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Title: Accelerated shedding of prions following damage to the olfactory epithelium

Running Title: Accelerated olfactory shedding of prions

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Abstract

In this study we investigated the role of damage to the nasal mucosa in the shedding of prions into nasal fluids as a pathway for prion transmission. Here we demonstrate that prions can replicate to high levels in the olfactory sensory epithelium (OSE) in hamsters and that induction of apoptosis in olfactory receptor neurons (ORNs) in the OSE resulted in sloughing off of the OSE from nasal turbinates into the lumen of the nasal airway. In the absence of nasotoxic treatment olfactory marker protein (OMP), which is specific for ORNs, was not detected in nasal lavages. However, after nasotoxic treatment that leads to apoptosis of ORNs both OMP and prion proteins were present in nasal lavages. The cellular debris that was released from the OSE into the lumen of the nasal airway was positive for both OMP and the disease-specific isoform of the prion protein, PrP^{Sc}. Using the real time quaking-induced conversion assay to quantify prions, a 100- to 1,000-fold increase in prion seeding activity was observed in nasal lavages following nasotoxic treatment. Since neurons replicate prions to higher levels than other cell types and ORNs are the most environmentally exposed neurons, we propose that an increase in ORN apoptosis or damage to the nasal mucosa in a host with a pre-existing prion infection of the OSE could lead to a substantial increase in the release of prion infectivity into nasal fluids. This mechanism of prion shedding from the olfactory mucosa could contribute to prion transmission.

Introduction

The presence of prion infectivity in bodily fluids and secretions has been proposed to be a source for prion transmission(10, 31, 32, 37, 41, 47, 49, 54). Breast milk from scrapie-infected ewes is the only secretion that has been linked to vertical transmission(36, 38). Placenta from prion-infected sheep or carcasses from deer and elk that succumb to chronic wasting disease in nature are likely to be important sources of environmental contamination(47, 49). However, horizontal transmission by either direct contact with infected hosts or indirectly through contamination of the environment has not been linked to any specific source of prion infectivity despite its role in transmission of chronic wasting disease in cervids and scrapie in sheep(27, 42). Low prion titers are found in blood, saliva, urine, and feces from prion-infected ruminants and rodents(18, 31, 32, 54) and although one or more of these sources could be involved in prion transmission, none of them has yet to be directly implicated in natural prion transmission. In addition, the disease specific prion protein, PrP^{Sc}, is below the level of detection in these bodily fluids using standard immunoassays, but in some cases, can be detected using PrP^{Sc}-seeding based amplification assays(13, 31, 40, 46).

An alternate hypothesis states that olfactory neurons in the nasal mucosa are a potential source of prion infection through the release of prions into nasal secretions(10, 17, 62). Since neurons replicate prions to significantly higher levels than other cell types, the prion titer in nasal fluids has the potential to be substantially greater than in other bodily fluids in which prions are shed from non-neuronal cells. Prion infectivity and PrP^{Sc} deposits in neurons and nerve bundles in the peripheral and central olfactory systems are found in both human prion disease and natural prion diseases of ruminants(4, 15, 17, 29, 30, 39, 62). In an experimental prion infection of hamsters the primary site of infection in the nasal mucosa is in the olfactory receptor neurons (ORNs), whose cell bodies and dendrites are located within the olfactory sensory epithelium (OSE)(10, 17). The total prion titer of nasal mucosa extracts was

reported to be only ~100-fold less than the amount of prion infectivity in the olfactory bulb(10). It was proposed that centrifugal spread of the prion agent from the central nervous system to the peripheral ORNs via the olfactory nerve resulted in high prion titers in the nasal mucosa because of the high number of neurons in the OSE(10). Furthermore, these studies demonstrated low-to-moderate levels of prion infectivity in lavages of the nasal cavity, which could be due to the localization of PrP^{Sc} to the terminal dendrites of ORNs at the border with the lumen of the nasal airway(10). Another mechanism of prion shedding from the nasal mucosa could be due to the continual turnover and replacement of ORNs throughout adult life(12, 23) since mature ORNs survive for approximately 30 to 40 days before undergoing apoptosis(28, 44). It has been proposed that prion infected ORNs could release PrP^{Sc} into nasal secretions following apoptosis and serve as a source of prion infectivity for prion transmission(10, 17, 62).

ORNs are also the most environmentally exposed subset of neurons, but despite nasotoxic insults olfaction is maintained through the regenerative capacity of ORN progenitor cells and cellular mechanisms to resist environmental stress. However, many environment-borne pathogens, toxins, and chemicals can induce apoptosis of ORNs leading to hyposmia or anosmia. Those insults that can induce apoptosis in ORNs include viruses, bacteria, fungi, bacterial cell wall components, mycotoxins, allergies, man-made chemicals dispersed in the environment, and tobacco smoke(3, 16, 22, 24, 25, 34, 43, 56, 60). Since these biological and environmental nasotoxic insults can occur often and multiple times, we investigated whether an increase in apoptosis of ORNs or damage to the OSE in a prion-infected host could increase the amount of prion infectivity released into nasal fluids. In the current study, infection of the OSE was established in hamsters infected with the hyper (HY) strain of the transmissible mink encephalopathy (TME) agent and acute sloughing of the OSE from nasal turbinates was observed after induction of apoptosis in ORNs. Analysis of olfactory marker

protein, a protein found only in ORNs(57), and the cellular and disease-specific isoforms of the prion proteins (i.e., PrP^{C} and PrP^{Sc}, respectively) revealed that these proteins were released from the nasal mucosa into the lumen of the nasal cavity for several days after nasotoxic injury. A rapid, highly sensitive, and quantitative prion seeding assay was used to measure the amount of prion agent in olfactory tissues and nasal lavages, and it revealed a 100- to 1,000-fold increase in prion seeding activity in nasal lavages following nasotoxic injury. These studies demonstrate that damage to the nasal mucosa results in a significant increase in the release of prion infectivity into the lumen of the nasal airway and suggest that conditions that induce apoptosis in ORNs or that disrupt the olfactory epithelium in prion infected hosts can lead to accelerated prion shedding from the nasal cavity.

Materials and Methods

Animal inoculations and tissue collection. All procedures involving animals were approved by the Montana State University IACUC and were in compliance with the *Guide for the Care and Use of Laboratory Animals*; these guidelines were established by the Institute of Laboratory Animal Resources and approved by the Governing Board of the U.S. National Research Council. Weanling, Syrian golden hamsters (Simonsen Laboratories, Gilroy, CA) were inoculated into the olfactory bulb with 2 µl of a brain homogenate from a normal hamster (i.e., mock infected group) or a HY TME infected hamster containing $10^{8.5}$ intracerebral lethal median dose per ml as previously described(7, 8). For intra-olfactory bulb inoculations a minor surgical procedure was performed as previously described(10). Following inoculation of the HY TME agent, hamsters were observed at least three times per week for the onset of clinical symptoms, which include hyperesthesia, tremors of the head and trunk, and ataxia. Animals were euthanized after the onset of clinical symptoms of HY TME.

