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Metabolic profiling of prions in the gastro-intestinal tract

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Metabolic profiling of prions in the gastro-intestinal tract

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Metabolic profiling of prions in the gastro-intestinal tract

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Abstract

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of debilitating neurodegenerative disorders that affect both humans and animals. They can be spread by horizontal transmission as seen in chronic wasting disease in deer and elk population. In addition, transmission to humans has also been documented which includes bovine spongiform encephalopathy in cattle to variant Creutzfeldt-Jakob disease in humans due to consumption of contaminated meat. The most probable natural route of transmission is by oral consumption of infectious material. Even though this mode of transmission has been known for a long time it is still unclear how the infectious material distributes *in vivo* shortly after ingestion and what is the metabolic stability in gastro-intestinal tissues. In this line, our hypothesis is that infectious prions resist gastro-intestinal digestion and directly cross the intestinal barrier after per-oral administration, distributing in blood and various organs. In order to test this hypothesis, we characterized the metabolic profile of prions in gastro-intestinal tract tissues by *in vitro* experiments and determined the initial distribution of infectious material within hours of ingestion *in vivo*. Our results showed the radiolabeled ^{125}I-PrP^{Sc} undergoes limited metabolic degradation -mostly in duodenum, jejunum and ileum tissue homogenates- as compared to stomach and colon homogenates where little degradation is observed. We have also separately shown that within two hours of oral

ingestion of prions in mice self propagating prions are detected in blood, brain and various tissues.

For my parents and family

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Abbreviations

AUC – Area under the curve

%ID/mL – percent injected dose per milliliter

°C – degrees Celsius

BCA – Bichinchoninic Acid Assay

BSA – bovine serum albumin

BSE – bovine spongiform encephalopathy

CJD – Creutzfeld-Jakob disease

cpm – counts per minute

CWD – chronic wasting disease

DTT – dithiothreitol

ECL – enhanced chemiluminescence

EDTA – ethylene diamine tetra-acetic acid

g – gram

g – gravity (acceleration due to)

h – hour(s)

HRP – horseradish peroxidase

HPLC – High performance liquid chromatography

I.C**. –** Intra-cerebral

I.P. **–** Intra-peritoneal

kDa – kilodalton

M – molar

mg – milligrams

min – minute(s)

mL – milliliter

mM – millimolar

MW – molecular weight

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate-buffered saline

PBST – phosphate buffered saline with 0.05%Tween-20

PMCA – Protein Misfolding Cyclic Amplification

pH – hydrogen ion concentration

PI – protease inhibitor

PK – proteinase K

P.O. – per oral

PrP – prion protein

PrP^{C}– normal isoform of the prion protein

PrP^{Sc}– disease-associated prion protein (Sc is for scrapie)

RML – Rocky Mountain laboratory

rpm – revolutions per minute

RT – room temperature

secs – Second (s)

S.E.M**. -** Standard Error of the Mean

SDS – sodium dodecyl sulphate

TSEs **–** Transmissible Spongiform Encephalopathies

UV – ultraviolet

V– Volts

WT – wild-type

w/v – weight in volume

µg – micrograms

µL– microliters

µM – micro molar

CHAPTER 1

Introduction

Prion diseases also known as transmissible spongiform encephalopathies (TSE's) are a group of infectious, debilitating and fatal neurodegenerative diseases affecting both animals and humans (Prusiner, 1998). Currently, there is no cure for these diseases and positive diagnosis is made after death by brain autopsy. In humans one in a million cases per year globally are reported and can be inherited due to mutation in prion protein gene (<5%), sporadic without any known etiology (>95%), or acquired (<1%). The latter comprises both human to human (iatrogenic) or animal to human (variant CJD) transmissions. Although rare, these diseases remain intriguing due to the unique nature of infectious agents composed largely of misfolded proteins known as prions (Prusiner *et al*., 1983). Prions were defined more than three decades ago by Dr. Stanley Prusiner as small proteinaceous infectious particles that resist inactivation by most procedures that modify nucleic acids (Prusiner, 1982). In contrast to other infectious agents, like bacteria and viruses, prions resist inactivation by many conventional chemical treatments, ultraviolet radiation, heat, and nucleases (Gajdusek *et al*., 1977; Prusiner, 1982). The infectious prion protein (PrP^{Sc}) forms as the result of a post-translational process that modifies the conformation of the endogenous cellular PrP (PrP^{C}) into PrP^{Sc} (Borchelt *et al*., 1990; Caughey & Raymond, 1991). As a result of the misfolding process, PrP^{C} that is composed mainly of alpha helices undergoes a conformational change to intermolecular beta sheet as reported in PrP^{Sc} (Pan *et al*., 1993). In various prion diseases the clinical characteristics differ but they all share a common neuropathology which includes prion protein aggregate deposition, dendritic and synaptic loss, extensive spongiform brain degeneration, atypical inflammation and widespread neuronal death (**Figure 1**)(Budka, 2003; Soto & Satani, 2011) .

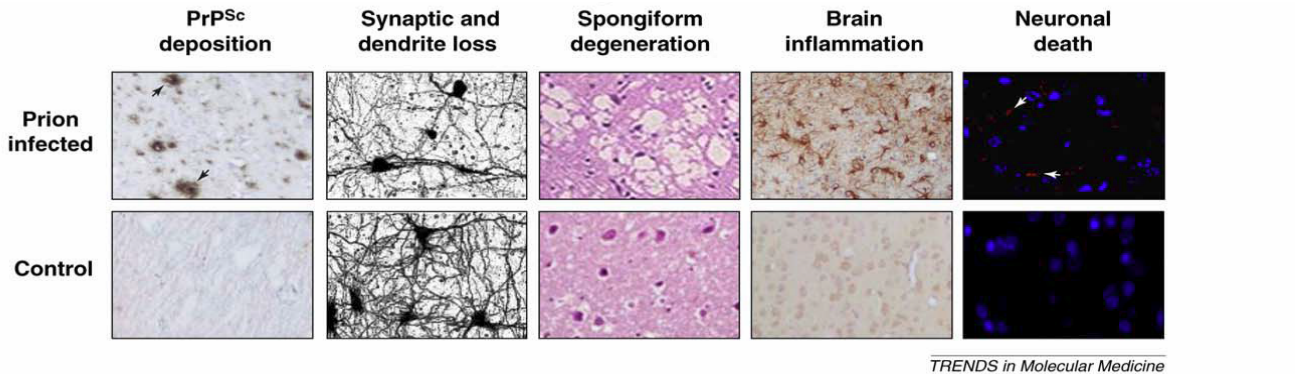

Figure 1. Multiple neurodegenerative pathways are involved in prion diseases. Brain abnormalities include PrP^{Sc} deposition, synaptic and dendrite loss, spongiform degeneration, brain inflammation and neuronal death. Prion deposition was determined by IHC staining with anti-PrP antibodies. The prion infected animal brain (upper) and normal uninfected (lower) is shown
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1. Transmission of prion diseases in animals and humans

Some prion diseases that are known to spread by oral transmission in animals include scrapie in sheep and goat, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) in cattle, Kuru and variant Creutzfeldt-Jakob disease (vCJD) in humans (Brown, 2009).

1.1 Animal Prion Diseases

1.1.1 Scrapie

Scrapie is the most studied TSE and its history goes back to 1700's where it was first reported in England by M'Gowan in 1710 (Plummer, 1946). This is a natural prion disease that can affect sheep and goat. Initial transmission studies done by Dammann in 1869, M'Fadyean in 1918 and Bertrand *et al*., in 1937 were negative as these studies failed to follow the long incubation period required for disease progression (Plummer, 1946). However, once the long incubation period for this disease was recognized it was shown to be transmissible to goats (Cuille and Chelle, 1936) and later by accidental transmission to sheep herd by administration of a contaminated vaccine (Gordon, 1946). Histopathologically, Brownlee in 1940 reported the presence of large vacuoles in medulla and spinal cord of all animals affected with scrapie (Plummer, 1946). Hallmark clinical symptoms of scrapie included intensive skin irritation caused by itching, rubbing and scratching which resulted in the animal rubbing itself against the posts, followed by motor and sensory impairments leading to ataxia or complete paralysis (Plummer, 1946). Environmental transmission was observed when unaffected sheep acquired the disease when grazed on pastures in previous contact with affected

animals (Plummer, 1946). To date, oral exposure of the contaminated agent has been shown to be a major route of entry for the infectious agent (Andreoletti *et al*., 2000; Greenlee & Greenlee, 2015; Gough K.C. 2010 and van Keulen *et.al*., 2002). However, recently, classical scrapie has been shown to be transmitted to cynomolgus macaque (which is the preferred animal model to study human prion diseases) after long incubation periods (Comoy *et al.*, 2015).

1.1.2 Chronic Wasting Disease (CWD)

Chronic wasting disease is a horizontally transmissible disease that has been reported to affect free ranging and captive deer, elk and moose populations in the United States, Canada and South Korea (Sohn *et al*., 2002). After its first appearance in Colorado in the 1960's, the disease quickly expanded (Kuznetsova, *et al.*, 2014). Clinical symptoms include excessive thirst, behavioral alterations and progressive weight loss followed by death in 2 weeks to 8 months (Williams, 1980). Although it is not conclusively known how CWD is transmitted in the natural environment, it has been shown that prion contaminated soil and plants can spread the infectious agent and cause disease (Johnson *et al.*, 2007; Pritzkow *et al*., 2015). CWD prions have been detected saliva, urine and feces of CWD infected deer by either *in vitro* PrP^{Sc} detection assays or *in vivo* infectivity bioassays (Haley *et al*., 2011; Henderson *et al.*, 2015). Oral exposure to contaminated material has been implicated as the most probable route for disease transmission (Denkers, Telling, & Hoover, 2011).

1.1.3 Bovine Spongiform encephalopathy (BSE)

BSE, also known as "mad cow disease", was first reported in Great Britain in the mid-1980's where more than 1000 cases of neurological disorder in cows were reported within a period of two years (Hope *et al.*, 1988). It was suspected that the most likely source of transmission in cattle was due to food-borne transmission (Parodi *et al*. 1990). This was later confirmed in 1996 when more than 160,000 cases of BSE were reported in the Great Britain with most likely source of origin being contamination of cattle's supplementary feed with meat and bone meal (MBM) from scrapie infected animals (Wilesmith *et al.* 1991; Anderson *et al.*, 1996). Since then, considerable measures were taken that resulted in significant reduction in BSE cases in cattle (Budka *et al.*, 2008). BSE may manifest clinically as changes in behavior that may include aggressiveness and motor impairment accompanied by weight loss. Even though BSE was suspected to arise from scrapie-contaminated MBM fed to cattle, the infectious agent responsible was found to be biochemically different than the one involved in scrapie (Stack *et al.*, 2002). Although there was a steady decline in BSE cases, a new variant form of Creutzfeldt-Jakob diseases was identified in 1996 that raised suspicions of disease acquired by consumption of meat that was contaminated with BSE infected cow (Will *et al*. 1996). Later transmission studies along with immunochemical analysis were able to confirm that the same agent involved in BSE in cows is also responsible for vCJD in humans (Collinge *et al.* 1996; Bruce *et al*. 1997; Hill *et al*. 1997; Weissmann & Aguzzi, 1997)

1.2 Human Prion Diseases

1.2.1 Kuru

Kuru was one of the first human prion diseases that were shown to be transmissible. This disease was first described by Gadjusek and Gibbs in 1959 in the fore people of Papua, New Guinea (Gajdusek & Zigas, 1959). The disease was characterized by cerebellar ataxia accompanied by tremor, choreiform and athetoid movements (Gajdusek & Zigas, 1959; Liberski P, 2012). It was believed to be spread due to a cannibalistic ritual that involved consumption of infected brains from the deceased. This was one of the first piece of evidence for oral transmission of prion diseases in humans. Later, in a breakthrough study, three chimpanzees intra-cerebrally challenged with brain extracts of kuru infected patients developed a disease similar to the one seen in humans (Gadjusek, 1966).

