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I would be grateful if you would consider the submitted manuscript “Differential expression of Prnp and Sprn in scrapie infected sheep also reveals Prnp genotype specific differences” for publication in Biochemical and Biophysical Research Communications

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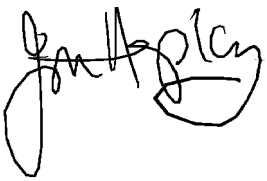

Differential expression of Prnp and Sprn in scrapie infected sheep also reveals Prnp genotype specific differences

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ABSTRACT

The central role for PrP in the pathogenesis of the transmissible spongiform encephalopathies (TSEs) is illustrated by the resistance of $Prnp^{0/0}$ mice to disease and by the inverse association of Prnp gene dosage with incubation period. Understanding the role of PrP^{C} in TSEs necessitates knowledge of expression levels of the Prnp gene during the development of disease. SSBP/1 scrapie shows a defined pattern of disease progression and here we show that Prnp and shadow of PrP (Sprn) are differentially expressed in different brain areas and lymphoid tissues. Counter-intuitively we found that there is no positive correlation between expression of Prnp or Sprn and patterns of disease progression. Prnp and Sprn expression levels are both influenced by Prnp genotype; although the scrapie-sensitive VRQ/VRQ sheep did not express the highest level of either. In addition, infection with SSBP/1 scrapie seems to have little effect on either PrP or Shadoo expression levels.

Keywords: Prion; scrapie; shadoo; PrP genetics; mRNA expression.

1. Introduction

The central role for cellular prion protein, PrP^{C} in the pathogenesis of the transmissible spongiform encephalopathies (TSEs) is illustrated by the resistance of $Prnp^{null}$ mice (which express no PrP protein) to disease and by the inverse association of the normal cellular PrP^{C} expression levels with incubation period in transgenic mice [1;2]. Furthermore in sheep scrapie, susceptibility and resistance to infection is largely controlled by polymorphisms of the PrP gene (Prnp) at codons 136 (valine, V, or alanine, A), 154 (arginine, R, or histidine H) and 171 (arginine, R or glutamine, Q,). Following experimental challenge with SSBP/1 scrapie by peripheral routes, VRQ/VRQ sheep have considerably shorter incubation period than VRQ/ARR sheep and ARR homozygotes are resistant. Furthermore, heterozygous animals of different breeds have variable incubation periods relative to homozygous genotypes, implying that factors other than PrP are influencing disease [3].

The prion gene (Prn) complex consists of two homologous genes [4], Prnp encodes the ubiquitously-expressed PrP and Prnd encodes doppel (Dpl), which is truncated at the amino terminus, is expressed almost exclusively in testes and heart [5] and seems to play little role in TSE pathology. However, in $Prnp^{null}$ mice Dpl is ectopically-expressed in the central nervous system (CNS) and, like amino-terminally truncated PrP (ΔPrP), causes neurodegeneration and ataxia [4]. A second homologue called shadow of prion protein (Shadoo or Sho), encoded by the Prnp-unlinked gene Sprn [6], is expressed in the CNS and, like full length PrP, protects the brain from the neurotoxic effects of Dpl and ΔPrP [7]. Consequently, Sho might be one of the factors, other than PrP, that influences disease progression.

Scrapie strains in mice are differentiated by their different and reproducible incubation times in defined mouse strains. In addition scrapie isolates in sheep exhibit characteristic patterns of deposition of disease-associated PrP^{Sc} in specific regions of the CNS and in lymphoreticular system (LRS) {Gonzalez, 2002 18162 /id}. SSBP/1 scrapie is a well characterised sheep scrapie strain and shows a sequential progression of PrP^{Sc} deposition [8], from lymph nodes, tonsils and

spleen in the LRS, to obex (medulla), thalamus and then cerebellum and frontal cortex in the CNS.

In this study we used real-time quantitative RT-PCR (qRT-PCR) to quantify PrP and Sho transcripts in peripheral lymphoid tissues and the brains of sheep experimentally infected with SSBP/1 scrapie. In this way we tested the hypotheses that; (1) differential expression of PrP and Sho explain the patterns of SSBP/1 scrapie disease progression, (2) expression levels of PrP and Sho are related to differential susceptibility to scrapie pathology and disease of different Prnp genotypes and (3) PrP and Sho expression levels change in relation to the development of disease.

