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Prions Impair Bioaminergic Functions through Serotonin- or Catecholamine-derived Neurotoxins in Neuronal Cells*[S]

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The conversion of the cellular prion protein, PrP^{C}, to an abnormal isoform, PrP^{Sc}, is a central event leading to neurodegeneration in prion diseases. Deciphering the molecular and cellular changes imparted by PrP^{Sc} accumulation remains an arduous task due to the small number of cell lines supporting prion replication. Here we introduce the 1C11 cell line as a new *in vitro* model to investigate prion pathogenesis. This cell line is a committed neuroectodermal progenitor able to differentiate into fully functional serotonergic or catecholaminergic neurons. 1C11 cells, which naturally express PrP^{C} from the undifferentiated state, can be chronically infected with various prion strains. Prion infection does not promote any noticeable phenotypic change in the progenitor cells nor prevent the onset of the serotonergic and catecholaminergic differentiation programs. Pathogenic prions, however, deviate the overall neurotransmitter-metabolism in both pathways by decreasing bioamine synthesis, storage, and transport, and enhancing catabolism. Noteworthy, oxidized derivatives of both serotonin and catecholamines are selectively detected in the differentiated progenies of infected cells and contribute to irreversible impairment in bioamine synthesis. Finally, the level of PrP^{Sc} accumulation, that of infectivity, and the extent of all prion-induced changes in infected cells appear to be correlated. The report of such specific effects of infection on neuronal functions provides a foundation for dissecting the events underlying loss of neuronal homeostasis in prion diseases.

Prion diseases or transmissible spongiform encephalopathies (TSEs)[3] are a group of fatal neurodegenerative disorders that includes scrapie and bovine spongiform encephalopathy in animals and Creutzfeldt-Jakob disease in humans (1). Although sharing some hallmarks with other neurodegenerative diseases such as Alzheimer or Parkinson disease, TSEs are unique in that they can have a genetic, sporadic, or infectious origin. An enigmatic feature of infectious TSEs is the high latency that may range between a few months up to several decades. The occurrence of various prion strains associated with different incubation periods, clinical manifestations, and neuropathological lesions further illustrates the complexity of the host-pathogen relationship in the TSE field.

A key event in TSE pathogenesis is the conversion of the cellular isoform of the prion protein, PrP^{C}, into an abnormal conformational variant called PrP^{Sc}, which stands for the scrapie isoform of the prion protein (1). The central role played by PrP^{C} in the development of prion diseases was first exemplified by the observation that PrP knock-out mice are resistant to TSE, whereas PrP-overexpressing (tga20) mice exhibit reduced incubation periods as compared with wild-type mice (2). Neurograft experiments carried out on $PrP^{-/-}$ mice using tga20 mice as brain tissue donors clearly demonstrated that the presence of endogenous PrP^{C} is mandatory for PrP^{Sc} to induce pathological alterations (3). Mallucci *et al.* (4) further showed that switching off PrP^{C} neuronal expression in infected mice just before the clinical phase blocks TSE pathogenesis, although abundant prion replication still occurs in extraneuronal tissues. These experiments, thus, outline that prions require neuronal PrP^{C} to exert their toxicity. This notion was recently reasserted and refined in a study based on transgenic mice expressing a glycosylphosphatidylinositol (GPI) anchor-less PrP^{C} (ΔGPI PrP) (5). Indeed, ΔGPI PrP infected mice were found to efficiently replicate scrapie and accumulate high levels of PrP^{Sc} in

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[3] The abbreviations used are: TSE, transmissible spongiform encephalopathy; 5,6-DHT, 5,6-dihydroxytryptamine; 5-HIAA, 5-hydroxyindolacetic acid; 5-HT, serotonin; 7,7′-D, 7,7′-bi-(5-hydroxytryptamine-4-one); CA, catecholamine; DA, dopamine; NE, norepinephrine; PrP, prion protein; PrP^{C}, cellular PrP; PrP^{Sc}, disease-associated scrapie PrP protein; SERT, serotonin transporter; T-4,5-D, tryptamine 4,5-dione; TPH, tryptophan hydroxylase; VMAT, vesicular monoamine transporter; Bt_2AMP, dibutyryl cyclic AMP; DTNB, 5,5′-dithiobis(nitrobenzoic acid); ROS, reactive oxygen species; GPI, glycosylphosphatidylinositol.

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their brains without any sign of clinical illness. As a whole, these data argue for a primary role of neuronal, GPI-anchored PrP^{C} in prion neuropathogenesis.

Despite this overall advance, unraveling the sequence of cellular and molecular events that lead to neuronal cell demise in TSEs still constitutes an ongoing challenge. Exploiting cell culture systems that sustain stable prion replication represents a valuable approach to tackle this issue. Over the past decade prion-infected cell lines of neural or nonneural origin have shed much light on PrP^{Sc} biogenesis, conversion, and trafficking (6). In addition, their use as tools to detect infectivity (7), screen therapeutic compounds (8), or evaluate immunotherapy-based strategies (Ref. 9 and references therein) has been widely acknowledged. However, they have provided but little insight into PrP^{Sc}-induced cellular dysfunction, as in most cases prion infection had no obvious impact on the cell phenotype. Prion replication was nevertheless found to alter cholinergic functions in PC12 pheochromocytoma cells (10). In GT1 hypothalamic cells, infection is associated with reduced viability (11) and increased susceptibility to oxidative stress (12). Increased rates of apoptosis were also recently observed upon infection of primary neuronal cultures derived from tg338 mice overexpressing ovine PrP (13).

Investigating how prions disrupt neuronal cell function may be notably hindered by the relative scarcity of cell systems of neural origin supporting prion replication to date. Here, we introduce the 1C11 neuroectodermal clone, which naturally expresses PrP^{C} (14), as a novel prion-permissive cell line. The murine 1C11 progenitor derives from F9 pluripotent embryonal carcinoma cells and behaves as a committed neuronal stem cell (15). This cell line has the unique capacity to differentiate upon induction into fully functional serotonergic ($1C11^{5\text{-HT}}$) or noradrenergic ($1C11^{NE}$) cells. In the presence of dibutyryl cyclic AMP (Bt_2AMP), the 1C11 progenitor adopts a neuronal morphology and starts expressing neuron-associated markers. Within 4 days, almost 100% of the precursor cells convert into $1C11^{5\text{-HT}}$ cells that display a complete serotonergic phenotype including serotonin (5-HT) synthesis, storage, catabolism, and transport (15). On another hand 12 days after the addition of Bt_2AMP in combination with dimethyl sulfoxide (DMSO), $1C11^{NE}$ cells implement a complete noradrenergic differentiation program (15).