1.2.2 Variant Creutzfeldt-Jakob disease (vCJD)

As mentioned previously, a novel form of human prion disease was reported in the mid- nineties coinciding with the time BSE was epidemic in Great Britain (Will *et al*.; 1996; Collinge J and Rossor, 1996). Variant CJD, also known as the human equivalent of BSE, is of public health concern as its mode of transmission is food-borne. Consumption of BSE-infected cattle meat was the most likely route for transmission into humans (Ward *et al*., 2006) The incidence of vCJD has continued to decline since the outbreak in mid-nineties due to the BSE related feed control policy that was adopted by the United Kingdom (Will, 2003) but of concern now is the secondary spread of the disease by iatrogenic transmission (blood transfusions,

contaminated surgical instruments) as the incubation period for CJD can span for decades. Clinical features involve sensory and psychiatric symptoms followed by ataxia, cognitive impairment and involuntary movements (Zeidler *et al*., 1997). Neuropathologically, along with the rest of prion disease pathology, PrP^{Sc} positive florid amyloid plaques are present (Will, 2003). As vCJD is spread by consumption of contaminated BSE infected meat, there is also considerable peripheral deposition of PrP^{Sc} as found in skeletal muscle (Peden *et al*., 2006) and the lymphoreticular system (Hilton *et al*., 2004).

1.2 Pathogenesis of prions after oral ingestion

Even though oral transmission for prion diseases has been known for a long time, as shown by naturally or by deliberate inoculation of infectious agents (Maignien, *et. al*., 1999) surprisingly very little work has been done to determine the immediate fate of the infectious agent after ingestion. Previous studies have shown the localization of infectious particles in Peyers' patches of ileum and mesenteric lymph nodes (Aguzzi 2003; Bergstrom *et. al* 2006) followed by retrograde transport to the brain via peripheral nerves. Presence of PrP^{Sc} in spleen and gut-associated lymphoid tissues (Andreoletti *et al*., 2000) suggests that even after metabolic processing of PrP^{Sc} in the gastro-intestinal track the infectious agent is not degraded and is transported across the intestinal barrier. Many studies have been done showing trans-cellular transport of prions across the intestinal epithelial barrier from the apical (mucosal) side to basolateral (serosal) side in cell monolayers (Heppner *et al*., 2001; Mishra *et al*., 2004). Caco-2 cellular models for intestinal barrier have shown that PrP^{Sc} can cross the barrier by endocytosis upon interaction with the laminin receptor (Morel *et al.*, 2005). On the other hand, it has also

been shown that M cells mediate transcytosis of prions across epithelial cell monolayers (Heppner *et al.*, 2001). In another study, Mishra *et al*. have shown that a prion-ferritin complex is trans-cytosed in Caco-2 cell monolayers (Mishra *et al.*, 2004). These studies point out that after crossing the basolateral side PrP^{Sc} is immediately absorbed by dendritic cells circulating in blood. Studies using infectivity and histological techniques have shown that the circulatory system does not play a major role in PrP^{Sc} transport and the infectious agent is directly taken up by the enteric nervous system (Maignien *et al*., 1999; Kimberlin & Walker, 1989). Also, rapid appearance of infectious agent in sub-mucosal lymphatics and not in Peyer's patches of intestine has been shown in isolated gut loops inoculated with prions (Jeffrey *et al*., 2006). These results suggest that most likely once ingested prion protein can be transported by both peripheral nerves and/or blood. However, most of these studies used methods and techniques that rely on detection of prions after peripheral replication and not the original material administered. It is important to note that neither conventional histology nor infectivity studies have the sensitivity to detect the immediate transport of PrP^{Sc} within minutes or hours after oral exposure. One breakthrough technique for detection of prions that has led to substantial advances in the past decade is the Protein Misfolding Cyclic Amplification technology (PMCA). PMCA technology effectively enables prion replication even from a single oligomeric particle of PrP^{Sc} (Saborio, Permanne, & Soto; 2001; Saá *et al.*, 2006). Unlike conventional techniques, the great sensitivity achieved from this technique to amplify otherwise undetectable amounts of PrP^{Sc} has allowed for detection of prions in various bodily fluids at both pre-symptomatic and post-symptomatic stages of the disease (Saa, P., 2006; Chen, Morales, Barria, & Soto, 2010;

Moda *et al*., 2014). In addition, by using this technique it has been recently reported that inoculated prions can be detected at short times after intraperitoneal administration in experimental hamsters (Chen *et al*., 2014). The schematics involved in the PMCA technique are shown in **Figure 2** and expanded in the Methods section. A detailed study to determine the distribution and metabolism of PrP^{Sc} immediately after oral ingestion can provide key evidence to determine the bioavailability of prions and development of therapeutic strategies.

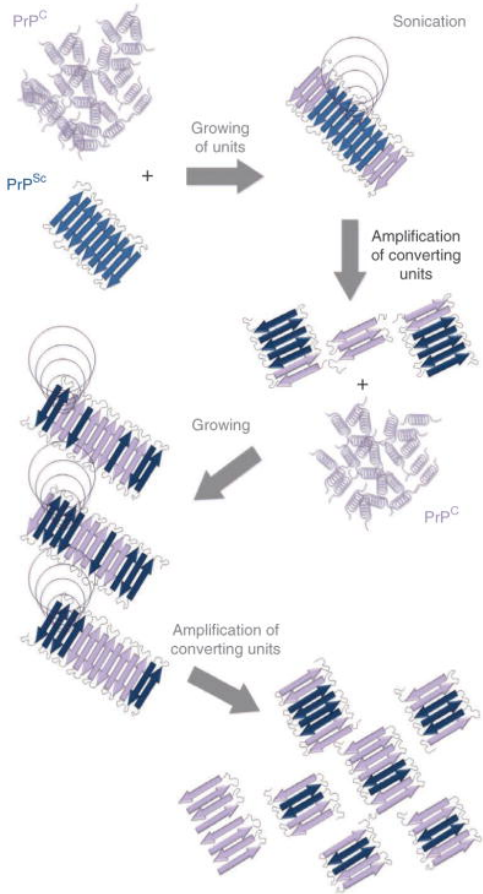

Figure 2: Schematic diagram for protein misfolding cyclic amplification. PMCA is based on the assumption that prion replication occurs by a seeding/nucleation model (Caughey, 2003), in which PrP^{Sc} seeds bind and misfold PrP^{C} by incorporating the protein into the polymer. In PMCA, PrP^{Sc} and PrP^{C} are mixed and incubated, allowing the misfolding of PrP^{C}, which permits the incorporation and growth of PrP^{Sc} aggregates. After incubation, samples are submitted to sonication in order to fragment PrP^{Sc} polymers, thereby generating new free ends suitable for continued prion replication. This process is cyclically repeated in order to produce an exponential amplification of PrP^{C} conversion (Morales, *et al*., 2012) (figure with legend reproduced with permission from Nature Publishing Group).

Hypothesis

Our hypothesis is that infectious prions resist gastrointestinal metabolism and directly cross the intestinal barrier after per oral administration, distributing in blood and various organs. In order to test this hypothesis, we will address following two aims: characterization of the metabolic profile of prions in gastrointestinal tract tissues by *in vitro* experiments and determination of the initial distribution of infectious material within hours of ingestion *in vivo*.

CHAPTER 2

Materials and Methods

Materials and Methods

Ethics statement

All animal experiments were approved by and conducted in strict accordance with guidelines of the Animal Care and Use Committee of the University of Texas Health Science Center in Houston and complied with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.1 Production of prion proteins in host animals:

2.1.1. Animal infection with Rocky Mountain Laboratories (RML) scrapie prions

The Inoculum for the infection study was prepared as follows;

Two mouse brains infected with RML prion strain were homogenized in 10mL Dounce homogenizer. A 10% homogenate was prepared in 1x PBS with protease inhibitor. The homogenate was spun down at 2000x*g* for 5 minutes. The supernatant was collected for inoculation in mice and debris was discarded.

2.1.1. Animal inoculation with RML prions

Twenty five female 129S2 mice at the age of 3-4weeks were purchased from Harlan laboratories (Houston, Texas). Mice were divided in groups of five in each cage and ears were punched for identification. They were housed in light and temperature controlled mouse prion room with 12 hours of light and dark cycles and free access to water and food. Each mouse was inoculated intraperitonealy with 100 µL of RML inoculum. After inoculation, mice were inspected daily for signs of prion disease. At seven months, the symptoms for early clinical stage of scrapie including piloerection, hunched posture and ungroomed appearance, were observed. At the terminal stages of disease which were defined by clinical signs of unsteady gate, ataxia, and limb

paralysis, mice showed 20% of body weight loss or complete paralyses were sacrificed. The brain was harvested from each mouse and frozen at -80°C for purification experiments.

2.2 Purification of PrP^{Sc}

Purification of PrP^{Sc} from the infected brain was performed as described previously (Hetz *et al.*, 2003) with modifications. A 10% brain homogenate (w/v) was prepared in 1xPBS with 20% Sarkosyl to precipitate prion proteins. The mixture was thoroughly sonicated using a probe sonicator (Bandelin Sonoplus) at power of 45% until it became clear. Then, the mixture was then centrifuged for 15 min at 5311 x *g* at 4°C (Beckman Coulter Allegra 25R). To the supernatant, 1/3 volume of 1X PBS/0.1%SB3-14 was added and ultra-centrifuged using T 70.1 rotor at 198,000 x *g* for 2h at 20°C (Beckman Coulter Optima XE-90 Ultracentrifuge). The resulting pellet was reconstituted with 1 ml of 1xPBS/10%NaCl/0/1%SB3-14, and sonicated until clear solution was obtained. One mL of the mixture was layered over 3 mL of 20% sucrose cushion made in 1x PBS/10% NaCl/0.1% SB3-14. This material was then ultra-centrifuged at 257,000 x *g* for 3h at 4°C using T 70.1 rotor. The pellet was dissolved in 1mL of 1xPBS/0.1%SB3-14 and extensively sonicated until the solution became clear. Then Proteinase K (PK) (Sigma-Aldrich) was added at the final concentration of 100µg/mL and the mixture incubated at 37°C for 2 h (Eppendorf Thermomixer R). The PK digestion process was stopped by adding 1mM of Pefabloc SC (Roche). Next, this material was sonicated and centrifugation with sucrose gradient for 1.5 h repeated. The final pellet was re-suspended in 300µL of sodium phosphate buffer (pH 7.4).

