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Context-dependent perturbation of neural systems in transgenic mice expressing a cytosolic prion protein

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  Figure S2: t-map for animals without Mn administration
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We analyzed the relationship between pathogenic protein expression and perturbations to brain anatomy and physiology in a genetic model of prion disease. In this model, the mouse line 1D4, neuropathology is promoted by accumulation of a cytosolic form of the prion protein (cyPrP). CyPrP distribution was determined and compared with anatomical magnetic resonance imaging (MRI) data, a form of functional MRI based on manganese labeling, and immediate early gene mapping with an antibody to c-Fos. Significant discrepancies between 1D4 and control mice became apparent well in advance of overt behavioral pathology in the mutant mice. Alterations to brain structure and function in the mutants varied among brain regions, however, and differed strikingly even among regions with the highest levels of cyPrP expression: In the cerebellum, gross neurodegeneration was accompanied by increased Mn$^{2+}$-enhanced MRI signal, raising the possibility that compensatory mechanisms act to preserve cerebellar function in the face of massive atrophy. In the hippocampus of 1D4 mice, no significant structural alterations were observed, but both Mn$^{2+}$-enhanced MRI and c-Fos data indicated perturbations to neurophysiology. In the neocortex, there were no clear neural activity differences between 1D4 and control animals, but mutant mice showed significant reduction in cortical thickness. Our finding that distinct combinations of anatomical and functional abnormalities accompanied cyPrP overexpression in different parts of the brain indicates the importance of context in conditioning effects of protein pathogens, and exemplifies the notion that neurodegenerative phenotypes extend beyond cell death and the immediate consequences of atrophy for particular neural systems.
INTRODUCTION

Late-onset neurodegenerative conditions such as Creutzfeldt-Jacob, Alzheimer’s, and Huntington’s diseases are in most cases caused by the expression of specific mutant proteins. Despite relatively widespread distribution across the brain, these proteins lead to degeneration only of select regions; the areas most affected differ between diseases (Gambetti et al., 1995; Palop et al., 2006; Castellani et al., 2007; Orr and Zoghbi, 2007; Kahle, 2008). The apparent contrast between generalized toxic protein distribution and region-specific neurodegeneration raises two questions: First, to what degree does the extent of brain disruption correlate simply with the level of pathogenic protein accumulation in different areas? Second, is neurodegeneration the only important result of mutant protein accumulation, or are there neural contexts in which expression leads to brain dysfunction prior to, or in the absence of extensive cell death?

Transgenic mouse models of neurodegenerative conditions provide a context in which to address systematically the connection between disease-related mutant protein expression and perturbations to neuroanatomy and function. Prion diseases are a family of neurodegenerative disorders in which the pathogenic agent is thought to be a misfolded form of the mammalian prion protein (PrP) (Prusiner, 1982; Aguzzi and Polymeridou, 2004), and for which a number of mouse models exist (Hsiao et al., 1990; Lasmezas et al., 1997; Chiesa et al., 1998; Ma et al., 2002; Cunningham et al., 2005). Cytosolic accumulation of PrP is particularly damaging in neuronal cell types (Ma et al., 2002; Rane et al., 2004; Wang et al., 2006; Rane et al., 2008). Transgenic mice expressing a cytosolically-localized form of PrP (cyPrP) display cerebellar atrophy and intense degen-
eration of cerebellar granular neurons, also observed in a model of heritable prion dis-
eases (Chiesa et al., 1998). The cyPrP-expressing mouse line 1D4 (Ma et al., 2002) is
particularly amenable to studies of the relationship between cyPrP accumulation and neu-
ropathology, both because of its reproducible cyPrP expression patterns, and because the
pathological symptoms of neurodegeneration, ataxia, and pruritis develop relatively
slowly (6-18 months after birth) without need for introduction of exogenous pathogens.

In 1D4 animals, cyPrP is broadly expressed from a modified PrP (half genomic)
promoter (Borchelt et al., 1996; Fischer et al., 1996), yet both the reported patterns of
neuronal atrophy and the most severe behavioral symptom (ataxia) appear to be specific
to the cerebellum (Ma et al., 2002). As a step toward understanding the interaction be-
tween cyPrP expression and neuropathology at the whole-brain level in 1D4 mice, we
used a novel combination of anatomical and functional mapping techniques to compare
transgenic and non-transgenic wild-type (WT) control mice. We examined the specific
hypothesis that neuropathological effects of cyPrP accumulation are conditioned by the
neuronal context in the brain, and we also investigated the possibility that pathological
changes in 1D4 mice extend beyond the previously observed cerebellar degeneration (Ma
et al., 2002). Our results revealed that cyPrP expression levels, as well as cyPrP-
dependent perturbations to anatomical and functional measures, varied strongly between
neural structures. Differences between 1D4 and control animals were evident in brain
regions including but not limited to the cerebellum, and appeared to affect brain physiol-
ogy as well as structure. The results support the general view that the neuroanatomical
environment can play a significant role in determining the consequences of mutant pro-
tein expression in neurodegenerative diseases.
MATERIALS AND METHODS

Animals

For MRI, c-Fos, and behavioral experiments, 1D4 mutant and non-transgenic littermate control mice were maintained on an inbred C57Bl/6 (Taconic, Hudson, NY) background in a pathogen-free barrier facility at the Whitehead Institute (Cambridge, Massachusetts). For cyPrP immunohistochemistry studies, 1D4 mice were bred onto a PrP knock-out background (Bueler et al., 1992) at Ohio State University. Experiments were carried out in accordance with NIH guidelines, following approval of the animal ethics committees of the Massachusetts Institute of Technology and Ohio State University.

Immunohistochemistry of cyPrP

Brains for PrP immunohistochemistry were fixed overnight by immersion in paraformaldehyde in PB (0.1 M phosphate buffer, pH 7.4), then embedded with paraffin and cut into 5 µm thick sagittal sections. Paraffin was removed by passages through xylenes followed by decreasing concentrations of ethanol. For epitope retrieval, sections were microwave irradiated in 10 mM citrate buffer, pH 6. After inactivating endogenous peroxidases with 3% H₂O₂ and blocking tissue samples in 4% normal goat serum, samples were incubated with the anti-PrP C SAF32 monoclonal antibody (Cayman Chemical, Ann Arbor, Michigan) with 2% serum in PBS, first for 1 h at room temperature and then over-
night at 4°C in a wet incubation chamber. Samples were then incubated for 1 h at room temperature with a biotinylated secondary antibody, followed by incubation for 1 h at room temperature with streptavidin-peroxidase (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA). Sections were photographed with varying exposure times in order to distinguish protein staining levels across a wide dynamic range (Fig. 1 and supplementary Fig. S1). Four transgenics and one control were analyzed.

