Scrapie-Induced Defects in Learning and Memory of Transgenic Mice Expressing Anchorless Prion Protein Are Associated with Alterations in the Gamma Aminobutyric Acid-Ergic Pathway\textsuperscript{V,Y,†}

Matthew J. Trifilo,1‡ Manuel Sanchez-Alavez,2‡ Laura Solforosi,3‡ Joie Bernard-Trifilo,3 Stefan Kunz,1 Dorian McGavern,1 and Michael B. A. Oldstone1,4*

Viral-Immunobiology Laboratory, Department of Immunology and Microbial Science,1 Molecular and Integrative Neurosciences Department,2 Department of Immunology,3 and Department of Infectology,4 The Scripps Research Institute, La Jolla, California 92037

Received 5 March 2008/Accepted 23 July 2008

After infection with RML murine scrapie agent, transgenic (tg) mice expressing prion protein (PrP) without its glycosylphosphatidylinositol (GPI) membrane anchor (GPI\textsuperscript{−/−}; PrP tg mice) continue to make abundant amounts of the abnormally folded disease-associated PrPres but have a normal life span. In contrast, all age-, sex-, and genetically matched mice with a GPI-anchored PrP become moribund and die due to a chronic progressive neurodegenerative disease by 160 days after RML scrapie agent infection. We report here that infected GPI\textsuperscript{−/−} PrP tg mice, although free from progressive neurodegenerative disease of the cerebellum and extrapyramidal and pyramidal systems, nevertheless suffer defects in learning and memory, long-term potentiation, and neuronal excitability. Such dysfunction increases over time and is associated with an increase in gamma aminobutyric acid (GABA) inhibition but not loss of excitatory glutamate/N-methyl-D-aspartic acid. Enhanced deposition of abnormally folded infectious PrP (PrPs\textsubscript{c} or PrPres) in the central nervous system (CNS) localizes with GABAA receptors. This occurs with minimal evidence of CNS spongiosis or apoptosis of neurons. The use of monoclonal antibodies reveals an association of PrPres with GABAA receptors. Thus, the clinical defects of learning and memory loss in vivo in GPI\textsuperscript{−/−} PrP tg mice infected with scrapie agent may likely involve the GABAergic pathway.

Transmissible spongiform encephalopathies represent the only known infectious protein folding disease (8, 29, 30). In these fatal neurodegenerative disorders of humans and animals, the normal cellular prion protein (PrP\textsubscript{c} or PrP\textsubscript{sen}), which is sensitive to protease K treatment, is misfolded from its normal cellular prion protein (PrP\textsubscript{c} or PrP\textsubscript{sen}), which is sensitive to protease K treatment, is misfolded from its predominant \(\alpha\)-helical form into a \(\beta\)-sheet-enriched structure that becomes resistant to protease K treatment (PrP\textsubscript{sc} or PrPres) (8, 29, 30). No diagnostic marker that signals the presence of infectivity in prion disease has been found. Instead, a firm diagnosis rests on identifying the characteristic neuropathologic changes, usually postmortem, in combination with immunohistochemically identified manifestations of abnormal PrPres on brain sections and on isolation from brain tissues tested by Western blotting.

In humans, the initial clinical indicators of Creutzfeldt-Jakob disease (CJD) include memory loss, behavioral changes and progressive dementia, and these signs resemble those in sheep infected in the wild with scrapie agent, in deer with chronic wasting disease, and in mice experimentally infected with scrapie agent (29). Following these early signs are marked manifestations of neurodegenerative disease, e.g., involving the cerebellum (ataxia or tremors) as well as the extrapyramidal (involuntary tremors, poverty of movement, or rigidity) and pyramidal (weakness or paralysis) systems.

The precise molecular and neuropharmacologic events underlying the early onset of these behavioral and memory dysfunctions are unclear. Further, in animal models the interval between deposition of large amounts of PrPres and clinical manifestations of neurodegeneration leading to death is often too brief for any long-term kinetic analysis of the progression of neurobehavioral breakdown or diminished learning/memory functions. The recent availability of the GPI\textsuperscript{−/−} PrP transgenic (tg) mice (9), i.e., tg mice lacking the glycosphatidylinositol (GPI) membrane anchor, provides an opportunity to evaluate behavior and memory dysfunction over a prolonged period of time while PrPres deposits continuously accumulate in the absence of clinical neurodegeneration; such mice live for 600 to 700 days. Further, the location of cellular PrP primarily to synaptic regions and nerve processes, as determined by cell fraction studies, in vitro culture experiments, in vivo immunohistochemical analysis by light and high-resolution electron microscopy, and use of green fluorescent protein-tagged PrP tg mice (1, 2, 11, 14), suggest a function for PrP in neural transmission. Since we know that a balance between the excitatory glutamate/N-methyl-D-aspartic acid (NMDA) pathway and the inhibitory gamma aminobutyric acid (GABA) pathway is a major factor in regulating synaptic transmission, identifying the function and expression of these pathways in the GPI\textsuperscript{−/−}/PrP tg model would be of interest.

Attempts to characterize learning and memory in PrP knockout mice have yielded variable results (27, 31, 36). However, the recent use of PrP knockout mice with backgrounds that were genetically equivalent indicated that their defect in...
cognitive learning was corrected by reconstitution of the PrP gene in neurons but not astrocytes; for this work, cell-specific promoters and genetic engineering were applied (13, 32). Knockout of the PrP gene in brains of adult mice resulted in minimal neurodegeneration, suggesting that responses critical to neuronal function are not lost in this situation (24, 36). Further, administering antibodies to PrPsen focally into the central nervous systems (CNS) of mice not infected with scrapie agent caused neuronal injury, suggesting that aggregation of PrPsen on the cellular membrane (presumably during disease caused by PrPres) signals a neuronal pathway leading to injury (34).

