Glutamate Excitotoxicity is Involved in the Induction of Paralysis in Mice after Infection by a Human Coronavirus with a Single Point Mutation in its Spike Protein

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ABSTRACT

Human coronaviruses (HCoV) are recognized respiratory pathogens and some strains, including HCoV-OC43, can infect human neuronal and glial cells of the central nervous system (CNS), and activate neuroinflammatory mechanisms. Moreover, HCoV-OC43 is neuroinvasive, neurotropic and neurovirulent in susceptible mice, where it induces a chronic encephalitis. Herein, we show that a single point mutation in the viral spike (S) glycoprotein (Y241H), acquired during viral persistence in human neural cells, led to a hind-limb paralytic disease in infected mice. Inhibition of glutamate excitotoxicity using an AMPA receptor antagonist (GYKI-52466) improved clinical scores related to the paralysis and motor disabilities in S-mutant virus-infected mice, as well as protected the CNS from neuronal dysfunctions, as illustrated by restoration of the phosphorylation state of neurofilaments. Expression of the glial glutamate transporter GLT-1, responsible for glutamate homeostasis, was down-regulated following infection and GYKI-52466 also significantly restored its steady state expression level. Finally, GYKI-52466 treatment of S-mutant virus-infected mice led to reduced microglial activation that may lead to improvement in the regulation of CNS glutamate homeostasis. Taken together, our results strongly suggest an involvement of excitotoxicity in the paralysis-associated neuropathology induced by a HCoV-OC43 mutant which harbors a single point mutation in its spike protein that is acquired upon persistent virus infection.
INTRODUCTION

Coronaviruses form a family of ubiquitous enveloped RNA viruses that induce respiratory, enteric and neurological diseases in several species (10). Human coronaviruses (HCoV) are respiratory pathogens responsible for upper and lower respiratory tract infections (49) and for the severe acute respiratory syndrome (SARS; 41). Possible involvement of HCoV other than SARS-CoV in more serious human pathologies was recently reviewed (49). Indeed, HCoV were associated over the years with the development of pneumonia, myocarditis, meningitis (16, 39) and occasionally for acute disseminated encephalitis (54). We have previously demonstrated that HCoV are neuroinvasive in humans, can infect and persist in human neural cells and can activate glial cells to produce pro-inflammatory mediators (3-5, 8, 14). Moreover, we have shown that wild-type reference HCoV-OC43 has neuroinvasive properties in mice, leading to chronic encephalitis (25) with accompanying disabilities (23). Given that murine coronavirus (MHV), a structurally related strain of the human coronavirus OC43 (HCoV-OC43), can cause neurodegenerative and neuroinflammatory disease in mice and rats (10), we hypothesized that HCoV-OC43 might be associated with neuroinflammatory and/or neurodegenerative human diseases. We have recently reported that a viral variant with four point mutations in its surface spike (S) glycoprotein, acquired during viral persistence in human neural cells (48), led to a drastically modified virus-induced neuropathology in BALB/c mice, characterized by a multiple sclerosis (MS)-like flaccid paralysis and inflammatory demyelination (24).

Glutamate is the major excitatory neurotransmitter of the central nervous system (CNS) that is involved in several neurophysiological functions. A disruption of its...
homeostasis can damage neurons, which may eventually lead to cell death (30). This pathological process, designated excitotoxicity, is able to induce degeneration of neural cells following an excessive stimulation of glutamate on its specific ionotropic receptors (AMPAr and NMDAr) (35). Activation of these receptors results in neural Ca\(^{2+}\) influx, which can mediate excitotoxicity by the means of a cascade of events involving free radical production, mitochondrial dysfunction, and the activation of several enzymes involved in normal cell development and function, resulting in damage to the cell membrane, cytoskeleton and DNA (42). Interestingly, excitotoxicity was reported to be involved in several neurodegenerative diseases such as Alzheimer’s disease or MS in humans (13). Glutamate re-uptake is necessary for the regulation of physiological extracellular glutamate concentrations and is mainly mediated by high-affinity sodium-dependent transporters. At least five different glutamate transporters expressed on neuronal or glial cells (GLT-1, GLAST, EAAC1, EAAT4 and EAAT5) have been well characterized (12) and up to 90% of the total glutamate re-uptake in the adult CNS is achieved by the glutamate transporter 1 (GLT-1), mainly expressed on astrocytes (50). In several neurological diseases, disruption of GLT-1 expression level was reported to be associated with alteration in glutamate uptake (52).

