Genetic heterogeneity versus molecular analysis of prion susceptibility in neuroblastoma N2a sublines

Stéphanie Chasseigneaux · Manuela Pastore · Janice Britton-Davidian · Elodie Manié · Marc-Henri Stern · Jacques Callebert · Josette Catalan · Danielle Casanova · Maxime Belondrade · Monique Provansal · Yonghua Zhang · Alexander Bürkle · Jean-Louis Laplanche · Nicolas Sévenet · Sylvain Lehmann

Abstract The neuroblastoma-derived cell line N2a is permissive to certain prion strains but resistant sublines unable to accumulate the pathological proteinase-K resistant form of the prion protein can be isolated. We compared for gene expression and phenotypes different N2a sublines that were susceptible or resistant to the 22L prion strain. Karyotypes and comparative genomic hybridization arrays revealed chromosomal imbalances but did not demonstrate a characteristic profile of genomic alterations linked to prion susceptibility. Likewise, we

showed that this phenotype was not dependent on the binding of PrPres, the expression of the prion protein gene, or on its primary sequence. We completed this analysis by looking using real-time quantitative PCR at the expression of a set of genes encoding proteins linked to prion biology. None of the candidates could account by itself for the infection phenotype, nevertheless sublines had distinct transcriptional profiles. Taken together, our results do not support a role for specific genomic abnormalities and possible candidate proteins in N2a prion susceptibility. They also reveal genetic heterogeneity among the sublines and serve as a guidance for further investigation into the molecular mechanisms of prion infection.

S. Chasseigneaux and M. Pastore contributed equally to the work.

S. Chasseigneaux · J. Callebert · J. L. Laplanche · N. Sévenet
EA 3621, Faculté de Pharmacie, Université Paris 5,
4 avenue de l'Observatoire, 75270 Paris cedex 06, France

M. Pastore · D. Casanova · M. Belondrade · M. Provansal ·
S. Lehmann (✉)
Institut de Génétique Humaine du CNRS, UPR 1142,
141, rue de la Cardonille, 34396 Montpellier cedex 5, France
e mail: sylvain.lehmann@igh.cnrs.fr

J. Britton Davidian · J. Catalan
Institut des Sciences de l'Evolution,
UMR 5554 Montpellier, France

E. Manié · M. H. Stern
INSERM U520, Institut Curie, 26 rue d'Ulm,
75248 Paris cedex 05, France

Y. Zhang · A. Bürkle
Department of Gerontology, SCMS,
Newcastle General Hospital, Newcastle University,
Newcastle upon Tyne NE4 6BE, UK

A. Bürkle
Chair of Molecular Toxicology, Department of Biology,
University of Konstanz, Box X911,
78457 Constance, Germany

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders that affect humans and animals [32]. They are characterized by spongiform degeneration of the brain, nerve cell loss, astrocytosis and sometimes amyloid plaques. The causal agent or prion, is believed to be a proteinaceous infectious particle consisting of a conformational variant of the normal host protein PrP^C [31]. Several strains of prions, with distinct biological properties, and with possible different conformation, have been characterized. In prion diseases, the key event in pathogenesis is the conversion of PrP^C into a protease resistant PrP isoform termed PrPres that is thought to occur at the cell surface or in the endocytic pathway [2]. Genetic forms of the disease in humans are all linked to *Prnp*, the host gene encoding the prion protein [32]. Presence of this gene is necessary for the development and the transmission of

the disease [5]. Nevertheless, differences in incubation time, for example in rodent models of experimental prion diseases or in natural scrapie in sheep, suggest that other genes modulate susceptibility to prions [19, 24].

Over the last decade numerous studies were performed using different approaches to identify cofactors involved in prion pathogenesis in natural or experimental prion diseases. To avoid the complexity of heterogeneous tissues like the brain, several cell culture models persistently infected with mouse-adapted scrapie strains have been developed and studied [13, 36]. A limited number of cell lines support prion replication *ex vivo*, and one, the N2a cell line, has been used uninfected and infected in studies to detect changes in gene or protein expression upon infection [6, 13].

Interestingly, N2a cells have been known for long time to be permissive to prions but the stability and the level of prion infection displayed a high variability between groups and laboratories. Bosque and Prusiner [3] subcloned N2a cells and isolated new sublines, some of which were highly susceptible to prions and some appeared to be resistant. The molecular basis of this phenotypic difference is as yet unknown but recent work used this approach to develop efficient *in vitro* prion assays [20]. We decided to compare gene expression patterns of six different N2a cell sublines, four susceptible and two resistant ones to the scrapie mouse-adapted prion strain 22L. Our aim was to identify molecular characteristics correlated with their susceptibility phenotypes. After karyotype analysis we rapidly were confronted to the issue of chromosomal differences between cell lines which could preclude genetic analysis. Chromosomal rearrangements were then explored using array-based comparative genomic hybridization (aCGH), first applied in this context to the mouse genome. We also focused on specific expression of 21 genes related to prion biology using real-time quantitative PCR (qRT-PCR). Taken together our results are important to progress toward the comprehension of the molecular mechanisms of prion susceptibility.

Results

Expression of PrP^C and infection of the N2a sublines

The amount of PrP^C whose expression level is a determinant for prion infection was evaluated by western blot in the different sublines. PrP^C displayed the same electrophoretic pattern and typical apparent molecular weights 22 to 37 kDa due to *N*-glycosylations in all sublines (Fig. 1). PrP^C abundance evaluated in three independent experiments after GAPDH normalization, was lowest and nearly identical in sublines R4 [$56 \pm 5\%$, of the value for

N2a, $P < 0.05$, Kolmogorov Smirnov (KS) test] and R10 ($57 \pm 3\%$, $P < 0.05$, KS test), highest in D11 ($170 \pm 6.5\%$, $P < 0.05$, KS test) and not significantly different in the other sublines (F1: $93 \pm 6\%$; H6: $108 \pm 12\%$; G9: $110 \pm 10\%$, NS, KS test).

