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Marked decrease of neuropeptide Y Y2 receptor binding sites in the hippocampus in murine prion disease

(autoinhibition/dentate gyrus/granule cells/pyramidal cells/scrapic)

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ABSTRACT **Using autoradiographic binding methodology with monoiodinated peptide YY together with the agonists neuropeptide Y (NPY) and NPY (13–36), as well as *in situ* hybridization with oligonucleotide probes complementary to the NPY Y2 receptor (Y2-R) mRNA, we have studied whether or not intracerebral prion inoculation affects Y2-Rs in male CD-1 mice. Monoiodinated peptide YY binding, mainly representing Y2-Rs, was down-regulated by 85% in the CA1 strata oriens and radiatum and by 50–65% in the CA3 stratum oriens 110–140 days postinoculation. In the CA3 stratum radiatum, where the mossy fibers from the dentate granule cells project, there was a significant decrease in PYY binding at 110–120 days. Y2-R mRNA, moderately expressed both in the CA1 and CA3 pyramidal cell layers and the granule cell layer in the dentate gyrus, showed a slight, but not significant, decrease in CA3 neurons 130 days postinoculation. The results indicate that the accumulation of the scrapie prion protein in the CA1–3 region strongly inhibits NPY binding at the Y2-Rs, which, however, is only marginally due to reduced Y2-R mRNA expression. The loss of the ability of NPY to bind to inhibitory Y2-Rs may cause dysfunction of hippocampal circuits and may contribute to the clinical symptoms in mouse scrapie.**

Prion protein (PrP^{C}) is a glycoprotein that is expressed at the cell surface of neurons and glial cells. Its scrapie isoform Prp^{Sc} is protease-resistant and is involved in the pathogenesis of several transmissible encephalopathies, including scrapie of sheep, bovine encephalopathy, as well as Creutzfeldt–Jakob disease, diseases characterized by spongiform degeneration of nerve cell processes (see refs. 1–3) and reactive astrocytic gliosis (4). In scrapie, internal organelles are eventually lost, and abnormal membrane accumulations and neuritic swelling can be detected (5, 6). The molecular and chemical consequences of pathological alterations that precede neuronal death are not known even though some evidence has accumulated suggesting involvement of abnormal Ca^{2+} influx in response to receptor stimulation (7), dysfunction of stress proteins (8), and programmed cell death (9, 10).

In some animals with scrapie and patients with Creutzfeldt–Jacob disease, death is not accompanied by spongiform degeneration or neuronal loss, which makes sublethal neurochemical alterations in the disease of special interest (11, 12). Previous studies have indicated disturbances in neurotransmission of acetylcholine, dopamine, and γ-aminobutyric acid (GABA) in scrapie-infected animal models (13–15). Neuropeptide Y (NPY) (16), an abundant, mainly inhibitory neuropeptide in mammalian brain (17–20), shows an aberrant mRNA induction in CA3 pyramidal neurons in the hippocampus weeks before the onset of neurological symptoms in the scrapie-infected mice (21). Because NPY in pyramidal CA3 neurons may inhibit glutamate release at the Schaffer collateral-CA1 synapses by binding to presynaptic NPY type 2 receptors (Y2-Rs) (22, 23), we decided to examine whether the increased NPY synthesis is associated with alterations in expression of NPY receptor binding sites in scrapie-infected mice. The recent cloning of a Y2-R (24–27) has permitted *in situ* hybridization studies (28) and detection of possible alterations in the corresponding mRNA levels.

MATERIALS AND METHODS

Animals. Two-month-old male CD-1 mice (Charles River Breeding Laboratories) were inoculated intracerebrally with either 30 μl of RML prion extract (Rocky Mountain Laboratories, Hamilton, MT) or 30 μl of diluent (5% BSA in Ca^{2+}-, Mg^{2+}-free PBS) into the right parietal lobe. In addition, untreated male CD-1 mice were used as normal controls.