The preparation was labeled as purified PrP^{Sc}. Western blot and silver staining was performed with the purified PrP^{Sc} to confirm the purity of the material. The concentration of PrP^{Sc} was determined by micro BCA protein assay kit (Pierce).

2.3 Western blot of Purified PrP^{Sc}

Ten µL of purified PrP^{Sc} was dissolved in 1x sample buffer with 1mM DTT (Life Technology Inc. LDS sample buffer), the mixture heated for 10 min at 100°C, briefly spun down and loaded on 12% NuPage Bis-Tris gel (Life Technologies Inc). Also, 3µL of protein ladder (Fisher BioReagents™ EZ-Run™ Pre-Stained Rec Protein ladder) was loaded. The gel was run for 20 min at 70V and 1.5 h at 135V with 1x MES SDS running buffer (NuPage). After SDS-PAGE, the proteins were transferred at constant current of 0.8A at 4°C to a nitrocellulose membrane (GE Healthcare Amersham™ Protran™ premium 0.45µm NC) for 1 h. The membrane was blocked for to prevent unspecific protein binding by treating with 2% milk in PBST 0.05% for 1 h at room temperature. Following blocking, the membrane was incubated with 6D11 monoclonal antibody (Covance) diluted at 1:2500 at 4°C overnight on shaker. Next day, the membrane was washed for 10 min three times in washing buffer at room temperature. Thereafter, the membrane was placed in washing buffer containing anti-mouse IgG Secondary antibody (Sigma Aldrich anti-mouse IgG) diluted at 1:3000, and incubated at room temperature for 1hr with gentle shaking followed by three 10 min washes with PBST 0.05%. The membrane was developed using ECL prime kit (GE Healthcare Amersham ECL prime western blotting).

2.4 Silver Staining of PrP^{Sc}

Following gel electrophoresis, silver staining was carried out using silver staining kit (Invitrogen SilverXpress silver staining kit). All steps were performed according to the instruction from the manufacturer.

2.5 Radiolabeling of purified PrP^{Sc}

Radiolabeling of purified PrP^{Sc} was performed by chloramine-T method with [^{125}I]Na (Perkin-Elmer), as described in previous reports (Gitlin *et al.*, 1958 ; Urayama *et al,*. 2004). Purified PrP^{Sc} (10 μg) was mixed with [^{125}I] Na (2 mCi) in 250 mM chloride-free sodium phosphate buffer (pH 7.4), and the protein labeling was initiated by adding chloramine-T (10 μg/reaction) into the mixture. After 1 min, the reaction was terminated by adding sodium metabisulfite (100 μg/ reaction). Each labeled agent was purified by Sephadex G-10 chromatography to remove free iodine. The labeled PrP^{Sc} was diluted with phosphate buffered saline and further centrifuged in albumin pre-coated Microcon filtration tube (Mw cutoff: 10 kDa) at 12,000 rpm for 30 min to further remove free iodine from the G-10 eluate. Labeled PrP^{Sc} was extensively washed by this procedure. Radioactivity was measured after each wash (Cobra II gamma counter, Packard). The radioactively labeled PrP^{Sc} preparations we used had > 90% precipitation with trichloroacetic acid (TCA) (Sigma).

2.6 Preparation of tissue homogenates for *in vitro* metabolism study

Female 129S2 mice at 8-10 weeks of age were fasted for 16 h prior to being sacrificed for gastro-intestinal tissue collection. Following tissues were obtained for homogenate preparation, stomach, duodenum, jejunum, ileum and colon. Ten % (w/v) tissue homogenate in saline were freshly prepared on ice each time of experiment

(prepared in a Precellys 24 device). Each homogenates was spun for 5 min at 3000 x *g* at 4°C (Beckman Coulter Allegra 25R). The supernatant was saved for the study.

2.7 Tissue metabolism of ^{125}I-PrP^{Sc}

Metabolic profiling of ^{125}I-PrP^{Sc} was investigated by following procedure. Tissue homogenate (1mL) was incubated with ^{125}I-PrP^{Sc} (3 x 10^5 cpm) for 1, 3 and 6h at 37°C on a thermomixer (Eppendorf Thermomixer R) shaking at 600rpm. Three hundred µL aliquot was sampled at designated time points, and the sample frozen in liquid nitrogen for HPLC analysis at later time. Saline was also incubated with ^{125}I-PrP^{Sc} for control experiments.

2.8 Size-exclusion HPLC experiments

The stability of ^{125}I-PrP^{Sc} in stomach, duodenum, jejunum, and ileum and colon homogenates was examined using HPLC with a size exclusion column. HPLC system used for this study consisted from Shimadzu UFLC system LC-20AB and the size exclusion column BioSep-SEC-S4000 (7.8 mm × 300 mm, Phenomenex, CA). The mobile phase consisted of 25 mM sodium phosphate buffer (pH 7.4) Tissue sample was centrifuged at 12,000 x *g* for 10 min (Eppendorf Centrifuge 5415D). The supernatant (300µL) was separated and loaded onto HPLC column. Fractions were collected at 1mL/min interval for first five min followed by 10-second intervals for 5-15 min at a flow rate of 1.0 mL/min, and the radioactivity in each fraction was detected using a gamma counter. TCA precipitation of each fraction was also performed. Molecular weight markers, including thyroglobulin (669kDa), aldolase (158kDa), conalbumin (75kDA), carbonic anhydrase (29kDa) and aptotinin (6.7kDa) (GE Healthcare Gel Filteration Calibration kit HMW) were employed to estimate the relationship between

the size and retention time A single peak for each marker was confirmed and the peak time defined as the retention time. In addition, the retention time of Iodine-125 was measured.

2.9 Data analysis and statistical analysis

The radioactivity normalized by % of injected amount in each eluate from size-exclusion chromatography was plotted against retention time. The areas under the curve (AUC) values were calculated from the fractions between 6.0 and 10.0 min, which represents intact ^{125}I-PrP^{Sc}. The radioactivity below 3 times of the background level (70 cpm) was excluded from the analysis which was seen after 11.0 min.
TCA precipitation was performed to estimate the levels of intact ^{125}I-PrP^{Sc}.

For TCA precipitation, following was done:

In each HPLC fraction 100µL of 1% BSA (Sigma Aldrich) prepared in DI water was added followed by ice cold 500µL of 30% TCA (Sigma Aldrich) prepared in DI water. This mixture was then centrifuged for 15 min at 4000 x *g* at 4˚C. The supernatant and pellet was separated and the radioactivity in each was measured.

The radioactivity in each pellet and supernatant and % of radiolabeled protein was calculated by the following formula:

TCA%= cpm in pellet/ (cpm in pellet+cpm in supernatant)*100

2.10 Statistics analysis

Statistical comparison was performed by one-way ANOVA followed by Dunnet's multiple comparison test against percent area under the curve (%AUC) from the pre incubated input ^{125}I-PrP^{Sc}.

Methods and materials for distribution of PrP^{Sc} *in vivo* after oral ingestion

2.11 Inoculum preparation

Two symptomatic (RML prion strain) infected mice were sacrificed by CO_2 inhalation and brains were harvested. Ten percent homogenate (w/v) was prepared in PBS supplemented with a cocktail of protease inhibitor (Roche Diagnostics). The homogenate was further processed as described previously (Morales *et al*., 2012). The homogenate was briefly centrifuged at 800 x *g* for 1 min at 4°C (Beckman Coulter Allegra 25R). The supernatant was collected and incubated with 20% sarkosyl (prepared in PBS) for 15 min at room temperature on rotator. The resulting sample was then ultra-centrifuged using T70.1 rotor at 100,000 x *g* for 1 h at 4°C (Beckman Coulter Optima XE-90 Ultracentrifuge). Supernatant was discarded and two volumes of 1x PBS + PI were added and ultra-centrifuged again at 100,000 x *g* for 30 min at 4°C. The final pellet was re-suspended and sonicated in one volume of PBS + PI and frozen at -20°C for Western blot analysis and animal inoculations.

2.12 Oral inoculation of PrP^{Sc} in mice

Three female mice 129S2 aged at 8-10 weeks were fasted for 16 h prior to inoculation. Each animal was given 500 µL of inoculum and culled after two hours. Blood (heparinized, 12.5 mg/mL), brain, spleen, liver, colon, intestines and stomach were collected. Extreme precaution was taken while tissue collection to prevent cross contamination. Surgical instruments, decontaminated by immersion in 2N NaOH solution for 24hrs, were used for organ collection. Each tissue had separate sets of surgical instruments. The blood after ficol (GE Healthcare) was then separated into

plasma, buffy coat and red cells as suggested by the manufacturer (GE ficol paque). Each tissue sample was frozen in -80°C for PMCA experiments.

2.13 Tissue homogenates preparation for PMCA

Forty percent tissue homogenates were prepared as described above and treated with 20% sarkosyl prepared in PBS and ultra-centrifuged as explained in section 2.11. The final pellet was re-suspended in 100µL of PMCA substrate (mouse) prepared in conversion buffer as previously described (Morales *et al*., 2012) and later on in this section.

2.14 Blood components processing

The plasma and buffy coat collected (400µL) was mixed with equal volumes of PBS + PI and ultra-centrifuged at 100,000 x *g* and 4°C for 1h. Two volumes PBS + PI were added to resulting pellets and ultra-centrifuged in the same conditions mentioned above for additional 30mins. Final pellets were re-suspended in PMCA substrate.

2.15 Protein Misfolding Cyclic Amplification

The PMCA protocol was carried out as reported previously (Morales *et al*., 2012). PMCA substrate (wild-type mouse brain homogenate) was prepared as described previously (Morales *et al*., 2012). 129S2 mice were sacrificed by CO_2 inhalation and brains were collected following perfusion with PBS + 5mM EDTA. Immediately after harvesting, the tissue was frozen in liquid nitrogen and stored in -80°C. PMCA substrate preparation was done as following: 10% brain homogenate (w/v) was prepared in conversion buffer (150mM NaCl, 1% Triton X-100 in 1X PBS) with PI. Tissue pellets that were re-suspended in PMCA substrate were submitted to PMCA (Morales *et al*., 2012). The PMCA was carried out in an automatic horn

sonicator (Qsonica). Samples (in individually capped 0.2 mL PCR tubes) were placed in PMCA tube holder placed on an automatic horn sonicator (Qsonica) filled with water at 37°C and submitted to 240 PMCA cycles for first round followed by two rounds of 96 cycles. Each cycles consisted of 29 min and 40 sec of incubation in water bath at 37˚C followed by 20 second of sonication at Amplitude 30 and power between 260-300W. At the end of each round serial dilutions were made by diluting 10µL of the resulting material into 90µL of fresh PMCA substrate. These new mixtures were subjected to additional PMCA rounds to increase amplification efficiency. After each round, 19 µL of sample were saved for Western blot analysis.

