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Toll-Like Receptor 3 (TLR3) Plays a Major Role in the Formation of Rabies Virus Negri Bodies

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Abstract

Human neurons express the innate immune response receptor, Toll-like receptor 3 (TLR3). TLR3 levels are increased in pathological conditions such as brain virus infection. Here, we further investigated the production, cellular localisation, and function of neuronal TLR3 during neuronotropic rabies virus (RABV) infection in human neuronal cells. Following RABV infection, TLR3 is not only present in endosomes, as observed in the absence of infection, but also in detergent-resistant perinuclear inclusion bodies. As well as TLR3, these inclusion bodies contain the viral genome and viral proteins (N and P, but not G). The size and composition of inclusion bodies and the absence of a surrounding membrane, as shown by electron microscopy, suggest they correspond to the previously described Negri Bodies (NBs). NBs are not formed in the absence of TLR3, and TLR3−/− mice—in which brain tissue was less severely infected—had a better survival rate than WT mice. These observations demonstrate that TLR3 is a major molecule involved in the spatial arrangement of RABV–induced NBs and viral replication. This study shows how viruses can exploit cellular proteins and compartmentalisation for their own benefit.

Introduction

Toll-like receptors (TLR) are innate immune receptors that recognise and respond to the presence of PAMPs (pathogen associated molecular patterns) encoded by pathogens [1]. TLR3 is a type I intracellular transmembrane protein that contains a large leucine-rich repeat (LRR) in the extracellular region and a Toll/Il-1 receptor homology (TIR) signalling domain in its cytoplasmic region. TLR3 can detect the presence of and respond to exogenous and endogenous RNA molecules: dsRNA of viral origin, mimicked by polyriboinosine-polyribocytidylic acid (polyI:C); mRNA; and ssRNA (polyinosinic acid) [2,3,4]. Upon ligand binding, TLR3 signals via a MyD88-independent signalling pathway involving the adaptor molecule TRIF/Ticam-1 [5,6]. TRIF can induce activation of IRF3 and NF-κB, notably through the interaction with TRAF6 and RIP1 [7,8,9,10,11,12,13,14]. TLR3-dependent activation leads to the expression of genes encoding proinflammatory cytokines, chemokines and IFN-α/β.

TLR3 is present within the central nervous system (CNS) [2]. High levels of TLR3 are found in glial cells [15,16] and neurons in disorders of the brain, neurodegenerative diseases and viral infections [17,18]. The high levels of TLR3 in the CNS suggest an important role in the response to neuronal injury and/or viral infection [19], which may involve mechanisms other than those limited to the innate immune response. Indeed, TLR3 has been described as a negative regulator of axonal growth [20].

In non-neuronal cells (dendritic cells (DCs) or epithelial cells), TLR3 is found in intracellular compartments — sometimes small perinuclear structures (300–500 nm), such as those observed after overexpression [21,22], or others identified as multivesicular bodies (MVBs) [16,21]. Intracellular localisation of TLR3 appears to be crucial for its activation [23,24]. In neuronal cells, TLR3 is mainly intracellular [16–18]. However, the precise intracellular localisation of TLR3 in neuronal cells is largely unknown.

Rabies virus (RABV) almost exclusively infects neurons, where it triggers interferon, inflammatory and antiviral responses [17,25]. The virus particle binds cell-surface receptors and follows the endosomal pathway, allowing the release of viral nucleocapsid (NC) into the cytoplasm. The life cycle of the virus then progresses in the cytoplasm, with transcription of the five N, M, P G and L viral genes and the replication of negative- and positive-polarity genomes [19,26]. Moreover, RABV infection induces the formation of cytosolic protein aggregates called Negri Bodies (NBs). NBs are characterized by the accumulation of viral NC proteins [27–29]; they also contain endothelial nitric oxide synthase (eNOS) [30]. NBs resemble inclusion bodies and/or aggresomes seen in several neurodegenerative disorders [31]. Aggresomes are defined as pericentriolar cytoplasmic inclusions resulting from the aggregation of misfolded and/or ubiquitinated proteins [32,33]. The aggresomal subcellular compartment sequesters proteins produced in excess following a cellular stress such as a viral infection. These proteins are then targeted to proteasomes and/or autophagy components present in this area. Some viruses may use aggresomes to enhance replication [34,35].

In this study, we further analysed the production, function and spatiotemporal location of neuronal TLR3, and notably its association to the endosomal compartment. We carried out these studies either in cultures of human neuronal cells (neuronal
Author Summary

Viruses are obligate parasites. The progression of their life cycle depends on their hijacking the cellular metabolism and machinery. Human neurons produce TLR3, a protein involved in early host defence mechanisms and the modulation of neuronal survival. Rabies virus is a neurotropic virus, infecting mainly neurons. In this study, we showed that rabies virus exploits TLR3 function to store viral proteins and viral genomic material in particular areas of the cell where virus multiplication occurs. We found that, during the course of infection, large (1–3 μm) spherical inclusions were formed within the region around the nucleus. These inclusions were composed of an inner core of aggregated TLR3 surrounded by a coat of viral proteins and genomic material. These inclusions were revealed to be the previously described Negri Bodies (NBs). In absence of TLR3, NBs were no longer formed and virus multiplication rate decreased. Mice deficient in TLR3 were more resistant to rabies and had lower levels of infection in their brains. This study shows how neurotropic viruses, such as rabies virus, hijack normal functions of neuronal proteins and use cell compartmentalisation to promote viral multiplication.

Results

Human Ntera-2clD/1, SK-N-SH, and NT2-N cells express a full-length TLR3 whose expression is not altered by RABV infection

Human post-mitotic neurons (NT2-N) or neuroblastoma cell line SK-N-SH) or in TLR3<sup>−/−</sup> mice using the pathogenic RABV strain CVS.