Autoradiographic Binding. At 10, 60, 110, 120, 130 and 140 days postinoculation, clinical signs were recorded. After 120 days of ataxia, lack of righting reflexes, kyphosis, tail rigidity, bradykinesia, loss of deep pain sensation, and paresis/paralysis were observed. Five mice per group were anesthetized and decapitated, and the brain was dissected and quickly frozen. Coronal sections (14 μm thick) were cut at the level of the hippocampus in a cryostat (Microm, Heidelberg, Germany). After air-dry, the sections were incubated in Hepes buffer (pH 7.4) for 60 min at room temperature, followed by preincubation for 20 min at room temperature in Hepes buffer containing 0.1% BSA (Sigma) and 0.05% Bacitracin (Sigma), in some cases with NPY (porcine; Bachem) or the Y2 agonist NPY (13–36) (porcine; Peninsula Laboratories) in Hepes buffer with BSA and Bacitracin. After rinsing, sections were incubated in monoiodinated peptide YY (^{125}I-PYY) (0.1 nM) (DuPont/NEN) or in ^{125}I-PYY plus 10^{-6} M of either NPY ligand for 60 min. Then, the sections were rinsed in ice-cold buffer, dried by a cold air stream, and stored in a dessicator over night. The slides were exposed to an autoradiography film (Hyperfilm-^{3}H, Amersham) for 3–6 days at −20°C, developed for 5 min in LX 24 (Kodak), fixed in AL4 (Kodak) for 13 min, and finally rinsed in running water and air dried.

***In Situ* Hybridization Histochemistry.** Five mice per group (as above) were anesthetized and decapitated, and the brains were frozen for *in situ* hybridization. Fourteen micrometer-thick sections were cut at the level of the dorsal hippocampus in a cryostat (Microm) and thawed onto ProbeOn microscope slides (Fisher Scientific). Three synthetic oligonucleotides

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Abbreviations: GABA, γ-aminobyturic acid; NPY, neuropeptide tyrosine; ^{125}I-PYY, monoiodinated peptide YY; PrP, prion protein; PYY, peptide tyrosine tyrosine; Y2-R, NPY receptor of type 2.

