

OLFACTORY BEHAVIOR AS AN INDICATOR OF
PRION INFECTION

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ABSTRACT

The current project sought to identify changes in olfactory-related behavior in hamsters infected with the HY transmissible mink encephalopathy (HY TME) strain of the pathological form of the prion protein. Experiment 1 was conducted to validate an olfactory preference paradigm for use with Syrian golden hamsters. An experimental group was induced with anosmia by treating them with methimazole. In an olfactory preference test in which the time subjects spent investigating attractive, aversive, and neutral olfactory stimuli were assessed, control animals spent a significantly longer amount of time investigating the attractive versus aversive scents. The methimazole-treated group did not demonstrate this pattern. Experiment 2 investigated changes in olfactory behavior as a result of prion infection. A group of hamsters was infected with HY TME and subjected to olfactory preference testing at four time points: 20, 40, 60, and 80 days post inoculation. In addition, parallel subjects were sacrificed and submitted to immunohistochemical analysis in order to examine the proliferation of HY TME throughout olfactory-related brain structures with the intention of relating behavioral changes to the progression of prion infection. Results indicated that HY TME subjects lost their ability to perceive the attractive scent early in the disease. However, avoidance of the aversive scent was retained until much later. The immunohistochemistry revealed an initial appearance of the pathologic prion at 20 days post inoculation in the glomeruli of the olfactory bulb. Widespread infection throughout all olfactory structures was observed at 40 days post inoculation and beyond. These results suggested a differential sensory loss to the olfactory stimuli that may have been due to initial infection in the glomeruli and later infection in other olfactory structures. These findings support the utility of discrimination paradigms for the diagnosis of prion diseases.

INTRODUCTION

In the mid 1990's a public health crisis emerged in the U.K. when cases of a strange, fatal neurodegenerative disease began to emerge in the human population. The hallmark of the disease was a rapid deterioration in motor and cognitive functioning with symptomology ranging from ataxia and weight loss to anxiety, depression, and sleep disturbances (Zerr & Poser, 2002). Though the exact vector was not immediately known, the disease bore a striking resemblance to a neurodegenerative disorder originally identified in the 1920's by Hans Gerhard Creutzfeldt and Alfons Jakob. Creutzfeldt-Jakob disease (CJD), as it later came to be known, was a transmissible spongiform encephalopathy (TSE) that generally affected individuals in the later stages of life and resulted in fatality rate of 100%. Though the cases in the U.K. were similar with regards to CJD symptomology, diagnosis was hindered by the presence of distinct differences between CJD and this mystery illness. To make matters worse, a well-known disease in the bovine population (bovine spongiform encephalopathy, BSE) was assumed to be a direct variant of the scrapie agent in sheep, and thus unlikely to be transmissible to humans (Bradely, Collee, & Liberski, 2006). This assumption was unfortunate; as BSE-infected material was the cause of infection in humans (Hill et al., 1997) and by 1995 the U.K. was recognizing a distinct class of CJD caused by exposure to the BSE agent (Will et al., 1996). This new disease was classified as variant Creutzfeldt-Jakob disease (vCJD).

The United States was largely spared from BSE-induced vCJD despite historically lax policies regarding the handling of non-ambulatory bovine. For example, in the state of Washington a downer cow later diagnosed with BSE was deemed fit for human consumption (Donnelly, 2004). There have also been at least two other experimentally confirmed cases of BSE in this country (Richt et al., 2007). Fortunately, lapses in food controls have yet to manifest any serious consequences for TSE's in humans. Nonetheless, the US exhibits a normative sample of the variant form of CJD relative to the rest of the world (National Institute of Health, 1996).

BSE to CJD

While the “Mad Cow” scare in Britain was not felt to the same extent in the US, the notoriety sparked by those events was the impetus for increased interest in TSE's, both popularly and scientifically. Understandably, less attention has been given to TSE's than to more ubiquitous fatal diseases such as cancer. CJD, in particular, is exceedingly rare with less than one death per million in the US population (NIH, 1996). However, given the lengthy asymptomatic incubation period, which is followed by a rapid deterioration and 100% fatality rate, a section of the scientific community has worked diligently to better understand the disease specifically, and prions in general.

The classic symptoms of CJD are withdrawal, depression, anxiety, sleep disturbances and other behavioral abnormalities. These are often accompanied by motor difficulties such as ataxia (Zerr & Poser, 2002). The psychological markers

are far from definitive, however, as a wide variety of other disorders, both psychiatric and physiological, also share these symptoms. This fact often confounds clinical diagnosis. Rapidly progressive dementia, myoclonus, and brain wave patterns (measured via EEG) can also be useful in identifying CJD (Kreutzschmar et al., 1996), though these too remain less than conclusive. Ultimately, diagnosis can be attained only post mortem by thorough neuropathological analysis of brain tissue (Master et al., 1979; Kreutzschmar et al., 1996).

The Prion Protein

As with all TSE's, CJD is believed to be caused by an abnormality in the normal form of the cellular prion protein (PrP^C) (Prusiner, 1991; Bradely et al., 2006; Colee et al., 2006; Prusiner, 1996). The term 'prion' was first coined by Prusiner (1982) to indicate the 'proteinaceous infectious only' particle that was discovered to be the agent responsible for scrapie; and while many variant strains of the infectious agent have been identified, prion is recognized as the general term for both the normal and abnormal forms. Though PrP^C exhibits widespread expression throughout the central nervous system, its exact function has yet to be determined. Among other things, it has been implicated in neural plasticity (Lauren et al., 2009), synaptic transmission (Collinge et al., 1994), ion channel gating (Quist et al., 2005), and memory formation (Coitinho et al., 2007). The abnormal form of the protein (PrP^{Sc}) is known to precipitate extensive neuronal damage as evidenced by the diseases previously described. While the exact mechanisms remain unknown, prion

diseases result from the post-translational misfolding of PrP^C into the PrP^{Sc} isoform (for a review see Nuvolone, Aguzzi, & Heikenwalder, 2009). Tissue deterioration, often in the form of vacuolation, is a result of the accumulation of the abnormal isoform and resulting conversion of PrP^C. It must be noted that in order for TSE's to propagate, the normal form of the protein must be present in order to serve as a substrate through which PrP^{Sc} can disseminate, thereby limiting the number of species that can be infected as well as the potential for interspecies transmission (Jones & Tuite, 2005).

Etiological classification indicates three routes of TSE infection. These include acquired, inherited and sporadic cases (Gains & LeBlanc, 2007). In acquired cases, subjects are generally infected via exposure to infected tissue, which can occur as a result of medical procedures or ingestion of infected organic matter—the latter being exemplified by the BSE epidemic of the 1990's. Iatrogenic TSE's arise as a result of transmission of infectious material through medical procedures such as corneal implants and blood transfusions and are often characterized by an unusually short incubation period (Zerr & Poser, 2002).

Inherited TSE's compose a smaller, though notable, proportion of the diseases in humans. Also known as familial or genetic, these types of prion diseases are a result of mutation in the gene that codes for the prion protein and often have differing clinical profiles and affected brain areas than acquired or sporadic cases (Gains & LeBlanc, 2007).

Sporadic TSE's cases account for nearly 85% of human prion diseases and are characterized by no clear antecedents with regard to genetic mutation or exposure to infected materials. The majority of sporadic cases are CJD, which tends to disproportionately affect individuals over the age of 50 (NIH, 1996).