Glutamate excitotoxicity can damage the cytoskeleton of axons in vivo, which may result in the slowing of axonal transport (1, 31). Neurofilaments (NF) are intermediate filaments constituting the neuron cytoskeleton and are involved in the stability of mature axons and the regulation of axonal transport rate. In physiological conditions, the heavy neurofilament proteins (NF-H) are predominantly phosphorylated in axons and nonphosphorylated in neuronal soma and dendrites (19). A shift of this NF phosphorylation...
state, which is represented by a loss of nonphosphorylated NF in soma and an increase of nonphosphorylated NF in axons are signs of neuronal injury (44). Evaluation of the NF phosphorylation state is a useful tool to monitor progressive axonal disabilities considering that the heavy NF-H proteins phosphorylation state has been shown to be a biomarker in neurodegeneration (37). We have already demonstrated that the NF-H phosphorylation state was altered following HCoV-OC43 infection of mice (24).

In the present study we demonstrate that only a single point mutation (Y241H) in the spike glycoprotein (S) of HCoV-OC43 is sufficient for the induction of motor dysfunctions and a paralytic disease in infected mice. Furthermore, infection of mice with this HCoV-OC43 S mutant induced a significantly stronger neuronal dysfunction and a significant decrease in the expression of the glutamate transporter GLT-1 on astrocytes compared to sham and wild-type virus infection. Therefore, the virus-induced pathological process appears to be driven by a glutamate excitotoxic mechanism, as blockade of AMPA receptor attenuated clinical scores related to the virus-induced paralysis and motor disabilities and partially restored physiological NF phosphorylation state and GLT-1 expression and reduced microglial cell activation.
**MATERIALS AND METHODS**

**Viruses.** The wild-type reference HCoV-OC43 virus (VR-759) was obtained in the 1980’s from the American Type Culture Collection (ATCC). The recombinant viruses of HCoV-OC43 (rOC/ATCC) were generated using the full-length cDNA clone pBAC-OC43FL and displayed the same phenotypic properties as the wild-type virus, as previously described (47). This recombinant virus was used as the reference control virus for all experiments. We introduced one or two point mutations at a time in the spike glycoprotein of HCoV-OC43, the D24Y and S83T mutations (corresponding recombinant virus designated rOC/U(S24-83)), the H183R and Y241H mutations (corresponding recombinant virus designated rOC/U(S183-241)), the H183R mutation (corresponding recombinant virus designated rOC/U(S183)) or the Y241H mutation (corresponding recombinant virus designated rOC/U(S241)) into the full-length cDNA clone pBAC-OC43FL by site-directed mutagenesis using the QuikChange® Multi Site-Directed Mutagenesis kit (Stratagene) as recommended by the supplier. Each cDNA clone was transfected into BHK-21 cells, amplified by two passages in the HRT-18 cell line and sequenced to make sure that only the introduced H183R and/or Y241H mutations were present and no other mutations appeared.

**Survival curves and clinical scores.** Female BALB/c mice (Jackson Laboratories) aged 22 days post-natal (DPN) were inoculated by the intracerebral (IC) route with $10^{2.5}$ TCID$_{50}$ of recombinant virus, as previously described (24). Groups of ten mice infected by each recombinant virus were observed on a daily basis over a period of 21 days post-infection (DPI) and survival and clinical scores related to motor dysfunctions were evaluated. Mice infected with rOC/U(S241) which presented motor dysfunctions were
evaluated and scored according to a scale based on experimental allergic encephalitis (EAE) clinical score (CS) evaluation (0-1: normal with no clinical signs; 1.5-2: partial hind-limb paralysis with a walk close to ground level; 2.5-3.5: complete hind-limb paralysis, 4-5: moribund state and death).