In the present work we demonstrate that 1C11 cells support the replication of various prion strains. Our results show that PrP^{Sc} accumulation does not prevent the entry of 1C11 cells into either neuronal differentiation program, but, however, triggers specific alterations of the overall neurotransmitter-associated functions in both $1C11^{5\text{-HT}}$ and $1C11^{NE}$ cells. In addition, prions promote the production of oxidized derivatives of either serotonin or catecholamines, which are critically involved in bioaminergic dysfunctions. Finally, the extent of all prion-induced changes in infected cells correlate with the level of PrP^{Sc} accumulation and that of infectivity.

EXPERIMENTAL PROCEDURES

Materials—Bt_2AMP, cyclohexane carboxylic acid, DMSO, trypan blue, GSH, dithiothreitol, cysteine, catalase, and superoxide dismutase were purchased from Sigma-Aldrich. [*Phenyl*-6′-^{3}H]paroxetine (20–25 Ci/mmol) and ^{125}I-labeled RTI-55 (2200 Ci:mmol) were from PerkinElmer Life Sciences. [^{3}H]Tetrabenazin (20 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO). Proteinase K and Pefabloc were from Roche Applied Science. Antibodies against tryptophan hydroxylase (TPH) were from Genway (San Diego, CA). Protein concentrations were determined by the BCA protein assay (Pierce).

Cell Culture—1C11 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% 5-HT-depleted fetal calf serum. The cells were induced to differentiate (i) along the serotonergic pathway by the addition of 1 mM Bt_2AMP and 0.05% cyclohexane carboxylic acid and (ii) along the noradrenergic pathway by the addition of 1 mM Bt_2AMP and 0.05% cyclohexane carboxylic acid and 2% DMSO (15). Cell viability was assessed by trypan blue staining exclusion assay, according to the manufacturer's protocol.

Ex Vivo Transmission and Subcloning of Infected Cells—10% brain homogenates were prepared in phosphate-buffered saline containing 5% glucose as described (16). About 1×10^{5} cells in a 35-mm culture dish were incubated for 4–5 h with 1 ml of brain homogenate diluted at 0.2% in serum-free Dulbecco's modified Eagle's medium. 1 ml of fetal calf serum-supplemented medium was then added. Cells were passaged at a 1:10 dilution every 3 or 4 days, and PrP^{Sc} was assayed by immunoblotting after 5 and 10 passages. Subcloning was performed at the first passage after the infection by limiting dilution (0.3 cell/6-mm titer well). The clones were then expanded, and the production of PrP^{Sc} was tested by immunoblotting.

Immunoblotting—Protein extraction and immunological detection of PrP^{Sc} by Western blotting in mouse brain homogenates or cell lysates were conducted as described previously (16). Briefly, 0.5 mg of total protein was digested with 10 μg of proteinase-K at 37 °C for 30 min supplemented with 1 mM Pefabloc and centrifuged at 20,000 $\times$ *g* for 45 min. The pellet was resuspended in 1× Laemmli sample buffer and boiled for 5 min before SDS-10% PAGE and electrotransfer on nitrocellulose membranes (Amersham Biosciences). PrP was detected using a mixture consisting of an equal volume of ascites of SAF 60, 69, and 70 antibodies (Commissariat à l'Energie Atomique, Saclay, France), namely SAF mix. Western blots were revealed with an enhanced chemiluminescence detection system (ECL, Amersham Biosciences).

Bioassay—Tga20 mice overexpressing wild-type Mo *Prnp*-a (kindly provided by Dr. Charles Weissmann) were inoculated intracerebrally with 20 μl of sample containing 0.1 or 10% of Fukuoka-1 brain homogenate or cell extracts (2×10^{6} cells). Cells were submitted to three freeze-thaw cycles, and suspensions were sonicated for 2 min (Cup-horn sonicator). Each sample was inoculated in 6 mice. Mice were monitored every 2 days, and the time at which they presented mouse-scrapie symptoms and terminal points were recorded as described previously (17).

Quantification of Enzymatic Activities—Determination of enzymatic functions was conducted on uninfected and infected cells after induction of serotonergic or catecholaminergic differentiation. Cells were plated onto 10-cm culture dishes and differentiated into serotonergic neurons for 2–4 days or into

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catecholaminergic neurons for 8 days. After 2 washings with cold phosphate-buffered saline, cells were scraped and collected by centrifugation (10,000 × *g*, 3 min, 4 °C). Enzymatic activities for neurotransmitter synthesis were measured radioenzymatically as in Levin *et al.* (18). Briefly, cell extracts were incubated for 30 min at 37 °C in an assay mixture containing 200 mM sodium acetate, pH 6.10, 1 mM ferrous sulfate, 2 mM 6-methyl-H_4-pterin, 40 mM 2-mercaptoethanol, 20 mM sodium phosphate, and 100 μM L-[^{3}H] tryptophan or L-[^{3}H] tyrosine to probe TPH and tyrosine hydroxylase, respectively. Enzymatic activities were determined by quantifying the production of $[^3H]_2O$ in a liquid scintillation counter and expressed as pmol/30 min/mg of cell protein extract.

Glucose-6-phosphate dehydrogenase activity was measured as described by Costa Rosa *et al.* (19). The assay mixture contained 100 mM Tris HCl, 5 mM $MgCl_2$, 0.5 mM NADP+, 1 mM glucose 6-phosphate at pH 7.6. Enzyme activity was assayed by following the rate of NADPH production at 340 nm and 30 °C and expressed as pmol/30 min/mg of cell protein extract.

Determination of Bioamines, Metabolites, and Oxidation Products—The contents in bioamines and related metabolites were measured by HPLC combined to electrochemical detection as described in Lin *et al.* (20) with modifications. Briefly, cells were homogenized in a solution containing 0.5 M acetic acid, 0.5 M sodium acetate, 0.4 M $NaClO_4$, and 4.7 μM guaiacol, used as an internal standard. Homogenization was performed in a Kontes ground glass Duall apparatus. Homogenates were centrifuged at 50,000 × *g* for 1 h at 4 °C. Supernatants were filtered through low speed centrifugation on a 0.45 μm BAS (West Lafayette, IN) polyacetate filter. Filtrates were stored at −80 °C until required. The levels of norepinephrine (NE), dopamine (DA), 5-HT, and related metabolites were measured using HPLC with a coulometric electrode array (ESA Coultronics, ESA Laboratories, Chelsford, MA). Quantifications were made by reference to calibration curves obtained with internal standards.