TSE Diagnosis

Without exception, prion diseases have a fatality rate of 100% and there are currently no effective means of TSE treatment in humans. In 2001 the anti-malarial drug, quinacrine, emerged as a potential pharmacotherapy for prion infection (Korth et al., 2001). However, human trials failed to indicate any benefits with regards to prolonged cognitive functioning or life expectancy (Geschwind, 2009). In fact, an in vivo study using a mouse model found a relationship between quinacrine treatment and the emergence of drug resistant prion conformations (Ghaemmaghmi, 2009). Currently, research involving gene therapy has produced some promising results (White et al., 2008).

The development and evaluation of TSE treatments can benefit from early detection and diagnosis. Unfortunately, the most informative feature of CJD is the associated vaculation and tissue atrophy in specific areas of the brain. However, these late markers of neuropathology represent a significant amount of degeneration for which therapy would be unlikely to be of any benefit.

Anecdotal evidence has suggested that the inability to smell (anosmia) is a common complaint of individuals suffering from CJD (Rueber, Al-Din, Baborie &

Chakrabarty, 2001). Indeed, anosmia has been widely demonstrated as a peripheral symptom of neurodegenerative diseases such as Parkinson's and Alzheimer's (Hawkes, 2003; Kovacs, 2004). Thus, olfactory dysfunction would not be an unlikely consequence of CJD and could serve as a useful diagnostic indicator. Zanusso et al. (2003) investigated the involvement of the olfactory system in sporadic CJD by examining the cadavers of individuals who had succumbed to the disease. In all cases PrP^{Sc} deposition was observed in the epithelium of the olfactory mucosa. This not only suggests the use of olfactory biopsies as a determinate of infection, but also supports the use of behavioral data to assess the disease. Since receptor neurons are located within the neuroepithelium, altered olfactory perception would be a logical consequence of atypical cellular activity at this location. While histological analysis has demonstrated observable levels of PrP^{Sc} infection in olfactory biopsies of living individuals (Tabaton et al., 2004), there has been little empirical research into olfactory system involvement as a behavioral manifestation. Rather than being an underappreciated nuisance presenting with a serious neurodegenerative disease, it may be that olfactory dysfunction could serve as a behavioral tool for TSE diagnosis. Considering the paucity of data regarding this type of sensory deficit in the human population, an examination of animal models of prion infection would be useful.

The Mouse Model

Mice represent a predominant animal species used in prion research because of the multitude of genetically identical inbred strains and the ability to manipulate

gene expression. By altering the gene that codes for the normal form of the prion protein (Prnp) the mechanisms and consequences of PrP^C can be examined. Perhaps one of the most interesting findings stemming from prion knockouts is the general lack of pronounced phenotypes, save for the inability to be infected with PrP^{Sc} (it must be noted that there are consequences of deleting Prnp, but they do not interfere with the normal life expectancy of mice unless mediating procedures are present (see Steele, 2007 for a review of Prnp knockout phenotypes). Generally, mice that are physiologically incapable of manufacturing the normal prion protein exhibit virtually no developmental defects, have normal life expectancies (Brandner et al., 2000) and until recently, no behavioral abnormalities had been reported within the literature.

While the localization of PrP^C within the mouse olfactory system has been experimentally verified (LePichon & Firestein, 2008), affects on behavior had been largely ignored. Recent evidence, however, has suggested that one of the overt expressions of Prnp knockouts is altered olfactory functioning (Kim et al., 2007). LePichon et al. (2009) found that Prnp null mice were slower to find a buried treat than were wild type controls. In addition, following repeated exposure (habituation) to a scent, the knockouts did not exhibit a renewed interest to a novel scent, whereas control animals did demonstrate an ability to discriminate between the two odors. Thus, expression of PrP^C appears to be requisite for normal olfactory functioning.

The Hamster Model

Hamsters have emerged as a suitable, if not superior, vehicle for in vivo studies of prion pathogenesis (Bartz, Kincaid, & Bessen, 2002; Bartz et al., 2005; Jones, 2008; Trevitt & Collinge, 2006). One particular advantage to researchers is the existence of several hamster-adapted strains of infectious prion, known as transmissible mink encephalopathy (TME). TME was originally discovered in mink in the 1940's. Infection of mink is believed to have been a result of transmission via BSE infected material. Two distinct strains of TME were subsequently identified: HY (hyper) and DY (drowsy). The HY strain is characterized by hyperexcitability, while the DY strain is characterized by lethargy and drowsiness. The benefits of the TME hamster model are the relatively short incubation period and high rate of PrP^{Sc} replication, especially in the case of the HY strain (Bessen & Marsh, 1992). In addition, the hamster exhibits the normal form of the prion protein that shares the same localization as primates (Sales et al., 1998), making it an efficient model with which to investigate prion-related phenomena.

Hamsters exhibit PrP^C localization in their olfactory system and when infected, show PrP^{Sc} deposition within the olfactory epithelium (Dejoia, Moreaux, O'Connell, & Bessen, 2006) similar to the pattern Zannusso et al. (2003) reported in CJD victims. Bessen et al. (2010) evaluated the pattern of PrP^{Sc} deposition in the hamster nervous system and observed the presence of the abnormal isoform within the olfactory receptor neurons. Not only do these findings support a speculated

pathway for horizontal transmission of TSE's, but also promote the use of olfactory measures to assess infection, as anomalies within sensory cells would likely interfere with perception.

Behavioral Olfactory Paradigms

Although hamsters represent an established model for studying the transmission of TSE's, behavioral paradigms for evaluating olfactory functioning are limited. Much of the past hamster olfactory research has been restricted to sexual odor discrimination paradigms that involve a complex variety of behavioral and endocrine system interactions. Using these paradigms would not be suitable for testing olfactory functioning in isolation.

Tests of olfactory functioning range from highly technical paradigms using sophisticated equipment (e.g. odorant dispersion apparatus and electrophysiological recordings from brain structures; Slotnick & Bodyak, 2002 or LePichon et al., 2009) to simple detection paradigms (e.g. hiding a cookie under bedding and recording the amount of time required to retrieve it; Edwards & Burge, 1973). Somewhere in between are preference paradigms. In a preference test, subjects are exposed to several odors and the amount of time spent investigating each is recorded. Odors investigated for a large amount of time are interpreted as 'attractive' while odors investigated for a smaller amount of time are 'aversive'. Often attractive and aversive odors are determined a priori, such that the ability to discriminate between the two can be ascertained. In these cases, a neutral stimulus

is often employed to give a baseline of investigative behavior. Thus, longer investigation of a pleasant scent relative the neutral stimulus is interpreted as an ability to perceive the attractant; while a shorter investigation of a noxious stimulus relative to the neutral scent is interpreted as an ability to perceive the aversive stimulus (for an overview of methods in rodent olfactory research see Slotnick & Schellinck, 2002). In this manner, the researcher can assess both the ability to discriminate between attractive and aversive stimuli as well as discrete sensitivity to each stimulus. Given subtle species differences between mice and hamsters, it is unknown whether olfactory behavior would be identical, which could prove problematic for the direct transfer of a successful mouse paradigm. Thus, it would be expedient to experimentally validate any olfactory assessment used for hamsters before launching a full-scale study.

The Current Research

Little literature exists regarding olfaction in prion-infected animals. In an effort to contribute to this knowledge base, the current project sought to identify prion-induced changes in olfactory-related behavior using the hamster model. In addition, PrP^{Sc} proliferation through olfactory structures was investigated. As the sense of smell is critical for rodents' survival, prion deposition in these areas should relate to empirical behavioral changes. For the purposes of the current project, the relevant areas of interest were identified with the aid of a comprehensive review of the rodent olfactory system (Shipley, McLean, & Ennis, 1995). The specific

structures examined were the olfactory bulb as well as the constituents of the primary olfactory cortex. Secondary areas involved in the modulation of olfactory processing were also examined.