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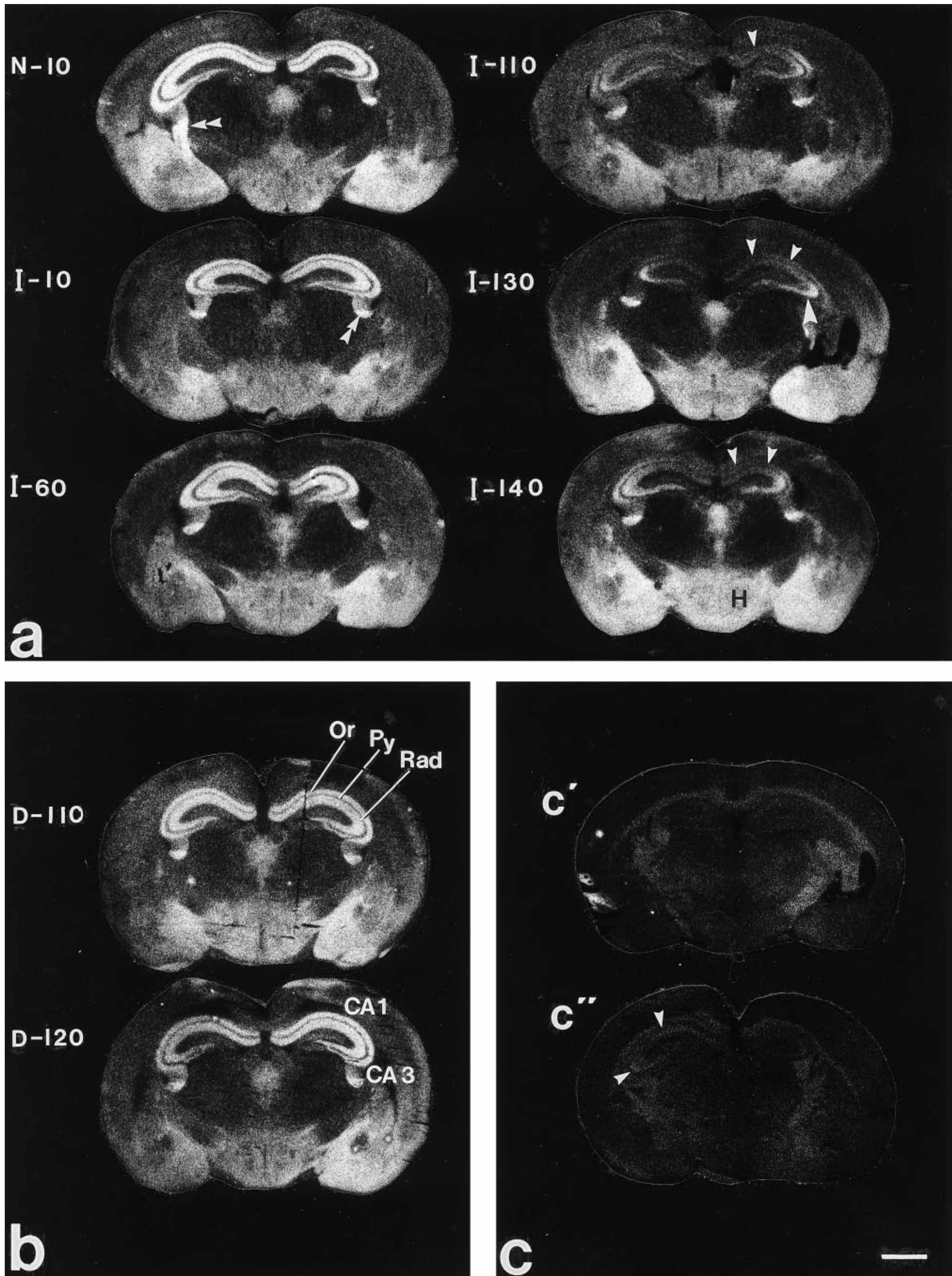

FIG. 1. (*a–c*) X-ray autoradiograms of coronal sections at the level of the dorsal hippocampus of normal (N-10), prion inoculated (I-10 to I-140), and diluent inoculated (D-110, D-120) mouse brains after incubation with ^{125}I-PYY. (*c*) Coincubation with NPY (c′) or the Y2 agonist NPY (13–36) (c″). (*a*) No differences can be observed between the control brain and brains 10 or 60 days after prion inoculation. However, 110 to 140 days after inoculation, there is a dramatic decrease in binding both in stratum radiatum and stratum oriens in the CA1 region (arrowheads), as well as in stratum

(Scandinavian Gene synthesis, Köping, Sweden), (*i*) TGCTT-GGAGATCTTGCTCTCCAGGTGGTAGACAATGCAAC, (*ii*) TGTGCCTTCGCTGATGGTAATGGTCACTTGCAG-CTCCAGGAC, and (*iii*) GAGTTTGTACTCCTTCAGGTC-CAGGACATGGCTGTCGA, complementary to nucleotides sequences of the intracellular loops 2 and 3 and extracellular loop 3 of the rat Y2-R (24), were mixed together and labeled at the 3′ end with (α-^{35}S) dATP (DuPont/NEN) by using terminal deoxynucleotidyltransferase (Amersham) and were purified through QIAquick Spin Columns (Qiagen, Chatsworth, CA) (specific activities 1–4 $\times$ 10^6 cpm/ng oligonucleotide probes). Sections were dried and hybridized and processed as described earlier (29), dehydrated, air-dried, and covered with Amersham β-max film, developed, and fixed. Sections then were dipped in NTB_2 nuclear track emulsion, exposed, developed, and fixed and were examined in a Microphot-FX microscope. For control purposes, an excess ($\times$100) of cold probe was added to the hybridization cocktail.