**Analysis of immediate early gene expression**

To assay immediate early gene expression, 6 mutant and 5 WT animals (12-16 mo) were either enriched for two hours in a novel, large cage containing a running wheel and assorted unfamiliar objects, or left in their home cage to serve as unenriched controls. Following the exposure period, animals were anaesthetized with avertin and perfused with paraformaldehyde in PB. Brains were removed and post-fixed overnight in the same fixative. Fixed brains were then immersed in 30% sucrose until they sank to the bottom, approximately two days later. They were then sectioned into 30 µm thick sagittal slices on a vibratome and stored in buffer (30% sucrose, 1% polyvinylpyrrolidone-40, 30% ethylene glycol, in PB). Prior to staining, slices were rinsed in PB. Following standard methods (protocol recommended by Calbiochem, Darmstadt, Germany), sections were incubated with blocking solution (8.5 mL 2.5% bovine serum albumin, 300 µL 10% Triton X-100, 200 µL goat serum, 1.5 mL Super Blocking Solution; Innovex Bioscience, Richmond, CA), and labeled with rabbit polyclonal anti-c-Fos antibody (Ab-5, 1:10,000
dilution of ascites; Calbiochem, Darmstadt, Germany) for 4 h at room temperature. Visualization was performed using biotinylated goat anti-rabbit secondary antibody, followed by streptavidin-peroxidase and DAB staining. Enrichment times of six hours resulted in poor c-Fos labeling, probably due to degradation of c-Fos synthesized after transfer to the enriched cage, and to reduced protein synthesis as the novelty of enrichment wore off at later times.

Analysis of c-Fos expression in the hippocampus was performed using histological sections corresponding to mouse brain atlas plates 110-116 (Paxinos and Franklin, 2000), examined through a 10X objective on a Zeiss Axiovert 200M microscope (Thornwood, NY). ROIs corresponding to the two dimensional cross sections through CA1/CA2, CA3, and DG, were defined with respect to boundaries in the atlas diagrams. Cortical c-Fos expression was analyzed using histological sections corresponding to atlas plates 110-116, photographed through a 5X objective on an Olympus BX50WI microscope (Center Valley, PA). A cortical ROI was defined by a 0.185 x 0.185 mm square, centered -2 mm from Bregma and 0.3 mm below the cortical surface (falling primarily within somatosensory cortex). ROI areas used for normalization were computed from digital images of the analyzed sections, using custom software in Matlab (Mathworks, Natick, MA). Data from each individual animal were averaged across 3-9 adjacent histology slides and normalized to obtain a single value for c-Fos positive cells/mm² for each ROI, and then averaged across animals to generate the values and statistical comparisons presented in the text and figures.

**Magnetic resonance imaging**
10 mutant 1D4 and 9 WT control mice were imaged to produce MRI data for group analyses. Of these animals, 7 mutants and 6 control animals were imaged between two and five months of age (mo) and between 7 and 12 mo. An additional WT animal was imaged at 3 mo with the contrast agent and then again 4 months later without injection of Mn^{2+} to ensure that no residual contrast agent was present that would affect the image intensity analysis. In addition, two WT and two 1D4 were imaged at 22-23 mo. 24 h before each MRI session, 37.7 mg/kg MnCl\(_2\) (30 mM MnCl\(_2\) in 154 mM NaCl solution) was administered by intraperitoneal injection. Animals were housed in their home cages (2-5 animals per cage) between MnCl\(_2\) injection and imaging. 5 WT and 5 1D4 animals were also imaged prior to MnCl\(_2\) injection, in order to obtain control data for Mn^{2+}-enhanced image intensity analysis. Immediately prior to scanning, each animal was anesthetized using 1-1.5% isofluorane and placed in a custom-made stereotaxic frame, which minimized head motion during the scans. A blanket was used to warm each animal during the experiments. Images of the whole mouse brain were obtained with cubic voxels (125 x 125 x 125 \(\mu\)m) on a 4.7 Tesla (T) MR scanner (Bruker, Ettlingen, Germany), using a \(T_1\) weighted 3D gradient echo sequence (\(TE/TR = 6/50\) ms) and a custom-fabricated surface coil for 200 MHz radiofrequency transmission and reception. A data matrix of 160 x 160 x 80 voxels was used to cover a 20 x 20 x 10 mm field of view; each image required 64 minutes to acquire.

An additional group of animals (5 WT, 5 1D4, all 12 mo) was imaged at higher magnetic field (9.4 T) and resolution, to obtain data showing a more precise distribution of manganese-enhanced contrast in the cerebellum. A custom made 400 MHz surface coil was used, along with a 3D gradient echo sequence (\(TE/TR = 6/50\) ms, matrix 400 x
400 x 150, resolution 50 x 50 x 100 µm, lower along the lateral-medial axis, with acquisition time 100 minutes per image).

**MRI image analysis**

Images were analyzed using custom routines running in Matlab (Mathworks, Natick, MA), in conjunction with freely available image processing tools: AIR (Woods et al., 1998), FSL (Jenkinson et al., 2002), Brainsuite (Dogdas et al., 2005), and Shiva (Laboratory of Neuroimaging, UCLA, California). Raw data were Fourier transformed and converted to an 8 bit Analyze format. Images were segmented using a semi-automatic procedure (corrected manually where necessary) to remove signal from tissue outside the brain. To measure anatomical features of the animals’ brains, defined landmarks were identified in the cerebellum, hippocampus, cortex, and olfactory bulb, following rigid-body alignment of each brain to a reference brain of comparable age and identical genotype. The alignment step served to eliminate small orientation differences that might otherwise have complicated the identification of equivalent landmarks in each animal.