The main olfactory bulb (OB) is the area in which afferent neural signals from the olfactory receptor neurons (ORN) are first processed. It is structured in a laminar fashion with several layers of principal cell types. The glomerular layer (Gl) is immediately inferior to the most superficial cell layer (olfactory nerve layer). Bearing distinctive morphology, the Gl is composed of ovoid structures containing dense neuropil surrounded by juxtaglomerular neurons. Incoming information from the ORN terminates into the Gl and synapse with mitral, tufted, and juxtaglomerular cells.

Inferior to the Gl is the external plexiform layer (EPL), which is a cell-poor but neuropil rich region. Composed mainly of mitral, tufted and granule cells, the axons in the EPL project mainly to areas of the OB, but also to the anterior olfactory nucleus and other cortical structures.

Deep to the EPL is the mitral cell layer (MCL). Predominately composed of mitral cell somas, the MCL is the main output center of the OB. Each mitral cell projects an apical dendrite into a single glomerulus, where it receives information from tens of thousands of ORN, effectively increasing sensitivity to odor detection.

The internal plexiform layer (IPL) is inferior to the MCL and is almost entirely composed of axons from mitral and tufted cells and dendrites from granule

cells. In addition, serotonergic axons from the raphe nucleus and noradrenergic axons from the locus coeruleus terminate in this region.

The deepest layer of the OB is the granule cell layer (Gr). Lacking axons, granule cells have dendrites in the EPL and may help to synchronize synaptic activity in the olfactory bulb. This function can be inferred from the reciprocal manner in which mitral cells make excitatory synapses into the dendrites of granule cells and the latter make inhibitory synaptic connections into the former.

Ipsilateral and contralateral projections from the OB innervate a group of structures collectively known as the primary olfactory cortex (POC). The current study was concerned with examining several areas within the POC. The anterior olfactory nucleus (AON) is a laminated structure partially composed of pyramidal cells and receives inputs from all other olfactory structures. Of all constituents of the POC, the AON also contributes the largest number of OB innervating neurons. The remaining cortical structures of interest include the piriform cortex (Pir), which contains numerous pyramidal cells and interneurons for ipsilateral and contralateral communication, the olfactory tubercle (Tu), a strip of cortex characterized by non-reciprocating enervation by the OB, and the taenia tecta (TT), a dense nucleus which predominately innervates the OB.

Two midbrain structures also contribute to olfactory functioning via neural modulation. The serotonergic dorsal raphe nucleus (DR) innervates the OB by way of the Tu and contributes to olfactory conditioned behaviors. The noradrenergic locus coeruleus (LC) also modulates olfactory processing with axons that innervate

the IPL and Gr layers. Estimates suggest that up to 40% of LC neurons project to the OB in rodents and that the LC may play a role in increased sensitivity to and heightened vigilance towards weak but important odors.

Proper interaction of all these structures is a requisite for normal olfactory functioning. Thus, if PrP^{Sc} were to compromise even part of the system, empirical behavioral changes would be a logical consequence. To test this hypothesis, HY TME was introduced into the OB of Syrian golden hamsters and these animals were compared to a mock-inoculated control group. While not necessarily a direct analogue of natural transmission, the OB was chosen as the inoculation sight as it represented the central olfactory structure with which to examine PrP^{Sc} and the resulting effects. Though the nasal cavity has been implicated as a route of horizontal transmission of TSE's, evidence suggests that infection does not occur via afferent spread from ORN to the brain but occurs via olfactory-unrelated peripheral routes (Sbriccoli et al., 2009). Thus, inoculation of the OB allowed for the most efficient time-course analysis of the behavioral ramifications of PrP^{Sc} proliferation throughout the olfactory nervous system. Given the preponderance of evidence regarding PrP^{Sc} deposition in olfactory structures and the comorbidity of olfactory dysfunction in neurodegenerative disease, it was expected that HY TME infected subjects would demonstrate altered olfactory performance relative to control subjects. In addition, immunohistochemistry was employed as a means to relate the presence of the abnormal prion protein within the system to observed behavioral changes.

An olfactory preference paradigm was used to examine the effects of prion infection on olfactory functioning. The logic guiding this model is that animals with normal olfactory function will spend more time investigating an attractive scent relative to a neutral scent (Slotnick & Schellink, 2002). Likewise, these animals will spend less time investigating an aversive scent relative to a neutral scent. In contrast, animals with compromised olfactory function would not be expected to exhibit this pattern of behavior, as they should be unable to discriminate between attractive, neutral, and aversive olfactory stimuli.

A preliminary investigation determined that vanilla extract was attractive to hamsters while 2-Methylbutyric acid (2-MB) produced an odor that was aversive. Thus, normal animals were expected to spend significantly more time investigating a vanilla stimulus relative to a 2-MB stimulus, while olfactory compromised animals were not expected to display this pattern.

Ethics Statement

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* (National Research Council, 1996).

EXPERIMENT 1

The purpose of Experiment 1 was to validate the olfactory preference paradigm for use with hamsters. Although olfactory preference paradigms have been successfully employed in various rodent models (Kobayakawa et al. 2007; Witt, Galligan, Despinoy, & Segal, 2009, no testing specific to hamsters have been reported. In the present study, a group of subjects was rendered temporarily anosmic with methimazole, a chemical found in thyroid medications and linked to reduced olfactory acuity. Methimazole induces sensory loss by inducing the sensory epithelium to dislodge from sub-mucosal structures within the nasal cavity (Sakamoto et al., 2007). This process occurs over the course of 24-48 hours and results in temporary anosmia. Regeneration of the sensory epithelium is typically achieved within 14-21 days.

Following treatment, an olfactory preference test was performed on the methimazole-treated subjects and a group of control subjects. It was expected that control subjects would spend significantly more time investigating the attractive versus the aversive stimuli relative to methimazole animals. To confirm that anosmia had been induced, two animals from each group were sacrificed following testing and nasal mucosa from each was collected and subjected to histological analysis.

Method

Subjects

Subjects were 20 male Syrian golden hamsters (Charles River Laboratories, Boston, MA). Age of the subjects at time of testing was approximately 12 weeks. The subjects were housed in groups of 3-5 in cages measuring 16.75" x 10.5" x 8.25" and were exposed to 12 hour light/12 hour dark light cycle. The average ambient temperature in the housing facility was 72° and the relative humidity was controlled at 25%.

Materials

Methimazole (Sigma-Aldrich Co., St. Louis, MO) was stored at room temperature and dissolved in DMSO/PBS immediately prior to use. Pure vanilla extract (Frontier Natural Products Co-op, Norway, IA) was used as the olfactory attractant. It was stored at room temperature and diluted to a 10% solution with deionized water immediately prior to use. The aversive olfactory stimulus was 2-MB (Sigma-Aldrich Co., St. Louis, MO). It was stored at room temperature and diluted to a 10% solution with deionized water immediately prior to use. Deionized water was used as the neutral olfactory stimulus.

Treatment

Experimental subjects (n=10) were given an intraperitoneal injection of methimazole diluted in DMSO/PBS at 125mg/kg. Controls (n=10) were given the

same dose, but the injections contained DMSO/PBS only. Behavioral testing was conducted 48 hours following treatment.

Testing procedure

Subjects were tested in normal light conditions approximately four hours before onset of their active phase (i.e. four hours before lights out). Testing occurred over two days with ten subjects tested each day.