Quantification. The measurements were performed on a Macintosh IIx computer, equipped with a Quick capture frame grabber board (Data Translation, Marlboro, MA), a Northern light precision illuminator (Imaging Research, St. Catherine's, ON, Canada), and a Dage–MTI CCD-72 series camera (Dage–MTI, Michigan City, IN) equipped with a Nikon 55 mm lens. Image processing was performed with Image software (courtesy of Wayne Rasband, National Institute of Mental Health, Bethesda, MD). ^{14}C-standards (30) or ^{125}I-standards (Amersham) were used for ligand binding autoradiography and *in situ* hybridization, respectively. Four to six separate measurements were made on two different sections of each brain and region studied. The data were analyzed with two-tailed unpaired *t* tests with STATWORKS software (Ver. 1.2, Cricket Software, Malvern, PA). For the analysis of the ^{125}I-PYY binding, sections from 110 and 120 days as well as from 130 and 140 days postinoculation were pooled, and these pooled values are labeled 115 and 135 days, respectively, in the Figures. The quantification method has been described in more detail elsewhere (31).

RESULTS

NPY Binding Sites. A pattern of specific ^{125}I-PYY receptor binding was seen in the dorsal hippocampus of noninoculated and diluent-injected mice. This pattern was similar to the one described by Dumont *et al.* (32) for Y2-Rs. Thus, an intense labeling was present in the strata radiatum and oriens of the CA1 and CA3 regions, extending into the hilus of the dentate gyrus, and the fimbria (and stria terminalis) were labeled strongly (Figs. 1 *a* and *b* and 2). In these areas, over 95% of binding sites was displaced by the full length peptide NPY (1–36) (Fig. 1*c*, c'), and ≈90% was displaced by the Y2 receptor ligand NPY (13–36) (Fig. 1*c*, c″), both at 10^{-6} M, indicating specific, mainly Y2-R receptor, binding.

No significant effect of prion inoculation on ^{125}I-PYY binding was observed at 10 and 60 days postinjection (Fig. 1*a*), nor were there any apparent effects of diluent injection at any time (Fig. 1*b*). At 110–120 and 130–140 days postinoculation, PYY binding in the CA1 strata radiatum and oriens decreased dramatically (Fig. 1*a*), and the quantitative evaluation revealed that binding was ≈15% of that of control mice (Fig. 2). At 110–120 days, the effect on PYY binding in the stratum radiatum CA3 was less pronounced (Fig. 1*a*), and a reduction to ≈65% of the control value ($P < 0.05$) was observed (Fig. 3), but at 130–140 days the change was not statistically different.