Methimazole can induce apoptosis in ORNs and this effect is dose and time dependent(26). To determine the appropriate dose of methimazole to disrupt the OSE in hamsters, groups of animals (N=3) were intraperitoneal inoculated with different doses of methimazole (0, 100, 125, 150, and 300 mg/kg) and tissues collected for histological analysis at 4, 8, 12, 24, and 48 hours post-treatment. Based on hematoxylin & eosin analysis of the OSE, it was determined that a dose of 125 mg/kg methimazole was the minimal dose that consistently resulted in at least a 50% disruption of the OSE by 48 hours after methimazole treatment.

After the onset of clinical symptoms of HY TME, nasal lavages were collected from the mock and HY TME groups every 24 hours for five consecutive days. These collection times are designated the 0-hour, 24-hour, 48-hour, 72-hour, and 96-hour nasal lavages. For nasotoxic treatment, hamsters were intraperitoneally inoculated with vehicle alone or methimazole at 125 mg/kg body weight in phosphate buffered saline (PBS) containing 10% dimethyl sulfoxide. Vehicle and methimazole treatment was administered after collection of nasal lavages from the 24-hour time point. After the 96-hour collection time, hamsters were euthanized and the olfactory bulb and nasal turbinate were removed and stored at -80°C. A modification of this experimental design included a vehicle and methimazole treatment after collection of the nasal lavage at the 0-hour collection time and two additional nasal lavage collections at 24-hour and 48 hour (Table 2, trial #2). Nasal lavages were collected in hamsters anesthetized with isofluorane by inserting a blunt end 25-gauge needle into the right nare and gently flushing with 2 ml of PBS and collection of the PBS lavage as it drains from the left nare. For collection of olfactory tissues for immunohistochemistry, hamsters in which nasal lavages were not collected, were perfused with periodate-lysine-paraformaldehyde (PLP) fixative, tissues dissected, and processed for embedding in paraffin wax as previously described(5, 9, 17, 45).

142 Three separate trials were performed for the experimental design described above. A
143 total of 16 mock infected hamsters and 24 HY TME infected hamsters were used for this study.
144 For histological and immunohistochemical analysis nine hamsters were fixed with PLP (three
145 mock and six HY TME hamsters) in addition to the hamsters used to optimize the methimazole
146 dose as described above. For western blot analysis of PrP and OMP, 31 hamsters (13 mock
147 and 18 HY TME hamsters) were used to collect nasal lavages at 24 hour time intervals and
148 olfactory bulb and the nasal turbinate were also collected from these hamsters and frozen at -
149 80°C until they were analyzed. Of the mock-infected hamsters, three were treated with vehicle
150 only and 13 were treated with methimazole. For the HY TME hamsters, there were eight in the
151 vehicle group and 16 were treated with methimazole. For histological, immunohistochemical,
152 and western blot analysis there was a minimum of three hamsters per group per treatment.
153 For OMP and PrP western blot analysis in hamsters treated with vehicle alone, four individual
154 hamsters were analyzed, while in the methimazole treatment group a total of eight individual
155 hamsters were analyzed (Table 1). For the RT-QuIC analysis of nasal lavages, a total of 68
156 samples were analyzed for the time to maximal ThT fluorescence with three to five hamsters
157 used for each time point (Figure 7). For measurement of median prion seeding dose (SD_{50}) in
158 nasal lavages, olfactory bulb, and nasal turbinates, three mock and three HY TME hamsters
159 were analyzed (Table 2).

160 **Tissue homogenization, western blot analysis, and immunohistochemistry.**
161 Frozen olfactory bulb and nasal turbinate were prepared in lysis buffer (i.e., 10 mM Tris-HCL,
162 pH 7.4, 150 mM NaCl, 1 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate and 0.5% ipegal)
163 containing 1X complete protease inhibitor (Roche Diagnostics, Indianapolis, IN) to
164 approximately 10% (weight per volume). Tissues were homogenized using a Bullet Blender
165 (Next Advance, Averill Park, NY) using either glass (olfactory bulb) or zirconium (nasal
166 turbinate) beads. Nasal lavage was prepared by methanol precipitation of protein and the

pellets were resuspended in PBS. The protein concentration in tissue homogenates and nasal lavage samples were measured using the micro-BCA assay (Pierce Protein Research, Rockford, IL).

Western blot analysis was performed in 12% MOPS NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes as previously described(5). For analysis of olfactory marker protein (OMP), 50 µg of protein from lysates of the olfactory bulb and nasal turbinate and 200 µl nasal lavage were used and detected with goat anti-OMP polyclonal antibody (Wako Chemicals, Richmond, VA) as previously described(10). For detection of prion protein, between 10 and 50 µg protein of olfactory bulb and nasal turbinate lysates and 200 µl nasal lavage were analyzed and immunodetection was performed with murine anti-PrP 3F4 monoclonal antibody as previously described(5, 9). Kodak 1D software (Eastman Kodak Company, Rochester, NY) was used to measure the OMP and prion protein signals on western blots. Within an individual western blot, the OMP and prion protein signal in the nasal turbinate was assigned a value of 100 and the level of signal in the olfactory bulb and nasal lavage samples were reported relative to the 100 value. The mean value from several trials is reported in Table 1.

For western blot of caspase 3, 50 µg of protein from lysates of the nasal turbinate were analyzed on NuPAGE gels and immunodetection was performed with rabbit anti-caspase 3 polyclonal antibody (product no. 9662, Cell Signaling Technology, www.cellsignal.com) that detects both procaspase 3 and cleaved (cl)-caspase 3 (Asp175). Western blots were stripped and reprobed with rabbit anti-actin polyclonal antibody. The apoptotic index was calculated by two approaches. One measured the ratio of cl-caspase 3 to the total caspase 3 (cl-caspase 3 + procaspase 3), and the second measured the ratio of the cl-caspase 3 to actin within an individual sample. Kodak 1D software was used to quantify band intensity on western blots. The values from individual hamsters (n=3 per treatment group) were used to compare different

192 treatments (e.g., vehicle and methimazole) and statistical analysis was performed using an
193 unpaired *t*-test (two-tailed). P values < 0.05 were considered significant.

194 For immunostaining, skulls containing the nasal cavity and olfactory bulb were collected
195 after PLP fixation, decalcified, embedded in paraffin wax and PrP^{Sc} and OMP
196 immunohistochemistry and immunofluorescence was performed as previously described(5,
197 45). In addition, immunofluorescence for cl-caspase 3 was performed using rabbit anti-cl-
198 caspase 3 (Asp175) polyclonal antibody (product no. 9661, Cell Signaling Technology,
199 www.cellsignal.com).