Habituation. Habituation consisted of 15 minutes in each of three cages and five minutes in a fourth cage (each cage measured 19" x 10.5" x 8.5"). Specifically, the first subject was placed in a clean habituation chamber. After 15 minutes, the first subject was transferred to the second habituation chamber, and the second subject was placed within the first chamber. Following another 15 minutes, the first subject was transferred to the third chamber, the second subject was transferred to the second chamber, and the third subject was placed in the first chamber. This process was repeated throughout the duration of the testing session with the exception that all subjects were habituated for only five minutes in the fourth and final chamber before the commencement of behavioral testing. The fourth chamber also served as the testing arena.

The habituation cages were placed sequentially on a lab bench and were separated by pieces of black poster board to prevent visual interaction between animals. The first three habituation cages were covered with standard housing lids while the testing chamber was covered with transparent plexiglas to allow sufficient

luminescence for testing purposes. To eliminate olfactory cues, all cages were thoroughly cleaned with ethanol between subjects, paying special attention to the corners of the apparatus.

Behavioral testing. The testing phase consisted of three trials, each 180 seconds in duration. For each subject, presentation order of the three olfactory stimuli was determined randomly. Olfactory stimulus solution (0.5 ml) was transferred via pipette to a piece of filter paper measuring 2 inches by 2 inches. Placement of stimuli was determined by the current position of the animal within the testing chamber such that the filter paper was placed at the end of the chamber opposite that of the subject. If the subject was in the middle of the apparatus, placement of the stimuli was left to the experimenter's discretion. Timing of the trial began immediately upon placement of the stimuli and concluded after 180 seconds.

In order to mitigate behavioral variability due to the presence of the investigator, video recording devices were placed at each end of the testing chamber. These cameras were connected to a computer that was positioned outside of the room and provided dual images of the testing session. The experimenter, with the aid of standard stopwatch, recorded the amount of time each subject spent investigating the stimulus. The dual, opposing images of the testing chamber provided the experimenter with an unobstructed view of the subjects' activities.

Investigative behavior was operationally defined as instances in which the subject's nose was within one centimeter of the filter paper and the subject was

actively sniffing the stimulus. Chewing and licking behavior was not recorded. Instances in which the subject sat upon or near the stimulus, but did not actively sniff the stimulus, were not recorded.

At the conclusion of each trial, the olfactory stimulus was removed and the area beneath it was wiped with ethanol. The cage was then fanned several times to remove any residual olfactory cues, the next randomly determined stimulus was introduced, and the second trial commenced. Intertrial intervals were approximately 30 seconds. After the third and final trial, the subject was removed from the testing chamber and returned to its home cage. The testing chamber was then cleaned with ethanol and habituation of the next subject in the testing apparatus began.

Histology

For collection of nasal mucosa (NM) tissues, hamsters were deeply anesthetized with isoflourane and exsanguinated via atrial incision. They were then intracardially perfused with periodate-lysine-paraformaldehyde (PLP) fixative and skulls were dissected and immersion fixed in PLP for 24-36 hours. Following decalcification by immersion in 10% formic acid, skulls were cut into coronal pieces and processed and embedded in paraffin wax. Using a microtome, NM was cut into 5 μm sections and slide-mounted.

Paraffin was removed from tissue sections with Clearite 3 (Richard-Allen Scientific, Kalamazoo, MI) and tissue was dehydrated in degrading alcohol baths.

Sections were stained with Hematoxylin (Richard-Allen Scientific, Kalamazoo, MI) and counterstained with Eosin (Richard Allen Scientific, Kalamazoo, MI) before being rehydrated in graded alcohols and cover slip mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA) for viewing with a Nikon Eclipse E600 microscope.

Results

Behavioral Testing

This experiment evaluated the utility of the olfactory preference paradigm. Investigation times for water, vanilla, and 2-MB were collected for each subject and the mean ($\pm$ SD) investigation times of each olfactory stimulus are depicted in Table 1.

Table 1

Investigation Means (SD) in Seconds

Condition	Olfactory Stimulus		
	Vanilla	2-MB	Water
Control	12.5 (10.3)	1.7 (3.3)	5.9 (5.0)
Nasal Toxic	4.4 (5.2)	3.3 (6.4)	6.1 (7.8)

Olfactory preference of hamsters treated with methimazole (Nasal Toxic, $n = 10$) or vehicle (Control, $n = 10$). Each subject was randomly presented with vanilla, 2-MB and water. Time spent investigating each stimulus over the course of 3 minutes was recorded.

To examine whether water constituted a suitable control stimulus, an independent samples t -test was conducted on the investigation times for this stimulus. Time spent investigating water did not differ between the two groups ($p > .05$). In subsequent analysis, subjects' water investigation time was subtracted from both their individual vanilla and 2-MB investigation times (Figure 1).

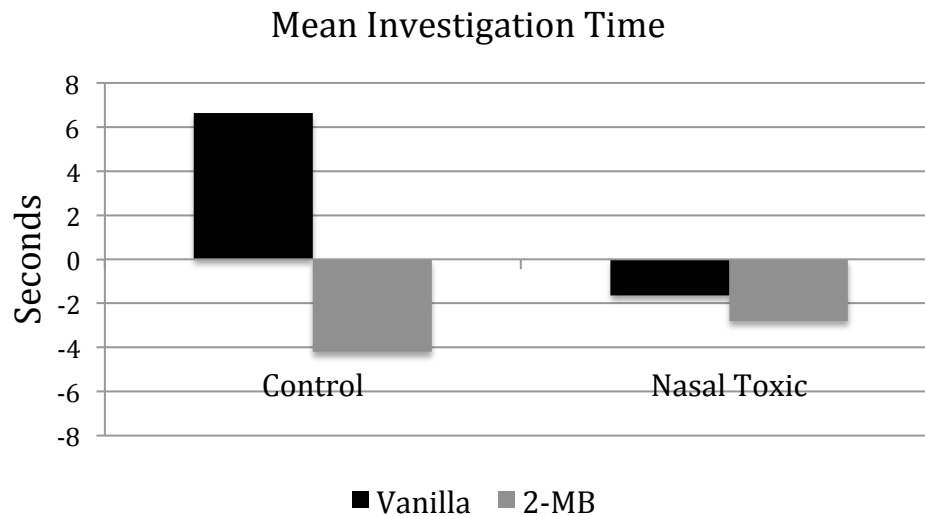


Figure 1. Exploratory preference scores for hamster treated with methimazole (Nasal Toxic, $n = 10$) or vehicle (Control, $n = 10$). Values represent investigation time of Vanilla or 2-MB from which exploratory time with water has been subtracted. Negative values represent aversive behavior and positive values indicate attractive behavior.

The difference in investigation of vanilla and 2-MB by treatment was evaluated using two planned comparisons (Bonferroni correction, $\alpha = .025$). Results indicated that the control group exhibited significantly different investigation times of vanilla and 2-MB ($t(9) = 2.98, p < .025$). The nasal toxic group did not exhibit this pattern ($t(9) = 0.40, p = .70$). This suggests that while the control group was able to discriminate between attractive and aversive scents, the group treated with methimazole was not.

Histology

Mucosal tissues from nasal cavities (nasal mucosa, NM) were collected and stained with hematoxylin and eosin (Figure 2). Control animals had intact sensory

epithelia, while the hamsters treated with methimazole exhibited extensive damage. The epithelial layer of treated subjects was degraded and/or detached from the sub-mucosal structures, which would impair the detection of olfactory stimuli. These histological observations were consistent with the impaired olfactory performance of animals treated with methimazole.