After infection with 22L of the different N2a sublines, the largest amount of PrPres was produced repeatedly by the previously described [50] susceptible subline D11 (Fig. 1). G9, H6 and R10 sublines accumulated PrPres and were considered also to be “susceptible”. PrPres was detectable in these lines for up to 15 passages indicating that they were able to maintain prion infection (not shown). Electrophoretic pattern of PrPres was identical in all sublines with, as expected, three major bands at 21, 25 and 30 kDa corresponding to un-, mono- and di-glycosylated isoforms. A minor band at 18 kDa was observed in D11 and corresponded to a truncated fragment already observed in infected N2a cells [27], which was more easily observed in this than other sublines due to the greater accumulation of PrPres. Two clones, R4 and F1, did not accumulate significant amount of PrPres after infection and were considered “resistant”. Prolonged exposure of the western blots (not shown) revealed however a faint band, only in R4, corresponding to the mono-glycosylated form of PrPres.

No obvious differences in growth, cell death or morphology between sublines were detected, with the exception of F1 which display abnormal giant cells. This is illustrated Fig. 1c where the surface immunofluorescent detection of PrP^C also revealed that even in the “most” resistant subline, PrP^C was readily detectable and present on the cell surface.

Interestingly, when the binding and internalization of PrPres to N2a, D11 and F1 was tested by incubation with 0.05% homogenates for 1 h on ice or 3 h on ice followed by 3 h at 37°C (Fig. 1d), F1 had repetitively slightly less signal. This could be related to its particular shape (Fig. 1c). However, this difference was not dramatic enough to explain the phenotype in agreement with similar experiments published recently [43].

Karyotype of N2a cell sublines

To evaluate the genetic variability among cell lines, karyotypes analysis was done on parental N2a, D11 and F1 cells (more than 20 individual cells for each were counted). The total number of chromosomes was increased in the parental N2a (Fig. 2) confirming the polyploidy already noted in the ATCC description. The mean number of chromosome was comparable in D11 and N2a, but was much higher in F1 with values of 102 ± 40 , 104 ± 35 , and 170 ± 43 , respectively. Regarding individual cells, around 15 % of N2a and D11 cells were tetraploid, while all F1 were at

Fig. 1 PrP^C and PrPres in N2a sublines. **a** Western blot of PrP^C using an N terminal antibody (SAF32). N2a sublines (D11, R10, G9, H6, R4, F1) were all derived from the parental cell line N2a. N2a58 are cells transfected with moPrP that over express the protein by a factor 3.5 [27]. Based on parental N2a expression, PrP^C level calculated on three independent experiments after GAPDH normalization were for the different sublines; D11: $170 \pm 6.5\%$, R10: $57 \pm 3\%$, G9: $110 \pm 10\%$, H6: $108 \pm 12\%$, R4: $56 \pm 5\%$ and F1: $93 \pm 6\%$. **b** After infection of the different sublines with 22L (0.01%), PrPres was detected by western blot (SAF mix) after PK digestion. PrPres relative intensities were the following D11: 100% (taken as

reference), R10: 62%, G9: 83%, H6: 31%, R4: <1% and F1: <1%. The data are representative of three or more independent experiments. **c** D11 and F1 cells were fixed, permeabilized, and analyzed by indirect immunofluorescence for PrP using SAF32. The protein was detected on the cell membrane of both sublines. The lower intensity in F1 matched the lower expression observed by western blot. A “giant” cells is observed in F1 and may correspond to cells with the highest chromosomal abnormalities. **d** PrPres associated with the cells was detected after incubation with 0.05% brain homogenates for 1 h on ice or 3 h on ice followed by 3 h at 37°C as described elsewhere [43]

least tetraploid, 8% of the cells having four times the normal number of chromosomes. The latter may correspond to the large cells observed in the cultures (Fig. 1c). The chromosomes were sorted by their morphological aspect revealing that, even though N2a and D11 had comparable total numbers, there were important differences in the % of metacentric chromosomes which results from abnormal fusions. As illustrated Fig. 2, the mean % of metacentric chromosome were about 14, 8 and 5 for D11, N2a and F1, respectively.

aCGH profiles of N2a sublines

We first established aCGH profiles to compare the parental N2a with the normal A/HeJ mouse strain DNA (Fig. 3), as this cell line was derived from a spontaneous neuroblastoma in this strain [16]. Twenty-three genomic gains and 16 genomic losses were found in N2a. Most of these alterations were also detected in the sublines (see below).

When N2a and sublines were analyzed (Fig. 3), diverse degrees of divergence were revealed, from highly modified profiles (D11, F1), minor changes (R10) to no detectable change (G9, R4), with no obvious correlation with their resistance or susceptibility to 22L. Indeed, G9 (susceptible) and R4 (resistant) sublines had almost identical aCGH profiles. Importantly, we also confirmed that all sublines had two *Prnp*^a alleles which sequencing of the coding region did not reveal any mutation or sequence divergence (not shown).

Selection of the genes analyzed using real-time PCR

To determine if some genes encoding proteins linked to prion biology had variable expression in susceptible or resistant sublines, 21 candidate genes distributed in three groups on the basis of previous studies were selected (Table 1). The first group contained *Prnp*, the major gene controlling susceptibility to prions [5], and the following

Fig. 2 Karyotypes of N2a, D11 and F1. **a** Micrographs of three karyotypes obtained after Giemsa staining. The higher number of chromosomes in F1 cells is evident. **b** Bar graph of the total number of chromosome in the three cell lines as follows: N2a: 102 ± 40 , D11: 104 ± 35 , and F1: 170 ± 43 chromosomes. **c** Ratio of metacentric chromosomes in 17 cells from each cell lines. This mean is for the different cell lines: N2a: 8%, D11: 14%, and F1: 5%