200 **Real time quaking-induced conversion (RT-QuIC) assay on olfactory tissues.** For
201 the RT-QuIC assay, olfactory bulb and nasal turbinate lysates were adjusted to a protein
202 concentration of one µg per µl, while nasal lavage samples in PBS were not adjusted for
203 protein concentration. RT-QuIC assay was performed as previously described(58). Briefly,
204 samples were pre-incubated in an equal volume of PBS containing 0.05% SDS and N2 media
205 supplement (Invitrogen, Carlsbad, CA) for 10 min. For dilution analysis, after pre-incubation
206 the samples were serially diluted 10-fold in PBS containing 0.28% SDS and 0.56X N2 media
207 supplement to a final dilution of 10^{-11}. An 8 µl aliquot of each sample dilution was used to seed
208 each RT-QuIC reaction in a 96-well microtiter plate (black plate with clear bottom). Each RT-
209 QuIC reaction included RT-QuIC buffer (10 mM phosphate buffer, pH 7.4, 300 mM sodium
210 chloride, 1 mM ethylenediaminetetraacetic acid tetrasodium salt), 10 uM thioflavin T (Sigma-
211 Aldrich Inc., Atlanta, GA), and hamster recombinant PrP 90-231 (0.1 mg/ml)(2, 58). All
212 samples were analyzed in quadruplicate RT-QuIC reactions using a BMG Fluostar Omega
213 plate reader (BMG Labtech, Cary, NC) at 42°C for 63 hr. 250 cycles (one cycle consists of
214 shaking at 700 rpm for 1 min and resting for 1 min) were performed, and ThT fluorescence
215 measurements were made every 15 minutes.

Spearman-Kärber analysis was used to calculate the median prion seeding dose (SD_{50}) per ml (nasal lavage) or µg protein (olfactory bulb and nasal turbinate), and SD_{50} was defined as sample amount giving positive responses in 50% of replicate RT-QuIC reactions(20). Positive RT-QuIC reactions were designated as those with ThT fluorescence that was greater than 200% of the average mock infected ThT fluorescence signal. To calculate the total SD_{50}, the SD_{50} per ml was multiplied by the total volume of the nasal lavage. For the olfactory bulb and nasal turbinate, the total SD_{50} was calculated by multiplying the SD_{50} per µg protein by the total amount of protein (µg) for each of the individual tissue lysates.

Results

Nasotoxic injury induces apoptosis of ORNs, disrupts the integrity of olfactory epithelium, and releases neuronal proteins into nasal airway.

Prior studies demonstrated PrP^{Sc} deposition in the OSE of prion-infected animals and prion infectivity in lavages of the nasal cavity suggesting that prion shedding into nasal secretions could have a role in prion transmission(10, 17, 35, 62). In order to investigate whether damage to the nasal epithelium can accelerate prion shedding from the nasal cavity, we induced apoptosis of ORNs in an animal model in which a pre-existing prion infection had been established in the OSE. To induce damage to the OSE hamsters were intraperitoneally inoculated with methimazole, a drug used for treatment of thyroid disorders that also can cause hyposmia, anosmia, and morphological changes to the nasal mucosa(26, 50). Methimazole is metabolized by a cytochrome P450 dependent pathway in duct cells of the Bowman's gland, which are located in the subepithelial layer of the nasal mucosa, and leads to the induction of apoptosis in olfactory neurons and detachment of the nasal epithelium(6, 50). In the absence of methimazole treatment the OSE remained intact and prominent immunostaining for olfactory maker protein (OMP), a protein expressed in ORNs, was

observed in ORNs in the OSE and in the nerve bundles of ORNs in the subepithelial layer (Figure 1A and 1B, data not shown). At 24 and 72 hours after methimazole treatment, the majority of the OSE was stripped from the nasal turbinates, while the subepithelial layer remained partially intact (Figure 1C and 1D, data not shown). OMP immunohistochemistry (IHC) revealed prominent signal in the nerve bundles of the subepithelial layer, but infrequent signal in the OSE since it was removed from the turbinates (Figure 1F). Cellular debris was observed in the lumen of the nasal airway and this was often positive by OMP IHC indicating that ORNs were released into the lumen of the nasal airway (Figure 1C, 1D 1E, 1F). Histological examination of the oral mucosa did not reveal disruption of the epithlelial mucosa or cellular changes following methimazole treatment indicating that there were not systemic changes to mucosal surfaces (data not shown).

Evidence that methimazole causes apoptosis of ORNs was determined by analysis of cleaved (cl) caspase 3, a terminal signal for apoptosis, in the OSE and extracts of the nasal turbinate. Western blot for cl-caspase 3 and procapase 3 in hamsters following vehicle treatment and 8, 16, and 24 hours after methimazole treatment revealed an increase ($P<0.001$, unpaired *t*-test) in the ratio of cl-caspase 3 to total caspase 3 as well as an increase in the ratio of cl-caspase 3 to actin at the three time points post-treatment (Figure 2A and 2B). Immunofluorescence analysis revealed that cl-caspase 3 positive cells were increased in the OSE of hamsters at 12 and 24 hours after methimazole treatment compared to vehicle treated hamsters (Figure 3A and 3B). The location of the cl-caspase 3 positive cells in the OSE was consistent with a distribution in ORNs and these cells also had condensed nuclei, which is indicative of apoptosis mediated cell death (data not shown). An increase in cl-caspase 3 immunofluorescence was also found in nerve bundles in the nasal turbinates, in the outer nerve layer of the olfactory bulb containing mainly axons of ORNs, and in glomeruli of the olfactory bulb, which are synaptic-rich structures containing nerve terminals of ORNs (Figure

266 3C and 3D). Unlike the cl-caspase 3 immunofluorescence in the OSE, the pattern in the outer
267 nerve layer and glomeruli of the olfactory bulb was not associated with distinct nuclei and is
268 consistent with localization to axons and nerve terminals of the olfactory receptor neurons.