Materials and Methods

Experimental animals, scrapie infections and tissue collection. New Zealand-derived Cheviot sheep of three different Prnp genotypes (VRQ/VRQ, VRQ/ARR and ARR/ARR) from the DEFRA breeding flock were placed in groups of five animals (three infected animals and two mock-infected controls) per time point. All were inoculated by subcutaneous injection with SSBP/1 scrapie or normal brain homogenate in the drainage area of the prescapular lymph nodes (PSLNs) [9]. Animal experiments were performed under an Animals (Scientific Procedures) Act 1986 Project Licence. Animals were killed by exsanguination under terminal anaesthesia and tissues were removed immediately post mortem. Dissected tissues were stored in RNAlater (Ambion, Huntingdon, UK) at –80˚C. Spleens were dissected and disrupted by passing through a fine steel sieve in ice-cold PBS containing 4 mM EDTA and then filtered through muslin. Follicular dendritic cells (FDC) were enriched using an Optiprep gradient (Nycomed Amersham, Little Chalfont, UK) with the cells at the 1.068 g/ml – PBS interface being collected after centrifugation at 600×g for 25 min at 20 °C. Cells were washed in PBS/EDTA and pelleted at 300×g, before they were lysed using 1 ml of RNAwiz (Ambion) per 10^7 cells.

RNA extraction and cDNA synthesis. Total RNA from brain tissue was prepared using RNeasy Lipid Tissue Mini Kit (Qiagen, Crawley, UK) and from lymphoid tissue using Ribopure Kit (Ambion). RNA was digested with DNase I, assessed using a RNA 6000 Nano LabChip on the Agilent 2100 bioanalyzer and quantified using a NanoDrop ND-1000 spectrophotometer. First strand cDNA synthesis used 0.5 µg of total RNA with 50 µM oligo$(dT)_{20}$, 40 U RNaseOUT, 200 U Superscript ™ III reverse transcriptase, 5× RT buffer, 25 mM $MgCl_2$ and 0.1 M DTT (Invitrogen, Paisley, UK). Genomic DNA contamination was assessed using a no reverse transcriptase control. For each sample three identical RT reactions were performed and prepared at the same time.

Construction of plasmid standards. Primer sequences, amplicon sizes and optimized conditions are listed in Supplementary Table 1. Amplicons were cloned using pGEM-T Easy kit (Promega, Southampton, UK), assessed by gel electrophoresis and sequenced. Purified plasmid DNA was linearized; plasmid concentration determined using a NanoDrop ND-1000 spectrophotometer and serial dilutions generated using a CAS-1200™ (Corbett Robotics, Sydney, Australia). Standard curves were prepared for all genes; 7-fold serial dilutions were made from 1 ng/µl. Copy numbers were calculated by; the molecular weight of vector plus insert (M) = size of plasmid and insert size bp × 660 g/mol per bp. Number of molecules per ng = $[(1\times10^{-9})/(M$ g/mol)] × $(6.023\times10^{23}$ molecules/mol). The Prnp primers amplified bp 783 – 914 encoding amino acids 207 – 251 (Genbank accession no. **NM_001009481**); the Sprn primers amplified bp 2033 – 2177 (Genbank accession no. **DQ870545**) located in the 5' untranslated region (33 bp) and the 5' of the coding region (111 bp). These primers were not associated with any known polymorphisms.

Quantitative real-time RT-PCR. qRT-PCR was performed using the Rotor-Gene™ 3000 (Corbett); all qRT-PCR reactions were prepared using a CAS-1200™. All reactions were performed in 10 µl final volume containing 5 µl cDNA at optimal dilution (1:20), 0.375 U FastStart Taq DNA Polymerase, 10× PCR reaction buffer, $MgCl_2$ at optimal concentration, 200

μM of each dNTP, 0.35 μl SYBR Green I (1/1000 in DMSO) and 0.5 μl of each primer at optimal concentration. The amplification profile used was the same for each gene except for annealing temperature (Supplementary Table 1): 5 min at 94ºC, 40 cycles of 15 s at 94ºC, 15 s at gene specific annealing Tm and 15 s at 72ºC, followed by a dissociation curve analysis program. Each sample was assayed in triplicate in the same run and each included a no-template control and a plasmid DNA standard curve for each gene. The dynamic range of the standard curve spanned five orders of magnitude and was used to derive copy number of the target sequence in unknown samples. The data were analyzed using Rotor-Gene 3000 Software v 6.1.81 (Corbett).