2.16 Western blotting of PMCA amplified material

Samples saved from each PMCA round were treated with 50µg/mL PK and incubated for 1 h at 37°C with shaking. PK reaction was stopped by addition of sample buffer supplemented with 1mM DTT and heated for 10 minutes at 100°C. Western blots were then done on each sample as described above in 2.3.

CHAPTER 3

Results

3.1 Purification and radiolabeling of PrP^{Sc} from infected brain, and characterization of size-exclusion HPLC chromatography

Figure 3 shows immunoreactivity and protein purity of purified PrP^{Sc} of RML prion strain obtained through experimental procedure (Section 2.2). The presence of PrP^{Sc} in SDS-PAGE and subsequent western blotting was confirmed by silver staining in gels and specific immunoreactivity probed by 6D11 monoclonal antibody. PrP^{Sc} bands were ranged within the molecular weights between 27kDa and 30kDa as reported previously (Bendheim *et al*., 2015). After PK digestion the electrophoretic mobility of PrP^{Sc} (**Figure 3**) shows a majority of monoglycosylated isoform of prion protein and size of about 21kDa for the unglycosylated band (Morales *et al.*, 2007).

The purity was confirmed by silver staining in which protein derived bands other than PrP^{Sc} were not observed. Silver staining and western blotting of PrP^{Sc} were conducted each time when purification of PrP^{Sc} from infected brain was performed, and results were reproducible.

Radiolabeling of PrP^{Sc} was performed with these preparations. Tricholoacetic acid assay showed >90 percent of purified PrP^{Sc} protein being successfully labeled with ^{125}I.

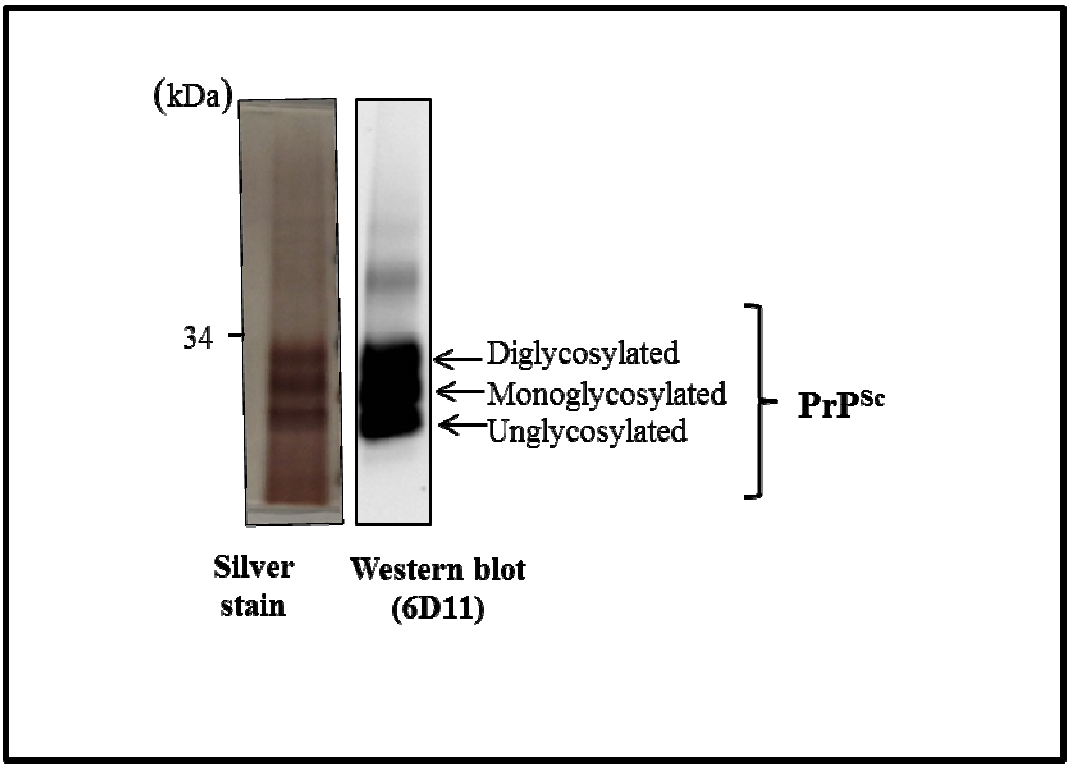

Figure 3. Purification of PrP^{Sc}. Silver staining (left) and western blot (right) of purified PrP^{Sc}

The size distribution of $^{125}I\text{-}PrP^{Sc}$ based on molecular weight was analyzed by size-exclusion radio-HPLC chromatography **(Figure 4A).** The HPLC eluate was reserved for TCA precipitation to estimate intactness of $^{125}I\text{-}PrP^{Sc}$. In addition, the retention times in size-exclusion column were characterized with high molecular weight markers (**Table 1**), For markers the retention times were; 7.35 minutes for thyroglobulin (669kDa), 9.45 minutes for aldolase (158kDa), 10.0 minutes for conalbumin (75kDa), 10.8 minutes for carbonic anhydrase (29kDa) and 12.0 minutes for aprotinin (6.5kDa). Also, the retention time for iodine-125 was 12.3 minutes (**Fig 4B**).

^{125}I-PrP^{Sc} (1x10^5 cpm) (now will be referred to as input ^{125}I-PrP^{Sc}) was injected onto the column and fractions were collected at the flowrate of 1mL/min from 1-5 min at 1 min interval and from 5-13 min at 10 sec intervals.

The radio chromatogram of input 125I-PrP^{Sc} (1x10^5 cpm) is shown (**Figure 4A**). ^{125}I-PrP^{Sc} was injected onto the column and fractions were collected at the flowrate of 1mL/min from 1-5 min at 1 min interval and from 5-13 min at 10 secs intervals. On x-axis is time in min and right y-axis is raw cpm (blue) and TCA normalized cpm (red) (**Figure 4A**). On right y-axis is percent of injected dose/mL (%ID/mL) plotted (**Figure 4A**). The chromatogram of ^{125}I-PrP^{Sc} spans between 6.0 -10.0 min showing various sizes ranging from 669 to 29 kDa (**Figure 4A**). Separately, iodine-125 was characterized in the column showing a retention time at 12.3min (**Figure 4B**).

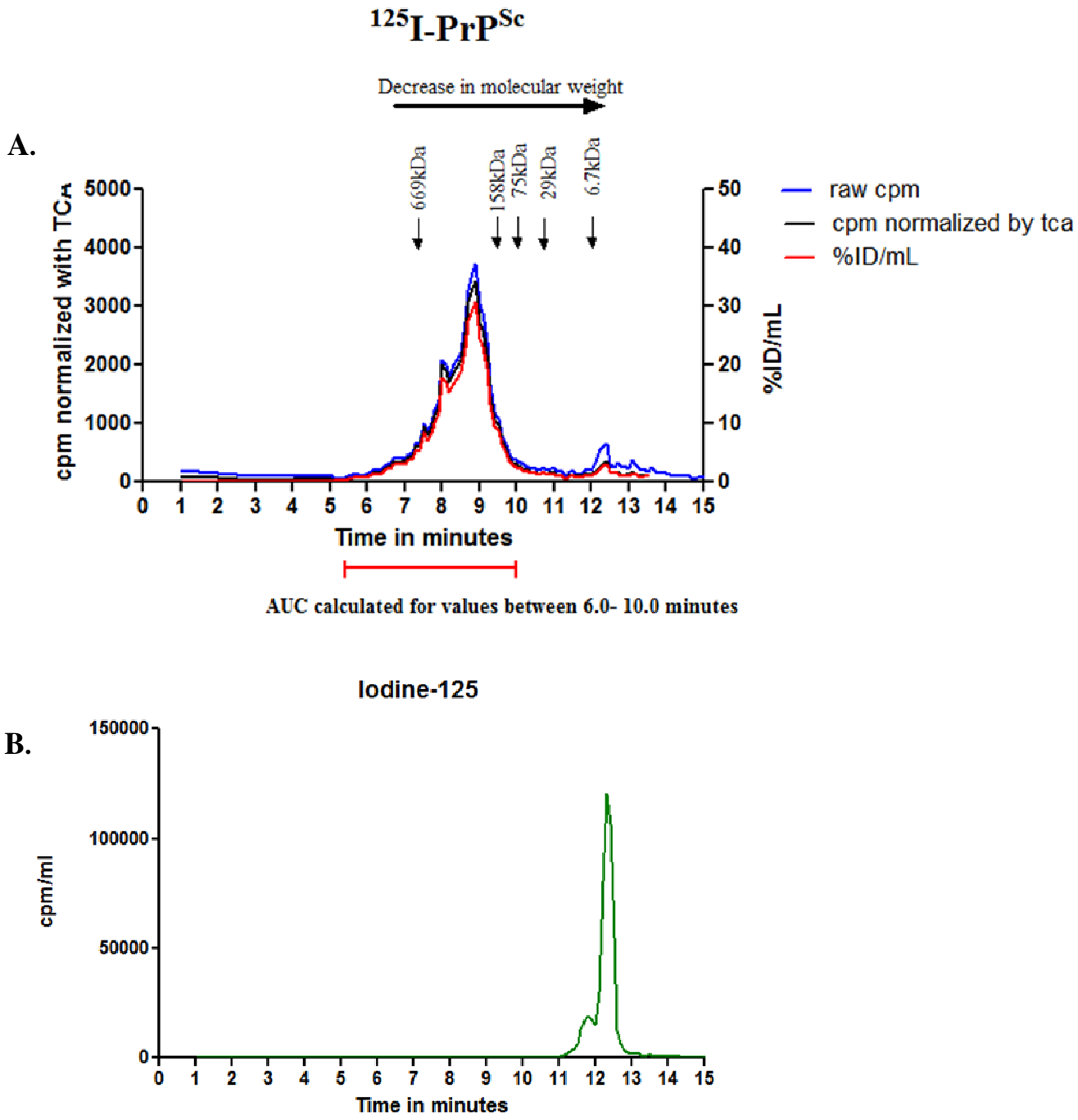

Figure 4. Radio-HPLC chromatogram of ^{125}I-PrP^{Sc} with molecular weight markers is shown. The area under the curve was analyzed for 6.0 -10.0 minutes as shown by red line under x-axis **(A)**

Retention time of iodine-125 was 12.3 minutes **(B)**

Protein	Molecular Weight (Da)	Retention time (minutes)
Thyroglobulin	669,000	7.35
Aldolase	158,000	9.45
Conalbumin	75,000	10.00
Carbonic anhydrase	29,000	10.80
Aprotinin	6,500	12.00

Table 1. High molecular weight markers retention times in Phenomenex Bio sep sec s4000 size exclusion column

3.2 Characterization of metabolic pattern of radioactively-labeled, PrP^{Sc} by trichloroacetic acid precipitation assay (TCA) and high-performance liquid chromatography (HPLC) *in vitro*

Next, freshly prepared tissue homogenates (stomach, duodenum, jejunum, ileum, and colon) were incubated with ^{125}I-PrP^{Sc} ($3x10^5$ cpm) for 1, 3 and 6 h and analyzed by radio-HPLC chromatography **(Figure 5)**. The %ID/mL of tissues was plotted with input ^{125}I-PrP^{Sc} (**Figure 5)**. As a processing control, ^{125}I-PrP^{Sc} was also incubated with saline. The radio-chromatogram of saline shows minimal changes in ^{125}I-PrP^{Sc} after 1hr (**Figure 5A).** The radio chromatogram of stomach, colon, and saline control (**Figure 5B, 5F** and **5A)** shows the major peaks between 6.0-10.0 min which was similar to the input ^{125}I-PrP^{Sc}. In contrast, the duodenum (**Figure 5C)** , jejunum **(Figure 5D)** and Ileum **(Figure 5E)** showed decrease in intact protein of ^{125}I-PrP^{Sc} compared to input ^{125}I-PrP^{Sc}, indicating that the amount of intact^{125}I-PrP^{Sc} was reduced in duodenum, jejunum and ileum tissue 1h after incubation. In addition to 1 h, for each

tissue homogenate samples collected at 3 h and 6 h were also injected in HPLC column and chromatograms were analyzed. Results were similar to the 1hr time point **(Figures 6 and 7)**.