269 To further investigate the release of ORNs into the nasal airway following methimazole
270 induced apoptosis of ORNs, nasal lavages were collected prior to and following methimazole
271 treatment and analyzed for OMP by western blot. In the first study, nasal lavages were
272 collected from age-matched, mock and clinical HY TME hamsters for five consecutive days at
273 24-hour intervals (0-hour to 96-hour time points). After the 24-hour collection point, hamsters
274 were treated with vehicle alone and after the 96-hour collection point, animals were culled and
275 the olfactory bulb and nasal turbinate were collected. Western blot revealed OMP in lysates of
276 the olfactory bulb and nasal turbinate, but OMP was undetectable in all of the nasal lavage
277 samples from the mock and HY TME groups (Figure 4A and 4C). This experiment was
278 repeated, but now hamsters were treated with methimazole to induce apoptosis of ORNs after
279 collection of the nasal lavage at the 24-hour time point. Similar to the findings for vehicle
280 treatment, OMP was found in the olfactory bulb and nasal turbinate lysates and was absent
281 from nasal lavages at the 0-hour and 24-hour collection points in mock and HY TME hamsters.
282 However, OMP was now present in nasal lavages from the 48-, 72-, and 96-hour collection
283 points in both the mock and HY TME groups (Figure 4B and 4D). This finding indicates that
284 methimazole treatment resulted in release of OMP-positive ORNs from the OSE into the lumen
285 of the nasal airway within 24 hours and this extended to at least 72 hours post-treatment. A
286 semi-quantitative analysis of the OMP signal in western blots revealed that in the absence of
287 methimazole treatment the OMP signal in the olfactory bulb ranged from equivalent levels up
288 to 1.3-fold greater than in the nasal turbinate, but the OMP signal in the nasal lavages was
289 <5% of the OMP signal in the nasal turbinate (Table 1). Following methimazole treatment, the
290 OMP ratio between the olfactory bulb and nasal turbinate did not change. However, in nasal

lavages after methimazole treatment (i.e., at the 24-hour collection point), the OMP levels consistently ranged between 25% and 35% of the OMP signal in the nasal turbinate in both the mock and HY TME groups at the 48-hour through 96-hour collections (Table 1). Prior to methimazole treatment (i.e., 0- and 24-hour collection points) the percentage of OMP in the nasal lavages was <5% of the nasal turbinate. These findings demonstrate that there was a significant increase in the amount of OMP released into the lumen of the nasal airway after nasotoxic treatment that causes apoptosis of ORNs and suggest that sloughing of the ORNs from the OSE results in release of neuronal proteins into nasal fluids.

Nasotoxic injury that induces apoptosis of ORNs removes prion proteins from olfactory epithelium and releases them into nasal airway.

To determine the fate of PrP^{C} and PrP^{Sc} in the olfactory system following an increase in ORN apoptosis and disruption of the OSE, these studies were extended to investigate the prion protein distribution in olfactory tissues and nasal lavages following nasotoxic injury. For these studies the nasal mucosa of mock and HY TME groups were analyzed for OSE tissue morphology and PrP^{Sc} deposition in the absence or presence of methimazole treatment similar to those described in Figure 1. In the mock and HY TME groups, the OSE remained intact and OMP was prominent in the OSE and subepithelial layers at 72 hours following vehicle treatment (Figure 5A, 5C, 5G and 5I). However, after methimazole treatment OMP was primarily found in nerve bundles of the subepithelilal layer since under these conditions the OSE was disrupted and partially stripped from the turbinates (Figure 5D, 5F, 5J, and 5L). OMP-positive cellular debris was also observed in the lumen of the nasal airway following methimazole treatment (Figure 5, see arrows). PrP^{Sc} immunofluorescence did not reveal PrP^{Sc} deposition in the OSE of vehicle or methimazole treated mock-infected hamsters (Figure 5B and 5E). In HY TME-infected hamsters a punctate PrP^{Sc} deposit pattern was observed in the OSE in vehicle treated hamsters (Figure 5H and 5I), and at a higher magnification, PrP^{Sc}

316 deposits were localized to the OMP-positive cell bodies and dendrites of ORNs (Figure 5I,
317 inset). PrP^{Sc} deposits were infrequently observed in nerve bundles in the subepithelial layer of
318 the nasal turbinates in HY TME hamsters. However, following methimazole treatment of HY
319 TME hamsters PrP^{Sc} was not readily found in the OSE, but PrP^{Sc} was occasionally found in
320 sloughed cellular debris in the lumen of the nasal airway (Figure 5K and 5L). Methimazole
321 induced apoptosis of ORNs and disruption of the OSE was consistent with a loss of PrP^{Sc} in
322 the nasal mucosa of HY TME hamsters.

323 A biochemical analysis of PrP^{C} and PrP^{Sc} in the olfactory system also resulted in a
324 redistribution of these molecules upon nasotoxic injury and an increase in apoptosis of ORNs.
325 Similar to our findings on OMP in the nasal cavity, PrP^{C} in mock-infected hamsters and total
326 PrP (i.e., the sum of PrP^{C} and PrP^{Sc}) in clinical HY TME-infected hamsters was found in the
327 olfactory bulb and nasal turbinate lysates, but not in the nasal lavages of vehicle treated
328 hamsters (Figure 6A and 6C). Quantification of the prion protein signal in western blots
329 indicated that the levels in the olfactory bulb were 1.3- to 1.7-fold higher compared to the nasal
330 turbinates, but the PrP levels in the nasal lavages were ≤5% of those in the nasal turbinates
331 (Table 1). Following methimazole treatment (e.g., after collection of the nasal lavage at the 24-
332 hour time point), PrP^{C} and total PrP were now observed in the nasal lavages at the 48-hour,
333 72-hour, and 96-hour time points (Figure 6B and 6D). Quantification of PrP signal revealed
334 that the levels in the nasal lavages at these three time points ranged between 10% and 57% of
335 that found in the nasal turbinates and that there was a stepwise increase in PrP levels with
336 each consecutive nasal lavage collection point (Table 1). The higher prion protein levels found
337 in nasal lavages of clinical HY TME hamsters compared to mock infected hamsters following
338 nasotoxic injury could reflect the higher levels of total prion protein found in the nasal turbinate
339 lysates of HY TME hamsters (Figure 6B versus 6D). The PrP^{C} and PrP^{Sc} migration pattern in
340 nasal lavages also appears distinct from the olfactory bulb derived prion proteins. The 27 kDa

341 PrP protein that predominates in the nasal lavages of HY TME hamsters was previously
342 described to be a PrP^{Sc} isoform found in the nasal turbinate and was distinct from brain
343 PrP^{Sc}(10), which has three major PrP^{Sc} polypeptides. These findings demonstrate that
344 apoptosis of ORNs induced by nasotoxic injury results in an increase in the release of prion
345 proteins into the lumen of the nasal airway.

346 Prion activity in nasal lavage increases up to 1,000-fold after increased apoptosis of ORNs due
347 to nasotoxic injury.