Amplified products were assessed by melt curve analysis; linearity and efficiency of qPCR amplification was determined for each primer pair using a standard curve generated by a dilution series of a pool of sample cDNAs for each tissue. Several genes were evaluated for suitability as endogenous reference genes for each of the different tissues using GeNorm v3.4 and NormFinder v 0.953 (Supplementary Table 1).

Normalized copy numbers were obtained by using the normalization factor determined by GeNorm for each tissue assayed based on the expression of multiple endogenous reference genes previously determined for each individual tissue (Supplementary Table 1). The expression levels were normalized by dividing the copy number derived from the standard curve by the calculated normalization factor for each individual sample. The geometric mean of the normalized expression levels for the three reverse transcriptions performed for each sample was used in analysis of expression levels of each gene.

For Prnp, the detection limit was 3 molecules but with the highest test linearity in the quantification range of 35.9 - 2.96×10^7 plasmid DNA molecules. The detection limit was 2 molecules for Sprn, with a quantification range of 36 - 4.21×10^6 molecules. qRT-PCR efficiencies and R^2 values were 100% for Prnp (R^2= 0.999) and 98% for Sprn (R^2= 0.998).

Statistical analysis. Datasets were tested for normal (Gaussian) distribution using the D'Agostino & Pearson omnibus K2 normality test. As the vast majority passed, normal

distribution was assumed for all and comparisons were completed using one-way analysis of variance (ANOVA) followed by Student's t-test ($P < 0.05$ for differences between groups was consider significant).

Results

Tissue specific expression of Prnp and Sprn transcripts

Expression levels of Prnp and Sprn in different tissues were examined in sheep of the VRQ/VRQ genotype (Table 1). This was related to the known pattern of disease progression with SSBP/1, where PrP^{Sc} deposition is first seen in the draining PSLNs, then spleen; and in the CNS, first obex and thalamus, then cerebellum and frontal cortex [9]. These data show that within the brain there is a hierarchy of expression of both Prnp and Sprn and that the pattern is similar for both Prnp and Sprn. In descending order this hierarchy is: frontal cortex>cerebellum>obex and thalamus. Table 1 also shows that Prnp is expressed at a level 100 to 1000 fold greater than Sprn. In PSLNs, Prnp levels are about 20 fold less that in obex but Sprn levels are approximately equivalent. In contrast, within processed spleen Prnp levels are 3 - 4 fold greater than PSLN but Sprn transcripts were below the level of accurate quantification.

Effect of Prnp genotype on Prnp and Sprn transcripts

Differences in levels of Prnp transcripts in sheep with distinct Prnp genotypes were assessed using obex, spleen and prescapular lymph node; spleen was replaced by frontal cortex for Sprn measurement because of the lack of detectable Sprn in spleen. Comparison of Prnp transcript levels in the three genotypes is shown in Fig. 1 and shows that the highest level of expression is in VRQ/ARR sheep. Within obex the levels of Prnp in VRQ/ARR sheep were 1.9×10^6 copies per 1

μg of RNA, significantly greater ($P \leq 0.05$) than both the VRQ/VRQ and ARR/ARR groups. In spleen the Prnp levels in the heterozygotes (3.7×10^6) were significantly greater ($P \leq 0.05$) than VRQ/VRQ sheep and in lymph node they were significantly higher ($P \leq 0.05$) than the ARR/ARR animals. Fig. 2 shows equivalent data for Sprn, and shows no significant difference in the expression level in frontal cortex between genotypes, but the Sprn level in lymph nodes was significantly higher in ARR/ARR animals than in VRQ/VRQ. In obex, Sprn transcripts in VRQ/ARR and ARR/ARR sheep were too low to be accurately quantified and therefore valid comparisons could not be made.

Effect of scrapie infection on Prnp and Sprn transcripts

The effect of SSBP/1 scrapie infection on Prnp and Sprn transcript levels was assessed in different tissues on days 25, 75 and 125 post-infection in VRQ/VRQ sheep and days 25, 75, 150 and 230 in VRQ/ARR and ARR/ARR animals. Transcript levels in mock-infected animals (C) were also measured, and data for Prnp in obex and lymph node are shown in Fig. 3. Analysis was performed comparing every time point with all others and also comparing early infection (days 25 and 75) with late infection (day 125, 150 and 230). These analyses showed that infection with SSBP/1 has no significant effect ($P>0.05$) on expression of either Prnp or Sprn in any tissue examined (frontal cortex, cerebellum, obex, thalamus, spleen and PSLN).