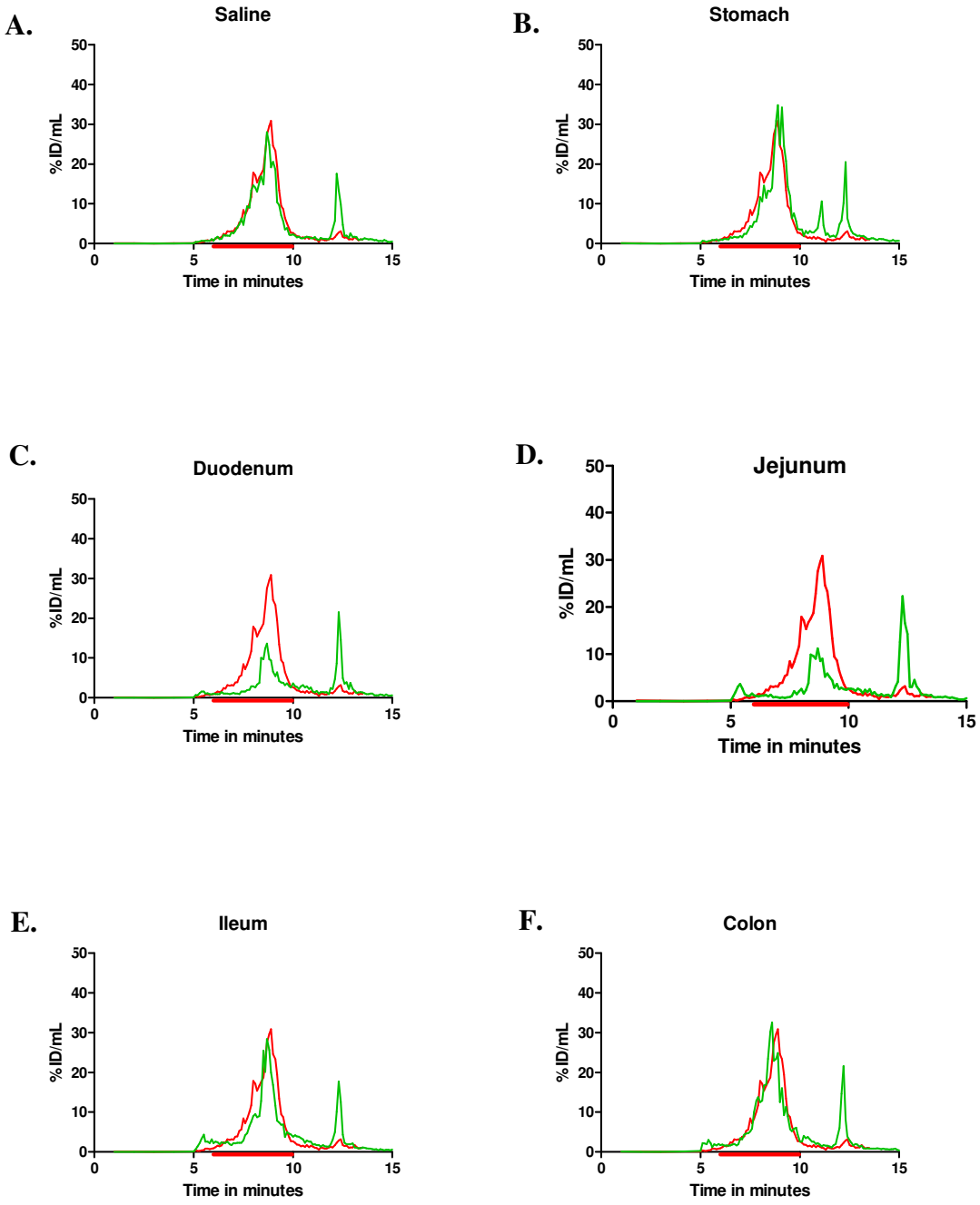

Figure 5. Radio HPLC-Chromatogram of tissue homogenates incubated with ^{125}I-PrP^{Sc} at 1hr. Saline only **(A)** , Stomach **(B)**, Duodenum **(C)** , Jejunum **(D)**, Ileum**(E)** and Colon**(F)** %Injected dose per mL (%ID/mL) of input ^{125}I- PrP^{Sc} (red) and %Injected dose per mL (%ID/mL)of tissue homogenates (green). The red line on x-axis of each graph marks the time (6.0-10.0min) measured for calculating total area under the curve.

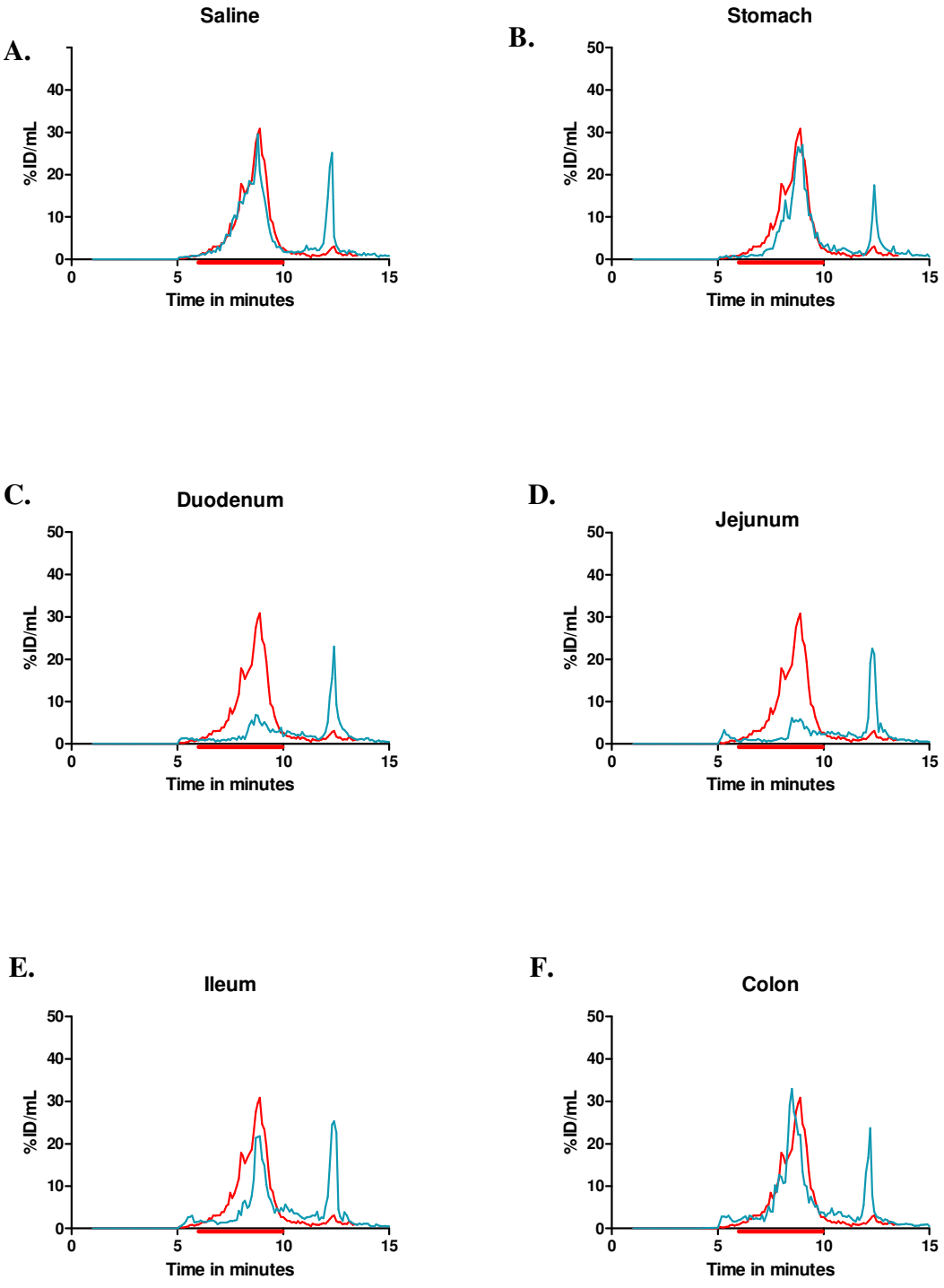

Figure 6. Radio-HPLC Chromatogram of tissue homogenates incubated with ^{125}I-PrP^{Sc} at 3hr. Saline only **(A)**, Stomach **(B)**, Duodenum **(C)**, Jejunum **(D)**, Ileum **(E)** and Colon **(F)** %Injected dose per mL (%ID/mL) of input ^{125}I-PrP^{Sc} (red) and %Injected dose per mL (%ID/mL)of tissue homogenates (blue). The red line on x-axis of each graph marks the time (6.0-10.0min) measured for calculating total area under the curve