To determine the chemical structure of oxidized derivatives of 5-HT or catecholamines (CA), collected chromatographic fractions were freeze-dried and analyzed by fast-atom bombardment mass spectrometry using a VG instruments Model ZAB-E spectrometer (Manchester, UK). ^{1}H and ^{13}C NMR spectra were obtained with a Varian model 300XL spectrometer. After identification, the levels of oxidized derivatives of 5-HT and CA were quantified as above according to calibration curves established with pure standards obtained from Sigma or kindly provided by P. Bonetti (Hoffmann-La Roche).

Radioligand Binding Experiments—The presence of vesicular monoamine transporter (VMAT) sites was assessed through tetrabenazine binding as in Mouillet-Richard *et al.* (15). [^{3}H]Paroxetine and ^{125}I-labeled RTI-55 binding to the 5-HT transporter (SERT) was carried out as in Launay *et al.* (21). Briefly, cells were harvested in phosphate-buffered saline containing a mixture of inhibitors (Roche Applied Science). Cells were then pelleted by centrifugation and resuspended in cold buffer A containing 4 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, pH 7.3. The supernatant was poured onto a 20% sucrose cushion and centrifuged at 100,000 × *g* for 90 min. The pellet containing crude membranes was resuspended in buffer B (75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, pH 7.3). Radioligand binding was performed on crude membranes (20 μg of proteins) in 50 mM Tris, pH 7.4 at 37 °C. A 30-min incubation period was followed by addition of ice-cold Tris HCl, pH 7.4 (5 ml, 10 mM). Samples were filtered on polyethyleneimine-treated filters, and radioactivity retained on filters was counted in liquid and solid scintillation counters (Packard Instrument Co.).

Treatment of TPH with Infected Cell Supernatant—TPH was purified from 1C11$^{5\text{-HT}}$ day-4 cells by affinity chromatography using agarose beads coated with antibodies against TPH2 (EC 1.14.16.4). TPH immobilized on agarose beads (about 3 μg) was incubated with 1 ml of 1C11Fk6$^{5\text{-HT}}$ day-4 supernatants for 15 min at 30 °C. Protective agents (50 μM GSH, 500 μM dithiothreitol, 1 mM cysteine, 20 mM DMSO, 100 units/ml superoxide dismutase, 100 units/ml catalase) when present, were added to TPH 10 min before incubation with infected cell supernatant. Beads were washed 3 times with 50 mM potassium phosphate buffer, pH 7.40, and TPH activity was assayed in duplicates.

DTNB titration of SH was carried out by the method of Ellman as modified by Riddles *et al.* (22). Briefly, TPH from 1C11$^{5\text{-HT}}$ day-4 cells exposed or not to 1C11Fk6$^{5\text{-HT}}$ day-4 supernatants was incubated with 50 μM DTNB in 50 mM Tris-HCl, pH 7.50, 6 M guanidine HCl. Absorbance was then recorded at 412 nm using a Cary 100 spectrophotometer (Varian Inc.).

Statistical Analysis—Statistics were performed on raw data using Student's *t* test. The chosen significant criterion was $p < 0.01$.

RESULTS

The 1C11 Neuroectodermal Cell Line Supports Prion Replication—As an attempt to infect the 1C11 cell line, precursor cells were incubated with brain homogenates (0.2%) from mice inoculated with five distinct mouse-adapted agents derived from scrapie: Chandler, 22L, 22A, and ME7 or a human TSE, Fukuoka-1 (23). After 4–5 h of incubation in serum-free medium, the inoculated cells were washed and passaged 5 times (3 weeks). Proteinase K-resistant PrP in the cell lysates was assayed by Western blot. Successful infections of 1C11 cells were obtained for the Chandler-, 22L-, and Fukuoka-1-infected cultures but not for the ME7 and 22A strains (Fig. 1*A*). In all infected cultures, PrP^{Sc} remained detectable after 10 passages (not shown). Notably, PrP^{Sc} signals obtained in the Chandler- and 22L-infected cells were lower than those observed in Fukuoka-1-infected cells. The later cells were selected for further studies and cloned by limiting-dilution to generate cultures with higher and stable PrP^{Sc} production (24). Two stably infected 1C11Fk clones producing elevated (#6) or moderate (#7) PrP^{Sc} levels (Fig. 1*B*) were chosen for further investigation. At this stage no obvious phenotypic differences (growth rate, viability) could be noted between the infected clones and the original 1C11 neuroectodermal cells when cultured in their undifferentiated conditions.

PrP^{Sc} from 1C11Fk Clones Is Infectious—An essential issue was then to assess whether the infected cells also produced infectivity. Pellets of uninfected 1C11 and infected 1C11Fk #6 and #7 cells passaged 7 times after subcloning were inoculated intracerebrally to tga20 mice. Mice ($n = 6$) inoculated with 2×10^6 control 1C11 cells remained healthy over more than 250

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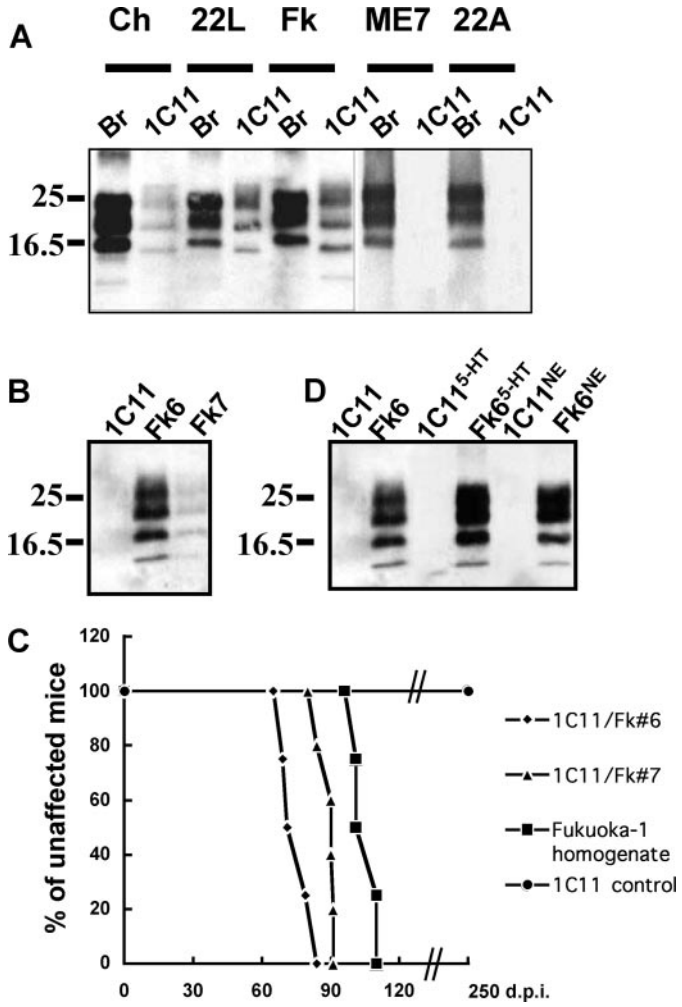