To determine MRI intensity differences between 1D4 mutants and controls, we performed an ROI-based analysis. A representative brain from our dataset was spatially coregistered to a high resolution volumetric brain image (Laboratory of Neuroimaging, UCLA), paired with a digital mouse atlas (Paxinos and Franklin, 2000). This coregistration consisted of a rigid-body alignment stage, followed by a nonlinear alignment, to yield a three dimensional reference brain segmented into major brain regions. Each additional brain from our dataset was then aligned onto the segmented reference; subfields of the hippocampus were segmented manually. Each segmented
image was inspected and corrected manually in order to ensure correct alignment of the atlas regions to the brain image. To reduce subject-to-subject variability, the voxel intensities of each spatially aligned brain image were normalized by the maximum of a Gaussian fit to each dataset’s intensity histogram (i.e. the mode of each image’s intensity distribution). Normalization constants determined from 1D4 and WT mice did not differ significantly (t-test \( p = 0.28 \)), suggesting that the normalization procedure did not introduce or mask systematic differences between the two mouse strains. Intensities normalized by the histogram maximum method were averaged over each brain region and used for subsequent analyses presented in the text and figures. To control for specific effects of the intensity histogram maximum-based normalization procedure, we performed additional analyses following normalization of each individual brain to the median or mean intensity from that brain. Normalization based on each image’s median intensity increased the intensity difference between mutant and WT cerebella and decreased the intensity difference between mutant and WT hippocampi, with respect to the histogram-based approach; both differences remained significant. An alternative normalization based on each image’s mean intensity also increased the intensity difference between mutant and WT cerebella and decreased the intensity difference between mutant and WT hippocampi; in this case, differences observed in the cerebellum became more significant, and differences observed in the hippocampus were no longer significant. Normalization using a phantom close to the mouse’s head proved to be less reliable than our use of an internal reference, since it was difficult to place the phantom close enough to the surface coil to experience equivalent sensitivity in each animal.
Findings from the ROI analysis were complemented by a $t$-value map characterizing image intensity differences between WT and 1D4 animals. Individual mouse MRI datasets were warped onto a common reference image, using a 12-parameter affine transformation implemented with the AIR software (Laboratory of Neuroimaging, UCLA). Each animal’s image was intensity-normalized by the maximum of its intensity distribution, as described above for the ROI analysis. Images were smoothed using a Gaussian kernel of 375 μm width (three voxels), to eliminate the influence of minor registration errors. Statistical parametric maps were then calculated to assess the statistical significance of voxel-level intensity differences. This procedure was performed on Mn$^{2+}$-labeled mice and, as a control, on mice imaged prior to Mn$^{2+}$ injection. Statistical maps were overlaid on a high resolution image to assist with anatomical interpretation.

**Behavioral assays**

Animals that were also imaged with MRI were tested for motor coordination and balance on a modified (Hockly et al., 2002) accelerating rotarod (Ugo Basile, Comerio, Italy). The rotation speed of the rod was increased from 4 to 40 rpm, over ten 30 s fixed-speed intervals. Performance was quantified as the time an animal remained on the rotarod before falling. All animals were trained for two days prior to testing and tested on a single day. An animal had to remain on the rod for at least 3 s for a trial to be valid. If an animal did not complete a valid trial within three attempts, the result of the trial was recorded as zero. For each animal, results of three trials separated by rest periods of two hours were averaged. In the 2-5 mo age group, 6 mutants (1 female, 5 male) and 7 con-
trols (all male) were tested; in the 7-12 mo age group, 5 1D4 (3 female, 2 male) and 5 WT animals (2 female, 3 male) were tested.

To test animals for differences in overall activity, a videographic analysis was performed on female mice (6 1D4, 6 WT; 10-11 mo) placed individually in a standardized “home cage” and filmed over a 16 h period. 24 behavioral properties [feeding, grooming, locomotion distance, etc. (Steele et al., 2007)] were quantified using commercial software (HomeCageScan, Clever Sys., Inc., Reston, VA). The average times spent on each activity during dark cycle hours (hours 2-6 after start of the video recording) were quantified for comparison between 1D4 and WT animals. In an initial experiment, animals were tested prior to manganese injections. To test whether Mn$^{2+}$ administration induced different behavioral effects on controls and mutants, five 1D4 and three WT were retested three weeks later, this time immediately following injection of 37.7 mg/kg MnCl$_2$. In the same experiment, an additional three 1D4 and four WT were imaged after administration of Mn.

**Statistical analyses**

Grouped data from multiple individuals were tested for normality using a Lilliefors test. Where appropriate, group means were then compared using a two-sided Student’s $t$-test. Non-normal data were assessed using a Mann-Whitney U test. Differences were judged to be significant if the null hypothesis of equivalence could be rejected with $p < 0.05/n$, where $n$ was the number of comparisons performed in parallel (e.g. over $n$ ROIs). In each case where mean values are reported in the text, reported error intervals are equal to the standard error of the mean. Standard error propagation methods were used where
appropriate. Statistical analyses were performed using the statistics toolbox in Matlab (Mathworks, Natick, MA). In evaluating the significance of intensity differences between 1D4 and controls for each voxel across the whole brain, a false discovery rate of 10% was used to determine the cutoff $t$-value for the $t$-map.

RESULTS

cyPrP is strongly expressed in neurons of the cerebellum, neocortex, and hippocampus of 1D4 mutants

We used immunohistochemistry to map PrP distribution in mice which express cyPrP (Ma et al., 2002) on a PrP$^c$ knock-out background (Bueler et al., 1992). This mapping was not possible directly in 1D4 mice, because the PrP-specific antibody could not distinguish the relatively small amount of cyPrP from endogenous PrP$^c$ produced in these animals. CyPrP expression patterns were reproducible between animals, but expression levels varied strongly between neural structures in the same animal. The mutant protein was expressed broadly throughout the brain and was most abundant in the outer layers of the neocortex (especially layer 2), in the granular layer of the cerebellum, and in the hippocampal formation (Fig. 1a). Control animals showed little or no antibody binding. These results indicated that dense accumulation of mutant protein could be found in brain regions not previously identified as sites of severe neurodegeneration, as well as in cerebellar structures where massive atrophy had previously been observed (Ma et al., 2002).
Cells with neuronal morphologies showed the greatest levels of cyPrP accumulation; cells with non-neuronal morphology did not seem to express detectable amounts of the protein (Fig. 1b-d). In the cerebellum, granule cells, Purkinje cells, and some molecular layer cells were cyPrP-positive. In the hippocampus, cyPrP was present in both pyramidal and non-pyramidal cells, and appeared to be particularly abundant in axons of CA1. Granular cells of the dentate gyrus were also strongly stained. In the neocortex, cells with morphological characteristics both of pyramidal cells and of interneurons showed cyPrP staining. In general, cyPrP-positive cells showed diffuse cytoplasmic distribution of the staining, including in axons. Nuclear staining was also observed, particularly in the granular layer of the cerebellum, where cyPrP was predominantly restricted to nuclei. Although patterns of cellular and subcellular cyPrP localization varied to some extent among brain regions, it was not the case that cell types thought to be most prone to cell death in the 1D4 model showed obviously more intense cyPrP staining in our analysis.