The sum of these observations combined with our findings that scrapie agent-infected (scrapie-infected) tg mice in which anchored PrP is removed from the neuronal membrane still form the folded PrP that incites disease and the manufacturing of infectious material but not overt clinical disease (9, 35) suggests the following outcome: PrP via an excitatory or inhibitory signaling pathway on the neuronal membrane could promote normal PrP function, whereas overstimulation of PrP caused by aggregation with PrPres presumably induces disease.

For this report we took advantage of the ability to separate PrPres buildup from chronic progressive CNS disease in scrapie-infected GPI−/− PrP tg mice. The continuous accumulation of abnormally folded infectious PrP in the CNS throughout the lifetime span of these animals in the absence of overt neurologically abnormal disease positioned us to study the effect of PrPres deposits on learning and memory as well as their influence on the excitatory and inhibitory neuronal pathways. We found that scrapie-infected GPI−/− PrP tg mice suffered marked deficits in learning and memory accompanied by severe loss in neuronal transmission as well as long-term potentiation (LTP) that increased over time. Further, in mechanistic terms, we noted that these deficits were associated with the inhibitory GABAergic pathway but not the excitatory glutamate/NMDA pathway.

MATERIALS AND METHODS

Scrapie inoculation of mice. tg mice lines 23 and 44, lacking a GPI anchor (GPI−/− PrP tg mice), were created and genotyped as described previously (9). When infected with murine scrapie, these mice developed PrPres amyloid deposits and infectious PrP material but not clinical neurodegenerative disease. C57BL/6 (wild-type) mice (obtained from the Rodent Breeding Colony at The Scripps Research Institute) were used for infectivity studies and as controls. Infection was induced by inoculating tg and wild-type mice intracerebrally at 6 weeks of age with 30 μl of a 1% suspension of brain tissue from infected with RML (Chandler) scrapie. The inoculum contained 0.7 to 1.0 × 107 50% tissue infectious doses of infectious scrapie agent.

Histopathology and immunohistochemistry. Brains were fixed in a neutral buffered 4% formaldehyde solution for 3 to 5 days, dehydrated, embedded in paraffin, and cut into 4-μm sections using a Leica microtome. Slides were deparaffinized and rehydrated in xylen and alcohol rinses, followed by immunohistochemical staining as reported previously (9, 35). Briefly, slides were autoclaved for 15 min at 201°C and 120°C for retrieval of PrPres antigen. Samples were blocked in 2% bovine serum albumin and stained either for PrPres using 3 μg/ml D13 (35) primary antibody or for GABAA receptor (GABAA R) β chain primary antibody. After an overnight incubation at 4°C or a 30-min incubation at room temperature, slides were rinsed with buffer and stained with secondary biotinylated goat anti-human or goat anti-rabbit antiserum diluted 1:200 for detection of D13 and GABAA R β chain, respectively. Streptavidin-conjugated rhodamine or horseradish peroxidase (HRP) followed by Ventana aminoethylcarbazol was used for immunofluorescence or light microscopy, respectively. Amyloid staining employed 1% wt/vol of Thioflavin S (MP Biomedicals, Costa Mesa, CA) in 50% ethanol. Slides were rinsed in twice in 50% ethanol and twice in water prior to mounting and coverslipping. The staining of PrPres and amyloid was observed using an Axiovert S100 (Zeiss) microscope.

Behavior: cued version of the Barnes circular maze. Here we used a modified form of the Barnes circular maze test (3, 13), in which mice learn to escape through a tunnel by recognizing a target cue that leads to the escape tunnel during sequential daily testing periods. This test differs from the standard Barnes test (3) in that the target cue is located behind the escape hole (13). Briefly, the circular maze consisted of an acrylic disk 75 cm in diameter, 0.5 cm thick, and elevated about 55 cm above the floor. Twenty holes, 5.0 cm in diameter, were placed 5.0 cm from the perimeter, and a black Plexiglas escape tunnel (7.5 by 9.0 cm) was placed under one of the holes. White noise (about 90 dB) and bright ambient light (100 W, 60 cm above the center of the acrylic disk) were used as negative stimuli. The apparatus was located in a procedure room where several visual cues (a white circle in the middle of a black square, a black diamond, and a black bar) were placed on the wall surrounding the circular maze. Prior to testing, the animals were placed in the blind escape tunnel for 4 min for habituation to the maze and then put in a cylindrical chamber in the center of the circular maze. After 10 s, the chamber was removed by using a pulley system and the mice were allowed to explore freely to find the hole with the escape tunnel. A video camera was secured at 70 cm above the platform surface, and two “blindfold” experimenters collected behavioral data for all mice tested.