**Infectious virus assays.** For each experimental condition, groups of three infected BALB/c mice were selected randomly and dissected every two days to monitor infectious virus production in brains and spinal cords. Tissues were processed for the presence and quantification of infectious virus by an indirect immunoperoxidase assay as previously described (27).

**AMPA receptor antagonist.** The specific non-competitive AMPA receptor antagonist, GYKI-52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride) was obtained from Tocris Bioscience and dissolved in Hank’s Balanced Salt Solution (HBSS; Invitrogen) at a final concentration of 300 µg/mL of HBSS (46). To investigate the effect of treatment with GYKI-52466, two groups of ten BALB/c mice infected with rOC/ATCC or rOC/US241 were treated intraperitoneally with 3 mg/kg body weight of GYKI-52466 at 12 h post-infection, then twice daily for three weeks or only with 100 µl of HBSS (vehicle) to normalize experimental stress conditions, twice daily, over a period of three weeks. To verify the non-cytotoxic effect of the GYKI-52466 solution, sham-infected mice received the same dose of AMPA receptor antagonist. As described in the literature, this pharmacological dose was reported to have no behavioral effects and to induce neuroprotective actions (20, 40).
Immunohistochemistry. Groups of three BALB/c mice either sham-infected or infected by each virus and treated with GYKI-52466 antagonist or vehicle were selected randomly and perfused with a solution of 4% paraformaldehyde at 10 DPI, which corresponds to the peak of viral replication in the spinal cord and the outcome of clinical scores related to paralytic disease. Lumbar segments from spinal cords were cryoprotected in 30% (w/v) sucrose, frozen at -20°C and processed for sets of 8 µm section size with a cryostat (MICROM HM 525). Axonal damage was investigated by assessing the heavy neurofilament (NF-H) phosphorylation state. Tissue sections were incubated with a mouse anti-nonphosphorylated neurofilaments monoclonal antibody (mAb) (SMI 311, 1/1000, Covance) or a mouse anti-phosphorylated neurofilaments mAb (SMI 312, 1/1000, Covance), or Mac-2 rat mAb (1/200, ATCC, Cedarlane) for 2 h at room temperature. Tissue sections were then washed and incubated with a secondary anti-mouse or anti-rat biotinylated antibody, before revealing with ABC Vectastain kit (Vector Laboratories) as previously described (24). Double staining for astrocytes and glutamate transporter GLT-1 fluorescence were investigated with primary antibodies: polyclonal rabbit anti-glial fibrillary acidic protein (1/1000, GFAP, Dako) and goat anti-glutamate transporter GLT-1 (1/500, K-16 sc-31582, Santa Cruz Biotechnology). Spinal cord sections were blocked with horse serum in PBS 1X, for 1 h at room temperature. Following incubation with both primary antibodies for 2 h at room temperature, sections were washed, then incubated with secondary fluorescent antibodies in the dark, for 2 h at room temperature: Alexa Fluor 488 anti-rabbit (1/1000, Invitrogen) and Alexa Fluor 568 anti-goat (1/1000, Invitrogen). After final PBS 1X washes, tissue sections were incubated 5 min at room temperature with 4,6-
diamidino-2-phenylindole (1/100, DAPI; Polysciences Inc.), then mounted with Immuno-
mount and observed under a fluorescence microscope.