FIGURE 1. ***Ex vivo* prion transmission to the 1C11 cell line.** *A*, detection of PrP^{Sc} in 1C11 cells inoculated with Chandler (*Ch*), 22L, Fukuoka-1 (*Fk*), ME7, and 22A mouse brain homogenates. Lysates from confluent cells, passaged 5 times after inoculation, were digested with proteinase K and subjected to Western blotting. Molecular mass markers indicated on the *left* are in kDa. *B*, clones were isolated from the 1C11 culture inoculated with Fukuoka-1 (*1C11Fk*). After 3 weeks of culture, 23 clones were isolated, expanded, and screened for PrP^{Sc} production. Two stably infected 1C11Fk clones were established that produce high (clone #6) or moderate (clone #7) levels of PrP^{Sc}. *C*, survival curves of Tga20 mice after intracerebral inoculation with 2×10^6 cells or 20 μl of the original Fukuoka-1 homogenate. Mice inoculated with 1C11Fk6 and 1C11Fk7 cells died at days 76 ± 3.5 and 89 ± 1.3, respectively, whereas those inoculated with control 1C11 cells remained healthy (>250 days post-inoculation (*d.p.i.*)). *D*, detection of PrP^{Sc} in prion-infected 1C11Fk6 cells induced toward the serotonergic ($1C11Fk6^{5\text{-}HT}$) or the noradrenergic ($1C11Fk6^{NE}$) differentiation program for 4 days. Uninfected 1C11 progenies are included as negative controls.

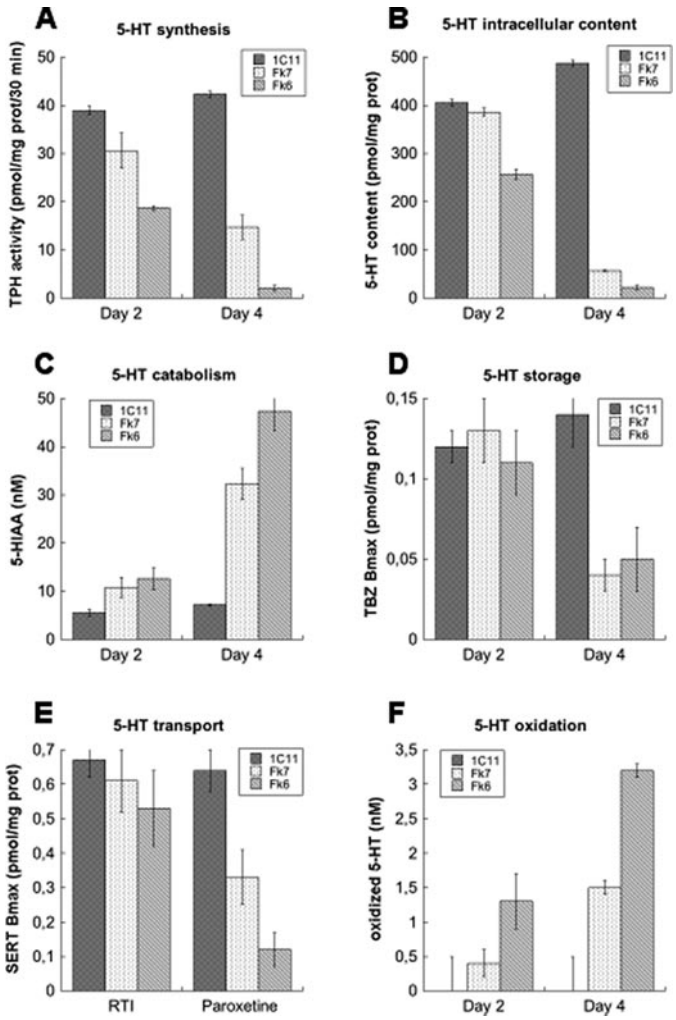


FIGURE 2. **Implementation of serotonergic functions in $1C11Fk^{5\text{-}HT}$ cells.** 1C11Fk6 and 1C11Fk7 cells were induced to differentiate along the serotonergic pathway for 2 or 4 days. 5-HT synthesis (TPH activity) (*A*), intracellular content (*B*), catabolism (5-HIAA concentration) (*C*), storage (VMAT tetrabenazine binding) (*D*), and transport (SERT RTI-55 and paroxetine binding) (*E*) were assessed in cell lysates. The concentration of 5-HT-derived oxidized species (*F*) was measured in supernatants. Control values were determined from $1C11^{5\text{-}HT}$ d2 or d4 cells. *Each bar* represents the mean ± S.E. of triplicate values.

days post-inoculation. In contrast, mice inoculated with 1C11Fk #6 and #7 cells died within a mean incubation time of 76 ± 3.5 (5/5 mice affected) and 89 ± 1.3 (6/6) days, respectively (Fig. 1*C*). As a comparison, the latencies of disease in tga20 mice upon inoculation with 20 μl of the original Fukuoka-1 homogenate diluted to 10 or 0.1% were 76 ± 0.7 (6/6) and 105 ± 2.4 (5/5) days, respectively. Thus, we conclude that 10^6 1C11Fk #6 cells carry approximately the same level of infectivity as 1 mg of crude Fukuoka-1-infected brain. Noteworthy, there was a 13-day delay between the incubation times with clones #6 and #7. It suggests that the #7 clone contains a 10-fold lower level of infectivity than the #6 clone, in agreement with the difference in the PrP^{Sc} contents of the two clones as estimated by Western blot (Fig. 1*B*). Altogether the above data show that 1C11Fk cells are chronically infected and produce infectious prions.

Prion Infection Impairs Serotonin Synthesis in $1C11Fk^{5\text{-}HT}$ Cells—Upon the addition of Bt_2AMP, 1C11 cells undergo serotonergic differentiation with a nearly 100% frequency (15). The acquisition of serotonin-associated functions in differentiating $1C11^{5\text{-}HT}$ cells is highly synchronous and follows a defined time schedule (15). Along the serotonergic pathway, 5-HT synthesis becomes detectable in $1C11^{5\text{-}HT}$ day-2 cells and reaches maximal levels in $1C11^{5\text{-}HT}$ day-4 cells (15).