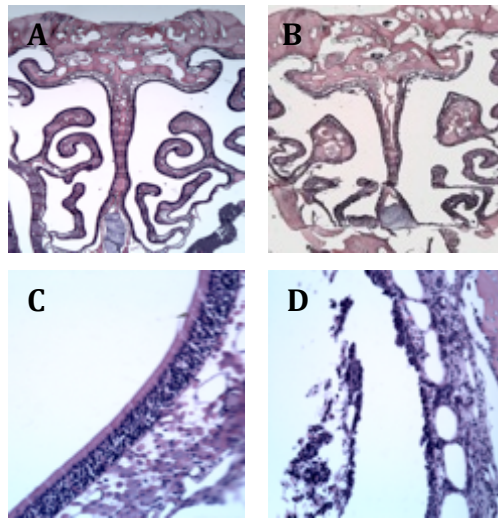


Figure 2. Low magnification (2X) of hematoxylin and eosin staining of nasal mucosa from representative control (A) and methimazole-treated (B) subjects. Higher magnification (20X) reveals deterioration of sensory epithelium in the methimazole treated subject (D) but not in control subject (C). Animals received methimazole (125 mg/kg) or vehicle (125mg/kg) 48 hours prior to sacrifice for histological assessment.

Discussion

The results of experiment 1 indicated that the preference paradigm was valid and could be used as means to assess olfactory functioning. Hamsters induced with anosmia were unable to discriminate between attractive and aversive odors. If the presence of PrP^{Sc} were to interfere with olfactory transduction and/or olfactory processing, it seems likely that a similar pattern would emerge in infected animals.

Although discriminatory deficits in prion afflicted subjects would be unlikely to be as pronounced as those in animals that have had their sensory structures destroyed (as did the animals in experiment 1), cellular malfunction should nonetheless manifest as empirical changes in behavior towards the olfactory stimuli.

Investigation of these effects would be best viewed under the auspices of a time-course analysis following inoculation of the HY TME agent.

EXPERIMENT 2

The purpose of Experiment 2 was to investigate prion-induced olfactory deficits using the olfactory preference paradigm developed in Experiment 1. A group of hamsters was inoculated with HY TME while another group was mock inoculated. Subjects were submitted to the preference test at four time points following inoculation: 20, 40, 60, and 80 days post-inoculation (DPI). It was expected that the control group would exhibit a normal attraction/aversion pattern of olfactory behavior. Experimental animals were not expected to exhibit this pattern. As no previous research exists to suggest the time-course of prion-induced olfactory dysfunction in hamsters, no predictions were made regarding the point in time at which olfactory deficits would be observed.

Two parallel groups of animals were used for histological analysis. The purpose of these animals was to investigate the course of prion proliferation throughout olfactory-related brain structures using immunohistochemistry. The magnitude of PrP^{Sc} infection was rated in each of the the relevant olfactory structures.

Method

Subjects

Subjects were 20 male Syrian golden hamsters (Simonsen Laboratories, Gilroy, CA) of which 10 constituted the experimental group and 10 the control

group. Age at the time of inoculation was approximately four weeks. Husbandry was identical to subjects in Experiment 1.

Materials

Olfactory stimuli were identical to those used in Experiment 1.

Treatment

Inoculations took place while hamsters were under general anesthesia, which was induced via i.p. injection of a ketamine/xylazine mixture. The animals' fur between the eyes was shaved and the area was scrubbed with betadine. A single incision (≈ 1 cm) was made and the skin was retracted. The subcutaneous fascia was cut and a single hole was made by gently twisting a 28-gauge needle into the incisive bone a few millimeters to the lateral side of the midline. A 30-gauge needle was inserted into the hole and subjects were intra-olfactory bulb (i.ob.) inoculated with 2 μ l of brain homogenate from either a normal hamster (control subjects, $n = 10$) or a HY TME-infected hamster (experimental subjects, $n = 10$) containing $10^{8.5}$ intracerebral median lethal dose (LD_{50}) per ml of the HY TME agent (for complete protocol see Bessen & Marsh, 1994). Following the procedure, the incision was closed using methacrylate glue and hamsters were given an intramuscular analgesic injection and monitored throughout recovery.

Testing procedure

All testing procedures in Experiment 2 were identical to those in Experiment 1 except for the following difference: Subjects were tested in low light conditions approximately two hours into their active phase (i.e. two hours after lights out).

Immunohistochemistry

For collection of tissues, hamsters were deeply anesthetized with isoflourane and exsanguinated via atrial incision. They were then intracardially perfused with periodate-lysine-paraformaldehyde (PLP) fixative and tissues were dissected (e.g. brain, olfactory bulb, nasal cavity) and immersion fixed in PLP (6 hours for brain and olfactory bulb; 24-36 hours for skulls containing NM). Decalcification of skulls containing NM was performed by immersion in 10% formic acid after which they were cut into coronal pieces and embedded in paraffin wax. Brains were cut into sagittal pieces prior to processing and embedding in paraffin wax. Using a microtome, all tissues were then cut into 5 μ m sections and slide-mounted.

Antigen retrieval was achieved by treatment with formic acid (99% wt. vol.) for 10 minutes. Following streptavidin and biotin blocking steps, tissue was incubated with anti-PrP monoclonal 3F4 antibody (1:6000, a gift from Victoria Lawson) overnight at 4° C. Sections were then incubated with horse anti-mouse biotinylated secondary antibody (1:400; Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes, followed by streptavidin-horseradish peroxidase (HRP) at room temperature for 20 minutes. PrP^{Sc} was visualized by localization of

HRP activity with DAB+ (Dako, Carpinteria, CA). Tissue sections were counterstained with hematoxylin and cover slip mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA) for viewing with a Nikon Eclipse E600 microscope (for protocol used see Bessen et al., 2010). A minimum of three animals per group per time point was analyzed with maximum intervals of 150 μ m for each specimen.

Results

Behavioral Testing

Investigation times for water, vanilla, and 2-MB were collected for each subject and the mean ($\pm$ SD) investigation times of each olfactory stimulus are depicted in Table 2. One subject in the mock (control) group did not approach any stimulus during the course of the entire experiment (12 trials total) and was subsequently removed from the analysis.

Table 2.

<i>Investigation Means (SD) in Seconds</i>				
Inoculum	Days Post Inoculation			
	<u>20</u>	<u>40</u>	<u>60</u>	<u>80</u>
Mock				
Vanilla	9.8 (7.2)	5.9 (5.3)	8.2 (7.3)	9.4 (8.5)
2-MB	1.4 (2.7)	0.4 (0.3)	0.5 (0.5)	0.4 (0.5)
Water	5.3 (6.0)	4.2 (3.2)	3.7 (3.2)	4.1 (2.8)
HY TME				
Vanilla	4.7 (5.3)	4.4 (4.2)	6.3 (4.9)	0.9 (1.1)
2-MB	0.1 (0.3)	0.5 (0.4)	2.1 (3.7)	1.2 (1.3)
Water	5.3 (5.5)	4.5 (3.2)	6.3 (6.7)	2.3 (2.5)

Olfactory preference of hamsters inoculated with vehicle (Mock, n = 9) or HY TME (n = 10). At each time point animals were randomly presented with vanilla, 2-MB and water. Time spent investigating each stimulus over the course of 3 minutes was recorded.

To examine whether water constituted a suitable control stimulus, a 2-way mixed model ANOVA was performed on the water data only, with days post-inoculation (DPI) as the within-subjects factor and condition as the between-subjects factor. Time spent investigating water did not significantly differ between the two groups ($F(1,17) = 0.03, p = .87$) nor did it significantly differ with regards to time of testing ($F(3,51) = 1.70, p = .21$). In subsequent analyses, subjects' water investigation times were subtracted from their respective vanilla and 2-MB investigation times (Figure 3).