A subset of cyPrP-expressing brain regions show size reduction in 1D4 mice

To determine whether the generalized distribution of mutant protein expression we observed was linked in turn to widespread anatomical abnormalities in 1D4 mouse brains, we analyzed MRI scans obtained from WT and 1D4 animals. MRI was performed on anesthetized animals, 24 h after intraperitoneal injection of MnCl₂ (37.7 mg/kg), a T₁ contrast agent shown previously to enhance visualization of neural architecture (Aoki et al., 2004). Whole brain images with 125 μm isotropic voxel resolution were acquired using a gradient echo pulse sequence on a 4.7 T scanner. A series of
physical landmarks was identified within each three dimensional image set (Fig. 2a), and
distances between pairs of landmarks were used to detect anatomical differences between
age-matched 1D4 and WT animals at either 2-5 or 7-12 months of age (mo).

Several of the brain features we measured were consistently smaller in 1D4 mutant
mice than in controls. For the older group (7-12 mo animals, Fig. 2c), we found that the
rostrocaudal extent (vector Cb2 in Fig. 2a) and the diameter at midline (vector Cb3) of
the cerebellum were lower by 28 ± 2% and 26 ± 2%, respectively, in 1D4 mutants ($n = 8$)
compared with controls ($n = 7$). These differences were significant, with $p < 0.001$ as
measured by a Mann-Whitney U test, where the threshold for significance under Bonfer-
roni correction for multiple comparisons was $p < 0.005$. The height of the cerebellum
(vector Cb1) was also smaller, by 13 ± 5% in mutants, but this difference was not statisti-
cally significant (n.s.; $p = 0.02$). The dorsoventral axis of the thalamus (vector Th1) was
also consistently shorter in 7-11 mo 1D4 animals, by 12 ± 2% ($p < 0.001$) with respect to
controls. In 7-11 mo 1D4 mice, the overall length of the cortex (vector Cx1) was shorter
by 10 ± 3%, but did not reach statistical significance ($p = 0.02$), whereas a measure of
cortical thickness (vector Cx2) was significantly lower, by 5 ± 1% in 1D4 mutants ($p =
0.004$). Significant changes were not found for distances measured in the hippocampus
($p > 0.8$), indicating that only a subset of brain structures that highly express cyPrP show
significant size reductions in 1D4 animals.

Some of the gross anatomical differences observed between cyPrP-expressing and
control mice at 7-11 mo were already apparent at 2-5 months after birth (Fig. 2b). Dif-
fences between 2-5 mo 1D4 ($n = 7$) and controls ($n = 7$) in the rostrocaudal extent and
height of the cerebellum (22 ± 3%, $p < 0.001$, and 12 ± 4%, n.s. with $p = 0.009$, respec-
tively) were comparable to those observed in older animals. Differences in the cerebellar diameter at the midline were somewhat less pronounced in 2-5 month olds than in older animals, however (21 ± 3% lower in mutants than controls, \( p < 0.001 \)). The overall length of the cortex was 9 ± 2% shorter in 2-5 mo mutant mice than in controls (significant with \( p = 0.002 \)), although at this age cortical thickness differences were not yet apparent. Cerebellar shrinkage appeared to be progressive: Between repeated imaging experiments, on average 4.4 ± 0.8 months apart, the cerebellar diameter at midline of individual 1D4 mutants decreased by 6 ± 2% (single test significant with \( p < 0.05 \)). In six of seven mutants, the cerebellar diameter at midline decreased by amounts ranging from 4-15%, whereas the cerebellar diameter increased in four out of six WT animals over an equivalent four month period. This suggests, as previously assumed, that some brain size differences between 1D4 and WT mice result from neurodegeneration, rather than developmental abnormalities.

Manganese-enhanced MRI signal is altered in the cerebellum and hippocampus of cyPrP mice

Is neuropathology in cyPrP-expressing mutant mice limited to deviations from WT anatomy, or are patterns of neural activity altered as well? Uptake of \( \text{Mn}^{2+} \), the contrast agent we used for enhancement of brain structures in our anatomical analysis, has been shown to indicate relative neuronal activity levels under a variety of conditions in rodents (Lin and Koretsky, 1997; Silva et al., 2004). To examine the ability of this method to detect activity-related differences between cyPrP transgenics and control mice, we therefore measured differences in the \( \text{Mn}^{2+} \)-enhanced MRI signal intensity between 1D4 (\( n = \)
and WT \( (n = 9) \) animals, including the animals assessed at 2-5 mo and at 7-12 mo group as well as two 1D4 and two WT aged 22-23 mo. In between Mn\(^{2+}\) injection and imaging (24 h), animals were housed in their home cages without special stimulation, in an effort to elicit “normal” patterns of behavior. Given the relatively slow uptake kinetics of the tracer (Lee et al., 2005), the relative intensity of MRI signal in scans performed after Mn\(^{2+}\) administration was assumed to reflect activity-dependent Mn\(^{2+}\) uptake averaged over the labeling period.

Mn\(^{2+}\) labeling data were analyzed using voxel-based and region of interest (ROI)-based approaches. Voxel-based analysis was performed on images spatially aligned to a reference brain. Each coregistered image was then intensity-normalized by the maximum of the scan’s intensity histogram, and smoothed over a Gaussian spatial kernel (375 µm width) in preparation for statistical analysis. A \( t \)-statistic was computed for each voxel to test the null hypothesis of signal equivalence between 1D4 and WT mice. A map of the computed \( t \)-values is presented in Fig. 3a, and provides an overview of signal intensity differences between Mn\(^{2+}\)-labelled 7-23 mo mutant and control animals. Significantly lower average MRI signal was observed in the hippocampus of mutant mice, with respect to WT (0.29 µL voxel volume with \( p < 0.001 \)). This suggests that Mn\(^{2+}\) uptake, and hence neural function, might be perturbed in the 1D4 hippocampus, identified as a site of cyPrP accumulation in the absence of neuronal atrophy. Significantly higher Mn\(^{2+}\)-enhanced signal, indicative of increased average neural activity, was observed in the cerebellum (0.75 µL voxels with \( p < 0.001 \)) of mutants. Significant signal differences were not observed in the neocortex, however, indicating that Mn\(^{2+}\)-dependent contrast changes did not arise in all brain regions with high levels of cyPrP expression. MRI sig-
nal differences in the cerebellum and hippocampus were not present between 1D4 ($n = 5$) and WT ($n = 5$) mice scanned before Mn$^{2+}$ injection, showing that the Mn$^{2+}$ enhancement effects in these regions did not arise from subtle differences in brain morphology or from registration errors (see supplementary Fig. S2). In contrast, apparent signal differences near the third ventricle and near the edges of the cerebellum were observed both before and after Mn$^{2+}$ labeling, and therefore most likely result from imperfect spatial coregistration.