The escape tunnel’s location varied randomly from session to session and a visual cue was located directly behind the hole with the escape tunnel. During the habituation period and between all test sessions, the maze and escape tunnel were cleaned with 10% alcohol to remove odor cues (13). The following items were measured in each trial: (i) the latency to start of exploration, which is the time interval (in seconds) between removal of the cylindrical chamber and the animal releases from the platform’s center; (ii) the time to find the escape tunnel, defined as the time between moving from the center of the platform and entering the escape tunnel; (iii) the number of errors, defined as searching holes without the escape tunnel; (iv) the first hole explored upon leaving the center of the platform; and (v) the search strategy, i.e., random, serial, and cued search strategies. The random search strategy involved continuous center crossings exploring incorrect holes and the edge of the platform until the mouse found the hole leading to the escape tunnel. The serial search involved a more rigid strategy in which the mouse reached the platform’s edge and searched every hole or every other hole in a clockwise or counterclockwise direction until finding the hole leading to the escape tunnel. The cued search strategy was the quickest way to escape, since the animal used cues to find the target hole. The criteria for determining the use of a cue strategy were that the first hole explored was located within two holes from the escape tunnel (cue field) and that escape followed by finding the target hole. Any animal that reached the spatial field and then explored holes that were not part of the cue field was scored as not using the cue search strategy. All animals were tested 1 h after the onset of darkness (7:00 p.m.). A trial session ended when the mouse entered the hole leading to the escape tunnel or when 300 s elapsed, whichever occurred first. A total of 42 trials (two trials per day, with each trial separated by 20 min) were performed on consecutive days.

Electrophysiological recording. For determination of auditory, visual, and brain stem evoked potentials, mice were anesthetized with isoflurane (induction, 4 to 5%; maintenance, 1.0% ± 0.2%) and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Body temperature was monitored and maintained at 37 ± 0.1°C by a feedback-regulated heating pad. A Grass clinical photostimulator model PS 22A was used to deliver a flash (100 flashes of 0.1-ms duration, 16 intensity and 1/s frequency) to generate visual evoked potentials. Mirrors were placed on the walls of the recording cage to ensure binocular stimulation. Auditory evoked potentials (AEPs) were obtained by a condensation stimulation produced by clicks with the following characteristics: for cortical AEPs, 100 clicks of 0.1-ms duration, 70-dB sound pressure level, and 1/s; for brain stem AEPs, 1,024 clicks and 10/s. Software from National Instruments (LabView, Austin, TX) was used to generate the stimuli and to collect the data. After behavioral studies and evoked potential studies, infected and uninfected GPI−/− PrP tg mice were divided randomly into two groups for electrophysiologic studies. In one group the dentate gyrus (DG) was tested, and in the second group the medial prefrontal cortex was tested.

Procedures for electrophysiological recording in the DG. In the same anesthetized preparation, population spikes, paired-pulse (PP) curves, and LTP were elicited in the granular layer of the DG as described previously (16). Briefly, the skull was exposed and holes were drilled to accommodate stereotactic placement of electrodes. The dura was opened over the recording area to facilitate positioning of a micropipette. Microelectrodes were stereotaxically oriented into the granular layer of the DG in the septal pole of the dorsal hippocampus (coordinates, 2.0 mm posterior and 1.0 mm lateral relative to bregma; 1.4 to 1.8 mm...
ventral from dura) (28). Extracellular evoked field potential activity was recorded using single 3.0 M NaCl-filled micropipettes (5 to 10 MΩ; 1 to 2 μm internal diameter). Field potentials were elicited in the DG by stimulation of the perforant path (0.4 to 1.5 mA; 0.10-ms duration; frequency, 0.05 Hz; one stimulus every 30 s) with insulated, bipolar stainless steel (130-μm) electrodes located in the angular bundle (coordinates, 3.6 mm posterior and 2.8 mm lateral relative to bregma; 2.0 to 2.5 mm ventral from dura) (28). A stimulus-response curve (threshold, half-maximum, and maximum) was determined for each electrophysiological preparation. The intensity of stimulation used to generate PP curves and LTP was set at the half-maximum level of stimulation. We used a PP stimulation protocol with interstimulus intervals ranging from 10 to 360 ms. To induce LTP, baseline activity was recorded for 10 min and then three high-frequency conditioning stimuli (HFS trains) (each consisting of five pulses at 400 Hz) were delivered and population spike amplitudes recorded for 60 min post-HFS.

Procedures for electrophysiological recording in the medial prefrontal area. The dura was opened over the recording area to facilitate positioning of the micropipette. Microelectrodes were stereotaxically oriented into the medial dorsal thalamic nucleus (0.8 mm posterior to bregma and 0.3 mm lateral to midline) and the medial prefrontal cortex (2.4 mm anterior to bregma and 0.4 mm lateral to midline) at a location generating a maximum amplitude of the prefrontal synaptic excitability.

Neurotransmitter receptor Western blotting. For detection of GABAA R, glutamate R, and NMDA R, brain tissue was placed in 2-ml polypropylene screw cap tubes (Biospec Products Inc., Batelleville, OK) filled up to 25% with sterilized glass beads containing 0.5 ml sterile 50 mM Tris-HCl (pH 7.4) with 1% Triton X-100, or 2, or 3; GABAA R or rabbit anti-GABAA R antibody D13 (recognizing amino acids [aa] 96 to 106) (5 μg/ml) to membranes and incubating overnight at 4°C. Membranes were rinsed with buffer and stained with secondary HRP-conjugated goat anti-human antisera diluted 1/20,000 for detection of PrP. Bands were detected using either the Pico or Phentom chemiluminescence detection kit.

