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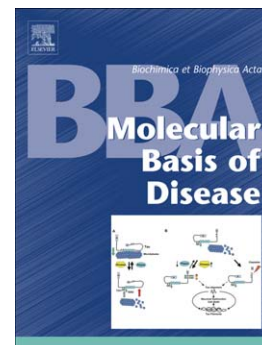
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Motor behavioral and neuropathological deficits in mice deficient for normal prion protein expression

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Summary

It has been difficult to reconcile the absence of pathology and apparently normal behavior of mice lacking prion protein (PrP), referred to as *Prnp*^{0/0} mice, with a mechanism of prion pathogenesis involving progressive loss of PrP^C-mediated neuroprotection. However, here we report that *Prnp*^{0/0} mice exhibit significant age-related defects in motor coordination and balance compared with mice expressing wild type *Prnp* on a syngeneic background, and that the brains of behaviorally-impaired *Prnp*^{0/0} mice display the cardinal neuropathological hallmarks of spongiform pathology and reactive astrocytic gliosis that normally accompany prion disease. Consistent with the appearance of cerebellar ataxia as an early symptom in an patients with Gerstmann-Sträussler-Scheinker syndrome (GSS), an inherited form of human prion disease, motor coordination and balance defects manifested in a transgenic (Tg) mouse model of GSS considerably earlier than the onset of end-stage neurodegenerative disease. Our results are consistent with a mechanism in which loss of normal PrP^C function is an important pathological component of prion diseases.

Introduction

The precursor-product relationship between the cellular and disease-associated forms of PrP, referred to as PrP^C and PrP^{Sc} respectively, is well-established for prion replication. In contrast, the mechanism by which prions elicit brain damage and the relative contributions of PrP^{Sc} accumulation and PrP^C depletion to this process remain unclear. Prion disorders are generally viewed as gain-of-function diseases with neuronal damage resulting from the accumulation of PrP^{Sc}.

However, certain examples of natural and experimental prion disease occur without accumulation of detectable protease resistant PrP^{Sc} [1-4] and the time course of neurodegeneration is not equivalent to the time course of PrP^{Sc} accumulation in mice expressing lower than normal levels of PrP^C [5]. Moreover, grafting experiments of PrP^C-expressing neuroectodermal tissue into *Prnp*^{0/0} mouse brains convincingly demonstrated that PrP^{Sc} is not toxic to cells that do not express PrP^C, even after long-term exposure [6].

While the prion resistant phenotype of *Prnp*^{0/0} mice and their inability to replicate infectivity [5, 7, 8] is in accordance with the prion hypothesis, *Prnp*^{0/0} mice have not been as definitive in resolving the physiological and possible pathological functions of PrP^C. Because the gene encoding PrP is highly conserved among species and is ubiquitously expressed, it was anticipated that *Prnp*^{0/0} mice would display overt phenotypic deficits that would provide information about essential physiological functions of PrP^C. In contrast to expectations, initial studies of *Prnp*^{0/0} mice produced in Zurich and Edinburgh

indicated that they developed and behaved normally [9, 10]. While *Prnp*^{0/0} mice subsequently produced in Nagasaki developed a progressive ataxia and Purkinje cell degeneration at ~70 weeks of age [11], these defects were found to be the result of inappropriate and neurotoxic expression of the PrP-like protein, Doppel (Dpl), in the central nervous system (CNS) rather than loss of PrP function [12, 13].

While subtle phenotypic defects in the Zurich and Edinburgh *Prnp*^{0/0} knockout mouse strains suggested roles for PrP^C in maintaining normal circadian rhythms [14], superoxide dismutase activity and protection from oxidative stress [15] and copper metabolism [16], the lack of overt phenotypic deficits raised the possibility that adaptive developmental changes might compensate for loss of PrP^C function in *Prnp*^{0/0} mice. Arguing against this possibility, Tg mice in which expression of PrP^C was suppressed in adult mice using a tetracycline-responsive trans-activator [17], or mice in which neuronal PrP was post-natally ablated [18], remained free of any abnormal phenotype.