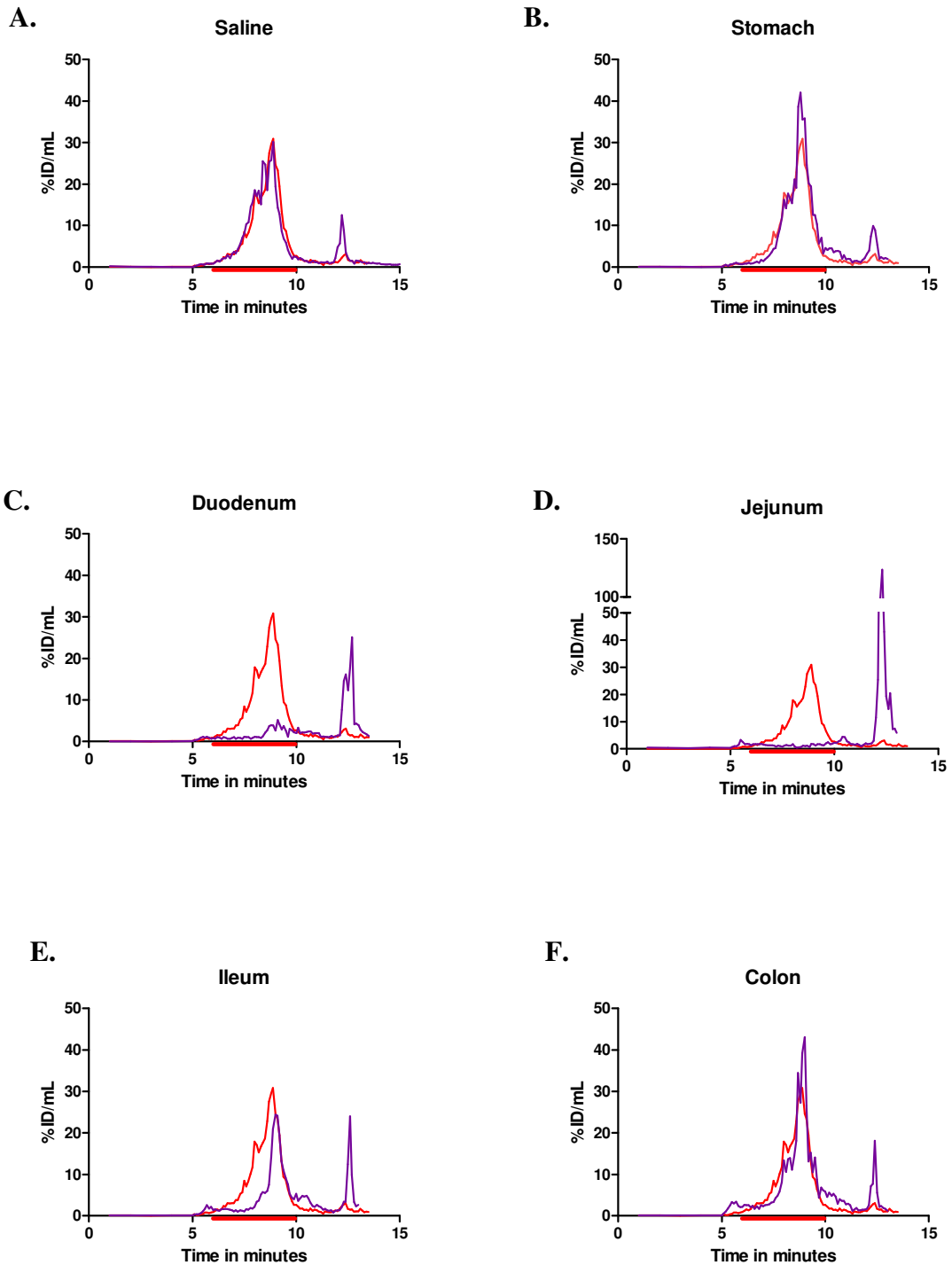

Figure 7. Radio-HPLC Chromatogram of tissue homogenates incubated with ^{125}I- PrP^{Sc} at 6hr. Saline only **(A)**, Stomach **(B)**, Duodenum **(C)**, Jejunum **(D)**, Ileum **(E)** and Colon **(F)** %Injected dose per mL (%ID/mL) of input ^{125}I-PrP^{Sc} (red) and %Injected dose per mL (%ID/mL) of tissue homogenates (purple). The red line on x-axis of each graph marks the time (6.0-10.0 min) measured for calculating total area under the curve

Table 2: %AUC in tissues is based on the AUC value compared to the input ^{125}I-PrP^{Sc} as 100 ± 20.7%

Time in Hours	Stomach	Duodenum	Jejunum	Ileum	Colon	Saline only
1	70.2 ± 16.2	36.3 ± 7.2	37.4 ± 7.1	56.2 ± 9.2	64.4 ± 16.5	60.4 ± 11.7
3	64.3 ± 11.2	29.3 ± 7.2	35.2 ± 8.1	42.3 ± 5.7	56.4 ± 19.3	64.0 ± 9.5
6	79.0 ± 14.2	28.5 ± 9.1	31.0 ± 11.2	42.1 ± 7.7	56.3 ± 22.1	67.5 ± 14.3

Mean ± SEM (n=3) are reported

The *in vitro* metabolite study to measure intactness of PrP^{Sc} in gastro-intestinal tissue homogenates at various time points was repeated by performing three separate experiments. The mean and SEM of % area under the curve (AUC) (measured between 6.0-10.0 min) for all tissues at different time points is shown **(Table 2).**

In table 2 the mean of %AUC (6.0-10min) along with its respective SEM is listed for each tissue. As seen by table 2 the change is metabolic profile for duodenum, jejunum and ileum is time-dependent and compared to input ^{125}I-PrP^{Sc}. The amount of reduction seen in graph duodenum, jejunum and ileum homogenates (**Figure 8**) are less than 50% as compared to the input ^{125}I-PrP^{Sc}.

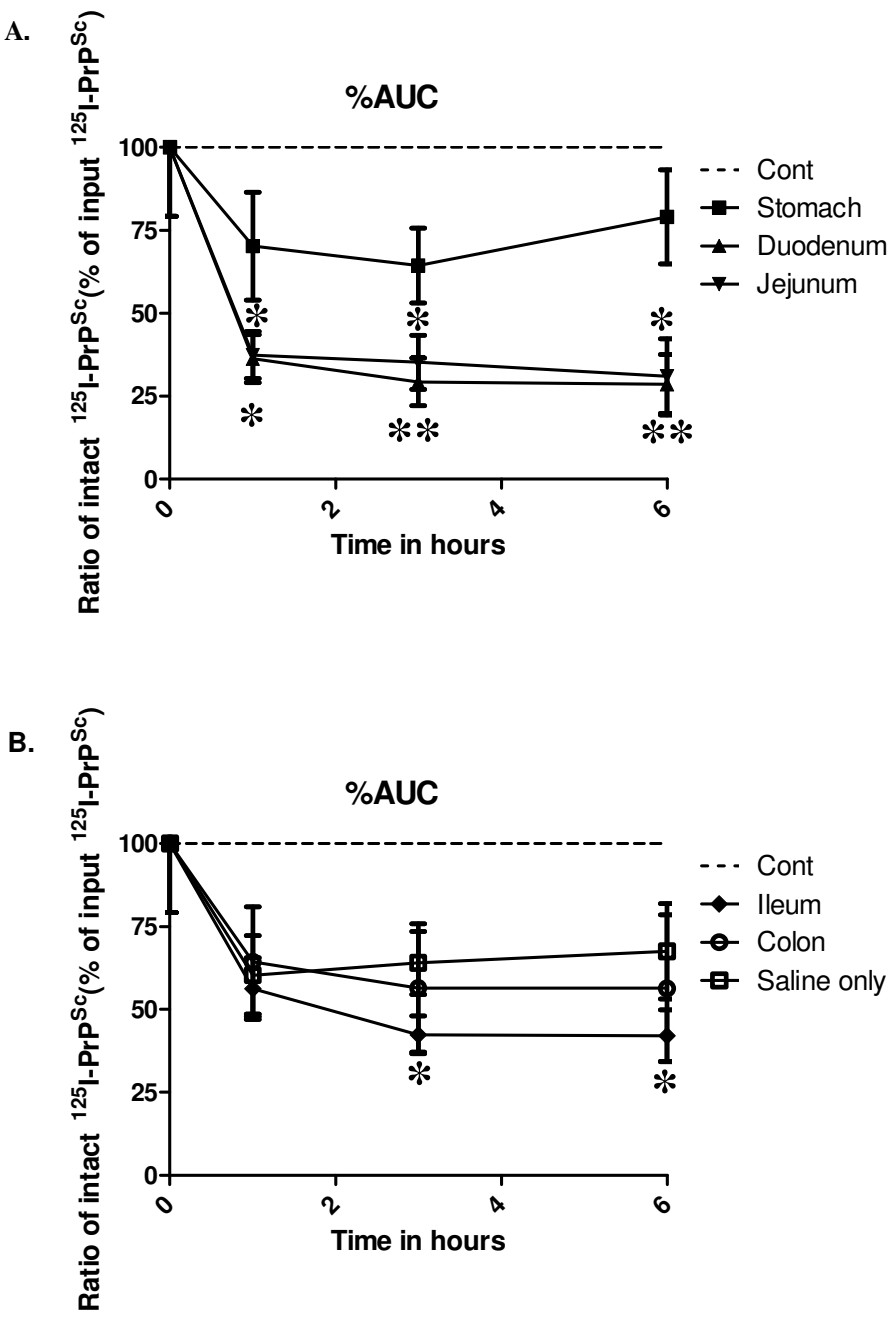

Figure 8. Figure 8. Gastrointestinal (GI) metabolite profile of ^{125}I-PrP^{Sc} after incubation with 10% mouse tissue homogenates. (**A**) The metabolic profile of stomach, duodenum and jejunum. **(B)** The metabolic profile of ileum, colon and saline only control. Each time point represents the mean value and standard error of the mean. All measurements were done in triplicates. Comparison of total area under the curve (6.0 – 10 min) of tissue homogenates incubated with ^{125}I- PrP^{Sc} all time points against input material (^{125}I- PrP^{Sc}) as 100% ± 20.7
Statistical comparison was performed using one-way ANOVA followed by Dunnet's multiple comparison tests
*p <0.05, **p<0.01

Summary

In-vitro metabolic profile of prions in gastro-intestinal tissues

Detailed analysis of metabolic profile of ^{125}I-PrP^{Sc} in gastro-intestinal tissues showed the amount of reduction in intact ^{125}I-PrP^{Sc} with time. The significant reduction is seen in the duodenum, jejunum and ileum, which is mediated through endogenous protease activity.

3.3 Detection of self-propagating prions in blood and tissues after per-oral inoculation using the protein misfolding cyclic amplification (PMCA) technology

As a preliminary approach to bio-distribution studies, we designed and performed experiments aimed to quantitatively detect self-replicating prions in blood and tissues of mice orally challenged with misfolded PrP. To study the immediate distribution of PrP^{Sc} *in vivo*, first we prepared a homogeneous inoculum suitable for our purpose (i.e. free from tissue debris). The material to inject in mice was prepared by mixing one volume of 10% BH and one volume of a 20% sarkosyl solution in order to disrupt membranes and isolate protein aggregates from unspecific bound molecules. After 15 min incubation at RT gently shaking, samples were ultra-centrifuged. Resulting pellets were washed and finally re-suspended in saline buffer using extensive sonication (see chapter 2). Western blots of 10% RML brain homogenate before and after treatment are shown in **Figure 9**. As seen in the figure, this treatment resulted in a minimal loss of PrP^{Sc}, data consistent with previously published results (Morales *et al*., 2008). Next, this inoculum was subjected to PMCA (see chapter 2 methods and materials) to set *in vitro* amplification conditions for this specific inoculum **(Figure 10).** Three PMCA rounds were done 48 h each round (see Chapter 2 for details). The initial samples, labeled as frozen (not submitted to PMCA) are shown **(Figure 10A).** As expected, they do not show positive signal in the Western blot due to their high dilution. After first round, 10^{-5} dilution of inoculum was detected **(Figure 10B)**. Following PMCA rounds revealed presence of misfolded prions in 10^{-10} brain dilution **(Figure 10C and Figure 10D)**. Also two negative controls containing 10% normal mouse brain

homogenate only were added to the PMCA reaction to control for inter-tube contamination. As expected, results were negative confirming true PrP^{Sc} detection.