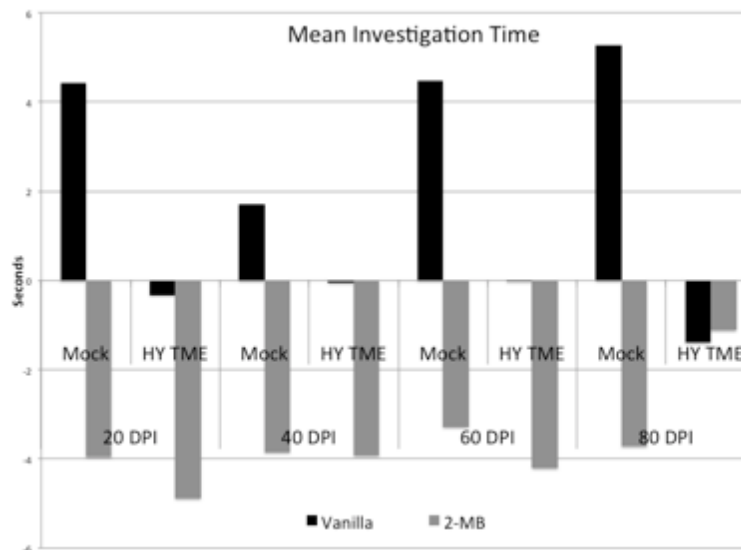


Figure 3. Exploratory preference scores for hamster inoculated with vehicle (Mock, $n = 9$) or HY TME ($n = 10$). Values represent investigation time of Vanilla or 2-MB from which exploratory time with water has been subtracted. Negative values represent aversive behavior and positive values indicate attractive behavior.

A 3-way mixed model ANOVA was performed on the water subtracted data with DPI and scent as within-subjects factors and condition as the between subject factor. There were significant main effects of both condition ($F(1,17) = 4.75, p < .05$,

$\eta_p^2 = .22$) and scent (Wilks' $\lambda = 0.34$, $F(1,17) = 32.88$, $p < .01$, $\eta_p^2 = .66$). The interaction of these two factors was also significant (Wilks' $\lambda = 0.74$, $F(1,17) = 5.98$, $p < .05$, $\eta_p^2 = .26$). In addition, the three-way interaction of DPI x scent x condition was marginally significant (Wilks' $\lambda = 0.63$, $F(3,15) = 3.00$, $p = .06$, $\eta_p^2 = .38$).

Given the graphical representation of the data (Figure 3) and the marginal three-way interaction with an observed medium effect size, vanilla and 2-MB investigation times were examined at 80 DPI. To examine whether mock and infected subjects exhibited differential investigation of the two stimuli two comparisons were conducted (Bonferroni correction; $\alpha = .025$). Results indicated that the control group exhibited significantly different investigation times of vanilla and 2-MB ($t(8) = 3.37$, $p < .025$), while the HY TME infected group did not ($t(9) = -0.60$, $p = .57$). This suggested that 80 days following inoculation the control group was able to discriminate between attractive and aversive scents. Infected animals were unable to make this distinction.

The significant two-way interaction of scent x condition in the omnibus ANOVA suggested that behavior towards vanilla versus 2-MB may have been fundamentally different for the groups across the entire time series analysis. To test this hypothesis, discrete two-way ANOVA were conducted for the vanilla data and the 2-MB data independently. In the vanilla ANOVA, condition served as a between subjects factor and DPI served as the within factor. The only significant effect to emerge was a main effect of condition ($F = 8.18$, $p < .05$, $\eta_p^2 = .33$), suggesting that investigation of the attractive scent differed for the two groups regardless of time. In

the 2-MB ANOVA, the factors were the same as the discrete vanilla analysis. In this case, however, no significant effects were observed. Thus, the data suggests that while vanilla investigation differed as a function of condition without respect to time since inoculation, both groups spent similar time investigating the aversive scent over the course of the experiment.

Histology

In order to investigate the spread of prion infection throughout olfactory related structures, immunohistochemistry (IHC) was used to visualize PrP^{Sc} deposition. At each of three time points (20, 40, and 70 DPI) three HY TME-infected and three mock-infected hamsters were examined for PrP^{Sc} signal in the structures of interest. Magnitude of infection was rated on a five-point scale: No PrP^{Sc} (--), rare PrP^{Sc} (+), mild PrP^{Sc} (++), moderate PrP^{Sc} (+++), and intense PrP^{Sc} (++++). No PrP^{Sc} was detected in any of the mock hamsters. PrP^{Sc} ratings for the HY TME subjects are depicted in Table 3.

Table 3

Magnitude of PrP^{Sc} infection in olfactory related structures

DPI	Olfactory Bulb					Primary Olfactory Cortex					Secondary	
	<u>Gl</u>	<u>EPL</u>	<u>MCL</u>	<u>IPL</u>	<u>Gr</u>	<u>AON</u>	<u>Pir</u>	<u>Tu</u>	<u>TT</u>	<u>Lo</u>	<u>LC</u>	<u>DR</u>
20												
S ₁	+++	--	--	--	--	--	--	--	--	--	--	--
S ₂	+++	--	--	--	--	--	--	--	--	--	--	--
S ₃	++	--	--	--	++	--	--	--	--	--	--	--
40												
S ₄	+++	++	+++	+++	++	++	++	++	++	++	++	+++
S ₅	++++	+++	+++	+++	+++	+++	+++	++	+++	++	++	+++
S ₆	+++	++	+++	++	+	+++	++	++	++	++	+++	+++
70												
S ₇	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
S ₈	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
S ₉	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Each S indicates a separate histological subject. Levels of PrP^{Sc} infection were rated on a five-point scale: none (--), rare (+), mild (++), moderate (+++), and intense (++++). All laminae layers of the OB were examined including the glomerular layer (Gl), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL), and the granule cell layer (Gr). Structures of the primary olfactory cortex included the anterior olfactory nucleus (AON), the piriform cortex (Pir), the olfactory tubercle (Tu), the taenia tecta (TT), and the lateral olfactory tract (Lo). Modulatory midbrain structures were also examined and included the locus coeruleus (LC) and the dorsal raphe nucleus (DR).

At 20 DPI, PrP^{Sc} was observed only in the OB. Within the glomerular layer of the OB, infection was of mild to moderate intensity. In one subject, rare infection was also observed in the granule cell layer. No infection was detected elsewhere in the brain at this time point (Figure 4).

At 40 DPI PrP^{Sc} infection had spread to all layers of the OB in varying magnitudes. Most layers exhibited mild to moderate infection, though intense infection was observed in the Gl of one subject. By 70 DPI intense infection was observed throughout the OB.