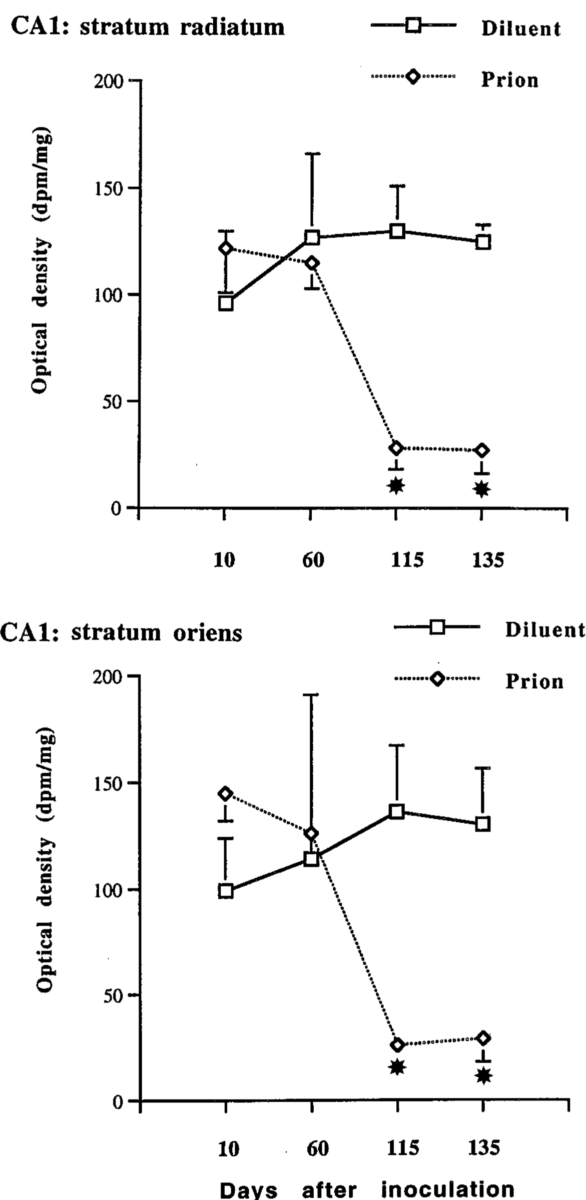

FIG. 2. Graphs showing quantitative evaluation of binding in stratum radiatum and stratum oriens of the CA1 region. Note dramatic decrease in binding at 115 (110/120) and 135 (130/140) days after prion inoculation in both layers. Sections from 110 and 120, as well as from 130 and 140 days, have been pooled for the measurements. Stars indicate significance at the level of $P < 0.01$. The data were analyzed with two-tailed unpaired *t* tests.

In the CA3 stratum oriens, ^{125}I-PYY binding decreased significantly ($P < 0.01$) to 35–50% at both late time points (Fig. 3) with no change in the hypothalamus (Fig. 4).

***In Situ* Hybridization.** Y2-R mRNA expression was clearly detectable in the pyramidal cell layer of the CA1–4 regions and in the granule cell layer of the dentate gyrus (Fig. 4 *a–c*). The strongest expression was measured in CA3 pyramidal cell layers, followed by the dentate gyrus. The expression in CA1 pyramidal neurons was on average 30% of that in CA3 neurons (Fig. 5). The mRNA was not detected in other hippocampal cell populations. At 110, 120, 130, and 140 days postinocula-

oriens of the CA3 region (big arrow heads). Note lack of apparent differences in the hypothalamus (H). Double arrowheads point to stria terminalis. (*b*) No changes in binding can be seen in brains injected with diluent. (*c*) Complete blockage of binding is seen after coincubation with cold NPY (c′) whereas a weak binding can be seen both in the CA1 and CA3 regions (arrowheads) after coincubation with NPY (13–36) (C″). (Bar = 800 μm.) All micrographs have the same magnification.