Protein extraction and Western blot analysis. Spinal cords from groups of three
mice selected randomly were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris,
pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM EDTA) supplemented
with the protease cocktail inhibitor 1X (P8340, Sigma, 100X). Lysates were cleared by
centrifugation for 5 min at 4°C at 17,000 x g and supernatants were aliquoted and stored at
-80°C. The BCA Protein assay kit (Novagen) was used to determine protein concentration,
according to the manufacturer’s protocol. Proteins (10 µg per sample) were separated on a
4-12% gradient gel Novex NuPage (Invitrogen), transferred to PVDF membrane
(Millipore, Immobilon-P Transfer Membrane) with the Bio-Rad Trans-Blot® Semi-Dry
Transfer Cell apparatus. Membranes were blocked with TBS buffer containing 1% (v/v)
tween (TBS-T) and 5% (w/v) non-fat milk at 4°C overnight, then membranes were
incubated with polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1/2000, Dako)
or guinea pig anti-glutamate transporter GLT-1 mAb (1/1000, AB1783, Millipore) or Mac-
2 rat mAb (1/100, ATCC, Cedarlane) or rabbit anti-GAPDH (1/1000, Santa Cruz
Biotechnology) for 1 h at room temperature. Membranes were washed three times with
TBS-T and then incubated with secondary antibodies for 1 h at room temperature: anti-
rabbit (1/1000, GE Healthcare UK) or anti-guinea pig (1/1000, Millipore) or anti-rat
(1/1000, Kirkegaard & Perry Laboratories) coupled to horseradish peroxidase and
detection was made by chemiluminescence using the Bio-Rad Immun-Star™ HRP
Substrate kit. Band detection and semi-quantification was made using the GeneSnap
software from Chemi Genius Syngene apparatus. ANOVA tests followed by post-hoc
Tukey’s analysis were performed to determine the statistical significances in the differences protein expression between different groups of mice using SPSS software version 16.0.
RESULTS

BALB/c mice infected with HCoV-OC43 containing the Y241H mutation in the spike glycoprotein (rOC/U S241) develop a hind-limb paralytic disease. We recently showed that a viral variant bearing four point mutations in its spike (S) glycoprotein (D24Y, S83T, H183R and Y241H), acquired during viral persistence in human neural cells (48), led to a modified virus-induced neuropathology in BALB/c mice compared to the reference HCoV-OC43 (rOC/ATCC) virus. This modified pathology was characterized by an MS-like flaccid paralysis with areas of demyelination in the spinal cord whereas mice infected by HCoV-OC43 (rOC/ATCC) induced only encephalitis (24). In order to further pinpoint the viral molecular determinants responsible for this modulation of virus-induced neuropathogenesis, we generated recombinant viruses that contained two S mutations at a time: D24Y and S83T (designated rOC/U S24-83) or H183R and Y241H (designated rOC/U S183-241) (Fig. 1A). Like in infection with virus containing the four mutations described above, infection of BALB/c mice by rOC/U S183-241 also led to a paralytic disease with small areas of demyelination in the spinal cord whereas rOC/U S24-83 induced encephalitis like rOC/ATCC. In order to investigate whether only one of the remaining mutations, either H183R or Y241H, was sufficient to induce the paralytic disease in mice, we generated recombinant viruses that harbored only one mutation at a time: H183R, designated rOC/U S183 or Y241H, designated rOC/U S241 (Fig. 1A). Whereas BALB/c mice infected with rOC/U S183 developed an encephalitis similar to what was observed after rOC/ATCC infection, mice infected with rOC/U S241 presented motor dysfunctions and paralytic disease. Neurovirulence of all recombinant viruses was evaluated following intracerebral inoculation of BALB/c mice (Fig. 1B). The survival curves of mice infected...
by the recombinant viruses bearing one, two, or four point mutations within the viral spike glycoprotein suggested a synergistic effect of these mutations on the mortality rate. Histological examination of infected CNS revealed that the primary target cell of the infection by all these recombinant viruses (rOC/US183-241, rOC/US183, rOC/US241) remained the neurons (data not shown), as previously described for rOC/US24-241 and rOC/ATCC (24). The rOC/US241 recombinant virus was associated with motor dysfunctions in infected mice. Therefore, we conclude that the Y241H single mutation was necessary and sufficient to generate the observed paralytic phenotype and focused the rest of the study on this recombinant virus to evaluate the involvement of this single Y241H mutation in paralytic disease, in comparison to the reference virus rOC/ATCC.