As well as its endosomal localisation, TLR3 targets large perinuclear aggregates in RABV–infected cells

Two days after RABV infection, the endosomal distribution of TLR3 was conserved (as shown in Figure 2A, left panel, and Figure 2B, lower panel). However, colocalisation coefficients between TLR3 and CD63 were slightly lower in RABV-infected than in NI cells (0.96 and 0.79 for NI and RABV-infected cells, respectively). This decrease may result from the appearance of a new site of TLR3 intracellular localisation. TLR3 became concentrated in large perinuclear aggregates in RABV-infected cells (arrow heads in Figure 2A and 2B). This observation was reproduced using three distinct TLR3-specific Ab (Sc-Q18, Sc-C20 and FITC-conjugated Ab IMG-315C) and in cultures of NT2-N (Figure S1) and SK-N-SH (data not shown). Perinuclear aggregates were not stained by anti-CD63 or anti-EEA1 Ab, or by OKT9, a marker of recycling endosomes. This suggests that the RABV-induced aggregates were distinct from the endosomal compartment. RABV-induced TLR3 aggregates were not associated with Golgi (GM130) and with the endoplasmic reticulum, ER (calnexin) (data not shown).

Thus, in neuronal cells, TLR3 protein was located mainly within the early/late endosomal compartment.

TLR3 has a canonical endosomal localisation in human neuronal cells

Intracellular localisation of TLR3 was further investigated in Ntera-2clD/1 cells by immunocytochemistry and confocal microscopy. In the absence of infection, TLR3 was found within small vesicles throughout the cytoplasm. Co-immunocytochemistry analysis using Ab directed against the NH2 terminal of TLR3 (Sc-Q18) and against either CD63, a marker of late endosomes and/or multivesicular bodies (left panels of Figure 2A), or a marker of early endosome (EEA1) (upper panels of Figure 2B) revealed that a proportion of TLR3 was associated with endosomes, mainly in late endosomal vesicles (colocalisation index of 0.53 and 0.68 for early and late endosomal markers, respectively; Figure 2C). A small proportion of TLR3 was found associated with Golgi (GM130) and with the endoplasmic reticulum, ER (calnexin) (data not shown).

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TLR3 proteins are associated with RABV N and P proteins in perinuclear inclusions formed by an inner TLR3 core coated with viral proteins

Confocal microscopy analysis of RABV-infected cells co-stained with Ab directed against TLR3 and RABV nucleocapsid NC
revealed that RABV NC was located in perinuclear inclusions, with the great majority displaying the same localisation pattern as TLR3-containing aggregates (Figure 3A, TLR3 in red, NC in green). Viral NC (comprising N, P, L proteins and the viral RNA genome) seemed to form a ring around a TLR3 core. Alternative splicing was not observed during infection, monitored by viral N protein (N) transcription (lower panel). M denotes molecular weight. (C) TLR3 protein was detected in RIPA lysates of non-infected (NI) and RABV-infected Ntera-2cID/1 cells using a mAb (cloneMG315-A) directed to the NH2 terminal of TLR3. Infection was monitored by detection of the viral N (N) and P (P) proteins. TLR3 protein (102 kD) was not upregulated by infection, as shown by quantification of TLR3 (histogram) using calnexin (Cx) as a standard (GeneTools, Syngene). The same results were obtained for proteins extracted using Phosphosafe extraction buffer. Graph was generated using GraphPad Prism. (D) Cytofluorimetry analysis showed that TLR3 has an intracellular location and cannot be detected at the surface of SK-N-SH cells. This distribution pattern was not modified by infection. The viral nucleocapsid (NC)—left panels—is strictly intracellular, and its detection was thus used as a control for membrane impermeability. The solid line corresponds to RABV-infected cells and the histogram to NI cells. TLR3 protein (right panels) was analysed by flow cytometry using a polyclonal antibody directed to the NH2 terminal of TLR3 (Sc-Q18) in NI and RABV-infected cells. Histograms correspond to secondary Ab and solid lines to TLR3 staining. Numbers represent the percentages of cells positive for TLR3 or NC proteins. These results are representative of at least 5 separate experiments.

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with anti-NC Ab (Figure 4C). NC aggregates were detected as early as 12 h pi, with an average diameter of 1.0 μm (Figure 4E). The size of aggregates increased as infection progressed, reaching an average size of 3.0 μm by 48 h pi (Figure 4D, left panel). Number of inclusions per cell was analyzed by Acapella software in 24 h RABV-infected SK-N-SH cells (Figure 4D, right panel). Majority of cells harbour one or two perinuclear TLR3 aggregates 24 h after infection. Later in the infection (48 h after infection and thereafter), the average number of inclusions per cell increases (as described below), suggesting that NB formation is a highly dynamic process.

These observations suggest that RABV triggers formation of perinuclear TLR3 aggregates—surrounded by a halo of viral N and P proteins—which number and size are modulated during infection.

TLR3/NC aggregates contain the RABV viral genome and are Negri Bodies (NBs)

TLR3 has been described for its ability to bind viral RNA. We carried out hybridisation experiments using a fluorescent probe specific for the RABV genome leader sequence to determine whether the TLR3/NC aggregates contained viral RNA (Figure 5). We did not detect fluorescence in the absence of probe (Figure 5B) or in NI cells (Figure 5D). The fluorescent probe stained perinuclear structures (Figure 5A–5C), corresponding to TLR3/NC aggregates, forming discrete ovoid structures visible in transmission images, as described above (Figure 3). Intensity of fluorescence was variable, suggesting that accessibility of the viral RNA may differ among aggregates. These observations show that TLR3/NC aggregates also contain RABV genomic material. Immunocytochemistry experiment using J2 mAb specifically recognizing dsRNA [37,38,39]—including endogenous cellular dsRNA [40,41]—revealed accumulation of dsRNAs in NBs (Figure S2).