To corroborate these findings, Mn$^{2+}$-enhanced MRI intensity differences between cyPrP-expressing 7-12 mo mutant and control animals were also examined using an ROI analysis of individual brain images. This analysis did not require spatial coregistration or smoothing, and was thus immune to artifacts from misregistration of multiple brains. Fig. 3c plots mean signal levels averaged across selected ROIs. Results were consistent with the $t$-value map, and showed that the MRI signal level was $3.4 \pm 1.0\%$ lower in the hippocampus of 1D4 mice ($t$-test $p = 0.001$; Bonferroni corrected significance threshold $p = 0.025$) with respect to nontransgenic controls, while the signal level was $3.9 \pm 1.0\%$ higher in the cerebellum ($p = 0.011$). Significant MRI signal differences were not observed in a cortical ROI or in the olfactory bulb. This analysis was repeated in 2-5 mo animals (Fig. 3b); differences at this age were qualitatively similar, but did not reach statistically significant levels. This suggests that discrepancies in Mn$^{2+}$ enhanced MRI signal between 1D4 and WT animals arise from progressive effects of cyPrP expression in a subset of brain regions with dense cyPrP accumulation.

Two cyPrP-rich structures with significant Mn$^{2+}$-enhanced signal changes, the hippocampus and cerebellum, were analyzed in greater anatomical detail. When broken
down by field (Fig. 3c inset), changes in the hippocampus appeared to be most pronounced in the dentate gyrus (DG; 6 ± 2% lower signal in 1D4, significant with $p < 0.006$). Mn$^{2+}$-dependent contrast changes in CA3 (5 ± 3% lower in 1D4 mice) and CA1 (1 ± 2% lower in 1D4 mice) were not statistically significant, although these regions showed cyPrP accumulation levels comparable to the other two subfields. We attempted to obtain cerebellar layer-specific information about MRI signal in 1D4 and WT mice by collecting images at higher resolution (50 x 50 x 100 µm) on a 9.4 T scanner (Fig. 4). Although unbiased layer-specific intensities were difficult to quantify, the high resolution images suggested that strong Mn$^{2+}$-enhanced contrast was produced in an internal layer, assumed to be the Purkinje cell layer, of both WT and 1D4 mice. In mutant mice, layers flanking the Mn$^{2+}$-enhanced Purkinje layer showed apparent reductions in thickness, consistent with earlier histological analysis (Ma et al., 2002). These results indicate that the greater mean MRI signal intensity recorded in the cerebella of 1D4 mice arises in large part from an increase in the proportion of highly Mn$^{2+}$-labeled cells, but that Mn$^{2+}$ staining of 1D4 Purkinje cells is strikingly conserved even in an environment characterized by massive cell death.

The blood-brain barrier is not disrupted in mutant mice

We performed a control experiment to determine whether differential Mn$^{2+}$ staining could have resulted from differences in blood-brain barrier (BBB) permeability between 1D4 and WT animals. The hydrophilic dye Evans Blue was injected intravenously in three 1D4 and three WT mice (12 mo). The dye distributed rapidly throughout the bodies of the animals, and was readily visible from the coloring of the extremities. Leakage
from the vasculature into the brain parenchyma was assayed by postmortem histology. No significant staining was revealed in either 1D4 or WT animals, indicating that both groups had intact BBBs (data not shown).

**Immediate early gene expression is perturbed in the 1D4 hippocampus**

Manganese staining is noninvasive and convenient to apply at a whole-brain level in live animals, but the relationship between Mn\(^{2+}\)-enhanced MRI signal and neural activity is not completely characterized. To investigate the effects of cyPrP expression on neuronal activity using an alternative measure, we therefore applied the technique of immediate early gene (c-Fos) immunohistochemistry on a separate group of animals (6 1D4 mutants and 5 controls, 12-16 mo). Expression of c-Fos was stimulated by transferring animals from their home cage to an enriched cage. This paradigm for rodents has been associated with activation of a broad range of subcortical and cortical areas, including the hippocampus (Wirtshafter, 2005), and was chosen because of its relative similarity to the home cage environment in which longer, 24 h Mn\(^{2+}\)-labeling studies were feasible. After two hours in the enriched environment, mice were sacrificed and brains were extracted for sectioning and staining.

All animals exposed to the enriched environment (n = 11) showed broad c-Fos expression (Fig. 5a); in contrast, animals maintained in their home cages (n = 2) showed only low expression levels (data not shown). Among the enriched animals, both the hippocampus and neocortex reliably showed foci of c-Fos accumulation (Fig. 5b), but the cerebellum showed only diffuse protein distribution. Quantitative assessment of c-Fos expression was therefore performed over ROIs defined within the hippocampus and neo-
cortex (primary somatosensory regions), by counting the number of c-Fos positive nuclei in each ROI and dividing by the ROI area. Unbiased delineation of the ROIs was established by comparing the stained histological sections to images from a standardized mouse brain atlas (Paxinos and Franklin, 2000). Although both hippocampal and neocortical ROIs showed robust immediate early gene expression, a significant difference between WT and 1D4 mice ($p = 0.023$) was found only in the hippocampus, where 1D4 mice showed $40 \pm 15\%$ higher c-Fos counts per square millimeter ($107 \pm 6$ cells/mm$^2$ for 1D4 vs. $107 \pm 10$ cells/mm$^2$ for WT). This difference was unlikely to arise from a difference in cell numbers, because the hippocampal dimensions in 1D4 mice were not greater than in WT animals (Fig. 2). The hippocampal ROI was subdivided into subfields for a more detailed analysis of c-Fos results; CA1, CA3, and DG subfields all showed increased staining in 1D4 animals with respect to controls, with the greatest relative increase visible in DG ($51 \pm 8$ cells/mm$^2$ for 1D4 vs. $25 \pm 5$ cells/mm$^2$ for WT; $p = 0.029$, with Bonferroni corrected threshold of $p = 0.017$). Taken together, these c-Fos expression results therefore supported MRI evidence for the finding that activity patterns are disrupted in the 1D4 hippocampus (where anatomy was not perturbed), but not in the neocortex (where thickness was reduced), despite the high cyPrP levels found in both of these structures.