Other lines of evidence contend that PrP^C provides cellular protective functions, raising the possibility that their disruption features in prion pathogenesis. Hippocampal cells from *Prnp*^{0/0} mice were more sensitive than cells derived from wild-type mice to serum withdrawal-induced apoptosis, an effect rescued by PrP^C or Bcl-2 [19], and cerebellar cells from *Prnp*^{0/0} mice were more sensitive to oxidative stress [20]. Also, overexpression of PrP^C rescued

Bax-induced cell death in primary human neurons [21]. A growing body of evidence suggests that cell surface-anchored PrP^C may provide a neuroprotective signaling function [22, 23] raising the possibility that impairment of this function is a feature of prion pathogenesis. Consistent with this notion, Tg mice expressing non-membrane tethered PrP, while they replicate prions and accumulate PrP^{Sc}, do not develop clinical prion disease [24].

A neuroprotective role for PrP^C raises the possibility that loss of normal PrP^C function in the brains of patients expressing mutant *PRNP* alleles is a component of the pathology of inherited prion disorders [25]. GSS is one example of an autosomal dominant inherited prion disease in humans. A common form of GSS is linked to a missense mutation resulting in replacement of proline to leucine at codon 102, referred to as P102L. The clinical and neuropathological features of GSS have been reproduced in Tg mice overexpressing a mouse PrP gene with the corresponding mutation at codon 101 (P101L), referred to as Tg(GSS) mice [4, 26, 27].

Here we show that Tg mice expressing null or missense mutant *Prnp* alleles exhibit an age-dependent motor behavior deficit suggesting a function for PrP^C in maintaining sensorimotor coordination. We also report that the brains of *Prnp*^{0/0} mice exhibit region-specific spongiform degeneration and reactive astrocytic gliosis which are neuropathological hallmarks of prion diseases. Impairment of sensorimotor function in Tg(GSS) mice prior to the onset of end-

stage neurodegenerative disease suggests that loss of PrP^C function is an early feature of prion pathogenesis.

Materials and Methods

Transgenic mice The Zurich strain of *Prnp*^{0/0} mice, which were originally maintained on a mixed 129/Sv and C57BL/6 genetic background, were subsequently repeatedly crossed with wild-type FVB animals to produce *FVB/Prnp*^{0/0} mice [28]. Breeding pairs were obtained from Stanley Prusiner by way of George Carlson at the McLaughlin Research Institute, Great Falls, Montana, and an inbred colony was established and maintained at the University of Kentucky. The production and characterization of Tg(GSS)22 mice, which were originally produced on a *FVB/Prnp*^{0/0} background, has been described previously [4]. The Tg(GSS)22 line, which is homozygous for the MoPrP-P101L transgene, was also maintained as an inbred colony.

Determination of spontaneous prion disease Groups of mice were monitored thrice weekly for the development of prion disease. The date of disease was recorded as the age at onset of progressive clinical symptoms of prion disease including truncal ataxia, hind-limb paresis, loss of extensor reflex and tail stiffening.

Histopathologic analysis Animals whose death was obviously imminent were euthanized and their brains taken for histopathological studies. Brains were

dissected rapidly after sacrifice of the animal and immersion fixed in 10% buffered formalin. Tissues were embedded in paraffin and 10 μm thick coronal microtome sections were mounted onto positively charged glass slides and stained with hematoxylin and eosin for evaluation of spongiform degeneration. Following inactivation of endogenous peroxidases by incubation in 3% H_2O_2 in methanol, peroxidase immunohistochemistry was used to evaluate the extent of reactive astrocytic gliosis using antibodies to glial fibrillary acidic protein. Detection was with Vectastain ABC reagents and slides were developed with diaminobenzidine.

Semi-Automated Lesion Profiling Paraffin-embedded mouse brains were sectioned coronally to areas corresponding to the five levels of the brain that contained the 16 mouse brain regions of interest. Brain sections were stained with hematoxylin and eosin. Images of the each of the 16 brain regions were captured using a Photometrics Cool Snap digital camera and a Nikon Eclipse E600 microscope. Each image was processed using Metamorph (Universal Imaging Corporation) version 6.2r6 software. The thresholding feature of this program was used to differentiate vacuoles from irrelevant background objects. Once a threshold was reached that maximized detection of true vacuoles, the software-processed vacuoles were measured using Metamorph's auto-trace region tool which automatically delineates well-defined vacuole edges, records the area of each vacuole and integrates the number of vacuoles per field. Lesion density, the number of vacuoles per field, was plotted on the y-axis with the

corresponding brain region on the x-axis using Prism GraphPad Prism 4 Software. Error bars represent the average number of vacuoles per field \pm the standard error of the mean (S.E.M.) in the brains of three age-matched mice per genotype.