Discussion

This study quantified Prnp and Sprn transcripts, in different areas of the CNS and LRS in sheep of defined Prnp genotypes and at times after SSBP/1 scrapie infection. The rationale for these studies was to examine the relationship between differential Prnp and Sprn expression and (1) the pattern of progression of SSBP/1 scrapie pathology; (2) differential susceptibility of

different Prnp genotypes to SSBP/1 disease and (3) time after SSBP/1 scrapie infection. In this study we measured transcripts and assume a direct, quantitative relationship between PrP/Sho protein and mRNA levels. At least two Prnp transcripts have been described resulting from alternative polyadenylation sites [10], which may have different stability rates and tissue distributions. However, the primers used were within the coding region and therefore amplified all possible variants.

Our data are consistent with the reports of Diaz-San Segundo [11] and Tichopad [12], and show a hierarchy of expression level of Prnp in the CNS; with thalamus and obex expressing the lowest level, then cerebellum with the highest level in frontal cortex. However, this contradicts Han [13] who report that obex expresses the highest level of Prnp. We also show a hierarchy in the LRS with Prnp transcript levels in spleen being approximately threefold those of prescapular lymph node. The only previous data on sheep Sprn expression used non-quantitative methods and showed that it is expressed within the CNS [14]. Here we show that Sprn is expressed at a level at least two orders of magnitude below Prnp but has a hierarchy of expression in the CNS regions identical to Prnp. However, in the LRS the hierarchy is reversed, the spleen expresses very little Sprn.

Despite the fact that incubation period of scrapie disease in transgenic mice is inversely related to transgene copy number [2], it is clear from these data that there is no positive relationship between quantitative levels of Prnp and Sprn expression and scrapie pathology. Within the CNS the regions showing PrP^{Sc} accumulation earliest are obex and thalamus, which express the lowest level of Prnp/Sprn of the four regions examined. Frontal cortex consistently expresses the highest levels of Prnp but shows no signs of pathology until terminal stages of disease [9]. These data show that tissues with the highest levels of Prnp and Sprn expression are the last areas to show Prp^{Sc} deposition.

The progression of pathology in the LRS might be explained by anatomy rather than quantitative expression of either Prnp or Sprn. Sheep were infected by SSBP/1 inoculation in the drainage area of the PSLNs, and it was these nodes that showed first signs of PrP^{Sc} accumulation. In naturally-infected sheep, lymph nodes and spleen seem to show pathology at approximately the

same time [15], with infection being disseminated within lymph and blood plasma [16;17] or associated with migrating cells [18]. Despite the fact that CNS is likely to become infected from the periphery via spinal nerves [19], anatomy is unlikely to be the simple explanation for the pattern of progression within the brain. Obex (medulla) is not apparently affected in some strains of ovine and murine scrapie [20;21] and non-stereotaxic intracranial inoculation of mice with different strains of scrapie results in strain-consistent pathological profiles [22].

Flow cytometry has previously shown that Prnp genotype influences the quantitative expression of PrP by different blood components [23], with the highest level of PrP^{C} being in scrapie-susceptible VRQ/VRQ sheep, the lowest in scrapie-resistant ARR/ARR sheep. These reports imply that susceptibility of different genotypes is positively linked to quantitative PrP^{C} expression; but our data and those of Garcia-Crespo [24] show that for obex and the LRS, Prnp levels are significantly highest in the heterozygote sheep with intermediate susceptibility. One explanation for these results could be that quantitative expression of PrP^{C} is controlled at the translation level and not by gene transcription. Recently, Morel [25] illustrated that the principal mechanism of scrapie resistance in Prnp allelic variants is through different efficiencies of conversion of PrP^{C} to PrP^{Sc}. Using MALDI-TOF of VRQ/ARQ sheep they showed that both variants are represented equally in PrP^{C} but that the VRQ variant predominates in PrP^{Sc}. Our data also show that Sprn expression levels in PSLN but not other tissues are highest in resistant ARR/ARR sheep. This implies the existence of a common mechanism controlling these genetically unlinked, but homologous genes.