**Wild-type and 1D4 mice show similar behavior in the cage environment**

Differences between 1D4 and WT animals measured by Mn$^{2+}$ staining or c-Fos immunohistochemistry could have arisen from discrepancies in the behavior of animals during the labeling periods, or from differences in the cerebral physiology required to sub-
serve similar behavioral patterns in mutant and WT mice. To distinguish these two possibilities, we performed a comprehensive survey of behavioral activity in a novel environment approximating the behavioral contexts used for Mn$^{2+}$ and c-Fos labeling studies, in which animals were not forced to perform specific tasks but were allowed to roam freely. Seven 1D4 and five WT 8-10 mo animals were studied, and 24 types of behavior were quantified using an automated video analysis system. This system can detect subtle behavioral differences prior to overt symptoms, even when small sample sets of mice are used (Steele et al., 2007). No significant differences between mutant and control animals were observed (supplementary Table S1), consistent with two previous experiments of larger sample sets (data not shown). To test whether Mn$^{2+}$ injection might have induced behavioral differences, we also performed the behavioral analysis on the same animals following intraperitoneal injection with 37.7 mg/kg MnCl$_2$. Both controls and transgenic animals showed reduced activity within 1-2 hours following Mn$^{2+}$ administration, but again no significant differences between the two populations were observed (see supplementary information).

The population of cyPrP expressing mice we examined performed equivalently to WT mice during “normal” behavior in a home cage environment, but this did not imply that the mutants were unrepresentative of the 1D4 phenotype. Mutant animals showed a clear behavioral difference from controls in the rotarod test, an explicit assessment of motor control applied commonly to characterize PrP-related neuropathologies in mouse models. In this test, 7-12 mo 1D4 animals scanned in our imaging experiments persisted for only $29 \pm 5$ s on a rotating rod, compared with $69 \pm 16$ s for controls ($t$-test $p = 0.035$). Rotarod performance in the 2-5 mo age group was also worse for mutants ($38 \pm$
10 s vs. 85 ± 10 s, p = 0.008). Motor test results from the scanned animals showed no dependence on gender and were in agreement with studies of an additional independent group of 1D4 (n = 8) and control (n = 10) mice (data not shown). A further incipient hallmark of disease in older (7-23 mo) mutants we examined was a low body weight, compared with littermate controls (32 ± 1 g vs. 40 ± 3 g, t-test p = 0.032, n = 5). Only a smaller and not statistically significant weight difference was observed at 3-5 months (29 ± 1 g vs. 33 ± 2 g, p = 0.06, n = 6). Although mutant animals used in our functional and anatomical experiments showed characteristic weight and motor coordination differences from wild-type animals, the videographic behavioral analysis suggested that these aspects of the 1D4 phenotype did not create behavioral differences that might have contributed to our Mn$^{2+}$ and c-Fos labeling studies. The perturbation of activity patterns we observed in 1D4 animals was therefore likely to arise directly from cellular or systems-level effects of cyPrP expression on neuronal function.

DISCUSSION

Methodology for surveying alterations to neural structure and function

In a study of the cytosolic PrP-expressing mouse line 1D4, we found that pathogenic mutant protein expression was broadly distributed throughout the brain. Transgenic mice displayed abnormalities in several brain structures, as assayed by anatomical MRI, a form of functional MRI based on Mn$^{2+}$ labeling, and c-Fos immunohistochemistry. Yet three structures with pronounced cyPrP levels in the 1D4 animals, the cerebel-
lum, the neocortex, and hippocampus, showed disparate combinations of structural and functional deviations from their equivalents in WT individuals. Among these observations, only the finding of neuronal atrophy in cerebellar granule cells had previously been revealed, by a conventional histological analysis (Ma et al., 2002). The combination of methods we applied here enabled us to show that cyPrP protein expression may perturb neuroanatomy, physiology, or both, and that the observed consequences of mutant protein expression appear to vary strongly across the brain.

High resolution MRI is becoming an established technique for characterizing transgenic mice (Anderson and Frank, 2007); in contrast, relatively few studies have applied manganese signal enhancement techniques for assessment of brain function and physiology in murine disease models (Angenstein et al., 2007). Our study therefore represents an exploratory application of this approach. The connection between Mn$^{2+}$-enhanced MRI contrast and neuronal activity has been repeatedly emphasized, but additional factors, such as BBB integrity and axonal transport, have also been shown to contribute (Pautler et al., 1998; Aoki et al., 2004). Because we observed similar retention of an intravascular tracer in 1D4 and WT mice, we were able to rule out gross differences in the BBB between the mouse strains used in our study. Differences in the overall health of individual cells could also have influenced Mn$^{2+}$ uptake, a particular concern in a neurodegenerative disease model. In the 1D4 mice, however, high resolution images of the cerebellum showed that the most pronounced Mn$^{2+}$ uptake was localized in Purkinje neurons, while the cells most prone to degeneration were in the granular layer. We therefore considered neuronal activity perturbation to be a likely explanation for Mn$^{2+}$-enhanced signal differences we observed in the hippocampus and cerebellum, and we explicitly
confirmed a disruption to activity patterns in the hippocampus using c-Fos immunohistochemistry. We could not tell whether perturbed activity patterns in 1D4 mice serve a functional role (e.g. compensating for limited neurodegeneration) or result from pathological conditions such as excitotoxicity. Although there remains a possibility that physiological differences unrelated to electrical signaling also contributed to the Mn$^{2+}$ labeling results, the value of Mn$^{2+}$-enhanced MRI as a minimally-invasive in vivo screen for neuropathology is supported by our findings of regionally-specific image intensity differences between WT and mutant mice, in some cases in the absence of obvious neurodegeneration.