348 To measure the amount of prion infectivity in olfactory tissues and nasal lavages we
349 used a quantitative prion seeding assay called real time quaking-induced conversion (RT-
350 QuIC). Previous studies demonstrated a correlation between median seeding dose (SD_{50})
351 using the RT-QuIC assay and hamster prion infectivity (median lethal dose, LD_{50}) using animal
352 bioassay when both were compared using serial endpoint dilution analysis(58). The RT-QuIC
353 assay is a rapid, highly sensitive, and quantitative method to measure prions that is based on
354 the ability of PrP^{Sc} to alter the conformation of recombinant PrP so that it can bind to thioflavin
355 T (ThT), which is a fluorescent molecule that specifically binds to amyloid proteins. An
356 additional RT-QuIC analysis was used for assessing the relative amount of prion seeding
357 activity, the time to maximum ThT fluorescence (i.e, length of time to reach saturation of the
358 fluorescent signal). There is an inverse correlation between the amount of prion seeding
359 activity and time to maximum ThT fluorescence. When the time to maximum ThT fluorescence
360 decreases, this indicates that there is an increase in the prion seeding activity. When the time
361 to maximum ThT fluorescence was used to measure prion seeding activity in nasal lavages
362 from five consecutive collections at 24-hour intervals from vehicle treated HY TME infected
363 hamsters (N=6), there was no difference among the nasal lavage samples (Figure 7). The
364 mean times ranged from between 34 to 39 hours, which was consistent with samples with low-
365 to-moderate levels of prion infectivity. These findings indicate that similar levels of prion

366 seeding activity were recovered at each consecutive time point. When HY TME hamsters
367 (N=6) were treated with methimazole after collection of nasal lavages at the 24-hour time point,
368 there was a significant reduction in the time to maximal ThT fluorescence in the RT-QuIC
369 assay from 30 to 37 hours at the 0-hour and 24-hour time points to 21 to 23 hours in the 48-
370 hour to 96-hour time points (Figure 7). This higher amount of prion seeding activity was
371 consistent with a higher level of PrP^{Sc} in nasal lavages following an increase in apoptosis of
372 ORNs in HY TME hamsters following nasotoxic injury (Figure 6D and Table 1).

373 In a second trial, methimazole treatment of HY TME hamsters (N=4) resulted in a >40%
374 reduction in the time to maximum ThT fluorescence from 31 hours for both the 0-hour and 24-
375 hour time points prior to methimazole treatment to 18 hours or less at the 48-hour to 96-hour
376 time points after methimazole treatment (data not shown). In both of these trials, 34 nasal
377 lavages from mock infected hamsters were assayed in quadruplicate and partial seeding
378 activity was found in one or two replicates from only two samples. Upon retesting of these
379 samples both were negative (data not shown). These findings indicate that there were
380 consistent levels of prion seeding activity in nasal lavages from HY TME hamsters that were
381 collected over a several day period and that there was an increase in prion seeding activity in
382 nasal lavages following nasotoxic injury. These experimental results were consistent with a
383 reduction of PrP^{Sc} in the OSE by IHC and an increase in the amount of prion protein in nasal
384 lavages by western blot after an increase in apoptosis of ORNs following nasotoxic injury.

385 The RT-QuIC assay was also used to measure the prion activity, or median seeding
386 dose (SD_{50}), of the olfactory bulb, nasal turbinate, and nasal lavages in the absence and
387 presence of nasotoxic injury. The total SD_{50} was defined as 1) the SD_{50} per ml in a nasal
388 lavage sample multiplied by the total volume of the nasal lavage, or 2) the SD_{50} per µg protein
389 of olfactory bulb and nasal turbinate multiplied by the total micrograms of protein in the
390 olfactory bulb and nasal turbinate lysate. The total SD_{50} of nasal lavages collected over five

consecutive days in vehicle treated HY TME hamsters ranged from $<10^{1.5}$ to $10^{3.2}$, but for an individual hamster the SD_{50} for each of the consecutive nasal lavages were within 10^1 SD_{50}, excluding samples in which no prion seeding activity could be measured (Table 2, H1635.3 and H1635.4). Similar amounts of total SD_{50} were measured in nasal lavages from HY TME hamsters at the 0-hour and 24-hour collection points prior to nasotoxic injury (Table 2, H1636.3 and H1636.4). In nasal lavage samples collected from four individual hamsters either without or prior to nasotoxic injury, 11 of 14 samples had a total SD_{50} between $10^{2.9}$ and $10^{3.2}$, which illustrates that the amount of prions recovered in nasal lavages was consistent over this time period. After methimazole treatment, the total SD_{50} in nasal lavages at the 48-hour through 96-hour time points was 100- to 1,000-fold higher than in nasal lavages prior to treatment (Table 2, H1636.3 and H1636.4; Figure 8). The total SD_{50} in nasal lavages following nasotoxic injury was between $10^{5.0}$ and $10^{5.7}$ in six of six nasal lavage samples collected from two individual hamsters. Much higher amounts of total SD_{50} were found in the olfactory bulb and nasal turbinate (as high as $10^{10.9}$ and $10^{9.6}$ SD_{50}, respectively) from both vehicle and methimazole treated HY TME hamsters (Table 2). In three of four HY TME infected hamsters, the total SD_{50} in the olfactory bulb was 10- to 100-fold greater than in the nasal turbinate. In a second trial, prion seeding activity was not detected in olfactory tissue and nasal lavages from a mock infected hamster, while the total SD_{50} in a HY TME infected hamster before and after methimazole treatment was consistent with the first trial. The nasal lavage collected prior to methimazole treatment had a total SD_{50} of $10^{3.0}$ and after methimazole treatment the total SD_{50} was 100- to 1,000-fold higher in each of two additional nasal lavage samples (Table 2). These findings indicate that there were low-to-moderate levels of prion infectivity in nasal lavages before nasotoxic injury and there was a significant increase in the release of prion infectivity into nasal lavages following nasotoxic injury to the OSE that causes apoptosis of ORNs.

Discussion

The release or shedding of prions from an infected host is an important pathway of horizontal prion transmission and the ability to establish infection in a naive host is strongly dependent on the level of infectious dose upon exposure. Although multiple sources of bodily fluids are likely to contribute to disease transmission, the current studies demonstrates that ORNs can be a source for the shedding of higher doses of prion agent than previously reported for other secretions. There are several findings to support a role for ORNs and damage to the OSE in the shedding of prions from an infected host. Firstly, the olfactory system is a common target for prion infection in several natural and experimental prion infections, and PrP^{Sc} has been found in the OSE, ORNs and/or the olfactory nerve(4, 10, 15, 17, 29, 30, 39, 62). Secondly, ORNs undergo continual turnover and programmed cell death, or apoptosis, throughout adult life(12, 23). It is conceivable that apoptosis of prion infected ORNs could release prion infectivity into nasal fluids especially since there is evidence for PrP^{Sc} deposition in terminal dendrites of ORNs that project into the mucus layer. ORNs are also the most environmentally exposed subset of neurons and there are many types of environmental factors that can cause stress to ORNs and induce apoptosis(3, 16, 22, 24, 25, 34, 43, 56, 60). An increase in ORN apoptosis can also disrupt the mucosal integrity of the OSE and cause a sloughing off of cells into the nasal airway. In this study we demonstrate that inducing apoptosis of ORNs in a prion-infected host resulted in immunodetection of PrP^{Sc} in nasal lavages and a 100- to 1,000-fold increase in prion seeding activity in nasal lavages.