This procedure was modified when PrPres-specific reagent was used initially for coimmunoprecipitation. Briefly, whole brains from scrapie-infected GPI−/− PrP tg mice were homogenized at 10% (wt/vol) in TBS (0.05 M Tris, 0.2 M NaCl, pH 7.4) containing 1% Triton X-100, diluted in an equal volume of TBS, and then rehomogenized and sonicated. Homogenates were clarified at 500 × g for 15 min at 4°C. A portion of clarified prion-infected homogenate was digested with proteinase K (50 μg/ml) for 1 h at 37°C. Phenylmethylsulfonyl fluoride was added to all samples to a final concentration of 2 mM. For each immunoprecipitation, human IgG 136–158 (26, 33) or b12 (7) at a final concentration of 10 μg/ml was incubated for 2 h at room temperature with an aliquot of brain homogenate containing ~1 mg of total protein in a reaction mixture adjusted to a final volume of 500 μl with assay buffer (TBS containing 1% Triton X-100). Tosyl-activated paramagnetic beads coupled to polyclonal rabbit anti-human IgG F(ab′)2 were added to the antibody-homogenate mixture and incubated overnight at 4°C. The beads were then washed four times in washing buffer (TBS plus 0.1% Tween 20) (TBS-X) and on the fifth wash, the collected beads were resuspended in 20 μl of loading buffer (150 mM Tris-HCl [pH 6.8], SDS, 0.3% bromophenol blue, 30% glycerol) and heated to 100°C for 5 min. Samples were then run on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature and blotted. PrP and GABAA R were detected, respectively, with mouse Fab D13 and rabbit anti-GABAA R α5 (Millipore) at 1 μg/ml. After five washes in TBST, blotted PrP protein was detected by incubation for 30 min at room temperature with an HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG (Pierce) diluted 1:10,000 in blocking buffer. Membranes were then washed five times in TBST and developed with enhanced chemiluminescence reagent onto film.

Immunoprecipitation. Brain tissues were placed in 2-ml polypropylene screw cap tubes (Biospec Products Inc., Burlington, CA) filled up to 25% with sterilized glass beads (1.0-mm-diameter beads; Biospec Products Inc.) containing 1 ml sterile 50 mM Tris-HCl (pH 7.4) with 1% Triton X-100, 150 mM EDTA, 150 mM NaCl, and protease inhibitor cocktail (PIC) (Roche Diagnostics, Indianapolis, IN). Tubes were placed into the Mini Beadbeater 8 (Biospec Products Inc.) and homogenized for 1 min. Subsequently, 500-μl portions of homogenates were transferred into sterile 1.5-ml Eppendorf tubes and centrifuged for 10 min at 1,000 × g to remove debris. Supernatants were incubated with 5 μg of either D13 monoclonal antibody (for PrP) or mouse immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA) as a negative control overnight at 4°C. Forty microliters of protein A-agarose (Millipore) was added for the final 2 h. Complexes were collected by centrifugation (10,000 × g for 5 min). Immune complexes were washed five times with Tris-buffered saline (TBS) containing PIC, eluted by boiling in 25 μl of reducing sample buffer, and processed for Western blot analysis. Samples were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels (20% for nitrocellulose membranes) and transferred into sterile 1.5-ml Eppendorf tubes and centrifuged for 10 min at 4°C. A portion of clarified prion-infected homogenate was digested with proteinase K (50 μg/ml) for 1 h at 37°C. The reaction was stopped by adding 0.1 M phenylmethylsulfonyl fluoride and cooling on ice for 15 min. After centrifugation at 70,000 × g for 1 h at 10°C, pellets were resuspended in 25 μl sample buffer, sonicated, and boiled for 5 min. All samples were run on SDS-polyacrylamide gels. Gels were blotted onto PVDF membranes and blocked for 1 h at room temperature in 5% powdered skim milk in sterile water. PrP was detected by adding human anti-PrP antibody D13 (recognizing amino acids [aa] 96 to 106) (5 μg/ml) to membranes and incubating overnight at 4°C. Membranes were rinsed with buffer and stained with secondary HRP-conjugated goat anti-human antisera diluted 1/20,000 for detection of PrP. Bands were detected using either the Pico or Phentom chemiluminescence detection kit.

For coimmunoprecipitation, human IgG 136–158 (26, 33) or b12 (7) at a final concentration of 10 μg/ml was incubated for 2 h at room temperature with an aliquot of brain homogenate containing ~1 mg of total protein in a reaction mixture adjusted to a final volume of 500 μl with assay buffer (TBS containing 1% Triton X-100). Tosyl-activated paramagnetic beads coupled to polyclonal rabbit anti-human IgG F(ab′)2 were added to the antibody-homogenate mixture and incubated overnight at 4°C. The beads were then washed four times in washing buffer (TBS plus 0.1% Tween 20) (TBS-X) and on the fifth wash, the collected beads were resuspended in 20 μl of loading buffer (150 mM Tris-HCl [pH 6.8], SDS, 0.3% bromophenol blue, 30% glycerol) and heated to 100°C for 5 min. Samples were then run on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature and blotted. PrP and GABAA R were detected, respectively, with mouse Fab D13 and rabbit anti-GABAA R α5 (Millipore) at 1 μg/ml. After five washes in TBST, blotted PrP protein was detected by incubation for 30 min at room temperature with an HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG (Pierce) diluted 1:10,000 in blocking buffer. Membranes were then washed five times in TBST and developed with enhanced chemiluminescence reagent onto film.
anchorless tg mouse provides a window of opportunity for analyzing the results of PrPres deposition alone (9, 35) over time. Figure 1A and B show both PrPres (red: Fig. 1A) and amyloid (green, Thioflavin S staining; Fig. 1B) in the brain of a GPI/H11002/H11002/H11002 PrP tg mouse 400 days after RML murine scrapie infection, and Fig. 1C shows the relative lack of apoptosis during the same period. At this time and over the next 200 to 300 days, these GPI/H11002/H11002/H11002 PrP tg mice were alive and had no clinical signs of the cerebellar, extrapyramidal, or pyramidal disorder typically associated with scrapie infection (100% of 46 mice tested). In contrast, all mice with an anchored PrP and inoculated with the same dose of RML scrapie had clinically severe neurodegenerative disease within 150 days and died by day 160th ± 6 after infection (mean ± one standard deviation; 24 of 24 mice).