The presence of viral N and P proteins and genomic material within the aggregates suggests that they could be NBs. NBs are found both in the neurons of rabid brain and in vitro in RABV-infected neuronal cells, in the form of accumulated viral material around the nucleus. Electron microscopy analysis of ultra-thin sections of SK-N-SH 48 h after RABV infection showed that amorphous aggregates (usually one or two per cell), with an average diameter of 3.0 μm and without a membrane, were indeed present and displayed perinuclear localisation (Figure 5E). We did not find such aggregates in NI cells (data not shown). We used electron microscopy to analyse cells treated with Triton X-100 (0.1%) and stained with Ab directed against NC or TLR3; cellular structures were not preserved. Aggregates were strongly stained (DAB accumulation) with both Ab, indicating they contained both TLR3 and RABV NC proteins (Figure S3).

Figure 2. TLR3 partially colocalises with the endosomal compartment of non-infected and RABV–infected Ntera-2clD/1 cells and relocates into perinuclear aggregates after RABV infection. Co-immunostaining of TLR3 (Ab sc-Q18, green) with late (A) and early (B) endosomal compartments using anti-CD63 and anti-EEA1 Ab (red), respectively, in non-infected (NI) (A: left panels, B: upper panels) and RABV-infected (A: right panels; B: lower panels) Ntera-2clD/1 cells. Nuclei are stained with Hoechst. TLR3 colocalises with CD63 in NI (A, Merge left, arrow) and in RABV–infected cells (A, Merge right, arrow). TLR3 protein additionally forms perinuclear aggregates that do not contain endosomal markers (A and B, arrowheads). Images were acquired using a Zeiss LSM 510 META confocal microscope. Projection of 8–10 confocal images is shown. Bars = 10 μm. (C) Colocalisation of TLR3 with endosomal markers: correlation diagrams and Pearson’s correlation coefficients (Rcoloc) are shown: TLR3 (Y axis) with either EEA1 (1) or CD63 (2 and 3) (X axis) are shown for NI (1–2) and RABV–infected cells (3). TLR3 shows very little association with the early endosomal compartment (1), but is strongly associated with the late endosomal marker in the absence of infection (2). Colocalisation with the late endosomal marker decreases following RABV infection (3). doi:10.1371/journal.ppat.1000315.g002

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Electron microscopy also showed that the aggregates were located in particular cell areas characterised by the accumulation of surrounding mitochondria (M), rough endoplasmic reticulum (rER) and, in some cells, ‘nests’ of viral particles (Figure 5F). These areas, with their particular composition resembled the viral factories previously described for other viruses. Thus, TLR3/NC aggregates may function as a storage or construction area for viral NC.

Overall, these observations suggest that TLR3/NC aggregates observed in RABV-infected neuronal cells correspond to previously described NBs.

Are NBs virus-induced aggresomes?

Due to their perinuclear localisation, size and protein composition, it is possible that NBs represented virus-mediated aggresomes. Aggresomes are associated with molecular chaperones such as Hsp70 and surrounded by a cage of cytoskeletal proteins such as vimentin and α-tubulin [32,33,42]. Thus, we studied the presence of aggresome characteristics in NBs. Examination of the distribution of α-tubulin and vimentin in RABV-infected cells revealed that a ring of α-tubulin indeed surrounded the NC-positive aggregates (Figure 6A, 1–3). However, vimentin was not closely associated with NBs (Figure 6A, 4–6). The chaperone Hsp70 was present at the periphery of the NBs (Figure 6B). Aggresomes are often formed at the microtubule organising centre (MTOC), which can be visualised using Ab directed against γ-tubulin [43]. Double staining of MTOC and NC proteins clearly demonstrated that NBs are not localized at the MTOC (Figure 6C).

When cells are lysed in the presence of detergent, aggresomes are retained in the insoluble (IS) fraction, together with cytoskeletal proteins such as vimentin, whereas proteins in the cytoplasm or associated with the ER — such as calnexin — appear in the soluble (S) fraction. S and IS fractions were prepared from Ntera-2cID/1 cells infected with RABV for 48 h, and were separated by SDS-PAGE. The IS fraction prepared from RABV-infected cells did not contain calnexin or α-tubulin. These proteins were present in the S fraction, as expected, with the IS fraction containing vimentin (Figure 6D, upper). Immunostaining revealed discrete NBs (positively stained for both RABV NC and TLR3) in the IS fraction (Figure 6D, lower), indicating that NBs are detergent-resistant structures.

Given the major role of microtubules in the formation of aggresomes, we analysed their role in the formation of NBs. We treated RABV-infected SK-N-SH cells (24 h pi) with colcemid (Figure 7A) — an inhibitor of microtubule polymerisation — diluted in PBS vehicle, or with vehicle alone or without any reagent. Effect of colcemid on size and number of NBs per cell was analyzed 24 h post treatment -corresponding to 48 h post infection- (Figure 7B and 7C). As shown in Figure 7B, colcemid treatment modified the size of NBs. Overall, in colcemid-treated cells there are more cells with large NB (range between 200 and 350 pixels) than cells exhibiting small NB (range 50 to 150 pixels).
In the meantime, colcemid treatment drastically modified the distribution of NB per cell (Figure 7C). In absence of treatment, 24 h after infection RABV-infected cells harboured 2 or 3 NBs per cell (as shown in Figure 4D). This number rises up to more than 7–8 NBs per cell 48 h after infection (Figure 7C). Colcemid treatment blocks this increase since colcemid treated cells have less NBs per cell than non-treated cells. These observations indicate that microtubule polymerisation play a role in the dynamic of NBs particularly after their formation, as suggested by the increase in number of NBs per cell between 24 and 48 h pi. These data support a role for microtubules in the outcome of NBs.