Behavioral Testing Rotarod testing was performed on groups of eight age-matched Tg(GSS)²², wild type FVB, FVB/*Prnp*^{0/0} mice using a Dual Species Economex Rotarod model #0207-003M (Columbus Instruments) Testing was initiated when mice were 60 days of age. Mice were tested within a conserved ~3 hour time period in the morning. Mice were trained for 3 consecutive days prior to the first official testing date by repeatedly placing the mice on the rod until they were able to remain on a rod rotating at a constant speed of 5 r.p.m./s. for 90 sec. In both training and official testing, during the first trial of the day, mice were placed on the stationary rod and allowed to become familiar with the environment for 1 minute before the rod motor was engaged. On each testing day, each mouse was tested for three trials with a 10 minute inter-trial resting period. For each consecutive trail, mice were allowed to become acquainted with the environment for 30 seconds. For testing, the rod motor was initially set at 5 r.p.m. with an acceleration rate of 0.3 r.p.m/sec for a maximum trial period of 180 seconds and performance was measured as latency to fall in seconds.

Results

Sensorimotor deficits are associated with loss of PrP function To evaluate whether loss of PrP function affected coordination and balance in *Prnp*^{0/0} mice we assessed performance on the Rotarod, a well-characterized behavioral task designed to assess motor function by requiring mice to ambulate on an accelerating rotating rod, with latency to fall as a measure of motor function. While there were no significant differences between the performances of young wild type FVB mice and *Prnp*^{0/0} mice that, apart from the *Prnp* null mutation, were otherwise syngeneic with FVB mice, after 95 d of age *Prnp*^{0/0} mice remained on the accelerating rod for an average of ~50% less time than wild type FVB mice over all testing dates between ~3 and 8 months of age. Differences in performance between *Prnp*^{0/0} and wild type FVB mice during this time were highly significant (Fig. 1).

Neuronal vacuolar degeneration in *Prnp*^{0/0}/FVB mice Microscopic examination of the CNS of patients or animals with prion disease typically reveals a characteristic triad of histopathological changes consisting of neuronal vacuolation and degeneration, which confers a microvacuolated or 'spongiform' appearance, and a reactive proliferation of glial cells. To assess whether the sensorimotor deficits we detected in older *Prnp*^{0/0} mice correlated with the presence of neuropathological lesions normally associated with prion disease, we assessed the histopathological profiles of multiple brain sections from *Prnp*^{0/0} mice and age-matched wild type FVB mice. To analyze vacuolar degeneration,

we developed a semi-automated modification of the lesion profiling system developed by Fraser and Dickinson [29, 30] and quantified neuronal vacuolation in 16 brain regions (Fig. 2A). Surprisingly, significant and reproducible spongiform pathology was present in the brains of *Prnp*^{0/0}/FVB mice at 6 months of age (Figs. 2 and 4). Vacuolar lesion profiling showed that the corpus colosum at the level of the hippocampus (region 7), the medial globus pallidus (region 10), the cerebellum (region 14), and the inferior cerebellar peduncle and spinal trigeminal tract (region 16) were particularly affected regions in *Prnp*^{0/0} mice (Fig. 2B). Representative examples of spongiform degeneration and coincident reactive astrocytic gliosis in the brains of *Prnp*^{0/0} mice at the level of the medial globus pallidus and cerebellum are shown in Fig. 3.

Sensorimotor deficits precede the onset of spontaneous

neurodegeneration in Tg(GSS) mice The neuromotor deficits and prion disease-related neuropathology in aged mice expressing a null *Prnp* allele suggests that loss of normal PrP^C function is an important pathological component of prion disease and raises the possibility that progressive loss of PrP^C function contributes to the pathogenesis of inherited prion diseases. To investigate whether motor behavioral impairments also occurred in mice expressing a missense *Prnp* allele, we evaluated the performances of Tg(GSS) mice of different ages and age-matched wild type FVB mice on the accelerating rotarod (Fig. 1). As reported previously [4], all Tg(GSS)22 mice overexpressing mutant mouse PrP-P101L, spontaneously developed a progressive neurological

disorder with a mean age of onset of 163 ± 8 d (\pm standard error of the mean, $n = 7$) (Fig. 3A). However, sensorimotor deficits manifested considerably earlier than the onset of these neurological symptoms. Significant impairments of motor coordination and balance in Tg(GSS)22 mice were detected as early as 60 d of age, which was 70 d earlier than the time at which the first Tg(GSS)22 mice spontaneously developed neurological signs, and >100 d earlier than the mean age of spontaneous disease in Tg(GSS) mice (Fig. 3A). The performance of Tg(GSS)22 mice on the rotarod progressively deteriorated. After 95 d of age, extreme deficits in rotarod performance were consistently observed in Tg(GSS)22 mice compared to wild type FVB mice until the end stage of disease, at which point the ataxic phenotype made it impossible for Tg(GSS)22 mice to remain on the rod. As a result of the sensorimotor deficits in *Prnp*^{0/0} mice, the differences in performance between Tg(GSS)22 and *Prnp*^{0/0} mice did not become significant until mice were 123 d of age which was only one week before the first Tg(GSS)22 mice spontaneously developed neurologic signs, and 40 d earlier than the mean age of spontaneous disease.