Bioassays were performed by injecting 500µL of this inoculum per orally in three wild type mice to study short-term distribution in various tissues.

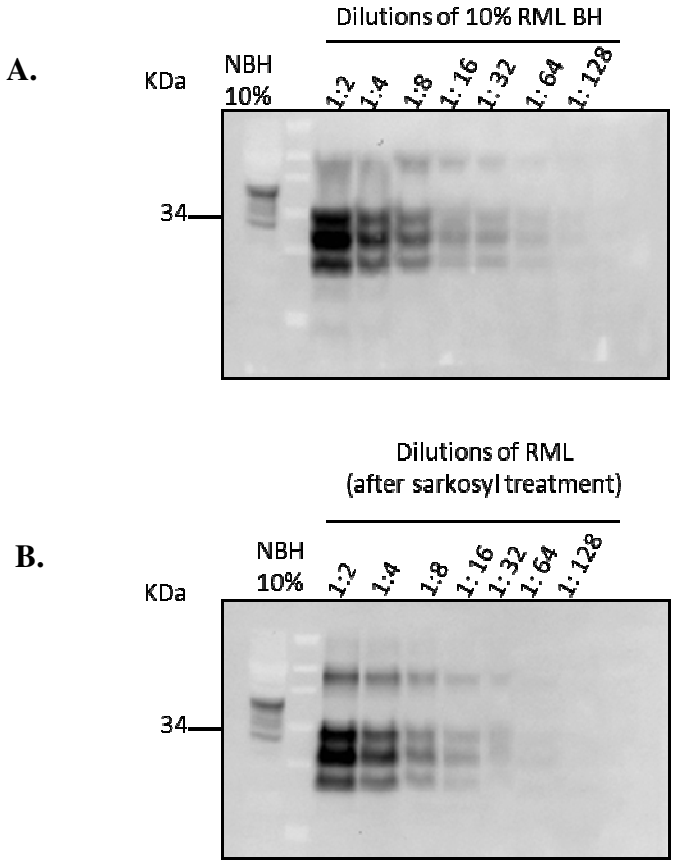

Figure 9. Western blot of 10% PrP^{Sc} (RML) brain homogenate dilutions (**A**). Western blot of 10% PrP^{Sc} (RML) brain homogenate dilutions after sarkosyl treatment (**B**). All samples were treated with PK 50µg/mL, 1 h at 37°C, shaking at 600rpm. First lane on both A and B is 10% normal mouse brain homogenate without PK treatment for reference.

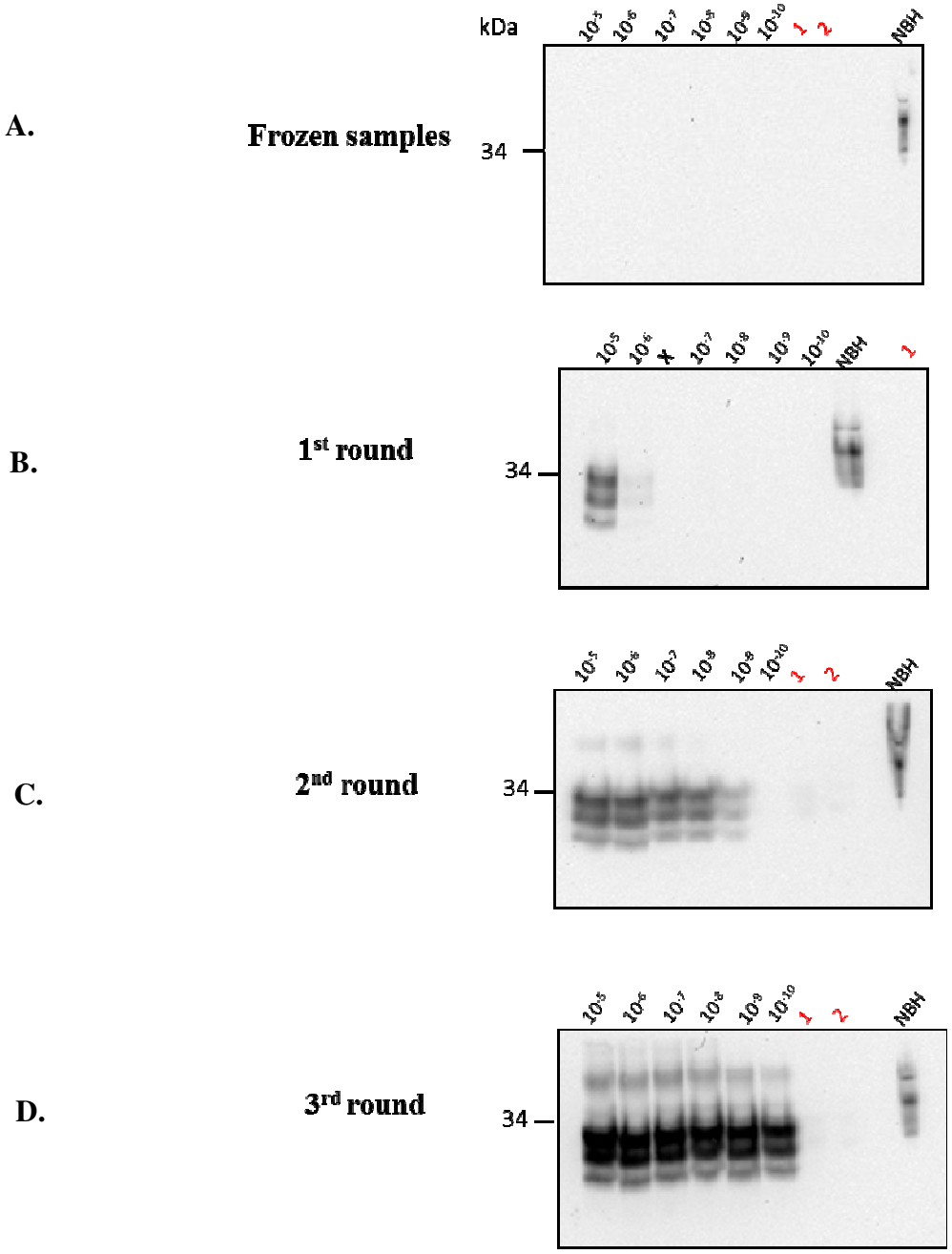

Figure 10. Western blot of "sarkosyl cleared" inoculum after PMCA. Dilutions of inoculum in normal brain substrate **(A)** not submitted to the PMCA reaction (frozen). PMCA amplification after first, second and third rounds **(B - D).** Labelled in red is the negative control on each gel (A-D).x means no sample in that lane (B).

Prior to inoculation, mice were fasted for 16h with free access to water in order to clean the gastro-intestinal tract from fecal material. Two hours after per oral administration

of prion containing material, the three mice were culled and blood, brain, spleen, liver, colon, intestines and stomach were collected respectively. For each tissue, either 10% (stomach, intestines, colon, liver, spleen, brain) or 40% homogenates (stomach, intestines, colon, liver, spleen, brain) (w/v) were prepared in PBS supplemented with PI and subjected to PMCA (see Chapter 2 for details). Before PMCA, Western blots were performed on 10% tissue homogenates (w/v) in order to assess for direct visualization of PrP^{Sc} by this method. As expected, none of the tissues analyzed showed positive signal for PrP^{Sc}, indicating that the concentration in each tissue was below the detection limit of conventional techniques **(Figure 11)**. Signals seen in the Western blots corresponded to unspecific bands as tested by incubating and developing the blot membranes with secondary antibody only. These non-specific bands are expected to be a consequence of blood components (data not shown).

After PMCA, positive signal was detected in plasma (3/3 animals), brain (3/3 animals and intestines (1/3 animal) **(Figure 12)**. These results show that self-replicating prions can directly cross the intestinal barrier and target different organs. The effective detection of PrP^{Sc} in brain and no other tissues is suspected to be a consequence of the PMCA conditions which has been standardized for this specific tissue. As seen, all negative controls (substrate only) in the PMCA stayed negative s suggesting absence of false positive signals in our specimens **(Figure 12)**.

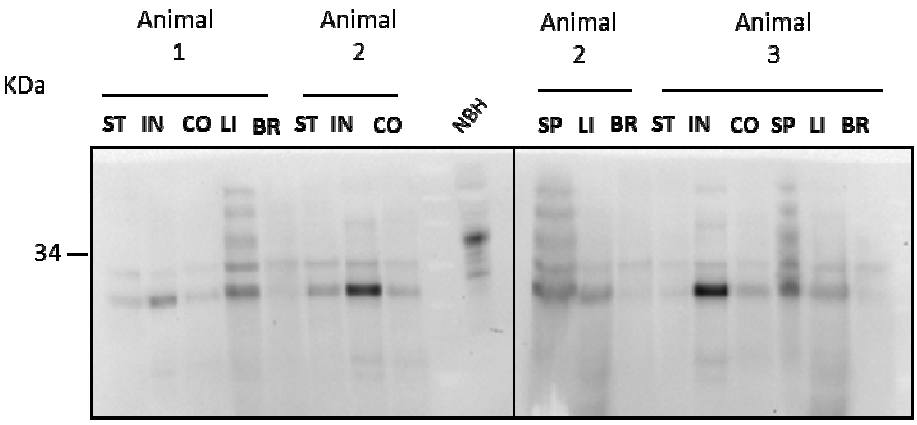

Figure 11. Western blot of 10% mouse tissue homogenates (before PMCA) taken two hours after per oral inoculation with sarkosyl treated PrP^{Sc} (RML) **(A and B)** ST: Stomach, IN: Intestines, CO: Colon, LI: Liver, SP: Spleen, BR: Brain