When exposed to inducers of the serotonergic pathway, 1C11Fk-derived cells, now referred to as $1C11Fk^{5\text{-}HT}$, kept producing PrP^{Sc} (Fig. 1*D*). To evaluate the ability of prion-infected 1C11Fk cells to implement a serotonergic phenotype, we measured the activity of TPH, the rate-limiting enzyme for 5-HT

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synthesis, and the 5-HT intracellular content after the addition of Bt_2AMP. As shown in Fig. 2, *A* and *B*, serotonin synthesis was detected in 1C11Fk$^{5\text{-HT}}$ cells at day 2. However, in 1C11Fk7- and 1C11Fk6-derived progenies, TPH activity reached only 79 and 48%, respectively, of the reference 1C11$^{5\text{-HT}}$ day 2 level (Fig. 2*A*). In parallel, a significant decrease (63% of control) in 5-HT content was recorded in 1C11Fk6$^{5\text{-HT}}$-infected cells as compared with uninfected cells (Fig. 2*B*). With 1C11Fk7$^{5\text{-HT}}$ cells, the 5-HT content was only slightly reduced (95% of control). The impact of prion infection on 5-HT synthesis was even more pronounced at day 4. Although TPH activity and 5-HT content normally increase between day 2 and day 4 of 1C11 serotonergic differentiation (Ref. 15) and Fig. 2, *A* and *B*), both parameters were significantly decreased in 1C11Fk6$^{5\text{-HT}}$ and 1C11Fk7$^{5\text{-HT}}$ cells. The TPH enzymatic activity measured at day 4 in 1C11Fk6$^{5\text{-HT}}$ cells represented only 5% that recorded in 1C11$^{5\text{-HT}}$-uninfected cells. Similarly, the 5-HT content of 1C11Fk6$^{5\text{-HT}}$ cells was reduced to 4.6% that of the reference 1C11$^{5\text{-HT}}$ day-4 level. Interestingly, TPH activity and 5-HT levels were less decreased in 1C11Fk7- than in 1C11Fk6-derived cells. Hence, the mean degree of enzymatic perturbation relates to the mean level of PrP^{Sc} accumulation. Although neurochemical analyses cannot be performed at the single cell scale, the extent of phenotypic alterations (>95% of reference level in 1C11Fk6$^{5\text{-HT}}$ cells) argues against the possibility that a small proportion of cells within the culture carry the overall infectivity and account for 5-HT synthesis impairment. We may instead conclude to a dose-dependent inhibitory effect of prion infection on serotonin synthesis. In contrast to the above findings, prion infection had no impact on the activity of unrelated enzymes such as glucose-6-phosphate dehydrogenase, the rate-limiting enzyme in the pentose phosphate pathway, throughout serotonergic differentiation (supplemental Fig. 1). Hence, 1C11Fk$^{5\text{-HT}}$-infected cells exhibit selective impairment in serotonergic functions. This first set of observations indicates that (i) 1C11Fk cells retain the ability to engage into serotonergic differentiation upon induction, (ii) prion infection hinders the implementation of an optimal TPH activity, (iii) prion-induced alterations are all the more severe since cells accumulate PrP^{Sc} and infectivity (compare Fk6 and Fk7) and, finally, (iv) defects in serotonin synthesis accentuate along the time course of differentiation.

The Overall Serotonin Functions of 1C11Fk$^{5\text{-HT}}$ Cells Are Disturbed—We next compared the extent of 5-HT catabolism in prion-infected 1C11Fk$^{5\text{-HT}}$ cells to that of uninfected 1C11$^{5\text{-HT}}$ cells. At day 4 the concentration of 5-hydroxyindolacetic acid (5-HIAA), a degradative product of 5-HT, was increased by 6.6-fold in 1C11Fk6$^{5\text{-HT}}$ cells (Fig. 2*C*). Again, the rise in 5-HIAA was less pronounced in 1C11Fk7$^{5\text{-HT}}$ than in 1C11Fk6$^{5\text{-HT}}$ cells (4.5-fold). According to Wolf *et al.* (25)[4], such an increase in 5-HIAA production may be accounted for by an exacerbated activation of the 5-HT degrading enzyme monoamine oxidase A. It may also relate to a deficiency in 5-HT storage in infected cells. This is indeed substantiated by the reduced abundance of the VMAT in infected cells, as assessed by [^{3}H]tetrabenazine binding experiments (Bmax in 1C11Fk6$^{5\text{-HT}}$ cells = 36% of control) (Fig. 2*D*).

The serotonergic program of 1C11$^{5\text{-HT}}$ cells is characterized by the induction at day 4 of a functional SERT, which ensures serotonin re-capture from the extracellular space (21). SERT activity is controlled by post-translational modifications. We have previously reported on pharmacological tools that allow discrimination between functional and nonfunctional forms of the SERT protein (21). Indeed, SSRI antidepressant (*e.g.* paroxetine) recognition by SERT is restricted to functional molecules. On another hand, the cocaine congener RTI-55 allows tracing of total (*i.e.* immature, functional, and over post-translationally modified) SERT molecules.

We were, thus, able to assess the SERT onset in infected cells. The total number of SERT molecules in 1C11Fk6$^{5\text{-HT}}$ and 1C11Fk7$^{5\text{-HT}}$ cells was measured through ^{125}I-labeled RTI-55 binding. It represented nearly 80 and 90%, respectively, of the number measured in uninfected 1C11$^{5\text{-HT}}$ cells (Fig. 2*E*). Such a result indicates that despite infection, most cells have kept the capacity to implement the SERT and to acquire a complete serotonergic phenotype. We next evaluated the ability of the SERT molecules to bind [^{3}H]paroxetine. With 1C11$^{5\text{-HT}}$ control cells, the maximal binding (Bmax) value was identical to the Bmax for ^{125}I-labeled RTI-55, as expected for maximal 5-HT uptake efficiency (21). With 1C11Fk6$^{5\text{-HT}}$ cells, the Bmax for [^{3}H]paroxetine was only 22% that of the ^{125}I-labeled RTI-55 Bmax value. Hence, we deduce that in 1C11Fk6$^{5\text{-HT}}$ cells, only 22% of the SERT molecules have the capacity to take up serotonin. With 1C11Fk7$^{5\text{-HT}}$ cells, the ratio of paroxetine to RTI-55 binding equaled 54%, indicating a milder impairment of SERT function. The above results suggest that in prion-infected 1C11Fk$^{5\text{-HT}}$ cells from 50% (clone Fk7) to 75% (clone Fk6) of the total number of SERT molecules have undergone post-translational changes that impair their functionality. Altogether, our data indicate that the overall serotonergic functions of 1C11$^{5\text{-HT}}$ cells (*i.e.* 5-HT synthesis, catabolism, storage, and transport) are affected by pathogenic prions.