Our results show that expression of Prnp or Sprn in different areas of the brain, prescapular lymph node or spleen was unaffected by SSBP/1 scrapie at any stage of infection. This contrasts with a previous report that indicates that scrapie infection causes an increase in expression level of PrP in ileal Peyer's patches follicles, but not whole ileal Peyer's patches ten months after oral infection [26]. This report also demonstrates that in infected ileal Peyer's patches there are PrP^{Sc} high and PrP^{Sc} low follicles, but that they express identical levels of Prnp. As with data in this report this indicates the lack of direct relationship between quantitative levels of PrP expression and scrapie pathology.

Acknowledgements

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Figure Legends

Fig.1. Expression levels of Prnp transcripts in the obex, spleen and prescapular lymph node of sheep with defined Prnp genotype. Data points are mean replicates from individual sheep, ± standard deviation and group mean; $n \geq 9$, but points not plotted if they fall outside the linear range of the calibration curve.

Fig.2. Expression levels of Sprn transcripts in the frontal cortex, obex and prescapular lymph node of sheep with defined Prnp genotype. Data points are mean replicates from individual sheep, ± standard deviation and group mean; $n \geq 9$, but points not plotted if they fall outside the linear range of the calibration curve.

Fig.3. Expression levels of Prnp transcripts in the obex and prescapular lymph node of sheep with defined Prnp genotype at times after SSBP/1 scrapie infection and in mock-infected sheep (C). Data are group means ± standard deviation.

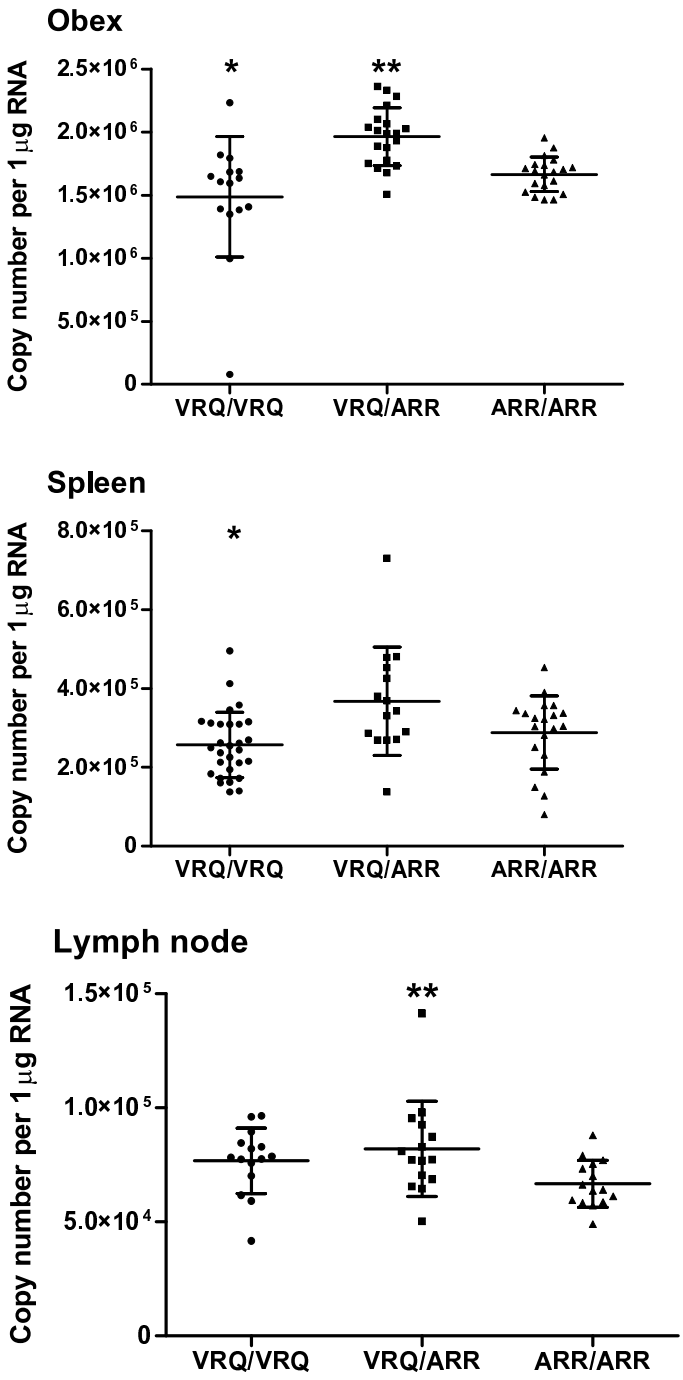
Table 1

Expression levels of Prnp and Sprn transcripts in the brain and peripheral lymphoid tissue of VRQ/VRQ sheep.