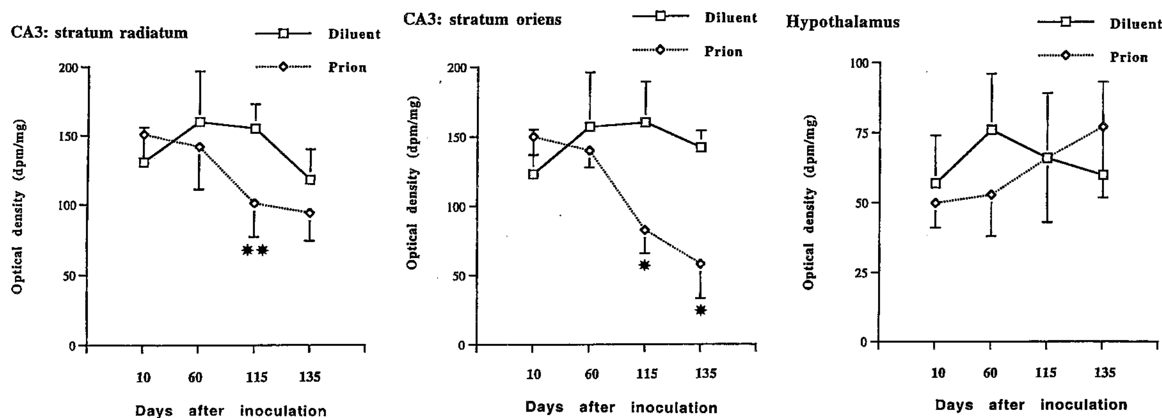

FIG. 3. Graphs showing quantitative evaluation of binding in strata radiatum and oriens of the CA3 region as well as in the hypothalamus. In the CA3 stratum radiatum, a significant change only was observed at 110/120 days whereas a significant decrease was obtained in stratum oriens both at 115 and 135 days. No significant changes were observed in the hypothalamus. Pooling of sections as described in Fig. 2. One star indicates significance at the level $P < 0.01$, and two stars indicate $P < 0.05$. The data were analyzed with two-tailed unpaired t tests.

tion, the mRNA levels in the CA3 and dentate gyrus regions tended to be ≈20% less than in control animals, but the difference was statistically significant only at 130 days in the CA3 pyramidal cells (Fig. 5). In the CA1 pyramidal cells, no differences in the Y2-R mRNA levels were detected between the inoculated and control mice (Fig. 5). None of the hybridization signals described above was observed after incubation with an excess of cold probe.

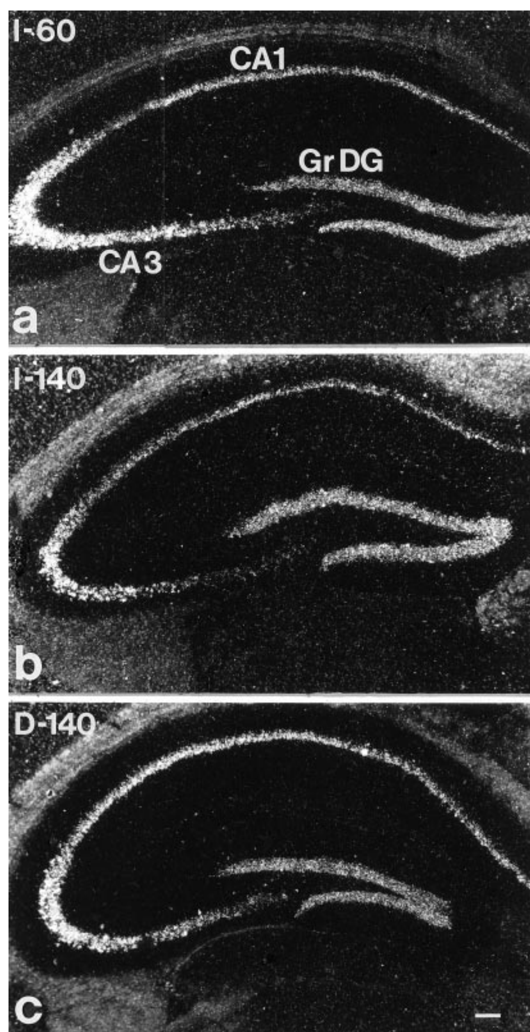

FIG. 4. *In situ* hybridization autoradiographs of coronal sections of mouse brains after prion inoculation at 60 (I-60) (*a*) and 140 days (I-140) (*b*) or 140 days after injection of diluent (D-140) (*c*) showing NPY-R2 mRNA. Y2-R mRNA is expressed at highest levels in the CA3 pyramidal layer. There are no marked differences in hybridization signals between prion (*a* and *b*) and diluent (c) inoculated mice. (Bar = 100 μm). All micrographs have the same magnification.