**Region-specific perturbation of brain areas with high levels of cyPrP**

Our combined anatomical and functional study indicated that the cerebella of 1D4 mice showed the sharpest deviations from WT mice. Relative cerebellar size in the mutants declined with age (from 2-5 mo to 7-12 mo animals), suggesting that the anatomical differences resulted from neurodegeneration, as opposed to developmental defects in the 1D4 animals. Previous ex vivo histological studies of these mice (Ma et al., 2002) showed that cerebellar degeneration is most pronounced in the granule cell layer; this was confirmed by our in vivo imaging studies. We further showed that these anatomical differences were accompanied by an increase in Mn$^{2+}$-enhanced MRI signal, which our results argue may be due in part to an increased proportion of manganese-enriched versus non-enriched cells in the 1D4 cerebellum. The distribution of MRI intensity suggested that Mn$^{2+}$ accumulation is most pronounced in the Purkinje cell layer, and that this accumulation persists in 1D4 animals, despite significant atrophy of the surrounding tissue.
The observed consistency of Mn\(^{2+}\) staining and basal behavioral activity between 1D4 and WT mice attests to the possibility that compensatory neural mechanisms preserve aspects of healthy cerebellar function under unstressed circumstances in cyPrP-expressing mice, even in the context of marked neurodegeneration. Consistent with this idea, only a specific neuromotor test of cerebellar function, but not a videographic analysis of home cage behavior, revealed movement-related deficiencies in the 1D4 mice we studied. Cerebellar c-Fos staining in our experiments was too diffuse to support or refute independently the hypothesis of compensatory neural changes. Other studies have documented hallmarks of cerebellar compensatory activity, however, in the context of cerebellar lesions (Yu and Eidelberg, 1983), and human motor disorders including early stage Parkinson’s disease (Mentis et al., 2003; Yu et al., 2007) and primary dystonia (Carbon et al., 2008), but not symptomatic Creutzfeldt-Jakob prion pathology (Henkel et al., 2002; Engler et al., 2003).

The anatomy of the hippocampal formation appeared to be unaffected in 1D4 mice, despite the high levels of cyPrP we found in this structure. In contrast, both Mn\(^{2+}\)-enhanced MRI and c-Fos immunohistochemistry analyses suggested that hippocampal neural activity in 1D4 mice differed significantly from that in WT mice. Apparently conflicting findings were obtained using the Mn\(^{2+}\) and c-Fos methods: Mn\(^{2+}\) staining suggested lower mean activity levels in the CA3 and DG subfields of 1D4 mice, whereas c-Fos labeling suggested higher activity in these regions, with respect to WT animals. These discrepancies were not truly contradictory, however, and could have arisen from a number of factors: First, the behavioral contexts for Mn\(^{2+}\) labeling and c-Fos expression differed because of the separate demands of each activity-mapping method. Mn\(^{2+}\) uptake
was measured during spontaneous steady-state behavior in the home cage, whereas c-Fos expression was induced by transferring mice to a novel enriched cage. It is possible that these two paradigms, though similar, evoke discrepant patterns of activity in 1D4 and WT mice. Second, the cellular events that lead to Mn$^{2+}$ accumulation vs. c-Fos expression are different, and only partially characterized. Mn$^{2+}$ is assumed to enter cells through voltage-gated calcium channels, and therefore may reflect neuronal firing rates or presynaptic activity; c-Fos induction is probably more closely related to postsynaptic events involved in synaptic plasticity (Clayton, 2000). The two methods might therefore be labeling different subsets of neurons, even in the context of similar neural activity. Third, the difference between c-Fos and Mn$^{2+}$ results may be partly due to factors unrelated to neural activity. In hippocampal brain slices from prion infected mice, changes in synaptic function have been shown previously to precede the development of lesions (Jefferys et al., 1994; Jeffrey et al., 2000; Chiti et al., 2006). Our finding of perturbed neuronal activity in the hippocampus of cyPrP-expressing mice may reflect a similar phenomenon, although a precise explanation for the patterns of activity modulation we observed would require more directed recording methods than we have applied here.

The neocortex of cyPrP-expressing mice showed high levels of anti-cyPrP immunoreactivity, comparable to those we observed in the cerebellum and hippocampus. Cortical anatomy, but not neural activity levels, appeared to be perturbed. Neocortical neurodegeneration, astrogliosis, and neuronal loss are hallmarks of the most common forms of prion disease (Gambetti et al., 2003). Disruption of cortical physiology, in the absence of neurodegeneration and gross behavioral abnormalities, has also been observed in a prion disease mouse model (Jefferys et al., 1994). It therefore seems surprising that
we did not observe any indication of neocortical neural activity differences between 1D4 and WT mice. It might be that the modest reduction of cortical thickness we observed in 1D4 mice arose not because of neurodegeneration, but from a reduction in mean cell size, without major consequences for neural circuit function. Another possible explanation is that the behavioral contexts we exposed animals to did not elicit strong enough modulation of cortical neural activity to be resolved as strain-dependent differences in our Mn$^{2+}$ or c-Fos labeling experiments. In the future, behavioral tests designed specifically to elicit cortical activity could be employed to test this possibility (Cunningham et al., 2003).

**Relevance to neurodegenerative disease**

Results presented here support the validity of cyPrP-based mouse models of prion disease like the 1D4 line. The PrP protein is normally attached to the outside of neurons, so the hypothesis that cytoplasmic PrP accumulation is involved in prion disease has been met by some skepticism (Roucou et al., 2003; Fioriti et al., 2005). Although recent experiments have begun to link events such as endoplasmic reticulum stress occurring during prion infection to the accumulation of PrP in the cytosol (Hetz et al., 2003; Kang et al., 2006; Orsi et al., 2006; Hetz et al., 2007; Rane et al., 2008), a persistent concern with the 1D4 model has been the contrast between the apparent cerebellar specificity of the previously described phenotype (Ma et al., 2002) and the propensity of natural prion diseases to affect multiple brain regions. In a partial resolution of this problem, we now report structural or functional abnormalities in the hippocampus and the neocortex, in addition to the cerebellum of 1D4 mice; all of these brain regions display higher levels of
cyPrP accumulation than unaffected brain regions. A new line of mice that express cyPrP in the forebrain also confirms the conclusion that cyPrP toxicity is not specific to cerebellar granule cells (Wang et al., 2009). Earlier studies may have failed to recognize cyPrP-dependent pathology in other brain regions in part because of the subtlety of the perturbations, and in part because the amount of cyPrP accumulation was deemed to be insignificant. More recent experiments suggest that cyPrP induces functional alterations and cell damage at concentrations far below the amount of PrP\(^C\) normally present in cells (W. Jackson and S. Lindquist, unpublished), explaining how pathogenic cyPrP levels could have been ignored in natural prion disease models. Data we present here suggest both that cyPrP neurotoxicity is more widely distributed throughout the brain than previously thought, and that the combination of assays we applied is more sensitive at detecting neurological consequences than conventional histological approaches have been.