In bodily fluids and mucosal surfaces of prion infected hosts the levels of PrP^{Sc} and/or prion infectivity are low compared to nasal fluids, extracts of nasal turbinates, and the OSE. Immunodetection of PrP^{Sc} has not been observed in urine, saliva, feces, blood, or breast milk from rodents and ruminants with prion infection(31, 38, 46, 54), but it can be detected using serial protein misfolding cyclic amplification (sPMCA)(31, 40, 46), which can amplify the level

441 of PrP^{Sc} by >10^9-fold so that it can be detected by western blot. Alternatively, the enhanced
442 QuIC assay, a modification of the RT-QuIC assay, can rapidly measure low levels of prion
443 seeding activity in blood of humans with Creutzfeldt-Jakob disease(48). The prion infectivity
444 levels in these fluids, when reported, are also very low(18, 31, 38). Despite the low amount of
445 infectious prions in most bodily fluids, saliva from CWD-infected deer has been used to
446 transmit CWD infection following experimental oral exposure of deer(41). Breast milk from
447 scrapie infected sheep can also transmit disease to lambs indicating that this is a natural route
448 of vertical transmission, although the source is not certain since PrP^{Sc} was not observed in
449 mammary tissue of scrapie infected sheep with otherwise normal health status(36, 38). In the
450 current study, methimazole induced apoptosis of ORNs resulted in the release of prion protein
451 that could readily be detected in nasal fluids by western blot. This is a significant finding in
452 light of previous studies that were unable to detect PrP^{Sc} in other bodily fluids in prion infected
453 hosts that have been implicated in prion transmission. The amount of prion seeding activity in
454 nasal lavages after methimazole treatment was between 10^3 and 10^5 SD_{50} below the prion
455 activity in the olfactory bulb. Previous studies demonstrate that the amount of prion titer in the
456 olfactory bulb is comparable to that of a HY TME-infected brain, or ~$10^{9.5}$ LD_{50} per gram of
457 tissue(7, 10). A comparison of the SD_{50} and LD_{50} using RT-QuIC assay and animal bioassay,
458 respectively, with brain homogenates of 263K scrapie strain illustrated a similar sensitivity of
459 these methods(58). Based on these studies we estimate the amount of prion infectivity in
460 nasal lavages after methimazole treatment to be approximately $10^{4.5}$ to $10^{6.5}$ LD_{50} in a 2 ml
461 lavage. We estimate that during collection of nasal lavages that we dilute nasal secretions by
462 approximately 100- to 1,000-fold. Therefore, we propose that a high concentration of prions
463 (i.e., >$10^{7.5}$ LD_{50} per ml) can be released in nasal secretions following damage to the nasal
464 mucosa in a host with prion infection of the OSE.

Additional evidence for nasal fluids as a potential source for new prion infections is based on our findings that there is a high level of prion infection in the nasal mucosa and previous studies that demonstrated PrP^{Sc} deposition in ORNs and the olfactory nerve in natural and experimental prion diseases(10, 15, 17, 62). In HY TME infection in hamsters, the amount of PrP^{Sc} in extracts of the nasal mucosa was within two-fold of the level in the olfactory bulb on a per protein basis in the absence of methimazole treatment, and the prion median seeding activity in these extracts were only 10- to 100-fold below the levels found in the olfactory bulb. In one hamster, the total SD_{50} was 10-fold higher in the nasal turbinate compared to the olfactory bulb. The slightly lower amounts of PrP^{Sc} and prion seeding activity in the nasal mucosa compared to the olfactory bulb indicate that the OSE also can support high levels of prion replication. These findings are consistent with natural prion diseases in scrapie-infected sheep in which the relative amount of PrP^{Sc} in the olfactory turbinates was ~10% of the level present in the brain. This is further evidence that high levels of PrP^{Sc} and prion infectivity are present in the olfactory mucosa in both natural and experimental prion diseases(15).

The targeting of prion infection to the nasal mucosa is likely due to the presence of an estimated 20 million olfactory receptor neurons in the OSE (in hamsters, for example), which is greater than the number of neurons estimated to be in the entire olfactory bulb(11, 51). These high levels of PrP^{Sc} and prion infectivity in the nasal mucosa have not been reported at the surface of other mucosal sites and is likely due to the distinct neuroanatomy of the olfactory system in which the axons of the ORNs (i.e., olfactory nerve) project into the olfactory bulb where they synapse with mitral and tufted cells in the synaptic-rich glomeruli (14). This pathway is the likely route of prion agent spread from the central nervous system to the OSE and ORNs (10). PrP^{Sc} deposition in the soma of ORNs and along the border of the OSE with the lumen of the nasal airway suggests that prions are in direct contact with nasal fluids. This

490 targeting of prion infection to ORNs could lead to release of prion infectivity due to the normal
491 turnover of ORNs and/or sloughing off of OSE into the lumen of the nasal airway due to
492 environmental factors.

493 In the current study, increasing apoptosis of ORNs and damage to the nasal mucosa in
494 a prion infected host resulted in a loss of the OSE, sloughing of the OSE into the lumen of the
495 nasal airway, and an increase in OMP and prion proteins as well as prion seeding activity in
496 nasal lavages. These findings indicate that significant levels of prion infectivity can be
497 released upon disruption of the nasal mucosa. There are many insults that are able to induce
498 apoptosis of ORNs (1, 19, 21, 25, 33, 52, 53, 55, 60, 61) and, in a natural prion infection these
499 could promote prion agent release into nasal fluids. This includes influenza A virus in which
500 virally-infected ORNs undergo apoptosis and, if the ORNs are prion-infected, rhinitis and
501 sneezing associated with flu symptoms could promote dissemination of prions into the
502 environment(43). Other ubiquitous microorganisms and potential pathogens that can induce
503 apoptosis of ORNs include *Staphylococcus aureus* and *Aspergillus fumigatus* as well as the
504 common bacterial cell wall component lipopolysaccharide(22, 25, 60). Molds produce
505 mycotoxins that can induce apoptosis of ORNs and man-made chemicals present in the
506 environment such as PCBs and DTT can breakdown into chlorinated hydrocarbons that also
507 can induce apoptosis of ORNs (3, 24). Other conditions can cause disruption of the nasal
508 mucosa or induce necrosis of ORNs. The most common are those that cause rhinosinusitis
509 including allergies, chronic illness, and other inflammatory conditions in the nasal cavity(1, 19,
510 21, 55, 59, 61). ORNs appear to have a unique niche among neurons in adults since they
511 normally undergo apoptosis and are exposed to biological and environmental insults that
512 shorten their lifespan. These features of ORNs along with their ability to replicate prions also
513 make them a good candidate for releasing high levels of prion infectivity into nasal fluids upon

514 damage, which can directly expose susceptible hosts and can contaminate the environment
515 with prion agent.