Fig. 3 Genomic profiles of N2a and its derived sublines. Each BAC spotted on the comparative genomic hybridization (aCGH) are represented by a dot. BACs were ordered on the x axis according to their position in the genome from chromosome 1 centromere on the left to the X chromosome telomere on the right. Chromosomes are separated by a vertical central line. The y axis corresponds to the Cy5/Cy3 ratio. Yellow, green, and red indicate genomic copy number normality, loss, and gain, respectively, as determined by the MANOR software. First aCGH array: N2a compared to normal A/HeJ mouse DNA, following arrays: sublines compared to parental N2a

genes encoding proteins functionally interacting with PrP^C. *Lamr1* encodes the laminin receptor, a cell surface receptor for PrP^C [34] whose presence is a determinant for prion propagation [41] making this gene a good candidate for the resistance phenotype. *N-cam1* encodes the neural cell adhesion molecule 1, the best-characterized member of the

calcium-independent adhesion molecules which interacts with PrP^C in lipid rafts resulting in downstream activation of Fyn kinase [37, 39]. *Stip1* encodes stress-inducible protein 1, a cell surface ligand for PrP^C that triggers neuroprotection [49]. *Cav1* was selected in this group because caveolin-1-dependent coupling of PrP^C to the

Table 1 List of the 21 genes whose expression was measured in the six sublines relative to that in N2a

Function	MGI accession ID	TaqMan reference	Chromosome	Gene	Protein	References
<i>Prnp</i> and genes encoding proteins interacting with PrP ^C	MGI:67769	Mm00448389	m1 2	<i>Prnp</i>	Prion protein	[31]
	MGI:102709	Mm00483057	m1 6	<i>Cav1</i>	Caveolin, caveolae protein 1	[25]
	MGI:105381	Mm00726662	s1 9	<i>Lamr1</i>	Laminin receptor 1 (ribosomal protein SA)	[18, 34]
	MGI:97281	Mm00456804	m1 9	<i>N cam1</i>	Neural cell adhesion molecule 1	[37, 39]
	MGI:109130	Mm00489584	m1 19	<i>Stip1</i>	Stress induced phosphoprotein 1	[49]
Genes encoding proteins modulated by PrP expression or conversion	MGI:1891917	Mm00457545	m1 2	<i>Ywhab</i>	14 3 3 Beta isoform	[14, 38]
	MGI:88395	Mm00483287	m1 2	<i>Chgb</i>	Chromogranin B	[33]
	MGI:88561	Mm00558321	m1 14	<i>Ctsb</i>	Cathepsin B	[51]
	MGI:88564	Mm00514439	m1 13	<i>Ctsl</i>	Cathepsin L	[51]
	MGI:95295	Mm00656724	m1 18	<i>Egr1</i>	Early growth response 1	[22]
	MGI:97172	Mm00809556	s1 8	<i>Mt2</i>	Metallothionein 2	[15]
	MGI:98331	Mm00456921	m1 2	<i>Snap25</i>	Synaptosomal associated protein 25	[42]
Genes encoding proteins that could modulate prion conversion and pathogenesis	MGI:109355	Mm00444008	m1 5	<i>Stx1a</i>	Syntaxin 1A (brain)	[42]
	MGI:98460	Mm00449779	m1 X	<i>Syn1</i>	Synapsin I	[42]
	MGI:109548	Mm00545742	m1 9	<i>Adam10</i>	A disintegrin and metallopeptidase domain 10	[45]
	MGI:99702	Mm00432050	m1 7	<i>Bax</i>	Bcl2 associated X protein	[7, 8, 30]
	MGI:88138	Mm00477631	m1 1	<i>Bcl2</i>	B cell leukemia/lymphoma 2	[30, 35]
	MGI:107739	Mm00438045	m1 8	<i>Casp3</i>	Caspase 3	[28]
	MGI:1916197	Mm00558321	m1 7	<i>Chst8</i>	Carbohydrate (<i>N</i> acetylgalactosamine 4 O) sulfotransferase 8	[1]
	MGI:98351	Mm01344232	g1 16	<i>Sod1</i>	Superoxide dismutase 1, soluble	[4, 23, 48]
	MGI:98834	Mm00441964	g1 11	<i>Trp53</i>	Transformation related protein 53	[28]

tyrosine kinase Fyn has been described [25]. *Ywhab* encodes the 14-3-3 beta isoform and its has been shown that 14-3-3 proteins could form a complex with PrP^C and Hsp60 [38] and they serve as diagnostic makers in the cerebrospinal fluid of patients with prion diseases [14].

The second group focused on genes encoding proteins modulated by PrP expression or conversion. Chromogranin B (*Chgb*) was detected in prion plaques and could play a role in neurodegenerative processes [33], while metallothionein expression was affected by the disease in human brain [15]. *Ctsb* and *Ctsl* encode the lysosomal proteases cathepsin B and L which were found to have a higher activity in D11 infected with the Chandler/RML prion strain suggesting that they may be actively involved in PrPres formation [51]. *Egr-1* can be considered to be a mediator of prion-induced gene transcription, coordinating subsequent waves of gene expression, and possibly

participating in the establishment of infection [2]. The following genes involved in synaptic function, *Snap25*, *Stx1a*, *Syn1*, encode proteins which had a modified expression matching the distribution of spongiform degeneration in prion animal model [39]. The third group was related to genes encoding proteins that could modulate prion conversion and pathogenesis. It is the case of *Adam10* which encodes a cell-surface disintegrin and metalloprotease that physiologically cleaves PrP^C in its internal amyloidogenic neurotoxic sequence essential for the pathogenic conversion into PrPres [45]. The link proposed between the genetic control of 4-*O*-sulfation of glycosaminoglycans and PrPres accumulation was the reason for the selection of *Chst8* [1]. The four genes *Bax*, *Bcl2*, *Casp3* and *Trp53* are well known mediators of apoptosis and cell survival. They have been involved in many instances in prion pathogenesis through functional or

direct interaction with prions [7, 29, 30, 35]. We also included *Sod1*, a gene involved in antioxidant systems to improve cell defenses which has been involved in prion normal function and pathogenesis [4, 23, 48].