Histopathologic analysis of brains from clinically healthy GPI/H11002/H11002/H11002 PrP tg mice infected with scrapie showed gliosis and minimal spongiosis. Immunohistochemical study using MAP-2 antibodies revealed a few focal areas of modest neuronal dendritic cell loss (Fig. 1D, right; compared to negative control left panel) throughout the entorhinal and cerebral cortex and the hippocampus. Considerable PrPres deposition also occurred in the hippocampus (Fig. 1E). CNS deposits of PrPres increased over time, as depicted in Fig. 1G, and by 500 days after scrapie infection far exceeded the amount of PrPres deposition found in scrapie-infected mice with anchored PrP that were...
sacrificed 160 days later or when moribund. When placed in an open field, the infected mice immediately began searching for cover (10 of 10 mice; day 350 postinfection) instead of initially standing still, i.e., freezing, as uninfected GPI/H11002/PrP tg mice did (Fig. 1F). The lack of a freezing behavior has been reported in sheep and in deer infected with scrapie (38), presumably reflecting a malfunction of the prefrontal cortex (25).

GPI/H11002/PrP tg mice infected with scrapie have marked deficits in learning, memory, and hippocampal electrophysiology. We then performed a series of cognitive training and electrophysiologic tests. GPI/H11002/PrP tg littermates were divided into two sex- and age-matched groups: one infected with scrapie and the other treated with vehicle. Both groups showed visual and auditory acuity. We then examined all mice 193 and 400 days after inoculation to chart their learning and behavior in a cued version of the Barnes circular maze described in Materials and Methods (3, 13) (Fig. 2A). The test objective was how well mice learned to navigate for escape by seeking a visual clue.

FIG. 2. GPI/H11002/PrP tg mice infected with scrapie have deficits in memory and hippocampal electrophysiology. (A) All 10 infected GPI/H11002/PrP tg mice (right) continued random exploration and failed to learn cued visual signals to escape. In contrast, all 10 uninfected control mice (left) used serial learning to seek the escape hole within 7 trials, and by the 12th trial all 10 mice used cued strategy to locate the escape tunnel. (B) Interstimulus interval studies show an increase in PPI for the infected GPI/H11002/PrP tg mice over that in controls. At 150 days after scrapie infection, GPI/H11002/PrP tg mice (one representative sample of five mice studied) show inhibition in the PP extending up to 60 ms (arrows), and by 350 days that inhibition lasted for 80 ms (arrows) with a total absence of PPF (up-and-down arrows) extended from baseline (dashed line). A recording of hippocampal DG neurons after perforant path stimulation is shown. (C) Absence of LTP in the infected GPI/H11002/PrP tg mice at 350 days after scrapie infection (bottom) despite its presence at 150 days after infection (top). (D) Increased excitability induced by bicuculline given in the lateral ventriciles of uninfected GPI/H11002/PrP tg mice but a negligible response in the GPI/H11002/PrP infected tg mice so treated (see arrows with bicuculline-treated mice). HFS of the perforant path elicited multiple spikes followed by long-duration depolarization (~140 ms) in all GPI/H11002/PrP uninfected controls. *, P < 0.001. GPI/H11002/PrP tg mice infected with scrapie displayed enhanced population spiking followed by a short duration of depolarization (~20 ms).
Noise (90 dB) and/or bright ambient light (100 W) was used as a stimulus. Visual clues located directly behind a hole leading to the escape tunnel were varied randomly from session to session. We then assessed the latency time to begin exploration and to find the escape tunnel, the number of errors made while seeking the escape tunnel, and the search strategy used. Strategies scored were classified as random to serial (strategy of searching every hole in a clockwise or counterclockwise manner) or cued (locating a visual cue for direct access to the escape tunnel) (Fig. 3). As cartooned in Fig. 2A (right) and with data provided in Fig. 3, after over 42 trials consisting of two trials per day with each trial separated by 20 min performed on consecutive days, all 10 scrapie-infected GPI−/− PrP tg mice continued random exploration and failed to learn the cued visual signals. In contrast, all 10 uninfected GPI−/− PrP tg mice successfully used serial learning to seek escape after only 7 trials, and within 12 trials all used the cued strategy to locate the escape tunnel (Fig. 2A [left] and 3). Thus, in addition to loss of a freezing-like response (Fig. 1F), GPI−/− PrP tg mice infected with scrapie, despite the absence of overt signs of neurodegenerative disease, displayed profound deficits in cognitive learning by 400 days postinoculation although not at 193 days postinfection.