TLR3 is necessary for the formation of NBs

TLR3 was found at the centre of NBs. Thus, we analysed the involvement of TLR3 in the formation of NBs in TLR3 silencing experiments. Plasmids encoding emerald GFP and miRNA targeting the TLR3 gene (miTLR3) were delivered by nucleofection (Amaxa program Q-001) into Hek293A Qbiogene cells. Non-specific miRNA (miNEG) was used as a control. Nucleofection efficiency (≥90%) was controlled using a plasmid encoding the GFP protein (provided by the Cell Nucleofector Kit V, Amaxa). Cells were infected with RABV 24 h after nucleofection. 48 h pi, RNA was extracted or cells analysed by immunocytochemistry. TLR3 mRNA levels were compared between miTLR3 and miNEG cultures. As shown in the left panel of Figure 8A, the level of TLR3 mRNAs was decreased by 60% in miTLR3 compared to miNEG cells. Cytofluorimetry analysis of TLR3 (sc-Q18 Ab) stained cells showed that these reduced mRNA levels were associated with a reduced intensity of fluorescence (Figure 8A, right panel), indicative of reduced TLR3 protein production in miTLR3 cultures. Moreover, the reduced TLR3 levels were accompanied by a 10-fold reduction of viral transcription.
and RABV N protein expression as shown by cytofluorimetry (Figure 8A, right panel), with N protein present in 77% of miNEG cells and 59% of miTLR3 RABV-infected cells.

Transitory nucleofected cells with the miTLR3 and miNEG vectors expressing emerald-GFP were assayed for expression of Em-GFP fluorescence by FACS (data not shown) and immunocytochemistry (Figure 8C), showing that the intensity of Em-GFP fluorescence is not homogenous among cells in the same population. Similar data were obtained on stable cell lines selected for resistance to blasticidin which all possess the expression plasmid but nevertheless exhibit a heterogenous Em-GFP fluorescence (data not shown). Cells were immunostained with anti-TLR3 (red) and anti-N-RABV mAb (blue) showing that the miTLR3 population of cells exhibits some TLR3-negative cells (Figure 8C, lower panel) in contrast to the miNEG population (Figure 8C, upper panel). In the TLR3-negative cells, NB formation is abolished. Immunocytochemistry analysis of individual cells (such as the green GFP cell on the right in Figure 8D) revealed that NBs — stained red for TLR3 and blue for RABV N protein — were not present in miTLR3 cells. These observations strongly suggest that TLR3 is required for NB formation and RABV N expression. To determine whether the cytoplasmic domain of the TLR3 protein encompassing the signalling TIR domain was involved in NB formation, we stable transfected HEK cells with a plasmid encoding a TLR3 gene with a deleted cytoplasmic domain (pZero-hTLR3-HA). Cells transfected with the pZERO-hTLR3 plasmid are therefore unable to signal via TLR3 because the absence of the TIR domain does not allow binding of TRIF to TLR3. As shown in Figure S4B, NBs were still formed and viral transcription was not modified in pZERO-hTLR3-expressing cells (transitory experiments). Similar data were obtained with stably transfected cell lines selected for their resistance to puromycin (data shown in Figure S4 are representative of both conditions). Furthermore, the adaptor TRIF could not be detected in NBs (Figure 4C), suggesting that activated TLR3 molecules were not present in NBs.

Thus, the ectodomain of TLR3 appears to be crucial for the formation of NBs. TLR3 may therefore favour virus multiplication. TLR3−/− mice have reduced susceptibility to rabies

Assuming that TLR3-NBs are required for virus multiplication, we would expect RABV infection to be impaired in the brain of TLR3-deficient mice (TLR3−/−) and subsequently these mice would be expected to have a higher survival rate than WT mice. We compared the progression and outcome of RABV infection in TLR3−/− with WT mice. TLR3−/− (n = 9) and control WT

(Figure 8B, left panel) and RABV N protein expression as shown by cytofluorimetry (Figure 8A, right panel), with N protein present in 77% of miNEG cells and 59% of miTLR3 RABV-infected cells.

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(n = 8) C57Bl6 mice were injected with a dose of virus expected to kill 3/4 of the mice. Mice were observed for 15 days post infection. At day 13 pi, only 37% of the WT mice were alive, whereas 66% of the TLR3$^{-/-}$ mice survived (Figure 9A). Progression of the disease in the two groups of mice is illustrated by the cumulative clinical curves (Figure 9B). Clinical score was significantly decreased in TLR3$^{-/-}$ mice compared to WT mice. Finally, RABV neuroinvasiveness was compared in these mice by measuring the amount of RABV genome in the brain of RABV-infected WT and TLR3$^{-/-}$ mice at day 11. Levels in NI brain were given a reference value of 1, for calibration. RABV genome level in TLR3$^{-/-}$ brain was markedly lower than in WT mice (20 times lower) (Figure 9C). All together, these strongly support a role of TLR3 in RABV infection.

**Discussion**

Our study demonstrates that TLR3 is localised in the cytoplasm of non-infected neuronal cells, where it is associated mainly with the endosomal compartment. TLR3 subcellular localisation is notably altered by RABV infection, with TLR3 found within viral-induced inclusions identified as RABV-induced Negri Bodies (NBs). The presence of TLR3 in the core of viral NBs, surrounded by a ring of viral N and P proteins, appears to be crucial for NB formation. This finding demonstrates a novel function of TLR3, a molecule mostly known for its immune functions.

In the absence of infection, reactivity with anti-CD63 antibody and weak signal from EEIA-expressing early endosomal structures showed neuronal TLR3 to be confined to the endosomal compartment, associated mainly to the late endosomes and multivesicular bodies. TLR3 is rarely detectable within the Golgi apparatus or at the endoplasmic reticulum. Indeed, we observed the canonical distribution pattern of TLR3 in human non-infected neuronal cells, as previously described for human DCs or fibroblasts [21,22].