These genes are scattered throughout the haploid genome, and their map positions cover 14 of the 20 chromosomes. Therefore, this selection of genes was extremely unlikely to introduce a bias as a consequence of their chromosomal locations. The thresholds for significance for qRT-PCR were defined based on the estimation of the error during experiments and were very similar for all the genes tested (not shown). We therefore applied a single pair of cutoff values, <0.5 and >2 (Table 2), which are standard for comparative studies based on qRT-PCR.

Gene expression in susceptible and resistant sublines

The expression of the 21 genes was measured in uninfected sublines using parental N2a as reference (Table 2). Statistical analysis using Kruskal Wallis test indicated that the

transcription level of each gene differed significantly between the six sublines ($P < 0.05$). Application of the threshold for significance (twofold up or down) reduced the number of genes with differential expression to a limited subset.

The variation of gene expression differed between the four susceptible sublines in terms of number and type of genes involved: 7 genes were differentially expressed in H6, 7 in R10, 6 in D11 and 10 in G9. Grouping the genes according to their function or to the localization of their products generated different types of transcriptional profiles among these susceptible sublines. For example, the genes affected were associated mainly with gene over-expression in D11 but mainly with down-regulation in G9 and H6. In the resistant sublines R4 and F1, 7 and 4 genes were differentially expressed relative to N2a.

The genes modified belonged to all functional categories but did not define a particular transcriptional profile associated with the phenotype of susceptibility or resistance. Only one gene out of the 21 tested, *Sod1*, did not reach the

Table 2 Relative expression levels of 21 candidate genes in the N2a sublines

Genes	N2a sublines					
	Susceptible				Resistant	
	D11	R10	G9	H6	R4	F1
<i>Prnp</i>	0.90 (0.62 1.30)	0.39 (0.34 0.44)	1.41 (1.25 1.60)	0.53 (0.40 0.72)	0.53 (0.45 0.62)	0.45 (0.33 0.62)
<i>Cav1</i>	ND	ND	ND	ND	ND	ND
<i>Lamr1</i>	1.19 (0.86 1.65)	1.47 (1.41 1.53)	0.40 (0.35 0.45)	0.42 (0.27 0.64)	1.26 (1.24 1.27)	0.73 (0.54 0.99)
<i>N cam1</i>	3.70 (3.31 4.14)	1.39 (1.08 1.79)	0.44 (0.37 0.53)	0.36 (0.28 0.46)	0.95 (0.76 1.18)	0.97 (0.64 1.77)
<i>Stip1</i>	1.04 (0.71 1.53)	2.56 (2.13 3.08)	0.53 (0.47 0.60)	0.63 (0.46 0.85)	2.42 (2.09 2.80)	0.68 (0.50 0.92)
<i>Ywhab</i>	1.36 (0.99 1.87)	0.86 (0.76 0.98)	0.36 (0.31 0.41)	0.91 (0.68 1.22)	0.69 (0.49 0.97)	0.73 (0.54 0.98)
<i>Adam10</i>	0.73 (0.52 1.01)	2.01 (1.87 2.16)	0.36 (0.32 0.40)	0.93 (0.69 1.26)	2.39 (2.12 2.69)	0.42 (0.28 0.63)
<i>Chgb</i>	2.19 (2.00 2.40)	0.97 (0.80 1.17)	0.68 (0.59 0.79)	0.28 (0.23 0.34)	0.79 (0.72 0.87)	0.74 (0.53 1.05)
<i>Chst8</i>	ND	ND	ND	ND	ND	ND
<i>Ctsb</i>	0.37 (0.32 0.42)	0.57 (0.49 0.67)	0.42 (0.33 0.53)	0.43 (0.37 0.50)	0.33 (0.30 0.35)	0.56 (0.41 0.76)
<i>Ctsl</i>	0.67 (0.48 0.93)	2.35 (1.86 2.87)	0.53 (0.46 0.62)	0.57 (0.45 0.71)	1.20 (1.05 1.37)	0.79 (0.55 1.16)
<i>Egr 1</i>	1.97 (1.52 2.57)	1.14 (0.92 1.42)	9.77 (8.78 10.87)	0.49 (0.45 0.52)	1.51 (1.36 1.67)	0.58 (0.37 0.88)
<i>Snap25</i>	1.38 (1.24 1.53)	0.11 (0.10 0.12)	0.12 (0.10 0.13)	0.65 (0.47 0.91)	0.14 (0.12 0.15)	0.21 (0.15 0.28)
<i>Stx1a</i>	2.32 (2.00 2.69)	0.54 (0.50 0.58)	0.97 (0.87 1.09)	0.48 (0.43 0.54)	0.65 (0.58 0.72)	0.73 (0.50 1.08)
<i>Syn1</i>	2.72 (2.14 2.45)	1.38 (0.93 2.04)	0.14 (0.09 0.20)	0.27 (0.22 0.35)	1.35 (1.21 1.52)	0.99 (0.79 1.24)
<i>Bax</i>	1.89 (1.82 1.96)	1.16 (1.06 1.28)	0.26 (0.22 0.32)	1.11 (0.91 1.35)	1.39 (1.08 1.79)	1.65 (1.21 2.25)
<i>Bcl2</i>	1.15 (1.04 1.26)	1.60 (1.50 1.72)	0.62 (0.53 0.72)	0.78 (0.64 0.95)	2.30 (1.79 2.96)	0.82 (0.58 1.17)
<i>Casp3</i>	0.50 (0.45 0.55)	0.27 (0.24 0.30)	0.29 (0.23 0.36)	0.82 (0.65 1.01)	0.36 (0.28 0.46)	0.45 (0.33 0.61)
<i>Mt2</i>	ND	ND	ND	ND	ND	ND
<i>Sod1</i>	1.50 (1.20 1.88)	1.31 (1.20 1.42)	0.85 (0.74 0.97)	0.69 (0.54 0.89)	1.36 (1.33 1.40)	0.52 (0.37 0.73)
<i>Trp53</i>	5.60 (5.00 6.27)	2.31 (2.07 2.38)	0.98 (0.87 1.10)	1.13 (1.03 1.23)	2.43 (2.06 2.88)	1.95 (1.40 2.72)