Next we measured the electrophysiologic transmission of evoked stimuli from the entorhinal cortex to the DG (Fig. 2B and C) and from the mediodorsal nucleus of the thalamus to the medial prefrontal cortex (see Fig. S1 in the supplemental material). From microelectrodes stereotaxically oriented into the granular layer of the DG, population spikes were recorded as negative potential superimposed on the positive-going field excitatory postsynaptic potential (EPSP)/inhibitory postsynaptic potential (IPSP) complex. The waveform consisted of relatively fast negative-going population spikes superimposed on the field EPSP/IPSP complex. An input/output curve was determined, and the intensity of stimulation used to generate PP response and LTP was set at the half-maximum level. PP curves were generated by testing various interstimulus intervals (0.010 to 1.0 s) of orthodromic paired stimuli. Representative samples are shown in Fig. 2B. At 150 days postinfection, uninfected GPI−/− PrP tg controls displayed a period of PP inhibition (PPI) during the initial 20 and 40 ms and a period of PP facilitation (PPF) from 60- to 80-ms interstimulus intervals, which in 80% of the mice extended up to 180 ms. In contrast, whereas the infected GPI−/− PrP tg mice displayed similar inhibition at 20 and 40 ms, inhibition extended up to 60 ms (n = 6, where the population spike amplitude was 24.3 ± 21.1 for scrapie-infected mice and 101.4 ± 12.39 for control mice [P = 0.008]).

When other matched groups of GPI−/− PrP tg mice infected with scrapie or not were tested at 350 days postinfection, the PPI increased dramatically in the infected mice. The uninfected GPI−/− PrP tg controls displayed PPI during the initial 20 and 40 ms and PPF at 60-, 80-, 180-, and 360-ms interstimulus intervals. In contrast, their infected counterparts showed an extended inhibition at 60 ms (n = 5, where the population spike amplitude was 16.8 ± 15.8 for scrapie-infected mice and 121.3 ± 18.6 for control mice [P = 0.002]) and 80 ms (41.4 ± 17.89 for scrapie-infected mice and 139.4 ± 12.14 for control mice [P = 0.001]). In addition PPF was absent at 180 ms (89.34 ± 7.6 for scrapie-infected mice and 117.4 ± 4.89 for control mice [P = 0.01]) and 360 ms (99.35 ± 10.2 for scrapie-infected mice and 101.5 ± 17.4 for control mice [P = 0.89]). At 150 days after scrapie infection, GPI−/− PrP tg mice had inhibited the PP response extending up to 60 ms (Fig. 2B). By 350 days, that inhibition lengthened to 80 ms, with an absence of PPF extended from the baseline to the peak amplitude of the population spike (Fig. 2B). Thus, defects were characterized by extended inhibition at longer intervals in scrapie-infected GPI−/− PrP tg mice in the absence of facilitation. Failure to achieve adequate levels of potentiation in the medial prefrontal cortex after extinction was reported to be associated with a deterioration of the fear response (18). Our corresponding findings for GPI−/− PrP tg mice infected with scrapie highlighted defects in synaptic efficacy in the medial prefrontal cortex possibly causing the loss of the freezing response (Fig. 1F).

The hippocampus is a region of the brain associated with learning and memory (20, 23). LTP that strengthens synaptic efficacy in the hippocampus has been considered a correlate of memory formation (19, 23). Owing to our finding of defective cognitive learning in scrapie-infected GPI−/− PrP tg mice (Fig. 2A) and heavy deposits of PrP in the hippocampus (Fig. 1E), we compared, using published techniques (13), LTP in the hippocampi of GPI−/− PrP tg mice infected with scrapie or not at 150 and 350 days postinfection following HFS (three trains consisting of five pulses at 400 Hz) in the perforant path. As recorded in Fig. 2C, at 150 days after scrapie inoculation and after HFS, infected mice had somewhat enhanced population spike amplitude (52% ± 3% from baseline) in the DG but less than that in uninfected controls (n = 6 per group; each dot represents a response elicited every 30 s) (P > 0.05). However, the differences in these values were dramatic at 350 days postinfection, when the infected mice had no further increase in change of population spike amplitude after HFS, whereas the control group underwent the expected increase of 1.5- to 2-fold (n = 5 per group; P < 0.0001). These results were confirmed in an additional experiment and duplicated when outcomes of stimulation from the medial dorsal nucleus of the thalamus were recorded in the mid-prefrontal cortex (see Fig. S1 in the supplemental material).

Abnormalities of synaptic transmission could result from deficits of excitatory pathways (glutamatergic/NMDA) or increased inhibitory inputs of the GABAergic pathway (4, 5, 12, 37). To evaluate the contribution of inhibitory regulation, we administered bicuculline methiodide, a competitive antagonist of the GABA receptor (5), directly into the lateral ventricles of GPI−/− PrP tg mice with scrapie infection for 350 days. Population spikes and EPSP/IPSP from the DGs of these mice were then compared to those of uninfected matched controls. Extracellular electrophysiological analyses revealed a greater susceptibility to bicuculline at increasing concentrations (5, 10, 20, and 50 ng/1 μL of artificial spinal fluid vehicle) in uninfected mice than in scrapie-infected mice. As shown in Fig. 2D, bicuculline given at 5 ng/μL induced a dramatic difference in excitability. Under this condition, a perforant path stimulation-induced enhancement of population spiking had a differential effect on the short-latency response, as well as long-latency, long-duration discharge (depolarization) in the uninfected controls, but little or no such increase in the GPI−/− PrP tg infected mice (Fig. 2D). HFS of the perforant path elicited multiple spikes followed by long-duration depolarization.
Under the same conditions, GPI PrP-infected mice had enhanced population spiking alone followed by short-duration depolarization (20 ms; \( P < 0.001 \)). Thus, the excitation-inhibition balance was significantly disrupted in the GPI PrP-infected mice at 350 days after scrapie infection. Further, 100% of uninfected controls (5/5) had tonic-clonic seizures that correlated with electrical depolarization at a higher dosage of 10
ng/μL. In contrast, infusion of 25 ng/μL of bicuculline into scrapie-infected GPI−/− PrP tg mice did not cause tonic-clonic seizures in four of five mice (80%). It is likely that the absence of 1 ng latency depolarization potential found in scrapie-infected GPI−/− PrP tg mice treated with bicuculline indicated a persistent inhibition that blocked excitatory feedback onto the granule cells.