516 In this study we demonstrate that high levels of prion infection can be established in the
517 nasal mucosa and that a substantial amount of PrP^{Sc} and prion seeding activity can be
518 released into nasal secretions following an increase in apoptosis of ORNs and disruption of the
519 OSE. These findings have several implications, 1) nasal secretions from prion infected hosts
520 could serve as a source of new prion infections through direct contact with susceptible hosts or
521 indirectly by contamination of the environment; 2) common insults to ORNs or the nasal
522 mucosa of a prion infected host could accelerate release of prion infectivity into nasal
523 secretions; and 3) nasal fluids may provide a noninvasive approach for diagnosis of prion
524 infection using the highly sensitive and quantitative RT-QuIC assay, perhaps in combination
525 with a procedure that induces partial shedding of the OSE.

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Figure Legends

Figure 1. Disruption of the olfactory sensory epithelium following nasotoxic injury. The nasal turbinates in the nasal cavity from mock infected hamsters were analyzed by hematoxylin and eosin (A, C) and immunohistochemistry (brown color) for olfactory marker protein (B, D) from vehicle (A, B) and methimazole treated hamsters at 24 hours after treatment (C, D, E, F). B, OMP is prominent in the olfactory sensory epithelium (OSE) and nerve bundles (NB) in the subepithelial layer (SE) in the absence of methimazole treatment. Following methimazole treatment the OSE was observed to slough off the nasal turbinates (arrowhead in C), but OMP immunostaining was still observed in nerve bundles of the SE (D). Some OMP immunoreactivity was observed in cellular debris in the lumen of the nasal airway (arrowhead in D). In panel C and D the 'E' and 'F' refer to areas that were enlarged and illustrated in panels E and F.

Figure 2. Apoptotic Index in nasal turbinates following methimazole treatment. The apoptotic index was measured as the ratio of cleaved caspase 3 (cl-caspase 3) to total caspase 3 (A), and the ratio of cl-caspase 3 to actin (B) by western blot as described in materials and methods. Post-methimazole (MTz) is the time point in hours at which nasal turbinates were collected for cl-caspase 3, procaspase 3, and actin analyses after methimazole treatment. Asterisk indicates a P value <0.001 (unpaired *t*-test) when comparing vehicle group to each methimazole group.

Figure 3. Distribution of cleaved caspase 3 following apoptosis of olfactory receptor neurons. Hamsters were treated with either vehicle alone (A) or methimazole (B, C, D) and the nasal turbinates (A, B) and olfactory bulb (C, D) were collected 12 and 24 hours following treatment for analysis of apoptosis by cleaved caspase 3 immunofluorescence (red). Cleaved

caspase 3 was infrequent in the olfactory sensory epithelium (OSE) and olfactory bulb of vehicle treated hamsters (A and data not shown). Following methimazole treatment cl-caspase 3 immunofluorescence was observed in the disrupted OSE (arrows in B) in a pattern consistent with deposition in olfactory receptor neurons (ORNs) as well as in the outer nerve layer (ONL) and glomeruli (*) in the olfactory bulb (C, D). The ONL is composed of the axons of the ORNs and the glomeruli are synaptic-rich structures containing the nerve terminals of ORNs as well as nerve terminals from neurons in the olfactory bulb. An increase in cl-caspase 3 in the OSE, ONL and glomeruli are consistent with apoptosis of ORNs following methimazole treatment. Nuclei are stained with ToPro 3 (blue). SE is the subepithelial layer and NA is the lumen of the nasal airway. Scale bar is 50 μm.

Figure 4. Western blot for olfactory marker protein in olfactory tissues and nasal lavages following nasotoxic injury. Tissues from age-matched, mock infected (A, B) and HY TME infected (C, D) hamsters in the presence of vehicle (A, C) or methimazole (B, D) treatment, which were administered after the collection of nasal lavages at the 24-hour time point (open and filled arrowhead, respectively). Nasal lavages were collected every 24 hours for five consecutive days and after the 96-hour collection, the olfactory bulb (OB) and nasal turbinate (NT) were also collected for OMP analysis. Fifty micrograms of protein for the OB and NT lysates and 200 μl of nasal lavage were analyzed by western blot using polyclonal anti-OMP goat antibody. The total amount of protein in nasal lavage samples varied from below the level of detection (i.e., < 1 μg/ml) to 70 μg protein. The numerical value under each nasal lavage lane indicates the total amount of protein (μg) analyzed. Nd refers to none detected or below the limit of detection. The marker (m) lane contains a polypeptide at 20 kDa.

Figure 5. Distribution of olfactory marker protein and PrP^{Sc} following disruption of the olfactory sensory epithelium. The olfactory sensory epithelium in the nasal cavity from mock infected (A to F) and HY TME infected (G to L) hamsters were analyzed by laser scanning confocal microscopy for olfactory marker protein (OMP, red)(A, D, G, J), PrP^{Sc} (green in B, E, H, K), and for both OMP and PrP^{Sc} (C, F, I, L). Panels A through C, D through F, G through I and J through L are the same field of view. Hamsters were either treated with vehicle alone (A to C and G to I) or with methimazole (D to F and J to L) and analyzed 72 hours after treatment. OMP is present in olfactory receptor neurons and immunofluorescence was observed in the OSE (white bar indicates the width of the OSE) and nerve bundles (*) in the vehicle treated group and primarily in the nerve bundles following methimazole treatment. OMP immunofluorescence in the OSE was less frequent following methimazole treatment and disruption of the OSE, but it could be observed separating from the epithelium (arrow in D, F, J, L) and in the lumen of the nasal airway (NA). PrP^{Sc} was not observed in mock infected hamsters, but was present in the OSE of HY TME hamsters in the vehicle group (H) and in the disrupted OSE of the methimazole treated HY TME hamsters (arrow in K, L). The nuclei (blue) of olfactory receptor neurons are packed at a high density in the OSE of the vehicle treated groups. Scale bar is 50 μm.

Figure 6. Western blot for prion protein in olfactory tissues and nasal lavages following nasotoxic injury. Tissues from age-matched, mock infected (A, B) and HY TME infected (C, D) hamsters in the presence of vehicle (A, C) or methimazole (B, D) treatment, which were administered after the collection of nasal lavages at the 24-hour time point (open and filled arrowhead, respectively). Nasal lavages were collected every 24 hours for five consecutive days and after the 96-hour collection, the olfactory bulb (OB) and nasal turbinate (NT) were also collected for PrP analysis. For the OB and NT lysates, 10 μg (B, D), 12.5 μg (C), or 50 μg

(A) of protein was analyzed while for the nasal lavage samples 200 µl were analyzed by western blot using monoclonal anti-PrP 3F4 mouse antibody. The total amount of protein in nasal lavage samples varied from below the level of detection (i.e., < 1 µg/ml) to 70 µg protein. The marker (m) lane contains polypeptides at 20, 30, and 40 kDa.