Our findings of apparent functional abnormalities in the absence of neuronal deterioration are also relevant for the assessment of mouse models of neurodegenerative diseases more broadly. Several models for Huntington’s and Alzheimer’s diseases involve distinct behavioral abnormalities and sometimes premature death of the animals, but neurodegeneration is often minimal or completely absent (Levine et al., 2004). Here we have shown new evidence of neuronal dysfunction prior to neurodegeneration in a model of prion disease. These results therefore continue to prompt the reassessment of neurodegenerative diseases in general as conditions that broadly perturb neuronal function, rather than simply killing cells (Palop et al., 2006). As in our mouse study, functional assays may be useful for characterization of degenerative diseases, and in clinical settings could help direct and monitor treatment regimens. Our results also reinforce the notion
that the interaction between pathogenic mutant protein expression and neurological phenotypes can be complex and highly dependent on the context of mutant protein expression in the brain. By identifying the determinants of context-specific effects identified in studies like ours, the mechanisms by which cyPrP and other pathogenic proteins induce perturbations to neuroanatomy and function may become more thoroughly understood.

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REFERENCES


FIGURE LEGENDS

Figure 1. Expression pattern of cyPrP in transgenic mice. Immunohistochemistry with an antibody against PrP was performed on sagittal brain sections from mice expressing cyPrP on a PrP<sup>C</sup> knockout background. No counter stain was used. Mutant protein expression varied strongly among brain regions (A), and could be visualized with multiple exposure times (see supplementary Fig. S1); scale bar = 1 mm. Knock-out mice without the transgene showed no staining (not shown). Closeup views are shown for regions of interest denoted by black boxes in (A). (B) In the neocortex, high cyPrP expression was observed, particularly in layer 2 (layers 1-4 numbered, scale bar = 100 µm for panels B-D). (C) The hippocampus showed high expression (dentate gyrus, DG, and CA1 subfields labeled). (D) Prominent cyPrP expression was also observed in the cerebellum; here, levels were highest in the granule cell layer (G), but sporadic expression was also observed in the Purkinje (P) and molecular (M) layers.

Figure 2. Comparison of brain anatomy between 1D4 and WT animals in two age groups. Macroscopic anatomical differences between 1D4 mutants and wild-type controls were quantified using MRI data from anesthetized animals. Characteristic landmarks were identified in brain images, and distances between pairs of landmarks were measured. Landmark positions are shown here superimposed on horizontal, coronal, and sagittal sections (top to bottom) through the Mn<sup>2+</sup>-enhanced T<sub>1</sub>-weighted MRI scan of a representative WT animal (A); image resolution 125 µm (isotropic), scale bar = 1 mm. Distances were quantified from the brains of 2-5 mo animals (B) and 7-12 mo animals.
Figure 3. Brain region-specific differences in manganese-enhanced MRI signal between mutant and control mice. $T_1$-weighted MRI scans were obtained 24 hours after intraperitoneal injection of the contrast agent MnCl$_2$ (37.7 mg/kg). During the labeling period, mice were maintained in their home cages, without additional stimulation. Images from 1D4 and WT animals were spatially normalized and used to compute a three-dimensional $t$-value map indicating signal differences between the two sets of animals (A). The top and bottom panels show sagittal and horizontal sections through the $t$-value map, respectively; relative positions of the sections are denoted by dotted lines. “Hot” colors indicate areas where mutant animals had significantly greater MRI signal than controls; “cold” colors indicate areas where mutant animals displayed hypointense signal. Both positive and negative $t$-values were thresholded using $p < 0.0054$ ($|t| \geq 3.19$, false discovery rate 10%) as a criterion for statistical significance. Independent analysis of manganese-enhanced MRI signal changes was performed by defining regions of interest (ROIs) in individual brain images, and averaging ROI-specific signal from multiple animals of each strain and age group. Results of this comparison are shown for ROIs in the the cerebellum (Cb), the neocortex (Cx) and the hippocampus (H), both in 2-5 mo animals (B) and in 7-12 mo animals (C). Insets in the bar graphs show average signal in the
hippocampus, broken down by subfield (CA1, CA3, and DG). Black and gray bars correspond to WT and 1D4 animals, respectively, with error bars denoting s.e.m.

**Figure 4. High resolution analysis of Mn
\(^{2+}\)-enhanced MRI intensity in the cerebellum.** Representative \(T_1\)-weighted images (\(TE/TR = 6/50\) ms) acquired on a 9.4 T MRI scanner, with 50 \(\mu\)m in-plane and 100 \(\mu\)m out of plane resolution. MRI signal from three adjacent sagittal slices around the midline was averaged to produce the images shown for individual WT (left) and 1D4 cyPrP transgenic (right) animals (both 12 mo). Both images show a narrow lamina of hyperintense signal winding through the cerebellar cortex, and probably corresponding to the Purkinje cell layer (P). Inner and outer gray matter regions correspond to the granular (G) and molecular (M) layers of the cerebellum, respectively. White matter (wm) appears hypointense. Scale bars are divided into 1 mm increments; comparison of the scale bars with WT and 1D4 sagittal cross sections indicates the pronounced reduction in cerebellar size observed in cyPrP-expressing mutants.

**Figure 5. c-Fos expression after exposure to a novel environment.** Expression of c-Fos was visualized using standard immunohistochemical methods. Foci of c-Fos accumulation were apparent in the hippocampus of a representative WT animal (A). The numbers of c-Fos positive nuclei per unit area were determined for WT (black) and 1D4 (gray) animals for ROIs in the neocortex (Cx) and hippocampus (H), including hippocampal subfields (inset). Error bars denote s.e.m. for each measurement, and asterisks identify comparisons for which differences between WT and 1D4 mice were statistically significant after Bonferroni correction.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5