**Association of PrP with the GABAergic pathway.** Lastly, the association between GABAA Rs and PrPsen or PrPres was explored by using coimmunoprecipitation and Western blot analysis as well as dual immunohistochemical staining with monoclonal antibodies to NMDA, glutamate, and GABAA Rs and PrP to compare responses from uninfected and scrapie-infected GPI−/− PrP tg mice. Uninfected tg mice inoculated intracerebrally with phosphate-buffered saline (PBS) and sacrificed at various times later failed to show either PrPres or any upregulation of NMDA, glutamate, or GABAA Rs compared to uninfected GPI−/− PrP tg mice. Similarly, when such tg mice were inoculated with scrapie and sacrificed at the corresponding times, no upregulation of either NMDA or glutamate R followed. In contrast, in such scrapie-infected mice GABAA Rs were upregulated, as illustrated in Fig. 4A and B. Western blot analysis revealed no differences between these infected mice and infection-free mice as to excitatory pathway (NMDA or glutamate) components (Fig. 4A). However, differences between the two groups were marked with respect to the inhibitory GABA pathway GABAA R subunits, specifically with GABAA R α5 (four of five mice, two of three mice shown) and GABAA R β (six of six mice, three of three mice shown) at 400 and 600 days after scrapie infection. The differential expression of GABAA β and α subunits (in our studies β1, 2, 3 and α1, α2, and α5) was confirmed by testing with monoclonal antibodies. All three β subunits collectively were upregulated, as was α5 but not α1 or α2, during scrapie infection (Fig. 4B).

Previously, GABAA Rs have been reported to display an extensive heterogeneity based on differential assembly of subunits into distinct receptor complexes (15). This extraordinary heterogeneity in the distribution of GABA R subunits is believed to provide flexibility in signal transduction (15). The enhanced GABAA R β subunit expression was also observed by immunohistochemistry and increased over time (days 300 to 700) after scrapie infection (Fig. 4C). In parallel, PrPres deposition grew over time. These events were observed in three mice sacrificed at each time point shown and occurred in consecutive sections from a single mouse at each time recorded in Fig. 4C. Coimmunoprecipitation was then performed to determine whether PrP was directly connected with increased expression of the GABAA R (Fig. 4D and E). Immunoprecipitation of brain material from uninfected tg mice with antibody against PrP (D13) followed by probing for selected subunits of the GABAA R clearly demonstrated that PrPsen is associated with GABAA R β (Fig. 4D, lane 2). This association of GABAA R β with PrP was markedly greater in brain tissue from infected tg mice tested at 400 days postinfection (P ≤ 0.001) than in that from uninfected tg mice (Fig. 4D, lane 3).

No such association was detected for receptors for NMDA or glutamate (data not shown). Similarly, when monoclonal antibodies against GABAA R β or GABAA R α5 were first used to precipitate brain material from GPI−/− PrP tg scrapie-infected mice 400 to 600 days after inoculation and the immunoprecipitate was probed against PrP (D13 antibody), PrPres was found.

For the results depicted in Fig. 4E, we used a reagent specific for PrPres to document and confirm coimmunoprecipitation with GABAA R. A PrPres-specific motif of aa 136 to 158 was grafted onto a human immunodeficiency virus (HIV) antibody molecule (b12) as reported previously (26, 33). Panel 1 of Fig. 4E shows that this PrPres-specific motif-engrafted antibody precipitated PrPres from the brain of a GPI−/− PrP tg mouse infected with RML scrapie but that the b12 antibody alone did not. Panel 2 of Fig. 4E shows that antibody against GABAA R α5 coimmunoprecipitated with PrPres. In contrast, the HIV antibody molecule (b12) without the grafted PrPres-specific motif of aa 136 to 158 failed to precipitate the GABAA R.

Dual immunochromic staining using specific monoclonal antibodies (Fig. 4F) shows GABAA R β (red) interlaced with PrPres deposit (yellow green). This interlacing of GABAA R with PrP was a common occurrence throughout the brain by 400 days after scrapie infection.

Earlier, electrophysiologic analysis of hippocampal brain slices from PrP knockout mice identified a reduction of GABAA R-mediated synaptic transmission and attenuation of LTP in vitro (10, 11, 24). Our results with GPI−/− PrP tg scrapie-infected mice in vivo complement these studies by revealing upregulation of GABAA R associated with enhanced deposition of PrPres and the likely association of PrPsen with GABAA R. Further, our findings support earlier studies by Lu et al. (22) and Bouzamondo-Bernstein et al. (6), who noted an increase in the total number of GABA-immunopositive neurons in brains of hamsters infected with scrapie. Additionally, GPI−/− PrP tg mice infected with RML scrapie did not develop the severe and selective loss of GABAergic neurons found by Guentchev et al. (17) in GPI-anchored PrP mice injected with either RML scrapie or CJD Fujisaki strain. Immunochromic studies of brains from CJD patients have produced varied results dependent on the area of the CNS sampled (17).