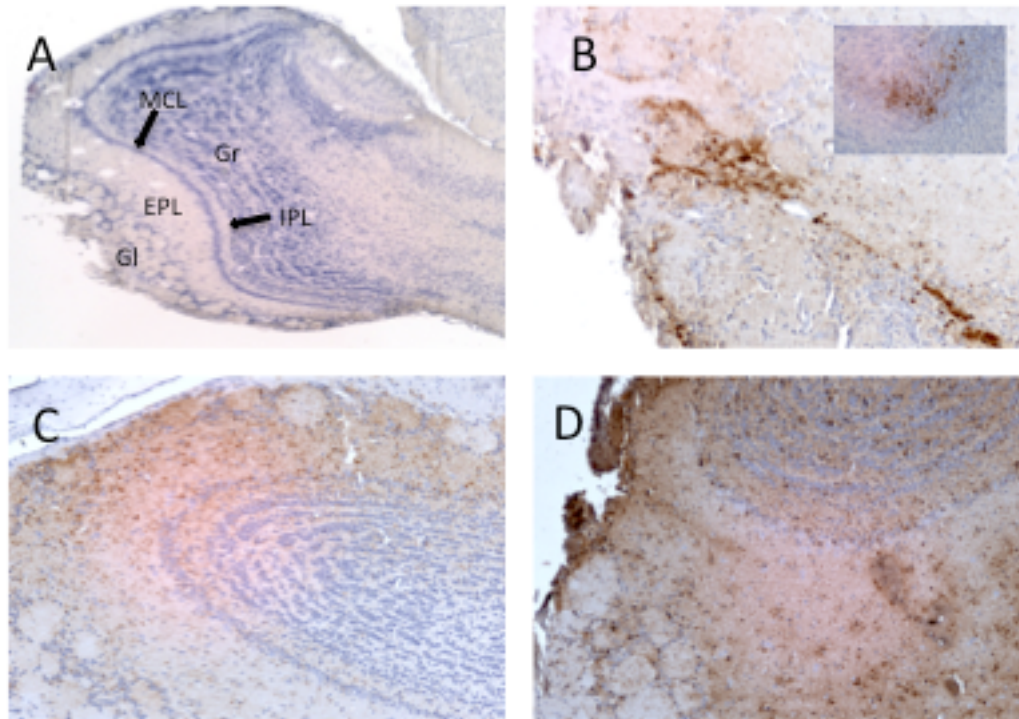


Figure 4. Low magnification Nissl stain (2X) of olfactory bulb (A). Labeled are the glomerular layer (Gl), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and granule cell layer (Gr). In the higher magnification (10X) IHC (brown chromagen with hematoxylin counterstain) punctate staining for PrP^{Sc} can be seen in the Gl (B) and Gr (inset) at 20 DPI. At 40 DPI (C) and 70 DPI (D) infection had spread to all cellular layers of the olfactory bulb.

Structures of the POC were negative for PrP^{Sc} at 20 DPI. At 40 DPI both the Lo and the TT were exhibiting mild infection. Mild to moderate levels were observed in the AON, the Pir and the TT at this time. At 70 DPI, all POC structures examined in this project were exhibiting intense levels of infection (Figure 5).

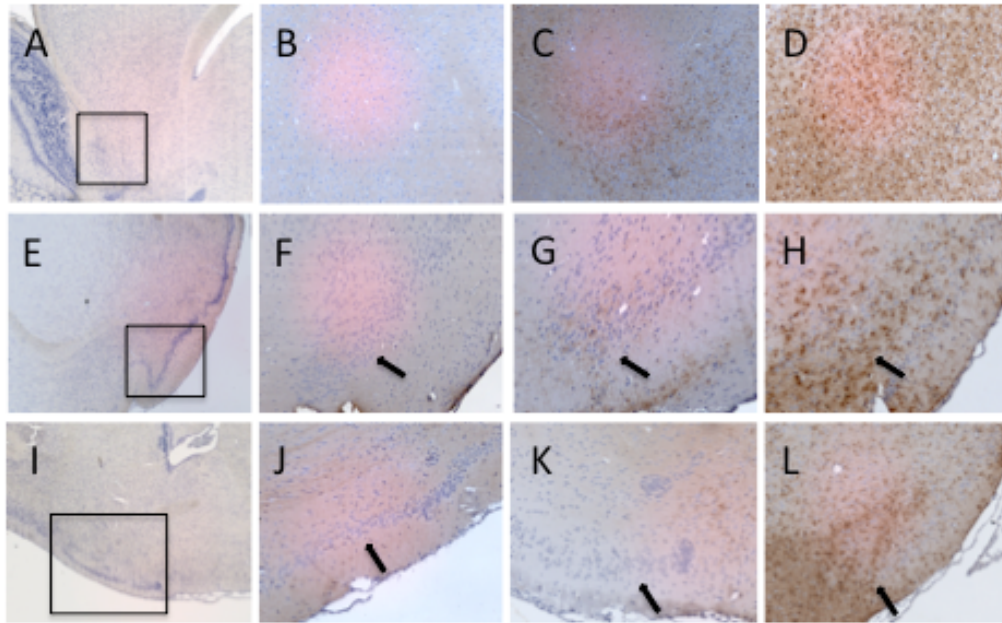


Figure 5. Low magnification (2X) Nissl stains of selected structures in the primary olfactory cortex: The anterior olfactory nucleus (AON, panel A), the piriform cortex (Pir, panel E) and the olfactory tubercle (Tu, panel I). Boxes indicate areas examined with higher magnification (10X). IHC (brown chromagen with hematoxylin counterstain) show no infection in the AON at 20 DPI (B), mild to moderate infection at 40 DPI (C), and intense infection at 70 DPI (D). No infection was present in the Pir at 20 DPI (F), mild to moderate infection at 40 DPI (G), and intense infection at 70 DPI (H). Arrows indicate the Pir. At 20 DPI no infection was detected in the Tu (J). Mild levels were present at 40 DPI (K) and intense levels at 70 DPI (L). Arrows indicate the Tu.

The secondary olfactory structures involved in the modulation of olfactory processing exhibited no infection at 20 DPI. At 40 DPI mild to moderate levels of infection were observed in the LC and moderate infection was found in the DR. At 70 DPI both of these structures were exhibiting intense levels of infection (Figure 6).

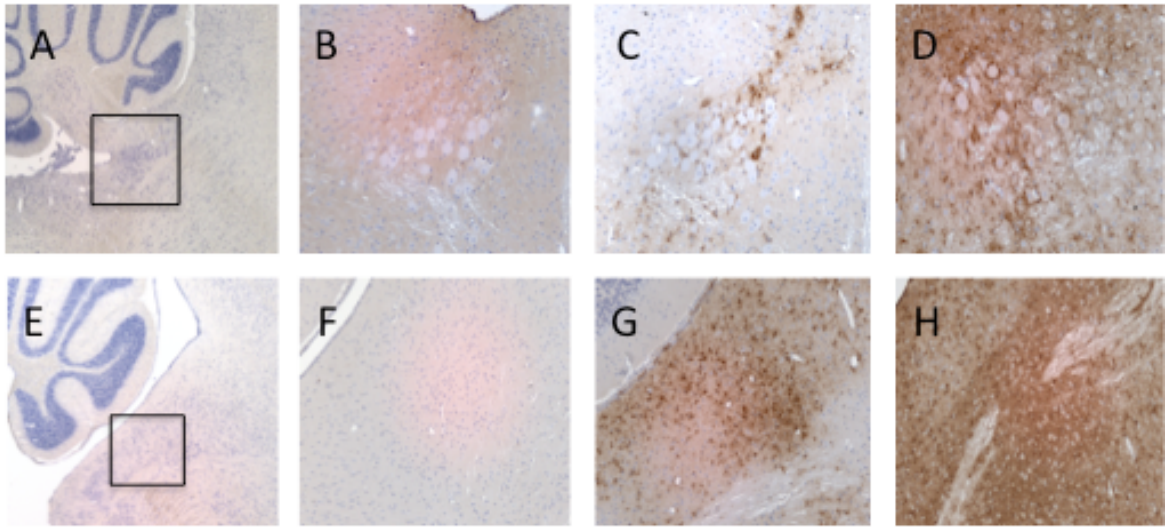


Figure 6. Low magnification Nissl Stain (2X) of brain structures involved in modulating olfactory processing: The locus coeruleus (LC, panel A) and the dorsal raphe nucleus (DR, panel E). Boxes indicate areas examined with higher magnification (10X). IHC (brown chromagen with hematoxylin counterstain) indicates no infection in the LC at 20 DPI (B) but mild to moderate levels at 40 DPI (C) and intense levels at 70 DPI (D). At 20 DPI the DR exhibits no infection (F) but moderate infection at 40 DPI (G) and intense infection at 70 DPI (H).