Kinetics of vacuolar degeneration parallel PrP^{Sc} accumulation in the brains of Tg(GSS) mice Previous studies using the PrP^{Sc}-specific monoclonal antibody (Mab) 15B3, showed that, while the levels of total MoPrP-P101L in the brains of Tg(GSS)22 mice remained constant as mice aged, substantial quantities of the pathological form of MoPrP-P101L accumulated immediately prior to the onset of clinical symptoms [4]. We were therefore interested to ascertain whether the

development of neuropathological lesions in the brains of aging Tg(GSS)22 mice followed similar kinetics.

Coincident with the accumulation of mutant MoPrP^{Sc}-P101L in the brains of sick Tg(GSS)22 mice, the appearance of severe spongiform degeneration and accompanying reactive astrocytic gliosis was abrupt and did not appear until the end stage of disease when Tg(GSS)22 mice displayed an overt clinical phenotype. The most profoundly affected brain regions, with more than 150 vacuoles occupying each observation field, were the primary motor cortex (region 3), striatum (region 5), the posterior mid-line of the cerebral cortex (region 6), and the corpus collosum at the level of the hippocampus (region 7) (Fig. 2 and Fig. 4 E and F). While vacuolation in the inferior cerebellar peduncle and spinal trigeminal tract (region 16) was relatively modest, lesions appeared earlier in this region and the degeneration was progressive.

Discussion

Initial behavioral tests of the Zurich *Prnp*^{0/0} mice, including swimming navigation, Y-Maze discrimination and the two-way avoidance shuttle box test, indicated that elimination of PrP expression did not compromise learning ability. Subsequent studies of learning and memory and associated hippocampal function using *Prnp*^{0/0} knockout mice have provided variable results [31-34].

Electrophysiological studies suggesting that GABA_A receptor-mediated fast inhibition and long-term potentiation were impaired in hippocampal slices from

Zurich *Prnp*^{0/0} mice [35, 36] were not independently confirmed [28, 37]. While progressive cognitive dysfunction is a feature of prion diseases in humans, physical problems such as ataxia, and changes in gait and posture are also typical characteristics. Therefore, in our behavioral assessments we used rotarod performance which requires postural adjustments for the maintenance of equilibrium on a rotating and accelerating rod. Rotarod performance is a commonly used task to assess motor coordination in genetically and pharmacologically modified mouse models and, while the ability to perform the rotarod task cannot be attributed to a single brain region, it has been shown to be a sensitive indicator of cerebellar abnormalities, for example in Lurcher mice [38]. While retinal degeneration in FVB mice caused by *Pde6b* gene mutations preclude tests of cognitive behavior that depend on vision, sight-impaired inbred mouse strains, including FVB, perform on the accelerating rotarod as well as strains that are not visually impaired, and FVB mice have been extensively used in rotarod studies [39, 40].

Cerebellar ataxia is an early symptom of GSS, preceding the onset of progressive dementia, and in some cases the clinical phase of disease may be as long as 10 years. The occurrence of sensorimotor deficits in Tg(GSS) mice similar to those observed in *Prnp*^{0/0} mice but considerably earlier than the onset of end-stage neurodegeneration, is consistent with a mechanism involving progressive loss of mutant PrP^C function. In patients with inherited prion diseases such as GSS, loss of PrP function is presumably exacerbated at later

times by the dominant-negative effects of mutant PrP^{Sc}. Indeed, our previous studies demonstrated that MoPrP^{Sc}-P101L accelerates prion disease in Tg(GSS) mice and that its accumulation is a late event in the course of disease [4]. Our studies suggest that the early behavioral abnormalities in Tg(GSS) mice serve as a preclinical predictor of prion disease that may be suitable for use in preclinical drug testing. Such approaches have been successfully applied using Tg models of Huntington's disease (HD) [41, 42] which exhibit behavioral anomalies as early as 3 to 4 weeks of age.