DISCUSSION

NPY and NPY Receptors in Normal Hippocampus. Two NPY receptors were first recognized on the basis of pharmacological experiments, the postsynaptic Y1-R and the presynaptic Y2-R (33). Here, we show that there is a high density of PYY, mainly Y2 binding sites in several layers of the hippocampal formation in the normal mouse brain, similar to earlier findings in rat (34–36) and in mouse (32). We confirm the Y2 identity by showing Y2-R mRNA in appropriate layers of this brain region with *in situ* hybridization, as shown by Gustafson *et al.* (28) in the rat. We have not considered here Y1-R mRNA or Y5-R mRNA, which, in the rat have been shown to be present in the hippocampal formation (37–39).

The present results suggest that Y2-R mRNA expression in the mouse hippocampus is restricted to the pyramidal cell layer and granule cells in the dentate gyrus but is not present in interneurons in other hippocampal laminal layers, known to mediate inhibitory interactions with principal pyramidal cells (40). Some of these interneurons contain NPY (17–19). Therefore, Y2-Rs are likely located presynaptically in axons of glutamatergic pyramidal and granule cells but not in NPY-containing nerve endings of local interneurons.

NPY Y2 Receptors in Mouse Scrapie. The present results demonstrate that ^{125}I-PPY binding, especially reflecting Y2-Rs presumably of the presynaptic type, is dramatically decreased in the hippocampus during the late phase of the development of the mouse prion disease but still before the manifestation of clinical symptoms. The decrease is most prominent in the strata oriens and radiatum of the CA1 region and is only marginally paralleled by a reduction in the expression of Y2-R mRNA. The mechanism(s) underlying this effect is not known.

A significant loss of NPY-R2 binding sites was seen in the stratum oriens of the CA3 region as well whereas Y2-R binding in the stratum radiatum of CA3 was unaltered. This is most likely due to the fact that mossy fibers originating from the granule cells in the dentate gyrus maintain Y2-R binding capacity even in the latest phase of disease development. The granule cells have been shown to express Y2-R mRNA (present results and ref. 28).

NPY has been reported to modulate higher cognitive functions (41–43) and anxiety (44), and in the hippocampus—the brain structure thought to have a crucial role in learning and

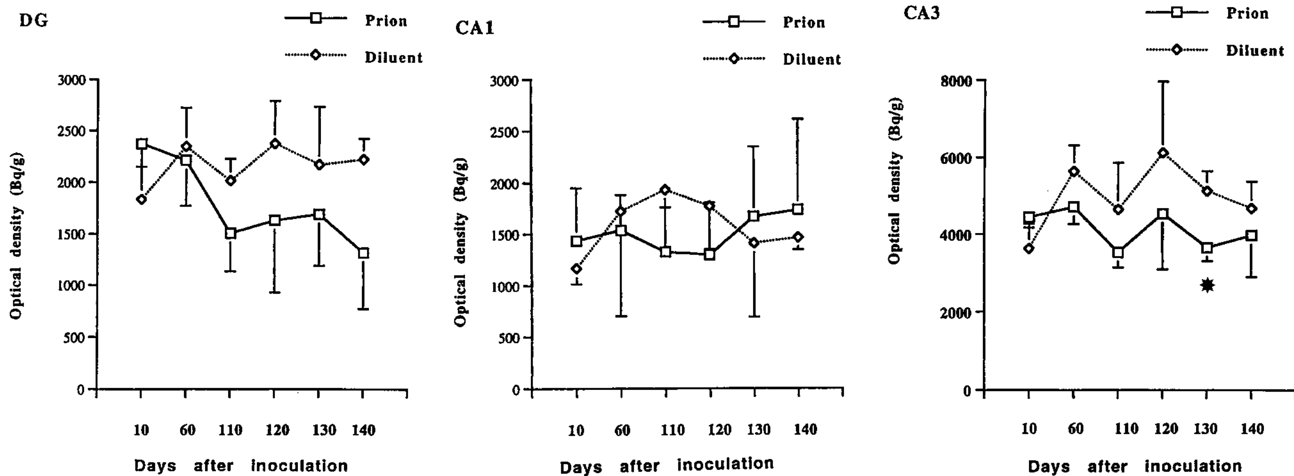

FIG. 5. Graphs showing quantitative evaluation of Y2-R mRNA levels in the dentate gyrus (DG) and in the CA1 and CA3 regions of the hippocampal formation after varying time intervals of prion inoculation or diluent injection. No significant differences can be observed except for a statistically significant decrease in the CA3 pyramidal cells layer 130 days after inoculation ($P < 0.01$). The data were analyzed with two-tailed unpaired t tests.