DISCUSSION

The major finding of this study was that prion-infected hamsters demonstrated altered olfactory performance relative to mock-infected control hamsters. This change did not manifest precipitously, but instead emerged as a loss of a behavioral response to an attractive scent early in the disease followed by a loss of a behavioral response to an aversive scent late in the course of the disease. The results indicate that, relative to mock-infected subjects, HY TME subjects behaved differently toward the vanilla stimulus beginning at 20 DPI and continued throughout the duration of the experiment. This pattern of apparent sensory loss was accompanied by a proliferation of PrP^{Sc} throughout the olfactory system. At 20 DPI when experimental subjects were demonstrating disparate behavior toward the attractive stimuli only, PrP^{Sc} deposition was limited to the Gl of the OB. The appearance of the abnormal form of the prion protein in this area has also been demonstrated in humans who had died from CJD (Zanusso et al., 2003). Taken together, these findings suggest that the appearance of PrP^{Sc} observed in the present study were consistent with observations of TSE's in the human population.

Although infected hamsters were not attracted to the vanilla scent, they exhibited an aversion to the 2-MB at several of the observation time points (20, 40 and 60 DPI). The observed PrP^{Sc} proliferation continued through these time points, with mild to moderate levels of infection in all olfactory-related structures at 40 DPI. At 70 DPI, immunoreactivity analysis of histological subjects indicated that all

olfactory structures were exhibiting intense staining for PrP^{Sc}. The HY TME group failed to demonstrate 2-MB aversion at 80 DPI. At this point infected subjects appeared to lose the ability to detect attractive or aversive scents, which would be indicative of anosmia. . In contrast, control subjects exhibited attraction towards vanilla and avoidance of 2-MB throughout the course of the experiment.

There are several potential explanations for the observation that HY TME hamsters were not attracted to vanilla early in the disease yet avoided the 2-MB throughout much of the disease progression. It is possible that, though they were unable to detect vanilla as an olfactory stimulus, animals were able to detect the 2-MB as an irritation to somatosensory nerve endings located in the olfactory membrane. Although investigation of PrP^{Sc} deposition in somatosensory nerve endings was not evaluated directly, this explanation seems unlikely as PrP^{Sc} deposition within the nasal and respiratory mucosal layer did not discernably change between 40 and 70 DPI. Thus, attenuation of the avoidant behavior would likely have been affected prior to 70 DPI.

Another potential explanation is relative sensitivity associated with the test odorants. It is possible that the dilutions of vanilla and 2-MB used in the experiment possessed different intensities and as such produced disparate behavioral responses over the course of the experiment. If vanilla were less aromatic than 2-MB, one would expect the infected subjects to demonstrate a loss of interest in vanilla earlier than they showed avoidance toward 2-MB. However, any gross loss of sensitivity should be accompanied by anomalies within the MCL of the OB (Meisami,

1989; Kim et al., 2007). This was not the finding of the current study as at 20 DPI PrP^{Sc} was detected only in the Gl and, in one subject, the Gr.

It is possible that the early appearance of PrP^{Sc} in the Gl of the OB caused a perceptual loss to one stimulus but not the other. In this case PrP^{Sc} deposition in the Gl of infected subjects hindered the sensory processing of vanilla but not 2-MB. At the earlier time points, this would explain the early loss of attraction towards vanilla but the retention of avoidance towards the 2-MB. Axons of ORN's expressing a specific odorant receptor typically converge on a very limited number of glomeruli (Vassar et al., 1994). Thus, a given odor activates a restricted area within the OB. If this particular area were compromised, then the subject may be effectively unable to perceive that specific scent. Indeed, mice that had toxically ablated glomerular regions in their OB exhibited differential perceptual behavior towards aversive stimuli as compared to controls. In some instances they completely lost their innate fear of predator odors (Kobayakawa et al., 2007). These findings demonstrated that a single type of odorant can represent a selective set of glomeruli and that the loss of one region within the Gl can manifest in the perceptual loss of a specific odor. While the determination of specific perceptual topography in the Gl was beyond the scope of this project, the literature suggests that the initial appearance of PrP^{Sc} within the Gl was responsible for the early disappearance of a behavioral response toward only one of the odors. Additional research is needed to test this hypothesis.

Infected animals eventually lost avoidance behavior for the aversive scent at 80 DPI. The reason for this delayed response relative to the attractive scent is not

apparent. One explanation is that total loss of discriminatory ability may have been due to the widespread proliferation of PrP^{Sc} within the Gr. Electrophysiological recordings of this area in rats have indicated that granule cells are critical for odor discrimination (Gheusi et al., 2000). While rare to moderate levels of infection were observed in this area at 40 DPI, it was not until 70 DPI that intense infection were observed. The data are consistent with the interpretation that prior to the 80 DPI observation point, the Gr was uncompromised and infected subjects were able to distinguish 2-MB from the other stimuli. At 80 DPI, however, levels of infection had reached sufficient magnitude in the Gr to interfere with synaptic activity resulting in the loss of discriminatory ability. A loss of aversion to 2-MB could also be due to malfunctions in other areas of the brain that related to processing the emotional quality of a scent. Since all olfactory-related structures were exhibiting intense levels of infection by 70 DPI, it is difficult to speculate about what area(s) could be responsible for the infected subjects' behavior at 80 DPI.

The present study used IHC to determine the course of PrP^{Sc} proliferation throughout the olfactory system. At 20 DPI infection was limited almost entirely to the Gl of the OB. By 40 DPI infection had spread to all primary olfactory structures examined, with mild to moderate levels of infection in all POC areas as well as the modulatory structures, the LC and DR. Thirty days later, infection had progressed to an intense magnitude in all olfactory related structures. These findings parallel another time course analysis in which PrP^{Sc} made a rapid appearance in all olfactory related structures, rather than appearing sporadically. Specifically, intranasal

administration of the 263K scrapie strain of the pathologic prion resulted in widespread olfactory system infection (Sbriccoli et al., 2009). Although this previous study utilized a different infectious agent, it is interesting that the pathological prion was absent from olfactory-related brain structures at one time point, and found in virtually all of them ten days later. Taken together, these findings suggest that once the olfactory system becomes involved in prion infection, the rate of PrP^{Sc} proliferation is rapid.

While olfactory deficits in mice lacking the normal form of the prion protein have been reported (Kim et al., 2007; LePichon et al., 2009), no study has addressed the olfactory consequences of infectivity of the pathological form of the prion. Thus, the current series of studies support the validity of olfactory performance as a behavioral marker of prion disease infection. These paradigms have utility as an early indicator of infection within a clinical setting, and behavioral index for the efficacy of any therapeutic intervention. The observation that infected subjects exhibited temporally differential loss of sensory ability to the attractive versus aversive scents may suggest the use of specific odors within a clinical diagnostic setting (i.e. a person suffering from CJD may be unable to perceive a specific odor). In addition, a discrimination task might be useful for identifying TSE induced olfactory deficits (i.e. a person suffering from CJD may be unable to distinguish between two scents).

Additional research is needed to further substantiate olfaction as a viable diagnostic indicator. While the preference paradigm used in the current project

showed differences between healthy and infected hamsters, an olfactory sensitivity paradigm may be able to measure subtler changes in olfactory acuity. In a sensitivity paradigm hamsters would be presented with varying dilutions of an attractive stimulus and their investigative behavior recorded. In this manner subjects' olfactory detection threshold could be assessed and related to TSE progression, which would further elucidate the prion induced olfactory deficits observed in this project.

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