Relative expression levels of 21 genes in the different N2a sublines are reported as multiples of that in parental N2a. Values represent the mean fold change $\pm$ standard deviation for triplicate experiments, and values are standardized to the 18S RNA internal control. mRNA levels half (<0.50) or double (>2) that of the reference are indicated in bold and bolditalics, respectively

ND not detectable

twofold change threshold in any of the sublines. Three genes, *Cav1*, *Chst8* and *Mt2*, had no detectable transcription in N2a or in any of the sublines ($Ct > 50$). The absence of *Cav1* in N2a has been reported previously [40] and our findings confirmed this at the transcription level and showed that subcloning had no effect on it.

Importantly, we verified the stability of the gene expression by studying sublines H6 and G9 over serial passages. Only three of 21 genes (*Snap25*, *Syn1*, *Egr-1*) underwent changes in expression levels during passaging (not shown). However, the general trend of the alterations to these genes (under-expression) was conserved relative to N2a (see Table 2). This suggests that the transcriptional profile of the sublines was generally stable enough during passaging to allow comparisons between sublines.

Comparison between aCGH data and relative gene expressions

Array-based comparative genomic hybridization analysis revealed substantial genomic alterations between sublines, with the exception of G9 and R4 when compared to N2a. The differences in gene expression measured by qRT-PCR prompted us to ask whether they were related to these genomic alterations, and in particular to gene deletion or amplification. Each of the 21 genes studied were mapped to chromosomal regions covered by, or in the vicinity of, a BAC, and we compared the qRT-PCR values with the genomic gains or losses as assessed by aCGH analysis for D11, R10 and F1. Only two over-expressed genes were located in regions of genomic gains on different chromosomes: *Chgb* and *Syn1* (D11) (not shown). Furthermore, none of the down-regulated genes were in regions of genomic loss. This suggested that the changes in gene expression mostly resulted from specific transcriptional regulation, rather than from alteration of gene copy number.

Discussion

N2a cell lines are widely used for ex vivo studies of prion propagation [44], for anti-prion drug screening [17], and as cell models for other neurodegenerative diseases [10]. Previous studies documented that neuroblastoma cells typically displayed a high degree of chromosomal instability, but did not demonstrate any recurrent pattern of chromosomal alteration [9, 46]. Isolation of N2a subclones, as well as use of different culture conditions, passage history, and cryptic infections by endogenous retrovirus may be responsible for important differences between cells. This may favor the emergence of spontaneous sublines with particular phenotypes. As a matter of fact, sublines of N2a susceptible to variable amounts of prion infectivity

and/or to different prion strains have been isolated [3, 20]. These cell lines have been qualified as “susceptible” or “resistant” based on their capacity to accumulate the pathologic proteinase-K resistant form of the prion protein, PrPres. Here, we describe six N2a sublines, four susceptible and two resistant to the mouse-adapted prion strain 22L. In an attempt to decipher the molecular mechanisms leading to these phenotypes, we compared these sublines and evaluated, to our knowledge for the first time, their transcriptional status for genes involved in prion biology.

No obvious difference in the rate of cell division between sublines that could account for various prion susceptibility [12] was observed, nor was the initial binding/internalization of PrPres dramatically different [43], and we therefore focused on the expression level of PrP^C whose presence is essential for prion propagation [5] and whose over-expression accelerates the disease and facilitates PrPres detection [11, 27]. After GAPDH normalization, western blot analysis revealed a lower PrP^C expression in R4 and R10 sublines and a higher one in D11. As already suggested [3], these differences could not explain by themselves prion susceptibility. mRNA *Pmp* levels were also analyzed in parallel (Table 2). As observed in many studies, there was no obvious correlation between mRNA and protein levels. Nevertheless, we observed that G9 and D11 had both the higher mRNA and PrPres levels in comparison with the other sublines. PrPres generation necessitates proper PrP^C biosynthesis and trafficking. The different sublines displayed apparently normal PrP^C glycosylation or surface expression even for the most resistant sublines (Fig. 1), and presented no mutation in its coding sequence that could alter prion conversion.

Before starting a full transcriptional analysis, we looked at the karyotype of N2a, D11 and F1 sublines. As expected, N2a had an increased chromosome number, similarly to D11 while F1 displayed a much higher number. The chromosomal distribution between lines was actually very different as revealed by the ratio of metacentric chromosomes. This prompted us to use, to our knowledge for the first time, a microarray-based CGH (aCGH) method to study N2a sublines. As expected, aCGH revealed chromosomal imbalances but did not indicate any characteristic profile of genomic alterations linked to susceptibility or resistance to prion, within the limits of the resolution of the array used. Moreover, two sublines with opposite phenotypes, R4 (resistant) and R10 (susceptible), had indistinguishable genomic profiles. Thus, the phenotype of the N2a sublines could not be easily explained by recurrent genomic alteration.