The subcellular distribution of TLR3 is markedly altered in RABV-infected cells, being localised in perinuclear inclusion bodies, the NBs. These NBs contain viral NC but do not include the envelope viral proteins [27]. Relocation of TLR3 during viral infection has only previously been described for respiratory syncitial virus (RSV). In epithelial cells, RSV infection increases the TLR3 levels and targets TLR3 to the membrane [44]. In neuronal cells, RABV infection did not increase TLR3 protein levels, or target TLR3 to the membrane. Given that RABV infection did not increase the total pool of TLR3 protein, it is possible that the TLR3 is recruited from pre-existing cytoplasmic-, ER- or endosome-
associated TLR3 by NBs. However, NBs were not stained by endosomal or ER markers, suggesting that endosome- or ER-associated TLR3 is not involved in NB formation.

NBs observed by electron microscopy did not reveal the presence of any membrane surrounding the NBs. Thus, it is unlikely NBs are autophagolysomes or lysosomes. Confocal microscopy and 3-D imaging revealed that NBs have a highly organised structure, with a TLR3-containing core surrounded by a halo of viral N and P proteins. The central position of TLR3 suggests that NB formation may be initiated by the aggregation of TLR3 molecules. A primordial role of TLR3 in NB formation is consistent with the absence of NBs from cells in which TLR3 had been silenced. TLR3 is a horseshoe-shaped solenoid with 23 leucine-rich repeats (LRRs) located in the ectodomain of the molecule. Similarly to other solenoid proteins including polyQ, TLR3 may have an intrinsic capacity to form aggregates [45]. NBs formed even in the absence of the cytoplasmic domain of TLR3, suggesting a major role for the TLR3 ectodomain in NB formation (Figure S4). PolyQ proteins such as mutant huntingtin protein form inclusions consisting of an inner dense core of aggregated huntingtin surrounded by a ring structure composed of sequestered cellular proteins [46,47]. Experiments involving the sequential expression of polyQ followed by expression of these cellular proteins led to the conclusion that the ring structure results from the subsequent recruitment of cellular proteins at the exterior surface of an initial polyQ core aggregate. The organisation of NBs may follow a similar pattern. In this case, NBs would result from the initial association of TLR3 molecules, followed in a second step, by the accumulation of viral proteins, forming a ring into which cellular proteins could also be inserted. This would be consistent with the concentric organisation of NB structure and observation of the cellular protein eNos is recruited in NBs in RABV-infected brain. However, the NB structure is distinct from polyQ-initiated aggregates. In particular, TLR3 in NBs is still accessible to Ab. This has not been observed for huntingtin. These observations suggest that the viral ring of protein in NBs is more porous than the ring surrounding the ordered huntingtin aggregate structure. Consequently, NB and polyQ aggregates may have distinct properties. The recruitment and sequestration of cellular proteins in polyQ aggregates may lead to the functional depletion of cellular functional proteins, possibly underlying toxic properties of these aggregates in the cell. This is not necessarily the case for NBs.

The nature of the interaction between TLR3 and viral proteins in NBs remains unclear, since attempts to coimmunoprecipitate TLR3 and NC have failed.

NBs mimic aggresomes but do not exhibit the full set of characteristics of these stress-induced cellular processes. NBs are associated to the chaperone Hsp70 and to the microtubule network, reminiscent of the two principle features of aggresomes [48]. However, the marked redistribution of vimentin fibres observed for example in GFP-250-induced aggresomes, and in Theiler’s or reovirus-induced aggresome-like inclusions [49,50] was not observed in RABV-infected neuronal cells. In contrast to most aggresomes,
such as those formed in HSV2 infection [51], NBs were not associated with the microtubule organising centre (MTOC).

The effect of inhibition of microtubule depolymerisation, by addition of colcemid, on the distribution and size range of NB may indicate that microtubules are required for NB outcome. The finding that NBs do not display all the characteristics of aggresomes has also been observed for inclusions formed in African swine fever virus (ASFV), which have been described to function as "viral factories". Viral factories are areas of cytoplasm where viral components and cellular components supporting viral replication are concentrated [51,52]. The possibility that NBs function as dynamic structures involved in viral multiplication, as proposed by Lahaye et al. (submitted), is supported by electron microscopy pictures showing that NBs are found closely associated with newly synthesised viral particles. TLR3 would thus be an essential component for virus multiplication. Consistent with this notion, we showed that NBs were absent from cells with TLR3 silencing and that TLR3-/- mice were less severely infected by RABV than WT mice. Further studies are now required to show whether RABV production is promoted by TLR3.

A major function of TLR3 is to sense and respond to viral infection. The presence of TLR3 in the core of NBs can be seen as an attempt to inactivate TLR3 function. Sequestration of TLR3 into NBs could reduce the cellular innate immune response. The absence of TRIF – adaptor of TLR3 - in NB could support the hypothesis that TLR3 molecules in NB are inactive-. The presence of viral RNA in NBs may reflect an interaction of TLR3 with viral components at an early stage of infection, when the virus enters the cell through the TLR3 decorated endosomal compartment. TLR3 could thus be involved in recruitment of viral NC from the endosomal compartment, before aggregation in NBs. However, the absence of endosomal markers within the NBs remains unexplained. In addition, the presence of dsRNAs in NBs may suggest that NBs can also sequester dsRNAs and thus may play a role in the innate immune response to RABV infection.