Tissue		**Copy number per 1 µg RNA ± SD**
Frontal Cortex	Prnp	$3.4 \times 10^6 \pm 5.1 \times 10^5$
	Sprn	$1.8 \times 10^4 \pm 7.9 \times 10^3$
Cerebellum	Prnp	$2.7 \times 10^6 \pm 6.1 \times 10^5$
	Sprn	$7.4 \times 10^3 \pm 1.6 \times 10^3$
Obex	Prnp	$1.5 \times 10^6 \pm 4.8 \times 10^5$
	Sprn	$2.5 \times 10^3 \pm 371$
Thalamus	Prnp	$1.5 \times 10^6 \pm 3.7 \times 10^5$
	Sprn	$3.1 \times 10^3 \pm 633$
Spleen	Prnp	$2.6 \times 10^5 \pm 8.3 \times 10^4$
	Sprn	307 ± 478 *
Lymph Node	Prnp	$7.7 \times 10^4 \pm 1.4 \times 10^4$
	Sprn	$3.0 \times 10^3 \pm 603$

* below level of accurate quantification

Figure


Obex
Copy number per 1 μg RNA
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
*
**
VRQ/VRQ
VRQ/ARR
ARR/ARR
Spleen
Copy number per 1 μg RNA
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6.0×10^5
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VRQ/VRQ