1C11Fk$^{5\text{-HT}}$ Serotonergic Progenies Produce Serotonin-derived Oxidized Species That Inhibit Tryptophan Hydroxylase—Because oxidative stress is a hallmark of TSEs (26, 27), we wondered whether prion infection would trigger the generation of oxidized bioamine derivatives in 1C11Fk-differentiated progenies. 1C11Fk$^{5\text{-HT}}$ cell culture supernatants were analyzed through HPLC coupled to electrochemical detection. Fractions in phase with redox potential deviation selectively obtained with infected supernatants were collected, dried, and analyzed by fast atom bombardment mass spectrometry and NMR. Chromatographic peaks 1, 7, and 10 were identified as tryptamine 4,5-dione (T-4,5-D), the dimer 7,7′-bi-(5-hydroxytryptamine-4-one) (7,7′-D), and 5,6-dihydroxytryptamine (5,6-DHT), respectively (Fig. 3). As shown in Fig. 2*F*, the global concentration of these 5-HT oxidation products was higher in 1C11Fk6$^{5\text{-HT}}$ than in 1C11Fk7$^{5\text{-HT}}$ supernatants. None of these compounds was detected in 1C11$^{5\text{-HT}}$ control cells (Fig. 2*F*). Of note, all three compounds identified in 1C11Fk$^{5\text{-HT}}$ supernatants are detected in central serotonergic neurons exposed to methamphetamine, where they are endogenously produced as a consequence of serotonin oxidation (28).

[4] S. Mouillet-Richard and J.-M. Launay, unpublished observations.

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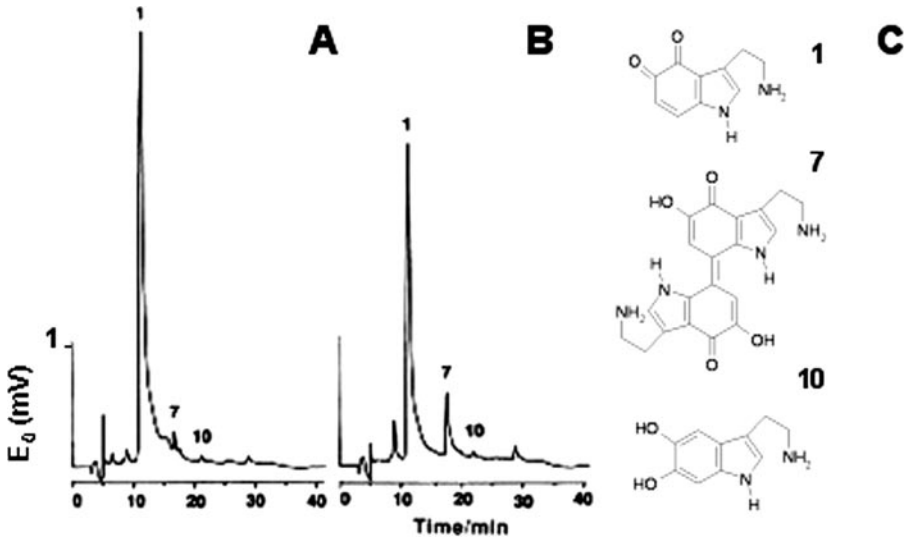
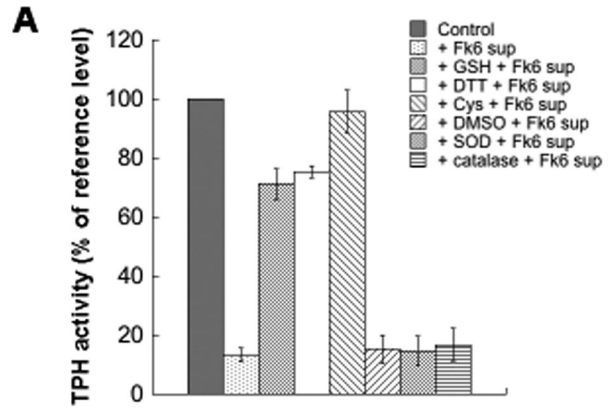


FIGURE 3. **Production of oxidized bioamines in 1C11Fk-derived serotonergic progenies.** Chromatogram profiles obtained with supernatants from 1C11Fk6$^{5\text{-HT}}$ (*A*) and 1C11Fk7$^{5\text{-HT}}$ (*B*) cells. *C*, peaks were identified as: *1*, T-4,5-D; *7*, 7,7′-D); *10*, 5,6-DHT.



B

SH titration by DTNB	
TPH control	12.35 ± 0.42
TPH + Fk6 sup	0.67 ± 0.21

FIGURE 4. **Impact of 1C11Fk$^{5\text{-HT}}$-derived 5-HT oxidation products on TPH activity.** *A*, TPH was isolated from 1C11$^{5\text{-HT}}$ day 4 cells and used to calibrate the control 100% activity. 1C11$^{5\text{-HT}}$-derived TPH was preincubated or not with the indicated protectant and exposed to 1C11Fk6$^{5\text{-HT}}$ supernatant. Remaining TPH activity is reported as % of the reference level. *DTT*, dithiothreitol; *SOD*, superoxide dismutase. *B*, free cysteines were titrated by DTNB in 1C11$^{5\text{-HT}}$-derived TPH, exposed or not to 1C11Fk6$^{5\text{-HT}}$ supernatant (*sup*). All values are the means ± S.E. of $n = 4$ experiments performed in duplicate.

5,6-DHT and T-4,5-D mediate the neurotoxicity of methamphetamine toward serotonergic neurons and are classified as serotonin neurotoxins (29). The production of these oxidized species in 1C11Fk$^{5\text{-HT}}$ cells, within the nanomolar range, did not, however, impact on cell viability according to trypan blue exclusion assay (data not shown).