Alternatively, given that TLR3 can trigger neuronal apoptosis [53], the sequesterisation of TLR3 into NBs in RABV-infected neuronal cells to escape TLR3-induced apoptosis. Tanaka et al, 2004 showed an uncoupling of a-synuclein-/ synphilin-1-positive aggregate formation and apoptosis [54]. They

Figure 8. TLR3 is required for the formation of viral NBs. Hek293A cells were nucleofected (QN-001 Amaxa Program) with Emerald-GFP-plasmids encoding specific miRNA for TLR3 (miTLR3) or control miRNA (miNEG). 24 h post-nucleofection cells were infected or not with RABV. (A) Efficiency of silencing was assayed at transcriptional level by quantitative PCR (left panel) and at protein level by cytofluorimetry (right panel). TLR3 mRNA levels in miTLR3–treated cells were 60% lower than in miNEG cells. Cytofluorimetry analysis showed that TLR3 levels (shift to the left) in miTLR3–treated cells were lower than in miNEG cells. The values are representative of at least 3 experiments. (B) TLR3 silencing decreases viral multiplication. Control cells (miNEG) and TLR3–silenced cells (miTLR3) were assessed for viral genomic material (left panel, Q-PCR) and RABV protein N levels by flow cytometry (right panel). RABV transcription was decreased by 80% after silencing and the percentage of cells producing N was reduced following TLR3 silencing. (C) The absence of NBs was demonstrated by immunofluorescence. TLR3–silenced cells were immunostained with anti–TLR3 (Sc-Q18) (red) and anti-viral N protein Ab (blue). Plasmid encodes emerald-GFP (green). NBs were detected in TLR3-positive cells but not in TLR3–silenced cells as observed in the miTLR3 population. Bars = 20 μm. (D) Enlarged area of C panels.

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showed that aggregation of α-synuclein-/synphilin-1 seemed to promote cell survival rather than cell death. In our model, sequestration of TLR3 could be a way for the virus to prevent a TLR3-mediated pro-apoptotic response to infection, as neuronal integrity is required for RABV propagation though the CNS. Promotion of neuronal survival and virus replication are not exclusive and could be complementary strategies. Preliminary results indicate that RABV strains promoting either neuronal survival or death display different effects on NB size. Further experiments will be required to understand the role of NBs in the control of neuronal death.

In conclusion, these findings describe a novel role for TLR3 and describe how viruses — in our case, RABV — hijack normal functions of neuronal proteins and exploit cell compartmentalisation to favour the progression of their life cycle.

Materials and Methods

Cells and virus

Human Ntera-2-c1D/1 cells (ATCC CRL, 1973), human SK-N-SH cells (ATCC HTB11) and HeLa293A (QBiogene) were grown in Dulbecco’s Modified Eagle Medium (DMEM) with Glutamax-I, high glucose and sodium pyruvate 100 nM (Invitrogen, UK), supplemented with 10% FCS-N (foetal calf serum for neuronal cultures, Invitrogen, U.K.), 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen). Human NT2-N cells [55] were differentiated from Ntera-2-c1D/1 cells by trans-retinoic acid and antimitotic treatment as previously described [56,57]. BSR cells (a clone of BHK21 baby hamster kidney cells) [58] were cultivated in DMEM supplemented with 8% FCS. Human grade III U373MG astrocytoma (ATCC HTB 17) cells were propagated in Dulbecco’s modified essential medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5% FCS, 2% sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C. The human microglial cell line CHME was cultivated as previously described [59]. The laboratory RABV strain CVS (ATCC vr959), a highly pathogenic strain causing fatal encephalomyelitis in mouse after intramuscular injection [60], was propagated as previously described [61]. Cells were infected at a multiplicity of infection (MOI) of 3 and cultivated for 12, 24 or 48 h at 37°C, in 5% CO2. HSV-1 strain KOS [62] was propagated in U373MG cells.

Mice

TLR3−/− mice were generated as described [2]. Six-week-old C57Bl6 from Janvier (St. Berthevin, France) or TLR3−/− female mice were inoculated intramuscularly in both hind legs, with 1×107 infectious particles of RABV. Disease progression was evaluated by scoring clinical signs and mortality. Mobility and mortality were scored as follows: 0 = normal mice, 1 = ruffled fur, 2 = one paralysed hind leg, 3 = two paralysed hind legs, 4 = total paralysis (defined as the total loss of mobility) and 5 = death. Daily clinical score was obtained by adding individual scores. Dead mice were reported in the clinical score of the day of the death and thereafter (cumulative scores). Disease progression was presented by a curve of cumulative clinical scores. Mortality was scored daily. Dead mice were counted on the day of death and thereafter (cumulative score). At day 11 after infection, groups of three mice were perfused with PBS. Brains were removed separately and stored at −80°C before being processed for RNA extraction. Animal housing and experimental protocols followed guidelines approved by the French Ministry of Agriculture and Ethical committee.
88) were from Sigma (Saint-Quentin Fallavier, France). Ab to Hsp70 (SFA-810, 1/200) was from Stressgen Bioreagents (Victoria, BC, Canada). Ab directed against calnexin (clone 37, 1/500) and EEAI (1/500) were purchased at BD Transduction Laboratories. Fc Block™ and CellFIX™ were from BD Pharmingen (BD, Franklin Lakes, NJ, USA). KaryoMAX® Colcemid® Solution in PBS, Alexa Fluor® 488-conjugated anti-IgG rabbit and Alexa Fluor® 594-conjugated anti-IgG mouse were from Invitrogen (Cergy-Pontoise, France); ProLong® Gold Anti-fade reagent with or without DAPI and Alexa Fluor® 594-conjugated anti-IgG goat were purchased at Molecular Probes (Eugene, Oregon, USA). R-phycocerythrin-conjugated streptavidin was purchased from Dako (Trappes, France). Ab against CD63 is a kind gift from Eric Rubinsteim (Inserm 602, Villejuif, France). Peroxidase-conjugated donkey anti-IgG mouse and AMCA-conjugated Streptavidin were from Jackson ImmunoResearch Laboratories (Suffolk, UK). For hybridisation experiments: RNA storage solution, proteinase K solution, deionised formamide, ultrapure water (Millipore, Billerica, MA). For RT–PCR, quantitative PCR 1 μl cDNA synthesized using the SuperScript II reverse transcriptase (RT) and 1 μl Taq DNA polymerase (QBiogene) were used for RT-PCR in a Pe2 thermal cycler (Hybaid Corp., USA). 35 cycles of amplification were performed as follows: 4 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, 10 min at 72 °C, and final cooling. Ribosomal 18S RNA was used as a housekeeping gene. Primers used for amplification of the TLR3 and 18S genes were synthesised by Eurogentec (Belgium), with the following sequences (5′→3′): forward-18S: CCTAGAGGGACAAGTGGCC; reverse-18S: ACGGTGAAGCTGAATGTA; forward-TLR3: GAGGGGTTTTTGAAGTAGAGA; reverse-TLR3: AAGTGCAATGAGGCCC. The primer pair used for TLR3 exon IV deletion is (5′→3′): Forward-exon4-TLR3: CTCCGAAGGTGTTTCCAGATTTG; Reverse-exon4-TLR3: TTTGAGGTACCTCACATTGAAAAGCC. For quantitative PCR, specific primers for TLR3 were from Qiagen (QuantiTect Primer #QT00007714). For amplification of RABV genome the following primers were used (5′→3′): F = GGAATTTCTCCG-GAAGACTGGACACGTATGAG; R = AGAATTTCCACCTA-AGCCTAGTGGAC. Real-time RT-PCR analysis was performed with an ABI Prism 7700 sequence detection system. Methods and relative quantification of gene expressions were carried out using the comparative method according to the manufacturer’s instructions. Sequence of neuronal TLR3 was determined and assigned the GenBank accession number DQ445682.