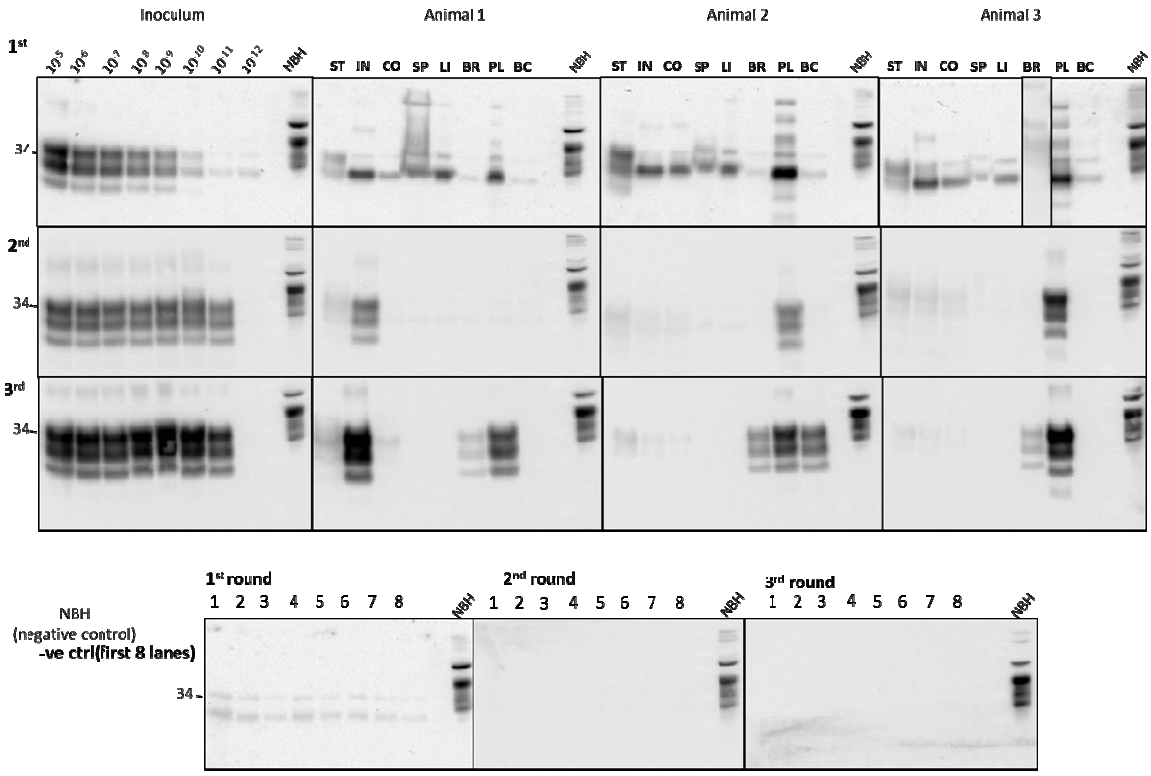

Figure 12. Western blot of tissue homogenates after PMCA amplification
All samples are treated with PK 50µg/mL, 1h 37˚C at 600rpm before gel electrophoresis. Three rounds done first round was five days followed by 48hrs round each. 50µL of 40% Tissue homogenate concentrated. Blood equivalent to 400µL of whole blood used (**A**)**.** Negative controls with just normal brain homogenate (**B**)
ST: Stomach, IN: Intestines, CO: Colon, LI: Liver, SP: Spleen, BR: Brain, PL: Plasma and BC: Buffy coat

Summary

Initial distribution of infectious material within hours of ingestion *in vivo*

In this section, we were able to partially purify the prion protein from terminally infected mice brains, prepare the inoculum from it and successfully inoculate animals via oral ingestion. Two hours after, animals were culled and various tissues, including blood, were collected and subjected to various rounds of PMCA. Our results show the efficient detection of self- propagating prions in plasma for all three animals inoculated **(Figure 12)**. Also, positive signal was seen in intestines (1/3) and brain (3/3) animals. These results signify that within extremely short time after ingestion of prions the self-propagating agents can spread through the gastrointestinal barrier and be taken up by blood.

CHAPTER 4

Discussion

4.1 Characterization of metabolic pattern of radioactively-labeled PrP^{Sc}

The gastro-intestinal tract serves as an important barrier as well as interface for the materials orally ingested. Much work has been done to study the transport of PrP^{Sc} from gut to brain (Kimberlin *et.a*l. 1989; Beekes *et.al*. 2000; Andreoletti et al. 2000, Shmakov *et.al*.2001; Bergström *et al*. 2006; Kujala *et al*. 2011). Surprisingly, very little is known about the metabolic profile of prions at short times after ingestion. Most of the methods used previously to determine this were not sensitive enough to detect the smallest changes in the size distribution of the PrP^{Sc} aggregate after digestion. Knowing this information can be vital in determining the mechanism and initial fate of the infectious agent and its bioavailability *in vivo*. In order to study PrP^{Sc} metabolism it is important to have a detailed and quantitative *in vitro* characterization of PrP^{Sc} stability in gastrointestinal tissue homogenates. This information can provide data about the maximal capability of each tissue to metabolize PrP^{Sc}. In this aim, we investigated PrP^{Sc} metabolism in various gastrointestinal tissues homogenates at different time points. To achieve this, the first step was to have a pure and efficient preparation of PrP^{Sc}. Various purification protocols have been reported that yield a variable degree of PrP^{Sc} purity. In our studies we modified previous methods to obtain an extremely pure preparation of PrP^{Sc} from infected mouse brain homogenates **(Figure 3)**. This is in agreement to a study that was just recently published showing purity of PrP^{Sc} comparable to that obtained in our preparations (Wenborn *et al*. 2015). Freshly prepared stomach, duodenum, jejunum, ileum and colon homogenates were separately incubated with biologically active radioactively labeled PrP^{Sc}. Samples were collected at 1, 3 and 6 hours and analyzed by HPLC to determine the degradation profile of the

radiolabeled protein. The difference in the profile of the radio-HPLC chromatogram of ^{125}I-PrP^{Sc} (input ^{125}I-PrP^{Sc}) was compared to the profile after incubation with GI tract tissue homogenates. Radiolysis and intactness was measured by TCA precipitation analysis coupled to HPLC characterization. Our results show for the first time that purified ^{125}I-PrP^{Sc} incubated in duodenum, jejunum and ileum homogenates undergoes a significant degradation (more than 50%) which could be due to endogenous proteases present in the freshly prepared tissue homogenates. This is in contrast to other tissue homogenates from the GI track, such as stomach and colon in which levels of ^{125}I-PrP^{Sc} underwent minimal reduction. It has been shown that the most probable site for PrP^{Sc} uptake after oral exposures are the intestinal lymphoid follicles (Payer's patches) (Beekes & McBride, 2000). Previous work has been done with gut loops from wild type sheep investigating the digestion of PrP^{Sc} in distal jejunum and ileum components at short time points ranging from 15 to 120min. Their results showed that PrP^{Sc} signal reduction is observed in both *in vitro* and *in vivo* intestinal environment (Dagleish *et al*. 2010). Studies done for 48 hours have shown almost complete degradation of ^{125}I-PrP^{Sc} incubated in a cocktail of alimentary track fluids (Jeffrey *et al*. 2006). However, these studies have just examined the digestion of PrP^{Sc} in distal jejunum and ileum and not in the duodenum and proximal jejunum. Also, these studies did not analyze a detailed profile of PrP^{Sc} degradation and unlike our highly sensitive experiments using radiolabeled PrP^{Sc} used less sensitive techniques like western blot for detection. Our results show that purified PrP^{Sc} appears as a broad peak with an estimated molecular weight of 310kDa, indicating of a small oligomer (8-10 monomers of PrP). After incubation with duodenum and jejunum extracts (**Figure 8**) this oligomer is rapidly

degraded. It has been shown previously that minimum infectious unit of PrP^{Sc} is a trimer of PrP^{Sc} (Groth, 1988), but the predominant species appear as large molecular weight fibrils and intermediate size oligomer in the range of what we observed in the present study. It is likely that large fibrillar aggregates of PrP^{Sc} were removed during the purification or the radio-labeling procedure. Nevertheless, it has been shown that the most infectious prion particles are oligomers of around 500KDa molecular weight (Silveira, 2005). In the light of our finding it would be interesting to investigate further the exact role of duodenal and jejunum in uptake and transport of prions from GI tract to the brain *in vivo*.

Detection of self-propagating prions in tissues after per-oral inoculation

The most natural route for prion transmission is by ingestion of contaminated materials. Previous results from our lab showed that prions intra-peritonealy injected in hamsters were detected in brains and spleens by PMCA within few days after inoculation, although at sub infectious levels (Chen *et. al*., 2014). Here, we investigated the initial uptake of prions in blood at shorter time points after oral challenge which is probably the most natural route for transmission of prion diseases. Much of the previous work has been done by infectivity bioassay to detect prions after long times after administration. Moreover, most of the techniques used for detection in these studies were not sensitive enough to detect minute quantities of prions within hours in various tissues *in vivo*. Here we showed that by employing the PMCA technology we can detect the tiny quantities of self-propagating prions in different biological samples after oral injection. Three mice were inoculated with RML prions (an established

mouse prion strain) and sacrificed after 2 h. The content of infectious prions in several organs related to prion uptake and transport were measured by PMCA **(Figure 12)**. Positive signals for PrP^{Sc} were detected in plasma of all animals orally inoculated with prions. Previous work has shown that infectious agent can reach the brain from blood because of vascular deposition of PrP^{Sc} seen in the basement membrane of endothelial cells of hypothalamus (Sílvia Sisó, González, & Jeffrey, 2010). Also, deposition of PrP^{Sc} in circumventricular organs in sheep was always observed regardless of the route of challenge suggesting that PrP^{Sc} can reach the brain by blood transport (S. Sisó, Jeffrey, & González, 2009). In agreement with a recent study (Elder *et al*. 2015), our results show that prions orally ingested can cross the gastro-intestinal barrier and be taken up in blood within just two hours after inoculation. Importantly, PrP^{Sc} found in blood and organs was intact and retained the capacity of self-propagating in a PMCA reaction **(Figure 12)**. Also, this is the first time self-propagating prions have been detected in brain after oral inoculation suggesting a direct transport of inoculated prions without peripheral replication. This information can be vital in understanding the pathway followed by prions immediately after ingestion and quantifying the amount taken up by various tissues. Also, work in this regard can help develop early interventional therapies and diagnostic tests for prion diseases.

CHAPTER 5

Conclusions and Future Directions

Conclusions and future work

In conclusion, as part of this work we have shown two findings that were not known previously in the field of prion diseases. First, our metabolic profiles of prions in gastrointestinal tract show that duodenum, jejunum and ileum have the capacity to degrade PrP^{Sc}. For future, it would be necessary to study this in an *in vivo* setting to map a distribution and degradation profile of orally ingested prions. Secondly, we have shown that following oral ingestion of prions the self-propagating agent reaches the blood and brain within just two hours. Future work in this regard can involve a detailed quantitative study including more time points and tissues. Also, it would be important to study the mechanisms responsible for PrP^{Sc} transport at the intestinal barrier and the subsequent distribution from blood to various tissues especially brain and what role does this play in the pathogenesis of the disease.

The above study also lays some of the groundwork to obtain a detailed knowledge of how prions are taken up, distributed, metabolized and cleared from the body which can provide an estimation of tissue risks and development of therapeutic strategies. More than likely in natural infection of prions the quantity of the infectious agent an organism is exposed to is very low, thus it is imperative to know the quantity and mechanism of the amount of the infectious agent is absorbed, metabolized, distributed and cleared in the body. The initial fate of the infectious agent could play a vital role in determining how the disease would manifest and progress with time.

The findings generated by bio-distribution and *in vivo* metabolite profiling can open novel avenues to develop therapeutic strategies aiming to prevent prions to get into the

brain or increasing prion elimination by enhancing metabolism. Also, our finding that self-propagating prions can reach blood and brain within two hours of oral inoculation opens up new avenues to further investigate mechanistically how infectious prions can be taken up by various tissues leading to a catastrophic disease in the brain and what other alternate transport pathways play a role in facilitating disease pathogenesis.

Chapter 8

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VITA

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