Because T-4,5-D has been reported to irreversibly inactivate TPH through covalent modification of cysteine residues (30), we evaluated the ability of 1C11Fk6$^{5\text{-HT}}$ supernatants to interfere with TPH activity. In these experiments 1C11$^{5\text{-HT}}$ control cells were used as the source of TPH (see "Experimental Procedures"). Upon incubation with 1C11Fk6$^{5\text{-HT}}$ supernatant, the activity of 1C11$^{5\text{-HT}}$-derived TPH was reduced by 87% (Fig. 4*A*). To get further insight into the mechanisms sustaining the inhibition of TPH activity, 1C11$^{5\text{-HT}}$-derived TPH was incubated with various protective agents before exposure to 1C11Fk6$^{5\text{-HT}}$ supernatants. As shown in Fig. 4*A*, TPH activity was not protected by DMSO, superoxide dismutase, or catalase, which scavenge hydroxyl radicals, superoxide, and hydrogen peroxide, respectively. Thus, we may rule out that loss of TPH activity upon exposure to 1C11Fk6$^{5\text{-HT}}$ supernatants is directly caused by ROS. In contrast, GSH or dithiothreitol partially rescued TPH activity from inhibition by 1C11Fk6$^{5\text{-HT}}$ supernatants and cysteine fully protected TPH from inactivation (Fig. 4*A*). Thus, we conclude that prion-infected cells produce reactive compounds distinct from ROS, which alter TPH activity through selective attack on SH groups. Upon incubation with 1C11Fk6$^{5\text{-HT}}$ supernatants, all 12 cysteine residues of TPH were in fact modified, as determined by titration of free SH groups with DTNB (Fig. 4*B*). As a whole, these results point to serotonin oxidation products as critical mediators of the damaging effects of prions on neurotransmitter-associated functions.

1C11Fk^{NE} Cells Display an Aberrant Noradrenergic Metabolism and Produce Oxidized Derivatives of Catecholamines—Having shown drastic alterations in neurotransmitter homeostasis upon prion infection along the serotonergic differentiation pathway, we wondered whether the noradrenergic progenies of 1C11Fk cells, referred to as 1C11Fk^{NE} cells, would also be affected by the presence of PrP^{Sc} (see Fig. 1*D*). We first compared the activity of tyrosine hydroxylase, the rate-limiting enzyme for NE synthesis, in 1C11Fk6^{NE} cells and in 1C11^{NE} control cells. As depicted in Fig. 5*A*, tyrosine hydroxylase activity was reduced by 95% in the infected cells. In addition, 1C11Fk6^{NE} cells exhibited dramatic reductions in the levels of L-DOPA (L-3,4-dihydroxyphenylalanine 13% of control), DA (4% of control), and NE (3% of control) (Fig. 5*B*). This reduction in CA content was accompanied by an increase (270% of control) in the level of the NE metabolite 3-methoxy-4-hydroxyphenylglycol (*MHPG*) (Fig. 5*C*). At last, in 1C11Fk6^{NE} cells the binding capacities of the vesicular transporter (VMAT) were below detectable levels, indicating defects in CA storage (Fig. 5*D*). 1C11Fk6^{NE} supernatants contained various catecholamine oxidation products, such as 6-hydroxydopamine, and tetrahydroisoquinolines (*e.g.* salsolinol) (Fig. 5*C* and data not shown). The noradrenergic progenies of the #7 clone also exhibited altered CA-associated functions. However, in 1C11Fk7^{NE} cells the impact of prion infection was less drastic than in 1C11Fk6^{NE} cells (Fig. 5). Thus, we may conclude to the dose-dependent effect of PrP^{Sc} on CA metabolism, similar to

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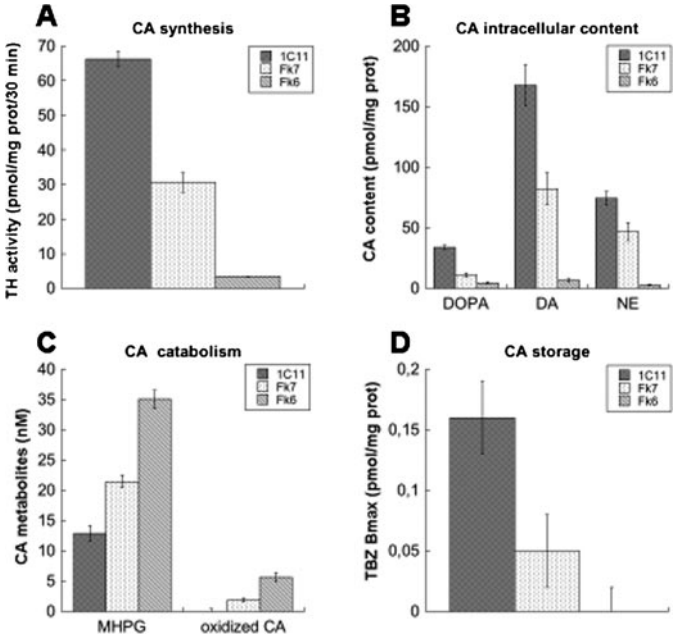


FIGURE 5. **Evaluation of noradrenergic functions in 1C11Fk^{NE} cells.** 1C11Fk6 and 1C11Fk7 cells were induced to differentiate along the noradrenergic pathway for 8 days. Cell lysates were collected to assess CA synthesis (tyrosine hydroxylase activity) (*A*), intracellular content (*B*), catabolism (3-methoxy-4-hydroxyphenylglycol (*MHPG*) concentration) (*C*), and storage (VMAT tetrabenazine binding) (*D*). The concentration of CA-derived oxidized species (*C*) was measured in supernatants. Control values were determined from 1C11^{NE} d8 cells. Each *bar* represents the mean ± S.E. of triplicate values. DOPA, L-3,4-dihydroxyphenylalanine.

the changes evidenced along the serotonergic differentiation pathway.

The above results emphasize that, although infected cells have kept the ability to express all biochemical hallmarks of serotonergic or catecholaminergic differentiation, the presence of PrP^{Sc} deviates the overall neurotransmitter metabolism specific to each pathway. In both cases bioamine synthesis and storage are decreased, and catabolism is enhanced. They point to bioamine-derived oxidized species as a selective consequence of prion accumulation in serotonergic or noradrenergic neuronal cells and a proximal cause of neurotransmitter dysfunction.

DISCUSSION

The present work introduces the 1C11 progenitor, which naturally expresses PrP^{C}, as a novel prion-susceptible cell line. The 1C11 clone efficiently replicates Chandler, 22L, and Fukuoka-1 prions. The two clones selected here from a Fukuoka-1-infected culture not only generate PrP^{Sc} but also produce infectivity, as demonstrated by animal assay (Fig. 1*C*). The incubation time of the disease in inoculated mice was inversely related to the level of PrP^{Sc} in the infected Fk clones, thus highlighting a correlation between PrP^{Sc} production and infectivity.

The 1C11 cell line used in our study exhibits the properties of a neuroectodermal stem cell. It expresses neuronal precursor cell markers and lacks neuronal functions (15). Noteworthy, prion infection of mouse fetal brain-derived progenitors was recently achieved (31, 32). That 1C11 precursor cells and neuronal stem cells sustain prion replication raises the possibility that prion accumulation may interfere with adult neurogenesis in TSE-affected animals or patients. Actually, recent data indicate that PrP^{C} positively regulates embryonic and adult neurogenesis (33). In view of the notion that prion pathogenesis involves some corruption of PrP^{C} normal function in neuronal cells (34), that PrP^{Sc} could affect neuronal stem cell differentiation in TSEs deserves consideration.