We then focused on specific genes linked previously to prion biology. Among the three groups studied (see “Results” section), we could not identify modifications specific to resistant sublines. This was the case for genes

whose active or silent state could influence the generation of PrPres (*Prnp*, *Lamr1*, *Stip1*, *Adam10*..), as well as for genes that would modulate the impact of prion replication on the fate of the cells (*Bax*, *Bcl2*, *Casp3*, *Sod1* ...). We obtained also western blot data to validate this lack of correlation for PrP^C or for the laminin receptor (not shown). A threshold for significance to filter the measurements generated by qRT-PCR was used and it is possible that as a consequence we failed to identify genes for which a small alteration in expression may have a significant impact on the phenotype of the sublines. In addition it is possible that some significant modifications may have a quantitative, but moderate impact on prion replication. For example, the 3.7-fold increased expression of *N-cam1* in D11 could modulate PrP biology and explain the high susceptibility of this clone. Although attractive, this possibility is speculative. Genomics studies using microarrays often generated expression profiles that could be linked to specific phenotypes. For logistic reasons, it was not possible to extend our study to hundreds of genes, and crucial genes directly controlling the phenotype (e.g., chaperones) may have escaped our analysis. We also analyzed our data using hierarchical clustering tools and could not demonstrate a statistical difference between resistant and susceptible cells lines (not shown). Nevertheless, we think that this gene sample, even though limited, allows a picture of the transcriptional state of the susceptible sublines to be drawn. Indeed, three ribotypes emerged among the susceptible clones all propagating prions *ex vivo*: one with mostly over-expressed genes as in D11, the second with mostly under-expressed genes as in G9 and H6, and a third, in R10 with an intermediate profile.

The absence of any obvious correlation between genomic alterations detectable by the aCGH analysis and the gene expression changes argued for transcriptional regulations specific to each subline. We did not find a characteristic gene transcriptional profile in the two resistant sublines R4 and F1 and they did not share either any remarkable and recurrent variation of expression of candidate genes, including *Prnp*, that could explain the low efficiency of prion propagation.

Clearly, further investigation is required to find out the reason why some sublines of N2a are resistant and others susceptible to prions. However, our results provide the first systematic study of concomitant genomic and gene expression changes in several N2a sublines with the following conclusion. Firstly, we demonstrated that susceptibility and resistance of the N2a sublines to 22L prions cannot be assigned to one or several recurrent genomic abnormalities, as indicated by aCGH. Secondly, we confirmed that the phenotype of sublines was not dependent on the expression of PrP^C or any changes in its primary sequence. Then, we refuted the direct involvement

of major gene candidates to explain prion susceptibility. Taken together, we believe that our data are important for further investigation into the molecular mechanisms of prion susceptibility.

Materials and methods

N2a sublines and PrP analysis

Six N2a sublines cloned from the same N2a parental cell line (named N2a, ATCC, number CCL-131) were analyzed. They were selected for their ability ("susceptible" subline) or inability ("resistant" subline) to accumulate PrPres after infection (Fig. 1) as demonstrated by western blot using a mixture of SAF60, SAF69, and SAF70 mAbs [27]. One of these lines, D11, was characterized by Zhang et al. [50] as susceptible; G9, H6, and F1 are unpublished sublines obtained by AB and colleagues during the same subcloning experiments and R4, and R10 were subcloned from N2a by MP, DC and SL. Sublines were obtained after cloning uninfected cells by limiting dilution as described [3]. The resistance or susceptibility of the cloned cells to the mouse-adapted scrapie strain 22L was assessed by infection with brain homogenate diluted to 0.01% in OptiMEM (Invitrogen, California, USA) as described elsewhere [27]. To confirm the phenotype of each subline toward 22L prion, four independent infection experiments were conducted. The experiment to test the binding of PrPres to the cells (Fig. 1d) was performed using 0.05% of brain homogenate as described in [43].

Cells were maintained in DMEM with glutamax, 1 mM sodium pyruvate and 1% L-glutamine supplemented with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO₂, 95% air and collected at confluence after 6–8 passages for RNA purification and protein analysis. PrP^C was detected in uninfected sublines by western blotting using mAb SAF32 (epitope 78–91) in three independent experiments. Band intensities were normalized to GAPDH signals after membrane stripping and reprobing. Relative intensities were calculated using Densylab software (Microvision Instruments) and N2a as the reference. PrP^C immunocytofluorescence was performed as previously described [21]. The PCR-amplified coding sequence of the *Prnp* gene was sequenced on both strands with a 3100 Applied Biosystems (Foster, California, USA) automated sequencer.

Karyotype of N2a cell sublines

Cells were grown up to 80% of confluence and then treated with 0.04 µg/ml colchicine for 1 h at 37°C and rinsed twice with PBS. Cells were then scraped and collected through centrifugation at 500g for 5 min at 4°C. Supernatant was

discarded and cells were lysed in hypotonic solution (75 mM KCl). After incubation at 37°C of at least 20 min 2 ml of fixative (3:1 = methanol:glacial acetic acid) was added to fix the pellet and followed by centrifugation at 1,200 rpm 10 min. Fresh fixative was added and the step repeated three times. Finally, nuclei were resuspended in 4 ml of fixative and stored at -20°C until needed. A drop of samples was released on chilled cleaned slides, dried and fixed on a Bunsen flame. Slides were stained for 4 min with filtered Giemsa solution and then rinsed profusely in running tap water for 1-2 min. After drying slides were observed at the microscope.

Mouse array comparative genomic hybridization

Array comparative genomic hybridization analysis was applied to N2a and five of its sublines (D11, F1, G9, R4, R10). A custom-made genome-wide mouse bacterial artificial chromosome (BAC) array was built using 932 BACs that were isolated, validated by end-sequencing and amplified by multiple displacement amplification. Results were analyzed with GenePix Pro 5.1 software and Cy5/Cy3 signal ratios were determined as described by Neuvial et al. [26]. The experimental design of the hybridization and the labeling protocol used have been submitted to the Gene Expression Omnibus database (GSE5374) and a full description of the CGH array has been deposited in the same database under accession number GPL3972.

Real-time quantitative PCR for quantification of mRNA levels

Total RNA was extracted from N2a and its six sublines (D11, F1, G9, H6, R4, R10) with the RNeasy mini kit (Qiagen, Oslo, Norway) and treated with DNaseI. qRT-PCR was performed using TaqMan Universal PCR Master Mix® and TaqMan Gene Expression Assays® (Applied Biosystems) for each cDNA following the manufacturer's instructions. The list of TaqMan Gene Expression Assays® is listed in Table 1. All cDNAs were amplified in triplicate using the Rotor-Gene 3000 with appropriate controls. Measurement of the amount of 18S RNA was used to normalize the values.