Flow cytometry

RABV-infected and mock-infected NTera2-1D/1 cells were washed once with phosphate-buffered saline (PBS) containing Ca²⁺Mg²⁺ (PBS Ca⁺⁺Mg⁺⁺), scraped (Cell Scraper, Corning) and pelleted in staining buffer (SB) (PBS, 1% inactivated FCS, 0.1% sodium azide, pH 7.5). Cells were fixed in 4% paraformaldehyde (PFA)/PBS for 30 min at 4 °C, and resuspended in permeabilisation buffer (PB) (PBS, 1% FCS, 0.1% sodium azide, 0.1% saponin). Cells were then incubated with FITC-conjugated anti-RABV NC Ab for detection of viral proteins and/or successively incubated with goat anti-TLR3 Ab (sc-109) followed by biotinylated anti-goat IgG Ab and finally with R-phycocerythrin-conjugated streptavidin. Cells were then washed with PB. For staining of non-fixed cells, cells were washed with SB instead of PB and fixed in CellFIX™ (BD Biosciences, USA). Cytofluorimetry was performed with a FACS-Calibur™ (BD Biosciences). Results were analysed using the CellQuest™ Pro (BD Biosciences) software.

Colcemid treatment

Cells were mock- or RABV-infected (at MOI 3) and treated or not at 24 h pi with colcemid (0.4 μg ml⁻¹) or vehicle alone (PBS). Cells were fixed at 48 h pi and immunostained for NC and tubulin proteins. Widefield observation was performed on Zeiss Apotome and Zeiss Axiovision 4.2 software for image acquisition. Nuclei were detected by DAPI staining and NB by anti-NC FITC Ab. Acapella software (PerkinElmer Acapella 2.0 (TM)) was used to delineate inclusions and measure of size of viral inclusions (area determined by number of pixels). The attribution of NB in each cell was computed by defining a region of interest around each nucleus. A set of negative controls has been used to fix the Acapella parameters (i.e. threshold or contrast adjustment). The different characteristics (area, position and number of NB for each cell) were saved and exported to Excel (Microsoft).

Immunocytochemistry

RABV- and mock-infected cells were washed once with PBS Ca²⁺Mg²⁺, fixed with 4% PFA for 30 min at room temperature (rT), washed again, and treated with gelatin (1% in water) for 5 min at 4 °C. The samples were then incubated for 20 min at rT in 0.3% Triton X-100-PBS and surface IgG receptors were blocked with a saturating buffer (ST) (2% bovine serum albumin and 5% FCS in PBS) for 30 min at rT followed by a 10 min incubation at 4 °C with Fc-block (1/50). Viral NC were detected by incubation with a FITC-conjugated rabbit Ab for 2 h at rT. Primary and secondary Abs were diluted in ST. For immunostaining, cells were incubated with secondary Ab, biotin/ streptavidin, followed by incubation with DAB, resulting in a brown coloration. Nuclei were stained with Hoechst 33342 for immunofluorescence analysis or haematoxylin for transmitted light acquisition. Slides were washed with PBS Ca²⁺Mg²⁺, using water for the last wash. Coverslips were mounted in Fluoromount-G™ or ProLong Gold Antifade reagent (+/- DAPI if Hoechst previously used). Widefield observation was performed on a Leica DM 5000B UV microscope equipped with a DC 300FX camera (×40 and ×63 objectives). Leica FW 4000 software was used for image acquisition and processing. Confocal images were acquired using a Zeiss LSM 510 META (version 3.2) on Axiovert 200 M with a Plan Achromat 63× and N.A. 1.4 objective. Confocal stacks were deconvoluted on Imaris® (Bitplane AG, Switzerland) software. Confocal images were compiled using serial Z-stacks 0.3 μm apart, representing at least two experiments. Co-localisation of TLR3 and organelle markers was analysed using the colocalisation finder and threshold

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protein was quantified using the Micro BCA Protein Assay RIPA buffer. Soluble and insoluble cellular protein fractions were separated using previously described protocols [33, 42]. Lysate protein was quantified using the Micro BCA Protein Assay Reagent. Proteins (20 μg) were loaded into a 10% Tris/Glycine SDS polyacrylamide gel with Kaleidoscope pre-stained standards (161-0324, Biorad). Proteins were separated and transferred to a Hybond-P PVDF membrane for 2 h (150 mA), followed by saturation for 2 h at rT in saturating buffer (PBS-Tween 0.1%, Milk 5%). Membranes were incubated with primary Ab overnight at 4°C, washed in PBS-Tween 0.1%, then incubated with secondary Ab coupled to horseradish peroxidase (1 h at rT). Membranes were washed again and analysed by chemoluminescence using the ECL® kit. Signals are acquired with a GBOX (Syngene) monitored by the Gene Snap (Syngene) software.