Our data show that PrP^{Sc} accumulation has no detectable effect on the phenotype of 1C11 undifferentiated cells. Prion replication does not alter the choice of fate of 1C11 cells nor does it hinder the implementation of serotonergic or noradrenergic functions. On another hand, prion infection has drastic consequences on the overall bioamine metabolism of differentiated 1C11Fk progenies. Our study takes advantage of the homogeneous and synchronous onset of neurotransmitter synthesis, catabolism, storage, and uptake in 1C11$^{5\text{-HT}}$ or 1C11^{NE} cells (15) and relies on pharmacological approaches to accurately quantify the overall phenotypic functions in infected cells. In both prion-infected 1C11Fk$^{5\text{-HT}}$ and 1C11Fk^{NE} cells we detect major impairments in bioamine synthesis, storage, uptake, and increases in serotonin or catecholamine catabolism. The prion-induced changes are comparable whether cells differentiate along the serotonergic or the noradrenergic pathway, adding to the significance of our observations. Besides, our quantitative analysis highlights a dose-dependent effect of prions on serotonergic or noradrenergic functions since the phenotypic alterations are all the more pronounced because the cells accumulate PrP^{Sc} and infectivity (Fk6 *versus* Fk7). It is of note that infection of 1C11 cells with Chandler prions promoted similar biochemical changes in differentiated cells (supplemental Fig. 2). Although unprecedented in *in vitro* prion infection studies, our overall observations are in agreement with the perturbations of monoaminergic functions reported in various experimental models of prion diseases (see Ref. 35 for review and references therein). Our conclusions also fit in with clinical (*e.g.* depression, (36)) or functional (*e.g.* reduced SERT availability (37)) evidence for dysfunction of the 5-HT system in TSE-affected patients.

Here, one major finding is the detection of serotonin and catecholamine oxidation products in the supernatants of infected cells. Serotonin and catecholamines are highly prone to oxidation and are normally protected against oxidative attack by storage into synaptic vesicles. The formation of oxidized derivatives in the differentiated progenies of 1C11Fk-infected cells likely involves alterations in 5-HT or CA storage. This idea is indeed substantiated by our observation that VMAT availability is reduced. Moreover, Wrona *et al.* (28) reported that exposure of cytoplasmic 5-HT to hydroxyl radicals yields 5,6-DHT and 4,5-dihydroxytryptamine, itself converting into T-4,5-D. The 7,7′-D compound then arises from dimerization of T-4,5-D (28). Similarly, defects in intracellular DA compartmentalization led to the generation of 6-hydroxydopamine and quinones and are thought to contribute to the pathogenesis of Parkinson disease (38). Because we detect these 5-HT- or CA-derived oxidized compounds in the supernatants of 1C11Fk$^{5\text{-HT}}$- or 1C11Fk^{NE}-infected cells, they are likely released through SERT- or NE transporter-mediated efflux (39).

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The formation of oxidized derivatives of 5-HT and CA clearly mirrors elevated ROS levels in infected 1C11Fk$^{5\text{-HT}}$ or 1C11Fk^{NE} cells. That prion infection alters the cellular redox state in 1C11Fk cells is in keeping with various *in vitro* and *in vivo* observations (26, 27, 40). An enhanced ROS level could simply stem from a deviation of the signal transduction pathways normally coupled with PrP^{C}, which involve the ROS-generating enzyme NADPH oxidase (41, 42). Such a scenario would fit in with the current view that prion injury is primarily caused by distortions of PrP^{C} normal function.

What are the molecular mechanisms responsible for the alterations in bioamine metabolism in 1C11Fk progenies? The generation of 5-HT and CA oxidation products appears to greatly contribute to neurotransmitter dysfunction. Our data indeed demonstrate that 1C11Fk6$^{5\text{-HT}}$-derived oxidized species have the potential to cancel TPH activity through modification of SH groups (Fig. 4). A modification of critical cysteine residues by CA-derived quinones may also account for tyrosine hydroxylase inactivation in 1C11Fk^{NE} cells (43). Because, T-4,5-D alters SERT function through reaction with sulfhydryl groups (44), we suspect oxidative attack on the SERT cysteines to be involved in transporter dysfunction as well. Actually, we observed that more than 50% of the nonfunctional SERT molecules in 1C11Fk6$^{5\text{-HT}}$ cells could revert to a functional state upon treatment with an SH reducing agent.[4] The same mechanism of SH oxidation may finally apply to the vesicular monoamine transporter, which contains multiple cysteine residues critical for tetrabenazine recognition (45). Thus, generation of 5-HT and CA oxidation products capable to react with SH groups may deeply contribute to neurotransmitter dysfunction. The production of such bioamine-derived oxidized species may also account for increased 5-HT or CA catabolism. 5,6-DHT concentrations below 1 μM can induce a rapid depletion of 5-HT associated with an increase in the level of 5-HIAA (46). The serotonin or catecholamine oxidation products act in a dose-dependent manner (46). At high concentrations the neurotoxins 5,7-DHT and 6-hydroxydopamine selectively damage serotonergic or dopaminergic neurons, respectively (29). Here, with 1C11Fk-derived progenies, these oxidized species exert toxicity at the level of neurotransmitter-associated functions only. The low concentration of these oxidation products in 1C11Fk$^{5\text{-HT}}$ and 1C11Fk^{NE} cells (Fig. 2*F* and 5*C*) may explain why the cells remain viable. Nonetheless, within the context of prion diseases latencies, chronic accumulation of bioamine-derived neurotoxins in infected neurons could progressively impair neuronal cell function and, in the long-term, lead to neuronal cell demise in TSEs.

Collectively, our data draw a link between prion infection, cellular redox imbalance, and dysfunctions in neurotransmitter metabolism. We may assume that the disruption of cell homeostasis involves prion-induced alterations in PrP^{C} normal signaling activity (42, 47). Because PrP^{Sc}-induced biochemical alterations coincide with the acquisition of neurotransmitter-related functions, the 1C11 cell line may be a valuable model to investigate into the time-sequence of cellular insults, which contribute to the TSE-associated neurodegeneration. Another challenge will be the identification of therapeutic agents with the capacity to antagonize PrP^{Sc} detrimental effects while preserving PrP^{C} normal function.

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Prions Impair Bioaminergic Functions through Serotonin- or Catecholamine-derived Neurotoxins in Neuronal Cells

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