Results were analyzed in a relative quantification study using the Rotor-Gene 6.0 software (Corbett Life Science) and the comparative $2^{-\Delta\Delta C_t}$ method [47]. Relative expression levels of the genes were assessed (1) in uninfected sublines using the parental cell line N2a as a reference, (2) in passages of sublines H6 and G9 with respect to passages $n - 3$ and $n - 1$, respectively. Non parametric Kruskal Wallis tests were used to compare gene expression between the different sublines. Changes in

gene expression were evaluated according to a calculated threshold for significance (see "Results").

Acknowledgments This work was supported by a grant from "GIS Infections à prions". Isolation of susceptible and resistant cell clones was supported by a grants from the EU Commission (EU PRIONS, QLRT 2000 01924; NEUROPRION, FOOD CT 2004 506579). The construction of the mouse BAC array was supported by grants from the "Carte d'Identité des Tumeurs (CIT)" program of the "Ligue Nationale Contre le Cancer". Anti prion antibodies were generously provided by J. Grassi and C. Creminon (CEA Saclay, SPI/CEA).

References

1. Barret A, Forestier L, Deslys JP, Julien R, Gallet PF (2005) Glycosylation related gene expression in prion diseases: PrPSc accumulation in scrapie infected GT1 cells depends on beta 1, 4 linked GalNAc 4 SO4 hyposulfation. *J Biol Chem* 280:10516-10523
2. Borchelt DR, Taraboulos A, Prusiner SB (1992) Evidence for synthesis of scrapie prion proteins in the endocytic pathway. *J Biol Chem* 267:16188-16199
3. Bosque PJ, Prusiner SB (2000) Cultured cell sublines highly susceptible to prion infection. *J Virol* 74:4377-4386
4. Brown DR, Schmidt B, Kretzschmar HA (1997) Effects of oxidative stress on prion protein expression in PC12 cells. *Int J Dev Neurosci* 15:961-972
5. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. *Cell* 73:1339-1347
6. Chich JF, Schaeffer B, Bouin AP, Mouthon F, Labas V, Larramendy C, Deslys JP, Grosclaude J (2007) Prion infection impaired functional blocks identified by proteomics enlighten the targets and the curing pathways of an anti prion drug. *Biochim Biophys Acta* 1774:154-167
7. Chiesa R, Piccardo P, Dossena S, Nowoslawski L, Roth KA, Ghetti B, Harris DA (2005) Bax deletion prevents neuronal loss but not neurological symptoms in a transgenic model of inherited prion disease. *Proc Natl Acad Sci USA* 102:238-243
8. Culpier M, Messiaen S, Hamel R, Fernandez de Marco M, Lili T, Eloit M (2006) Bax deletion does not protect neurons from BSE induced death. *Neurobiol Dis* 23:603-611
9. Doh-ura K, Perryman S, Race R, Chesebro B (1995) Identification of differentially expressed genes in scrapie infected mouse neuroblastoma cells. *Microb Pathog* 18:1-9
10. Ehehalt R, Keller P, Haass C, Thiele C, Simons K (2003) Amyloidogenic processing of the Alzheimer beta amyloid precursor protein depends on lipid rafts. *J Cell Biol* 160:113-123
11. Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C (1996) Prion protein (PrP) with amino proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* 15:1255-1264
12. Ghaemmaghami S, Phuan PW, Perkins B, Ullman J, May BC, Cohen FE, Prusiner SB (2007) Cell division modulates prion accumulation in cultured cells. *Proc Natl Acad Sci USA* 104:17971-17976
13. Greenwood AD, Horsch M, Stengel A, Vorberg I, Lutzny G, Maas E, Schädler S, Erfle V, Beckers J, Schatzl H, Leib Mosch C (2005) Cell line dependent RNA expression profiles of prion infected mouse neuronal cells. *J Mol Biol* 349:487-500
14. Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG (1996) The 14-3-3 brain protein in cerebrospinal fluid as a marker for