Electron microscopy

Confluent RABV-infected or mock-infected SK-N-SH cells were cultured for 48 h at 37°C, in 5% CO2 on untreated 4 mm2 square glass coverslips. Cells were fixed for 30 min at 4°C in a PFA/glutaraldehyde solution. Culture medium was removed and immediately replaced by fixative solution with 2% freshly depolymerized PFA + 2% Glutaraldehyde in 0.1 M PBS. Subsequent treatments were performed at 4°C. Cells were rinsed three times in PBS and post-fixed for 30 min in 1% osmium tetroxide in milliQ water and rinsed. They were dehydrated in graded concentrations of ethanol followed by pure acetone before being infiltrated with Araldite. The resin was cured for 3 h at 37°C, then 36 h at 61°C. Ultrathin sections were obtained with a Reichert Ulturract. Sections were counterstained (or not) with uranyl acetate and lead citrate and observed on a Jeol 1010 electron microscope. Electron microscopy was also performed on cells fixed in 3% PFA, permeabilised with 0.1% Triton x100, incubated with anti-RABV NC or anti-TLR3 (sc-H125) antibodies and revealed with DAB. Under these conditions, Abs can access intracellular targets, but cell structures are poorly conserved.

In situ hybridization

Probe was designed to target the leader sequence genome of the RABV strain SAD B19 (5’ ACCAGATCAGAAAAACAGACATTGACATTTG 3’). RNA probe was synthesised by Dharmacon and a fluorescein molecule was added at the 3’ end. RNA probes were prepared according to Dharmacon’s protocol and kept at –20°C. In situ hybridization was performed as described above. Stable cell lines were established by addition of selection antibiotics (blasticidin or puromycin) to culture medium; cells were passaged over several weeks (≥5 weeks).

Overexpression of a TLR3 deletion construct

pUno-mcs and pZERO-hTLR3-(deltaTIR) plasmids (Invivogen) were amplified according to Invivogen protocol and purified using JetStar kit Plasmid purification MAXI kit (Genomed). DNA was quantified and plasmids used for nucleofection of Hek293 cells (Amaxa program Q-001) (5 μg plasmid for 1×106 cells). 48 h post-nucleofection cells were incubated with RABV and 24 h post-infection cells were harvested for total RNA extraction. Q-PCR was carried out as described above. Stable cell lines were obtained by selection of antibiotic resistant clones.

Data analysis

Graphs (Figures 1C, 4D, 7B, 7C, 8A, 8B, 9, S4) and Student’s T tests (Figures 4D left, 7B, 7C, 9, S4) were generated and performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). For mice experiments, collected data were plotted for comparison of Kaplan-Meier survival curves and statistical analysis was performed with MedCalc software.

Supporting Information

Figure S1 TLR3 protein in NT2-N. Intracellular localisation of TLR3 in human post-mitotic neuron (NT2-N) cells. NI and RABV-infected NT2-N were immunostained with TLR3 and NC Abs. In the absence of infection (NI), TLR3 is localised in small vesicles throughout the cytoplasm. In RABV-infected cells, TLR3 and viral NC proteins are assembled in well-defined ovoid cytoplasmatic structures (arrow). N = nuclei. Bars = 5 μm.

Found at: doi:10.1371/journal.ppat.1000315.s001 (1.50 MB TIF)
**Figure S2** dsRNA can be detected within TLR3-positive viral NBs. Intracellular detection of dsRNA was assessed using J2 Ab (red) in non-infected (NI, upper panels) and RABV-infected SK-N-SH (NI, lower panels). NBs are detected with anti viral NC Ab (24 h pi), dsRNA is stained in both NI and RABV-infected cells and within the viral NBs, mainly in the coronary. 

Found at: doi:10.1371/journal.ppat.1000315.s002 (1.71 MB TIF)

**Figure S3** NBs are TLR3/NC aggregates. Electron microscopy section of 48h-RABV infected SK-N-SH showing typical NBs containing viral NC (A) and TLR3 (B) proteins. NB = Negri Bodies. B.w = base of culture well. C.m = culture medium. Bar = 5 μm in A and 2 μm in B. 

Found at: doi:10.1371/journal.ppat.1000315.s003 (1.70 MB TIF)

**Figure S4** TIR is dispensable for NBs formation. TRIF - TLR3 adaptor - cannot be detected in NBs. (A) Overexpression of a TIR-deleted form of TLR3 (encoded by the pZERO-hTLR3-HA plasmid from Invivogen) was assessed by RT Q-PCR (left panel) in Hek293A cells. An average fold increase of 25 for TLR3 mRNA in cells transfected with pZERO plasmid was obtained compared to Hek293A cells. (B) Overexpression of a deleted form of TLR3 does not alter the expression of endogenous TRIF. SH (lower panels). NBs are detected with anti viral NC Ab (24 h post infection) showing typical NBs (green). Nuclei (blue) were stained with DAPI. Bar = 10 μm. (C) Immunostaining of RABV-infected SK-N-SH with an anti-TRIF (green), an anti-RABV P protein (red) Ab and Hoechst (Nuclei, blue) revealed that TRIF is not located within viral NBs. Bar = 5 μm. 

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**Author Contributions**

Conceived and designed the experiments: C. Préhaud, M. Lafon. Performed the experiments: P. Ménager, P. Roux, F. Megret, J. Bourgeois, A. Le Sourd, M. Lafage. Analyzed the data: P. Ménager, A. Danckaert, M. Lafon. Contributed reagents/materials/analysis tools: M. Lafage. Wrote the paper: P. Ménager, M. Lafon.

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