Our study of GPI−/− PrP tg mice infected with RML murine scrapie documents that PrPres deposition within the frontal cortex and hippocampus despite the animals’ lack of obvious neurodegenerative disease coincides with a disruption of learning and memory as determined by cued Barnes testing and defects in LTP. These dysfunctions in learning and memory do not correlate with neuronal death, dropout, or apoptosis. Instead, the faulty learning and memory are likely associated with increased inhibition via the GABAergic pathway, according to results stemming from the addition of bicuculline during LTP studies in vivo. Supporting that conclusion are outcomes from direct immunochromic analysis of neurotransmitters and neurotransmitter profiles within brains of scrapie-infected and uninfected GPI−/− PrP tg mice. Finding PrPres deposition and upregulation of GABAA R β and α5 subunits in the same loci of the brain suggests a possible direct interaction between these molecules. The association of PrPsen and GABAA R β subunits, although of a low intensity, suggests that the normal PrP molecule may function in vivo with the GABAergic pathway, which becomes overexcited during PrPres deposition. Presumably the result would be a decreased capacity for learning and memory in the scrapie-infected host. The previous failure of Kannenberg and associates (21) to find a physical associa-
FIG. 4. Association of PrP with the GABAAergic pathway. (A and B) Results from Western blotting using a series of monoclonal antibodies to detect NMDA or glutamate R (A) or GABA R (B) from individual uninfected or RML scrapie-infected 400- to 450-day-old GPI/H11002/H11002 PrP tg mice. The expression of the NMDA or glutamate R was similar in the uninfected or scrapie-infected mice shown and in four additional age- and sex-matched uninfected or scrapie-infected mice (not shown) studied at 600 to 700 days postinfection. In contrast, a marked increase in expression of GABAA Rα5 and GABAA Rβ subunits but not of GABAA Rα1 or α2 occurred at both time points (total of four to six mice per time period). (C) Paired sagittal brain sections taken from one uninfected mouse at 450 days old and stained for PrPres (upper section on the left) or for GABAA Rβ (upper section on the right) expression. No PrPres was detected by Western blotting, indicating background staining for PrPsen. Tissues were fixed and processed for antigen retrieval as outlined in Materials and Methods. This quenches excessive GABA signal and allows immunochemical comparison with PrPres. The increasing deposition of PrPres (left sections) and increasing expression of GABAA Rβ (right sections) are shown in sections taken 300, 400, 600, and 700 days after scrapie infection from representative GPI/H11002/H11002 PrP tg mice. Paired 5-μm sections separated by not more than 10 μm were taken at the indicated times from each infected mouse. Results were similar for three additional infected mice sacrificed at each of the times shown as well as for four mice sacrificed at 500 days postinfection. In contrast, two uninfected GPI/H11002 PrP tg mice inoculated with PBS and sacrificed 500 and 700 days later failed to show either PrPres or upregulated GABAA R by either immunohistochemical testing or Western blot analysis. (D) Association of PrP and GABAA Rβ in uninfected and infected tg mice (day 400) is shown by immunoprecipitation with anti-PrP monoclonal antibody (D13) followed by immunoblotting for the presence of GABAA Rβ. Interaction between PrPsen and GABA Rβ is detectable in the brains of uninfected tg mice (lane 2). Brains from infected tg mice contain significantly more (P < 0.001) GABAA Rβ associated with PrP (lane 3). Ig isotype matched controls are shown in lane 1. Whole-brain homogenates (5 and 10 μl; lanes 4 and 5, respectively) served as a positive control for the presence of GABAA Rβ. (E) Specific coimmunoprecipitation of PrPres with GABA Rα5 from scrapie-infected GPI/H11002 PrP tg mice. The PrPres-specific antibody made by grafting PrP aa 136 to 158 onto the HIV monoclonal antibody b12 (first two lanes) precipitated GABAA R, but the control HIV b12 antibody missing the PrPres graft (lanes 3 and 4) did not. Whole-brain homogenates from scrapie-infected GPI/H11002 PrP tg mice were treated (+) or not (−) with proteinase K. Antibodies were then precipitated with polyclonal rabbit anti-human IgG F(ab′)2; linked to paramagnetic beads. Precipitates were analyzed by Western blotting for the presence of PrP (panel 1) and GABAA Rα5 (panel 2). Brain homogenate (lanes 5 and 6) was included as a positive control for the presence of PrP and GABAA Rα5. (F) Localization of upregulated GABAA Rβ and PrPres deposits by immunohistochemistry. Overlaid (60×) adjacent brain sections (within 5 μm) stained for the presence of GABAA Rβ (red) or PrPres (green). Only areas directly adjacent to PrPres deposits displayed increased GABAA R staining.
tion between cellular PrP and GABAA R likely reflects the sensitivity of the protein purification scheme that they employed. The GABAA R is a ligand-gated CI− ion channel. Our attempts to evaluate CI− ion channels using patch clamps on hippocampal slices from adult GPI−/− PrP tg mice that had PrPPrs deposits failed technically because of the age of the specimen used. Nevertheless, our results documented here indicate the worthiness of evaluating the CI− ion channel and possible pharmacologic applications to control GABA or the CI− ion channel in humans with prion disease.

ACKNOWLEDGMENTS

This is publication no. 18827 from the Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA. This research was supported by U.S. Public Health Service grant AG004342 and NIH training grants NS041219 (to M.J.T.) and AI07354 (to J.B.T.).

We thank Floyd Bloom, Iustin Tabarean, and Tamas Bartfai of TSRI and Bruce Chesbro of NIH, Rocky Mountain Laboratory, for helpful discussions and Iustin Tabarean for patch clamp studies. The GPI−/− PrP tg mice were generated in collaboration with Bruce Chesbro.

REFERENCES


