluating anti-prion compounds.

BRIEF SUMMARY

The formation of an abnormal isoform of the prion protein, PrP^{Sc} plays a central role in prion propagation. Investigations of the intracellular localization of PrP^{Sc} in prion-infected neuroblastoma cell lines have advanced our understanding of the cellular mechanisms underlying prion propagation. However, these neuroblastoma cell lines are not suitable for studying neurodegeneration, and prion-infected cell culture models closer to the brains of prion-infected animals are required for the elucidation of mechanisms for prion propagation and neurodegeneration. In this study, I explored the utility of primary neuronal and astrocytic cell cultures for analyzing the mechanisms of prion propagation. Here, I showed a remarkable difference in the localization of PrP^{Sc} and staining patterns of PrP^{Sc} in primary neuronal and astrocytic cultures. PrP^{Sc} was detected on the surface of live primary neurons as string-like stains with mAb 8D5 that recognize the N-terminal part of PrP (amino acid 31-39). In contrast, intracellular granular PrP^{Sc} stains, which were similar to those observed in N2a neuroblastoma cells infected with prions, were mainly detected in primary astrocytes with mAb 132 recognizing PrP amino acid 119-127. The majority of PrP^{Sc} in primary astrocytes was N-terminally processed. Treatment of prion-infected primary neurons with anti-PrP mAb 31C6, which binds to PrP^C on the cell surface and is well-known to inhibit PrP^{Sc} formation in prion-infected neuroblastoma cells, appeared to inhibit the formation of string-like PrP^{Sc} stains on the surface of primary neurons. Immunoblotting also revealed the decrease of PrP^{Sc} level in mAb31C6-treated primary neurons. Treatment of prion-infected primary astrocytes with mAb 31C6 reduced the size of granular PrP^{Sc} stains. Although the localization of PrP^{Sc} in primary neurons and astrocytes is quite different, these results suggest that blocking PrP^C on the cell surface affects PrP^{Sc} formation both in primary neurons and astrocytes. These findings suggest that primary neuronal and astrocytic cultures are useful models for studying prion propagation, transmission, and the evaluation of anti-prion therapeutics, as a model closer to the brain.

CONCLUSION

Prion diseases are fatal neurodegenerative disorders caused by the misfolding of PrP^C into protease-resistant, β -sheet-rich PrP^{Sc}, which aggregates into fibrils via seeded conversion and accumulates in the central nervous system. The generation and accumulation of PrP^{Sc}, which is synchronous with prion propagation, in the brain, varies across brain regions in prion-infected animals. However, mechanisms for the uneven distribution of PrP^{Sc} and their involvement in neurodegeneration and neuronal death remain poorly understood. Investigation of mechanisms underlying region-dependent prion propagation and neuronal death will contribute to the elucidation of neuropathobiology of prion diseases. Thus, in my thesis, I investigated the mechanism of brain region-dependent prion propagation and neuronal death using *in vivo* and *ex vivo* experiments. I also showed the utility of primary neuronal and neural cultures to analyze prion propagation as *ex vivo* models.

In Chapter I, prion propagation and neuronal death were analyzed using mice and primary neuronal cultures infected with the prion 22L strain. Primary neurons derived from the cortices, thalami, and striata resemble the proportion of excitatory and inhibitory neurons in the corresponding mouse brain regions, suggesting the utility of primary neurons for analyzing brain region-dependent prion propagation. The prion 22L strain propagates more efficiently in excitatory neurons than inhibitory neurons and excitatory neurons in the thalamus are vulnerable to prion infection. PrP^{Sc} accumulation was less intense in the striatum, where GABAergic inhibitory neurons predominate, compared to the cerebral cortex and thalamus, where glutamatergic excitatory neurons are predominant, in mice intracerebrally or intraperitoneally inoculated with the 22L strain. PrP^{Sc} stains were observed along the needle track after stereotaxic injection into the striatum, whereas they were also observed away from the needle track in the thalamus. Consistent with inefficient prion propagation in the striatum, the 22L prion propagated more efficiently in glutamatergic neurons than GABAergic neurons

in primary neuronal cultures. RNAscope *in situ* hybridization revealed a decrease in *Vglut1*- and *Vglut2*-expressing neurons in the ventral posterolateral nuclei of the thalamus in 22L strain-infected mice, whereas no decrease in *Vgat*-expressing neurons was observed in the adjacent reticular nucleus, mainly composed of *Vgat*-expressing interneurons. These results demonstrate that the excitatory neurons in the thalamus are vulnerable to prion infection.

In Chapter II, I showed remarkable differences in the localization of PrP^{Sc} and staining patterns of PrP^{Sc} in primary neuronal and astrocytic cultures. PrP^{Sc} was detected on the surface of live primary neurons as string-like stains. In contrast, intracellular granular PrP^{Sc} stains, which were similar to those observed in N2a neuroblastoma cells infected with prions, were mainly detected in primary astrocytes. Treatment of prion-infected primary neurons with anti-PrP mAb 31C6, which binds to PrP^C on the cell surface and is well-known to inhibit PrP^{Sc} formation in prion-infected neuroblastoma cells, appeared to inhibit the formation of string-like PrP^{Sc} stains on the surface of primary neurons. Immunoblotting also revealed the decrease of PrP^{Sc} level in mAb31C6-treated primary neurons. Treatment of prion-infected primary astrocytes with mAb 31C6 reduced the size of granular PrP^{Sc} stains by 17 days of treatment. Although the localization of PrP^{Sc} in primary neurons and astrocytes is quite different, these results suggest that blocking PrP^C on the cell surface affects PrP^{Sc} formation both in primary neurons and astrocytes. These findings suggest that primary neuronal and astrocytic cultures are useful models for studying prion propagation, transmission, and the evaluation of anti-prion therapeutics, as a model closer to the brain.

In my doctoral dissertation, I elucidated excitatory neuron-prone prion propagation and the vulnerability of thalamic excitatory neurons to prion infection. Furthermore, I showed the potential of primary neuronal and neural cultures as models closer to the brain for detailed analysis of prion propagation and neurodegeneration in different cell types. This study will provide new insights into future research addressing the mechanism of neuropathobiology of prion diseases and prion-related neurodegenerative disorders.

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