Our results raise the question of why the neuropathological deficits reported here were not previously detected in *Prnp*^{0/0} mice. Seminal studies indicated coarse vacuolation in the Ammons horn of the hippocampus in about one third of Zurich *Prnp*^{0/0} or wild type mice analyzed [9]. Interestingly, histopathological analysis of older Zurich *Prnp*^{0/0} mice at 33 or 57 weeks post-inoculation with the Chandler prion isolate lacked vacuolation or gliosis in the molecular and pyramidal layers of the telencephalic cortex or the ventromedial thalamic nucleus [7]. In other studies, scrapie-specific neuropathology was not detected in the brains of *Prnp*^{0/0} mice ~500 days post-inoculation with mouse adapted RML scrapie prions [43].

While the original Zurich strain of *Prnp*^{0/0} mice were maintained on a mixed 129/Sv and C57BL/6 genetic background, the *Prnp*^{0/0} mice used in these studies were derived by repeated crossing with wild-type FVB animals [28]. The

resulting *Prnp*^{0/0} mice were used for the generation of Tg mice because of the large pronuclei and breeding performance of the FVB strain. In the current studies we compared mice expressing wild type and mutant *Prnp* alleles on syngeneic FVB/NJ (FVB) backgrounds. Our results raise the possibility that additional genetic factors may influence the development of spongiform degeneration; it may therefore be of considerable interest to assess the effect of the *Prnp* disruption in a variety different of inbred backgrounds. Interestingly, deficiencies in other genes, notably *Mahogunin* in *Attractin* (formerly *mahogany*) result in similar age-dependent spongiform neurodegeneration but without accumulation of PrP^{Sc} [44, 45]. Both *mahoganoid* and *Atrn* mutations affect coat color similar to *Agouti*. To explain the phenotypic overlap in *Mahogunin* and *Attractin* deficiencies, He and colleagues proposed a model in which neuronal accumulation of an unknown substrate(s) which is normally targeted for proteosomal degradation resulting from the E3 ubiquitin ligase activity of Mahogunin, results in progressive vacuolation, astrogliosis, and neuronal cell death [45]. While PrP is not ubiquitinated by Mahogunin, our results raise the intriguing possibility that PrP, *Atrn* and Mahogunin function in the same pathway to preserve neuronal viability.

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Figure Legends

Fig. 1 Longitudinal analysis of motor and balance coordination of wild type FVB and *Prnp*^{0/0} mice on the rotarod. Groups of 8 age-matched wild type FVB mice (filled squares) and *Prnp*^{0/0} mice (open squares) were subjected to rotarod behaviour analysis as described. Each mouse was tested three independent times on each test date. The mean latency to falling \pm SEM was recorded for mice in each group on each date. Significant differences (p value < 0.05) between the mean performances of mice of different genotypes at each time point was established using an unpaired, two-tailed t-test with asterisks indicating the degree of significance between genotypes.

Fig. 2 Histopathological profiles of *Prnp*^{0/0} and wild type FVB mouse brains Using a semi-automated modification of the lesion profiling system we quantified neuronal vacuolation in 16 different brain regions shown in **A**. Images are adapted (with permission) from [46]. Regions are as follows: 1: forceps minor corpus collosum; 2: anterior midline cerebral cortex; 3: primary motor cortex; 4: para terminal body; 5: striatum; 6: posterior midline cerebral cortex; 7: corpus collosum at the level of the hippocampus; 8: hippocampus; 9: thalamus; 10: medial globus pallidus; 11: hypothalamus; 12: tectum (midbrain); 13: midbrain; 14: cerebellum; 15: dorsal medulla; 16: inferior cerebellar peduncle and trigeminal tract. **B** The mean (\pm SEM) number of vacuoles per field in each of 16 regions of the brains of three wild type FVB mice (solid bars) or three age-matched (at 6 months) *Prnp*^{0/0} mice (open bars) are shown. Significant

differences (p value < 0.05) between the mean vacuolation score in each brain region was established using an unpaired, two-tailed t-test with asterisks indicating the degree of significance between genotypes.