memory processes—this peptide is known to inhibit glutamate release at the Schaffer collateral synapses on CA1 neurons through presynaptic Y2-Rs (22, 23). Our results showing decreased Y2-R binding in the CA1 striatum radiatum indicate that NPY-mediated inhibition at these synapses may be strongly attenuated. These findings, together with our finding of increased NPY mRNA synthesis in CA3 pyramidal neurons but not in principal NPY-containing interneurons (21), suggest that pyramidal neurons are the primary target neurons for scrapie infection in the mouse hippocampus.

Previous studies with small rodent models have indicated decreased glutamic acid decarboxylase activity and binding to GABA type A receptors in certain brain areas as well as increased numbers of GABA-immunoreactive neurons in the hippocampus (13–15). Taken together with our results of increased expression of NPY mRNA in CA3 pyramidal cells (21) and the loss of NPY binding sites in the CA1 and CA3 regions as shown here, these findings suggest that scrapie prions interact with inhibitory neurotransmission in the hippocampus. The present results suggest that loss of NPY receptor binding is one candidate factor to cause dysfunction of hippocampal circuits and may contribute to the clinical symptoms in mouse scrapie.

The Functional Role of Prion Protein. The normal function of the neuronal cell surface protein PrP^{C} is still not well characterized, and mice homozygous for disrupted PrP genes show normal development and behavior (45). It has been reported that synaptic inhibition is depressed significantly in the hippocampus in PrP null mice, possibly due to a dislocation of GABA type A receptors (46), which in general terms would be in agreement with the present findings. However, PrP^{C} has been directly tested by analyzing for PrP^{C}—GABA type A receptor complexes could not be demonstrated (47). Also, Lledo *et al.* (48) found a normal neuronal excitability and synaptic transmission in the hippocampus of PrP-deficient mice. Recent results show that both PrP^{C} and PrP^{Sc} are present in caveolae-like domains (49), indicating that these structures may represent the site of formation of PrP^{Sc}. Such a process could influence trafficking of receptor proteins, leading to a decreased ability for the NPY ligand to bind to one of its receptors.

NPY and Hippocampal Pathology. It is well known that expression of neuropeptides in hippocampus, in particular NPY, changes in response to various types of injuries, including seizure activity (see ref. 50). Thus, kainic acid treatment (51, 52), pentylentetrazol kindling (51, 53), electric kindling (54, 55), spontaneous epilepsy (56), and cocaine administration (57), induce aberrant expression of NPY and NPY mRNA in hippocampal neurons. The induction of NPY may serve to protect hippocampal neurons against the cytotoxic effects of glutamate, and the increase in NPY mRNA levels in CA3 pyramidal neurons in mouse scrapie was interpreted in a similar way (21). The present findings of a decreased binding further emphasize the plasticity of NPYergic mechanisms in hippocampal pathology. It is possible that the up-regulation of NPY in CA3 pyramidal cells (21) leads to excessive release of NPY and to internalization of the Y2-R and that the internalized receptor does not bind ^{125}I-PYY. Alternatively, the apparent inaccessibility of the Y2-R for binding to Y2-Rs in the CA1 region, as shown here, may, via a feedback mechanism, lead to up-regulation of NPY synthesis in CA3 pyramidal cells (21). Interesting to note, Röder *et al.* (58) have shown an increased PYY binding in CA3 strata radiatum and oriens after 6 and 24 h after limbic seizures and a strong reduction after 7 and 30 days, suggesting a role for NPY receptors in the pathology of chronic epilepsy in rats.

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