- transmissible spongiform encephalopathies. *N Engl J Med* 335:924–930
15. Kawashima T, Dohura K, Torisu M, Uchida Y, Furuta A, Iwaki T (2000) Differential expression of metallothioneins in human prion diseases. *Dement Geriatr Cogn Disord* 11:251–262
 16. Klebe RJ, Ruddle FH (1969) Neuroblastoma: cell culture analysis of a differentiating stem cell system. *J Cell Biol* 43:69
 17. Kocisko DA, Caughey B (2006) Searching for anti prion compounds: cell based high throughput in vitro assays and animal testing strategies. *Methods Enzymol* 412:223–234
 18. Leucht C, Simoneau S, Rey C, Vana K, Rieger R, Lasmezas CI, Weiss S (2003) The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie infected neuronal cells. *EMBO Rep* 4:290–295
 19. Lloyd SE, Uphill JB, Targonski PV, Fisher EM, Collinge J (2002) Identification of genetic loci affecting mouse adapted bovine spongiform encephalopathy incubation time in mice. *Neurogenetics* 4:77–81
 20. Mahal SP, Baker CA, Demczyk CA, Smith EW, Julius C, Weissmann C (2007) Prion strain discrimination in cell culture: the cell panel assay. *Proc Natl Acad Sci USA* 104:20908–20913
 21. Mange A, Crozet C, Lehmann S, Beranger F (2004) Scrapie like prion protein is translocated to the nuclei of infected cells independently of proteasome inhibition and interacts with chromatin. *J Cell Sci* 117:2411–2416
 22. Marella M, Gaggioli C, Batoz M, Deckert M, Tartare Deckert S, Chabry J (2005) Pathological prion protein exposure switches on neuronal mitogen activated protein kinase pathway resulting in microglia recruitment. *J Biol Chem* 280:1529–1534
 23. Milhavel O, McMahon HE, Rachidi W, Nishida N, Katamine S, Mange A, Arlotto M, Casanova D, Riondel J, Favier A, Lehmann S (2000) Prion infection impairs the cellular response to oxidative stress. *Proc Natl Acad Sci USA* 97:13937–13942
 24. Moreno CR, Lantier F, Lantier I, Sarradin P, Elsen JM (2003) Detection of new quantitative trait Loci for susceptibility to transmissible spongiform encephalopathies in mice. *Genetics* 165:2085–2091
 25. Mouillet Richard S, Ermonval M, Chebassier C, Laplanche JL, Lehmann S, Launay JM, Kellermann O (2000) Signal transduction through prion protein. *Science* 289:1925–1928
 26. Neuvial P, Hupe P, Brito I, Liva S, Manie E, Brennetot C, Radvanyi F, Aurias A, Barillot E (2006) Spatial normalization of array CGH data. *BMC Bioinformatics* 7:264
 27. Nishida N, Harris DA, Vilette D, Laude H, Frobert Y, Grassi J, Casanova D, Milhavel O, Lehmann S (2000) Successful transmission of three mouse adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild type mouse prion protein. *J Virol* 74:320–325
 28. Paitel E, Alves da Costa C, Vilette D, Grassi J, Checler F (2002) Overexpression of PrPc triggers caspase 3 activation: potentiation by proteasome inhibitors and blockade by anti PrP antibodies. *J Neurochem* 83:1208–1214
 29. Paitel E, Fahraeus R, Checler F (2003) Cellular prion protein sensitizes neurons to apoptotic stimuli through Mdm2 regulated and p53 dependent caspase 3 like activation. *J Biol Chem* 278:10061–10066
 30. Park SK, Choi SI, Jin JK, Choi EK, Kim JI, Carp RI, Kim YS (2000) Differential expression of Bax and Bcl 2 in the brains of hamsters infected with 263K scrapie agent. *Neuroreport* 11:1677–1682
 31. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. *Science* 216:136–144
 32. Prusiner SB, Scott MR, Dearmond SJ, Cohen FE (1998) Prion protein biology. *Cell* 93:337–348
 33. Rangan CM, Haik S, Faucheux BA, Metz Boutigue MH, Fierville F, Fuchs JP, Hauw JJ, Aunis D (2003) Different chromogranin immunoreactivity between prion and a beta amyloid plaque. *Neuroreport* 14:755–758
 34. Rieger R, Edenhofer F, Lasmezas CI, Weiss S (1997) The human 37 kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med* 3:1383–1388
 35. Roucou X, Giannopoulos PN, Zhang Y, Jodoin J, Goodyer CG, LeBlanc A (2005) Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF 7 cells. *Cell Death Differ* 12:783–795
 36. Sandberg MK, Low P (2005) Altered interaction and expression of proteins involved in neurosecretion in scrapie infected GT1 1 cells. *J Biol Chem* 280:1264–1271
 37. Santucci A, Sytnyk V, Leshchynska I, Schachner M (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J Cell Biol* 169:341–354
 38. Satoh J, Onoue H, Arima K, Yamamura T (2005) The 14-3-3 protein forms a molecular complex with heat shock protein Hsp60 and cellular prion protein. *J Neuropathol Exp Neurol* 64:858–868
 39. Schmitt Ulms G, Legname G, Baldwin MA, Ball HL, Bradon N, Bosque PJ, Crossin KL, Edelman GM, DeArmond SJ, Cohen FE, Prusiner SB (2001) Binding of neural cell adhesion molecules (NCAMs) to the cellular prion protein. *J Mol Biol* 314:1209–1225
 40. Shyng SL, Heuser JE, Harris DA (1994) A glycolipid anchored prion protein is endocytosed via clathrin coated pits. *J Cell Biol* 125:1239–1250
 41. Simoneau S, Haik S, Leucht C, Dormont D, Deslys JP, Weiss S, Lasmezas C (2003) Different isoforms of the non integrin laminin receptor are present in mouse brain and bind PrP. *Biol Chem* 384:243–246
 42. Siso S, Puig B, Varea R, Vidal E, Acin C, Prinz M, Montrasio F, Badiola J, Aguzzi A, Pumarola M, Ferrer I (2002) Abnormal synaptic protein expression and cell death in murine scrapie. *Acta Neuropathol* 103:615–626
 43. Uryu M, Karino A, Kamihara Y, Horiuchi M (2007) Characterization of prion susceptibility in Neuro2a mouse neuroblastoma cell subclones. *Microbiol Immunol* 51:661–669
 44. Vilette D (2007) Cell models of prion infection. *Vet Res* 39:10
 45. Vincent B, Paitel E, Saftig P, Frobert Y, Hartmann D, De Strooper B, Grassi J, Lopez Perez E, Checler F (2001) The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester regulated normal cleavage of the cellular prion protein. *J Biol Chem* 276:37743–37746
 46. Warter S, Hermetet JC, Cieselski Treska J (1974) Cytogenetic characterization of C1300 neuroblastoma cells. *Experientia* 30:291–292
 47. Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real time quantitative reverse transcriptase polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* 270:41–49
 48. Wong BS, Pan T, Liu T, Li R, Gambetti P, Sy MS (2000) Differential contribution of superoxide dismutase activity by prion protein in vivo. *Biochem Biophys Res Commun* 273:136–139
 49. Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiarini LB, Nomizo R, Freitas AR, Cabral AL, Lee KS, Juliano MA, de Oliveira E, Jachieri SG, Burlingame A, Huang L, Linden R, Brentani RR, Martins VR (2002) Stress inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J* 21:3307–3316
 50. Zhang Y, Poirier GG, Burkle A (2002) In situ analysis of cellular poly(ADP ribose) production in scrapie infected mouse neuroblastoma cells. *Histochem J* 34:357–363
 51. Zhang Y, Spiess E, Groschup MH, Burkle A (2003) Up regulation of cathepsin B and cathepsin L activities in scrapie infected mouse Neuro2a cells. *J Gen Virol* 84:2279–2283