Fig. 3 Spongiform degeneration and astrocytic gliosis in the brains of *Prnp*^{0/0} mice The extent of spongiform degeneration in the brains of *Prnp*^{0/0} and age-matched (6 months) wild type FVB mice was determined in hematoxylin and eosin stained brain sections, while peroxidase immunohistochemistry was used to evaluate the extent of reactive astrocytic gliosis using antibodies to glial fibrillary acidic protein (GFAP). **A - D** Hematoxylin and eosin staining, **E - H** GFAP immunohistochemistry of brain sections through the medial globus pallidus (region 10) and cerebellum (region 14) of *Prnp*^{0/0} and wild type FVB mice. A and E: medial globus pallidus of *Prnp*^{0/0} mice; B and F: medial globus pallidus of wild type FVB mice; C and G: cerebellum of *Prnp*^{0/0} mice; D and H: cerebellum of wild type FVB mice. Bar = 100 microns.

Fig. 4 Sensorimotor deficits precede the spontaneous onset of neurodegenerative disease in Tg(GSS)22 mice. **A** Groups of 8 age-matched wild type FVB mice and Tg(GSS)22 mice were subjected to rotarod behaviour analysis as described. Each mouse was tested three independent times on each test date. The mean latency to falling \pm SEM was recorded for mice in each group on each date. Filled squares signify the performance of FVB mice, while open circles signify the performance of Tg(GSS)22 mice. Significant differences

($P < 0.05$) between the mean performances of mice of different genotypes at each time point was established using an unpaired t test with asterisks indicating the degree of significance between genotypes. **B** Spontaneous neurodegenerative disease in Tg(GSS)22 mice. Open circles signify Tg(GSS)22 mice; filled squares signify the performance of wild type FVB mice, none of which ($n=8$) developed disease in the same time frame; and, in accordance with previous results [27], spontaneous disease did not occur in Tg(MoPrP)4112 mice overexpressing wild type mouse PrP (open squares) up to 590 d of age ($n=5$ mice).

Fig. 5 Kinetics and regional distribution of spongiform degeneration in the brains of Tg(GSS)22 mice The extent of spongiform degeneration in the brains of spontaneously sick Tg(GSS)22 mice was determined in hematoxylin and eosin stained brain sections. For comparison, regions are shown where little or no spongiform pathology (**A**: cerebellum – region 14), or consistently high amounts of spongiform pathology (**B**: posterior midline cerebral cortex) was detected. In **C**, peroxidase immunohistochemistry was used to evaluate the extent of reactive astrocytic gliosis in the posterior midline cerebral cortex region using antibodies to glial fibrillary acidic protein. **D** Neuronal vacuolation was quantified in 16 different brain regions of Tg(GSS)22 mice of different ages. Blue symbols: 6 months of age; black symbols: 5 months of age; magenta symbols: 4 months of age; green symbols: 3 months of age; red symbols: 2 months of age; orange symbols: 1 month of age.