Figure 7. Real time quaking-induced conversion (RT-QuIC) assay of nasal lavage from HY TME hamsters. The time to maximum thioflavin T (ThT) fluorescence in the RT-QuIC assay was measured in nasal lavage samples collected every 24 hours for five consecutive days from HY TME hamsters following vehicle (open bar) or methimazole (closed bar) treatments, which were administered after the collection of nasal lavages at the 24-hour time point. Nasal lavages from three to five hamsters were assayed at each time point for both treatment groups. Samples that did not reach maximum ThT fluorescence by 63 hours were not included in the analysis, but this only consisted of samples from either the vehicle or pre-methimazole treatment groups. At each collection point, the values for the vehicle and methimazole groups were compared using a paired t-test (two-tailed), *P<0.01 and **P<0.001.

Figure 8. RT-QuIC assay of olfactory tissues from HY TME infected hamsters. The RT-QuIC assay was used to measure ThT fluorescence for a 63 hour duration in individual reactions seeded with serial dilutions of olfactory bulb (A), nasal turbinate (B), nasal lavage at the 0 hour collection point (C), and nasal lavage at the 48 hour collection point (and 24 hours after methimazole treatment)(D). Samples were assayed neat and serially diluted 10-fold (-f) to 1,000-fold (1K-f), 1,000,000-fold (1M-f), and 1,000,000,000-fold (1B-f). Normal hamster brain (NBH) was used as a negative control and 100 femtograms of PrP^{Sc} from a 263K scrapie infected hamster brain was used as a positive control in the RT-QuIC assay. Four replicates wells were assayed for each sample and each data point on the curve is the average of

replicates. The corresponding median prion seeding dose (SD_{50}) for these tissues (H1636.3) can be found in Table 2. Representative panels of the RT-QuIC assay are illustrated.

Tables

Table 1. Levels of olfactory marker protein and prion protein in the olfactory bulb, nasal turbinate, and nasal lavage by western blot analysis.

			Relative Percent of OMP and PrP Signal[3]							
Methimazole Treatment[1]	Infection Type	Analysis[2]	Olfactory bulb	Nasal turbinate	Nasal lavage, time 0 hr	24 hr	48 hr	72 hr	96 hr	Trial, N =
Vehicle only	mock	OMP	132	100	4	3	2	2	1	2
		PrP	130	100	3	0	0	0	0	1
Vehicle only	HY TME	OMP	103 ± 2	100	2 ± 0	1 ± 0	1 ± 1	1 ± 1	2 ± 1	3
		PrP	176 ± 43	100	5 ± 2	2 ± 2	2 ± 1	0	1 ± 1	3
After 24 hr	mock	OMP	142 ± 30	100	0	0	30 ±11	36 ± 8	26 ±10	3
		PrP	223	100	4	3	10	27	51	2
After 24 hr	HY TME	OMP	101 ± 19	100	0	1 ± 1	30 ± 8	30 ± 6	25 ±12	5
		PrP	302 ± 29	100	3 ± 1	1 ± 0	20 ± 6	37 ± 7	57 ±15	6

[1] Nasal lavages were collected at time = 0 and 24 hours later. Immediately after the 24-hour collection point, hamsters were intraperitoneally inoculated with methimazole (125 mg/kg) in PBS/DMSO or with vehicle alone. After treatment, nasal lavages were collected every 24 hours for three additional collection points (i.e., 48, 72, and 96-hour). The olfactory bulb and nasal turbinate were also collected at the 96-hour time point.

[2] For western blot of olfactory marker protein (OMP), fifty micrograms of protein was used for the olfactory bulb and nasal turbinate extracts, while for total PrP between 10 and 50 micrograms of protein was analyzed. Analysis of nasal lavage samples was performed following precipitation of proteins from 200 microliters at each time point. Intensity of western blot signals was measured using Kodak 1D software.

[3] The nasal turbinates were assigned a relative value of 100 and values for all the other samples were expressed as a percentage of 100. Mean percentage values are indicated for OMP and PrP for each treatment group. In treatment groups with three of more trials (N≥3), the standard error of the mean was included (±SEM).

Table 2. PrP^{Sc} median seeding dose (SD_{50}) in olfactory tissues from HY TME infected hamsters.

Trial 1		Total SD_{50}[1]			Total SD_{50}				
		Nasal Lavage			Nasal Lavage			At 96 hr	
Hamster	Infection	Collection,		Methimazole[2],	Collection Time,			Nasal	Olfactory
Group	Type	0 hr	24 hr	24 hr	48 hr	72 hr	96 hr	turbinate	bulb
H1635.3	HY TME	$<10^{1.5}$	$10^{1.9}$	no	$10^{2.7}$	$10^{2.5}$	$10^{2.5}$	$10^{9.6}$	$10^{10.9}$
H1635.4	HY TME	$10^{2.9}$	$10^{3.2}$	no	$10^{2.9}$	$10^{2.9}$	$10^{3.2}$	$10^{9.5}$	$10^{10.8}$
H1636.3	HY TME	$10^{2.4}$	$<10^{1.5}$	yes	$10^{5.2}$	$10^{5.0}$	$10^{5.2}$	$10^{8.0}$	$10^{10.3}$
H1636.4	HY TME	$10^{2.9}$	$10^{3.2}$	yes	$10^{5.5}$	$10^{5.7}$	$10^{5.2}$	$10^{9.5}$	$10^{8.8}$

Trial 2		Total SD_{50}		Total SD_{50}			
		Nasal Lavage		Nasal Lavage		At 48 hr	
Hamster	Infection	Time,	Methimazole[2],	Collection Time,		Nasal	Olfactory
Group	Type	0 hr	0 hr	24 hr	48 hr	turbinate	bulb
H1641.3	mock	$<10^{1.5}$	yes	$<10^{1.5}$	$<10^{1.5}$	$<10^{1.5}$	$<10^{1.5}$
H1644.3	HY TME	$10^{3.0}$	yes	$10^{5.7}$	$10^{5.2}$	$10^{8.5}$	$10^{9.9}$

[1] Median seeding dose (SD_{50}) was measured by endpoint dilution using the RT-QuIC assay and calculated by the Spearman-Kärber method(20). Total SD_{50} was calculated as described in materials and methods. Each SD_{50} value is from a single hamster in which sequential nasal lavages were collected at the indicated time points.

[2] Nasal lavages were collected at time = 0 and 24 hours later in trial 1 and at t =0 in trial 2. Immediately after the 0-hour (trial 2) or 24-hour (trial 1) collection point, hamsters were intraperitoneally injected with methimazole (125 mg/kg) in PBS/DMSO or with vehicle alone. After treatment, nasal lavages were collected every 24 hours for two or three additional collection points (i.e., 48, 72, and 96-hour). The olfactory bulb and nasal turbinate were also collected at the 48-hour (trial 2) or 96-hour (trial 1) time point.

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