VRQ/ARR
ARR/ARR
Lymph node
Copy number per 1 μg RNA
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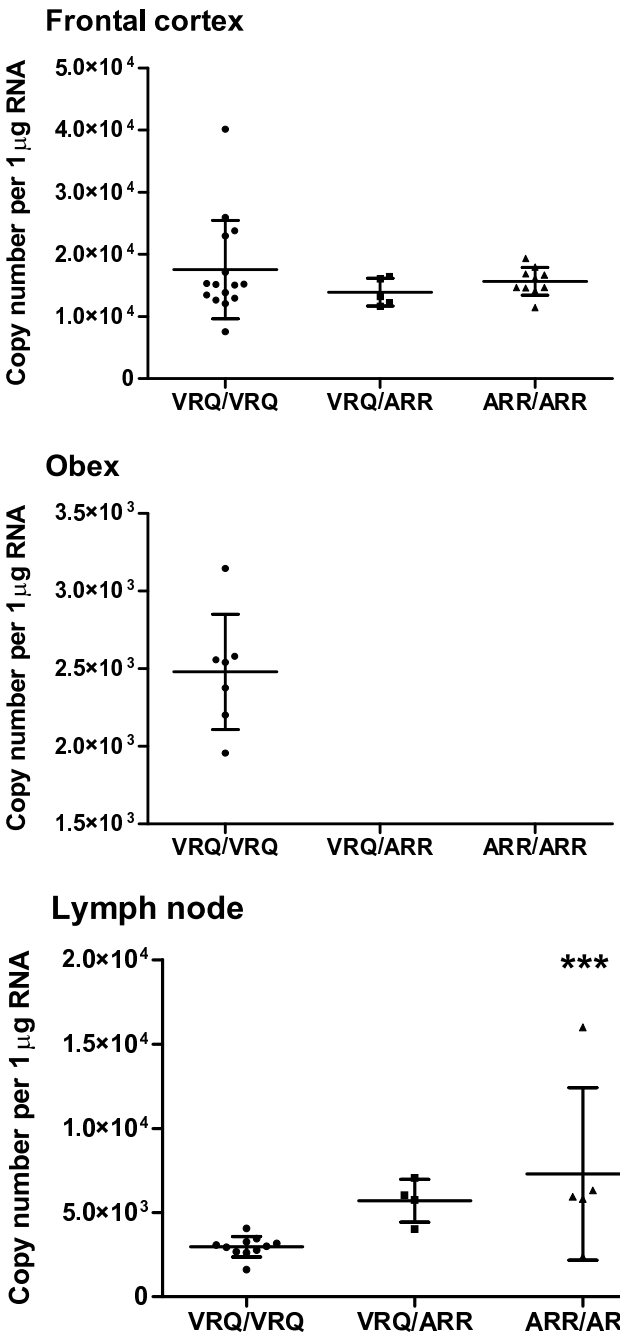
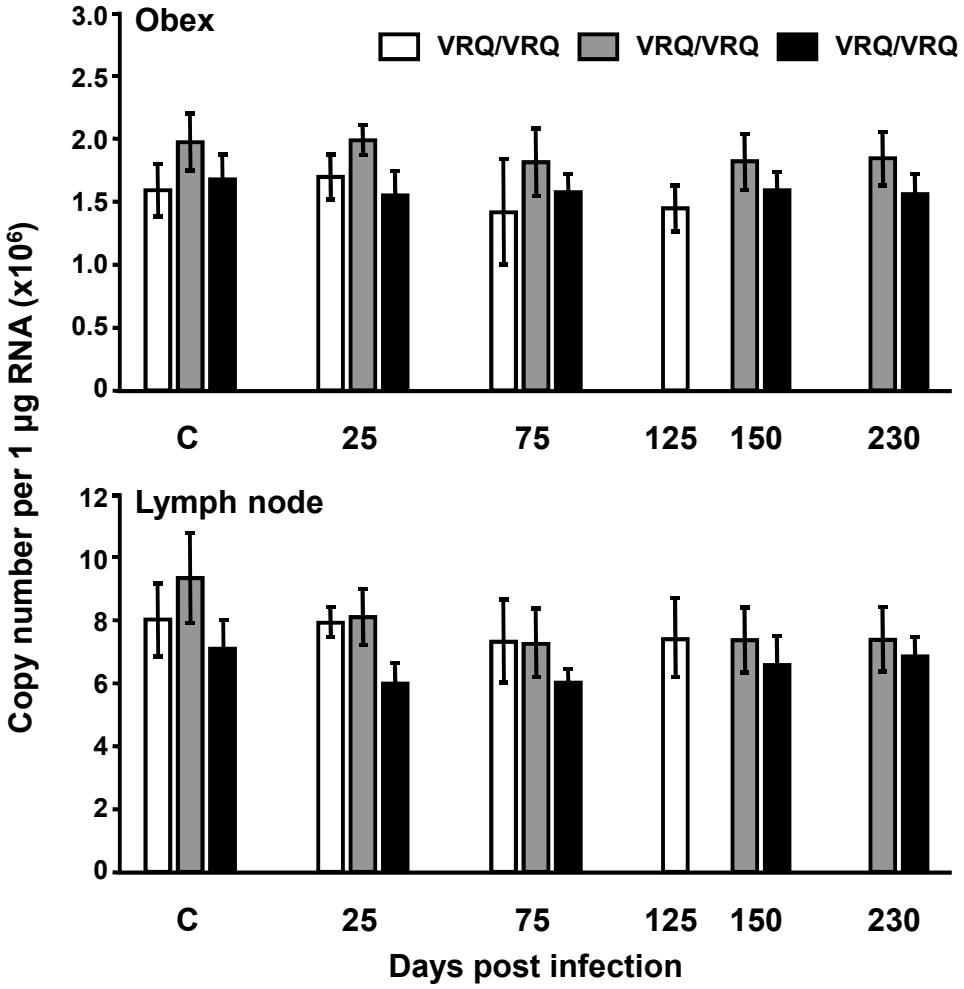
VRQ/VRQ
VRQ/ARR
ARR/ARR

Figure


Frontal cortex
Copy number per 1 μg RNA
5.0×10 4
4.0×10 4
3.0×10 4
2.0×10 4
1.0×10 4
0
VRQ/VRQ
VRQ/ARR
ARR/ARR
Obex
Copy number per 1 μg RNA
3.5×10 3
3.0×10 3
2.5×10 3
2.0×10 3
1.5×10 3
VRQ/VRQ
VRQ/ARR
ARR/ARR
Lymph node
Copy number per 1 μg RNA
2.0×10 4
1.5×10 4
1.0×10 4
5.0×10 3
0
***
VRQ/VRQ
VRQ/ARR
ARR/ARR

Figure


Obex
VRQ/VRQ
VRQ/VRQ
VRQ/VRQ
3.0
2.5
2.0
1.5
1.0
0.5
0
C
25
75
125
150
230
Lymph node
12
10
8
6
4
2
0
C
25
75
125
150
230
Copy number per 1 µg RNA (x10⁶)
Days post infection

Electronic Supplementary Material (online publication only)

Click here to download Electronic Supplementary Material (online publication only): Supplementary Table 1.doc