Fig. 1

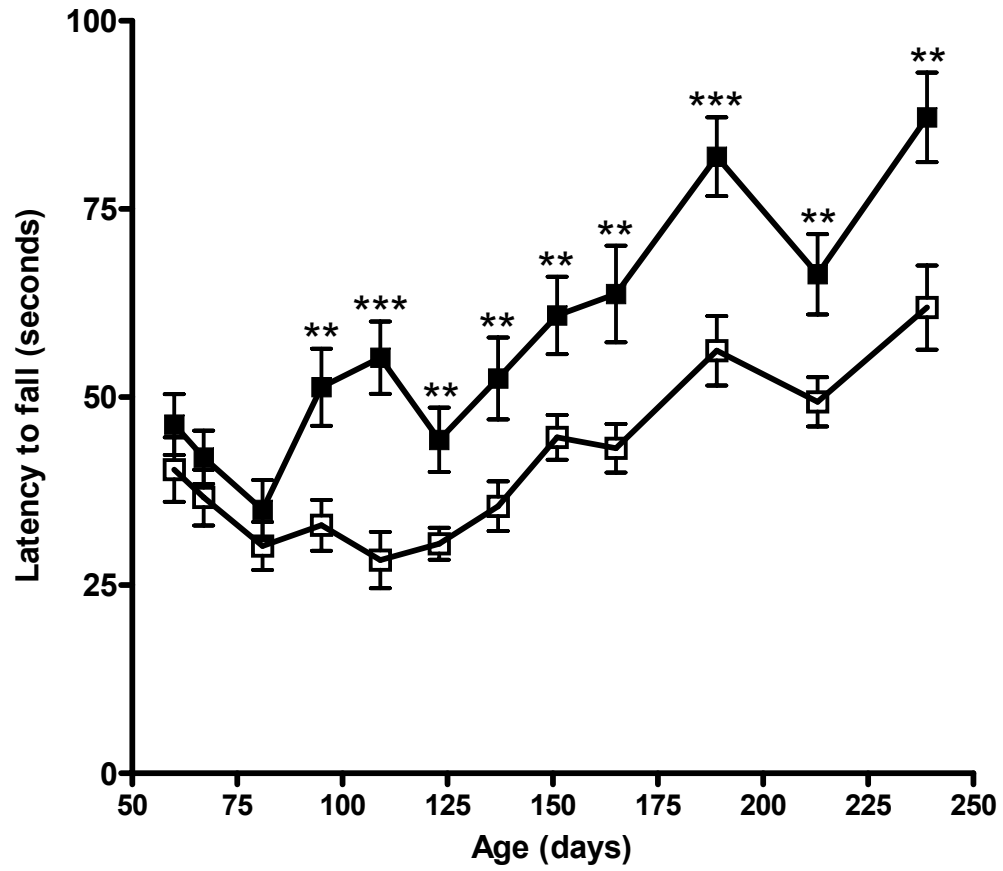
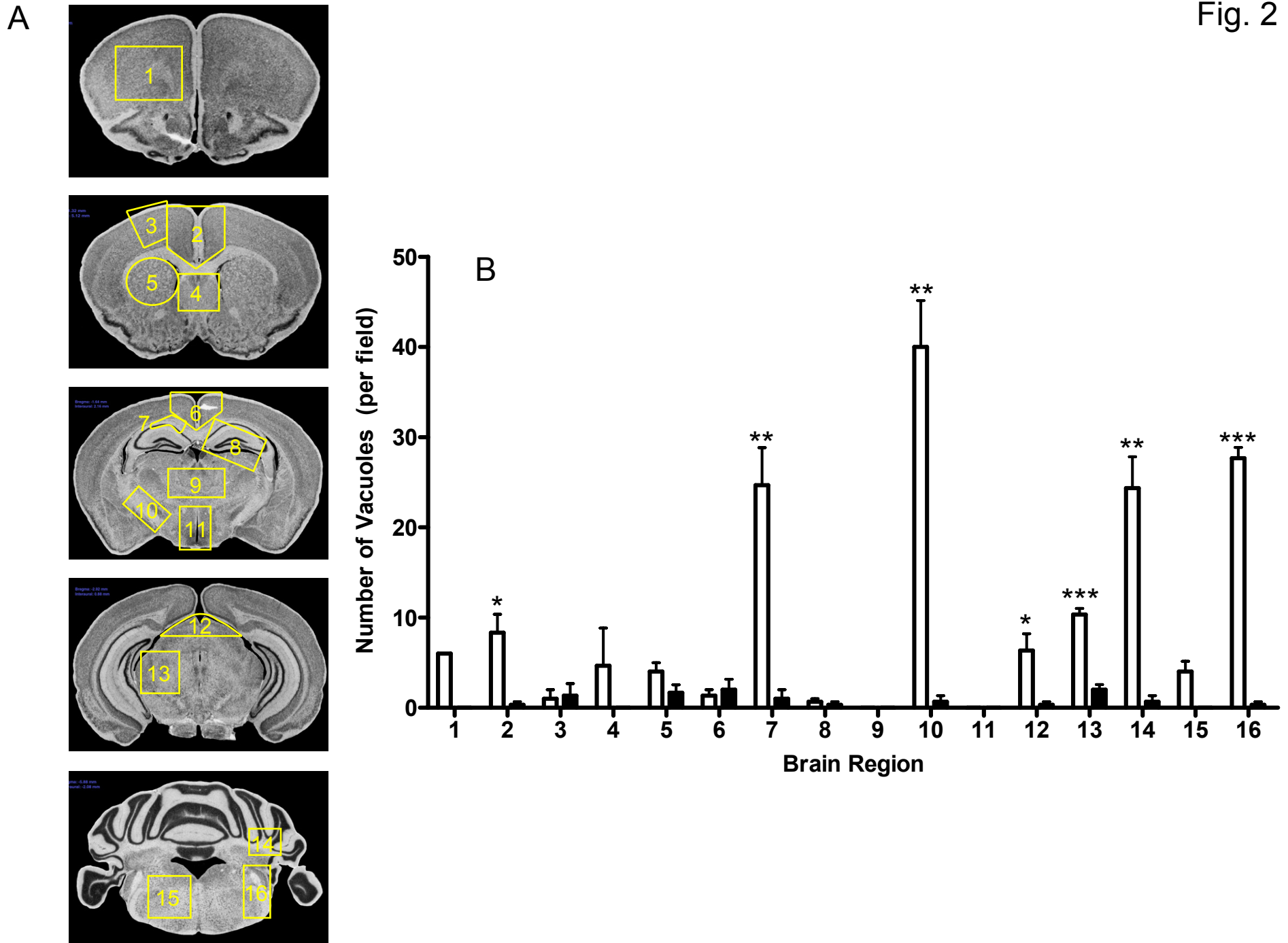


Fig. 2



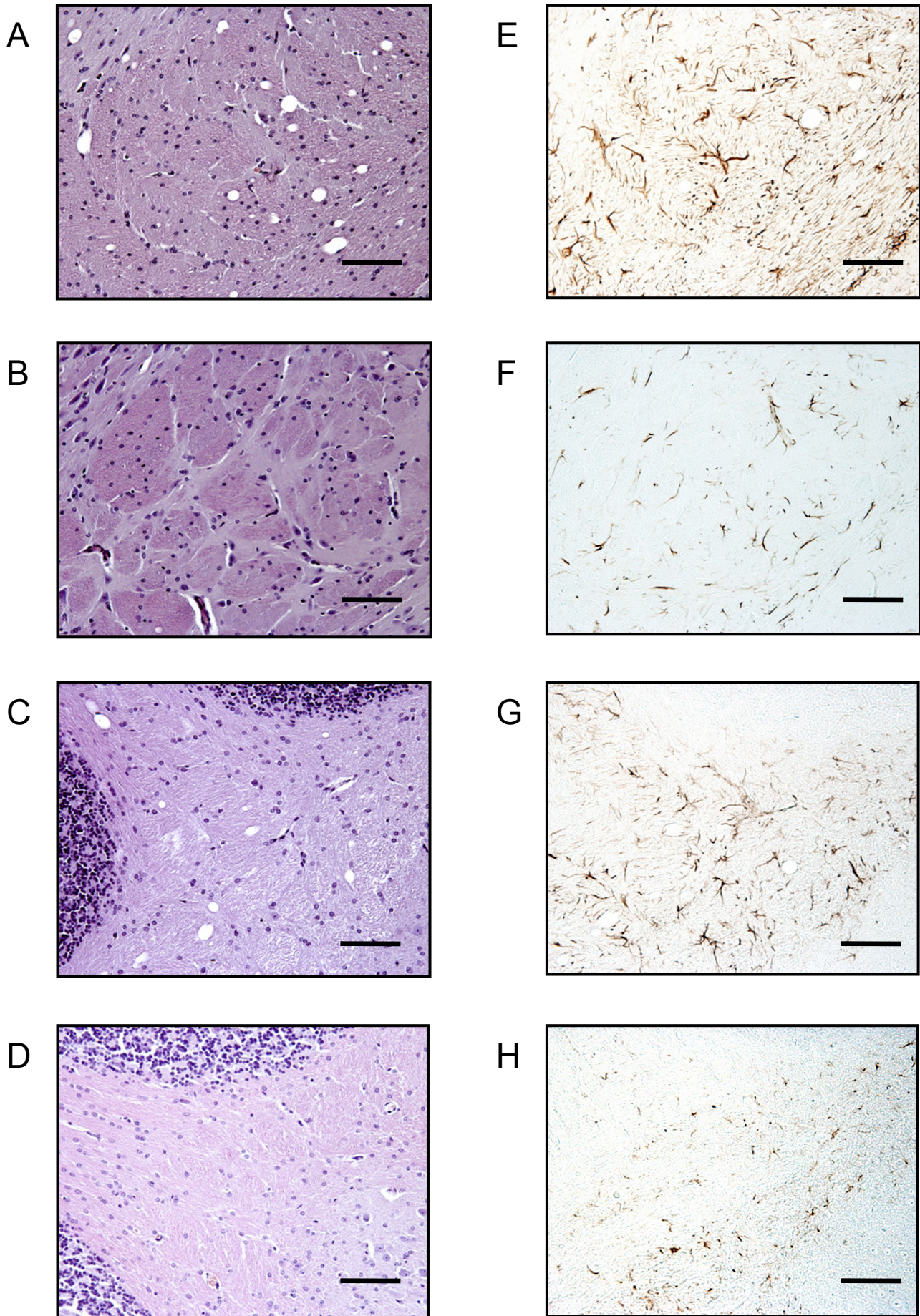


Fig. 4