To determine whether the difference in pathology induced by rOC/US241 and the reference rOC/ATCC could be related to differences in kinetics of replication in the CNS, brains and spinal cords were harvested and infectious virus titers were assayed every two days for a period of 22 DPI. Even though both viruses replicated to similar extents in the CNS and the highest level of infectious virions were found at 10 DPI in brains as well as in spinal cords, rOC/US241 remained in the spinal cord for a longer period of time compared to rOC/ATCC. Indeed, infectious rOC/US241 virus was still detectable at 15 DPI whereas rOC/ATCC infectious virus was never detectable beyond 12 DPI (Fig. 2). Even though viral persistence was not evaluated, we have already reported that viral RNA of wild-type reference HCoV-OC43 (ATCC VR759; 23) and recombinant viruses rOC/ATCC (which has exactly the same genomic sequence as the virus ATCC VR759) and rOC/US24-241 (24) persist in mice for several months post-infection.
AMPA receptor antagonist (GYKI-52466) treatment attenuates mice motor dysfunctions and severe paralysis induced following infection by rOC/U5241. In order to characterize a possible involvement of excitotoxicity in rOC/U5241-induced hind-limb paralysis, mice infected by rOC/U5241 were treated with the AMPA receptor antagonist GYKI-52466 or vehicle (HBSS). GYKI-52466 treatment did not affect survival rate of mice infected by either rOC/ATCC or rOC/U5241 (Fig. 3A) but it attenuated clinical scores related to motor dysfunctions and paralytic disease induced following rOC/U5241 infection (Fig. 3B). Indeed, fewer mice presented mild paralysis (CS 1.5-2) and they recovered more rapidly when treated with GYKI-52466 compared to control vehicle-treated mice. Moreover, whereas 20 to 30% of mice infected by the rOC/U5241 virus and treated with vehicle presented severe paralysis (CS 2.5-3.5), only 5-10% of the mice treated with GYKI-52466 (with exception of 12-13 DPI) fell in this category (Fig. 3B). Furthermore, recovery was more rapid following treatment by GYKI-52466. The worst symptoms of motor dysfunctions were observed at 10 DPI, and disappeared totally in the following 6 days of GYKI-52466 treatment, whereas motor disability persisted in vehicle-treated mice. Indeed, as soon as 18 DPI, surviving mice infected and treated with AMPA antagonist recovered completely with no more detectable motor CS. On the other hand, 20% of the infected mice treated with vehicle only were still presenting severe paralysis (CS 2.5-3.5) at 21 DPI. Viral replication in brains and spinal cords was assessed in mice infected by rOC/U5241 or rOC/ATCC and treated with GYKI-52466 or vehicle. For both recombinant viruses, treatment with GYKI-52466 did not modify viral replication (Fig. 3C).

AMPA receptor antagonist treatment reduces neuronal dysfunction in mice infected by rOC/U5241. Spinal cords from mice infected by rOC/U5241 or rOC/ATCC and
treated with the AMPA receptor antagonist (GYKI-52466) or vehicle were harvested at 10 DPI (time of motor dysfunctions and severe paralysis) to evaluate whether a neuronal alteration associated with excitotoxicity was underway in infected mice. Neuronal dysregulation was investigated by evaluating the axonal neurofilament phosphorylation state. Using SMI 311, an antibody against nonphosphorylated NF-H proteins, we found that infection of mice with rOC/ATCC and rOC/US241 resulted in abnormal loss of soma nonphosphorylated NF-H in the spinal cord grey matter (GM) compared to sham-infected mice. This modification in the phosphorylation state was much more pronounced following infection with rOC/US241 compared to rOC/ATCC (Fig. 4, SMI 311 GM). On the other hand, spinal cord white matter (WM) of mice infected with rOC/ATCC and rOC/US241 showed an abnormal presence of nonphosphorylated NF-H, and axonal swelling in WM was more important following rOC/US241 infection compared to rOC/ATCC (Fig. 4, SMI 311 WM). Staining with SMI 312 antibodies against phosphorylated NF-H proteins showed a low level of axonal phosphorylated NF in the spinal cord white matter following infection by rOC/US241, compared to rOC/ATCC- or sham-infected mice. Moreover, treatment with GYKI-52466 of mice infected by both recombinant viruses partially restored the physiological NF-H phosphorylation state.

Glutamate transporter 1 (GLT-1) expression is down-regulated in mice infected by rOC/US241 and up-regulated following AMPA receptor antagonist treatment. Consequently, as glutamate excitotoxicity may be due to a problem in this recapture, we decided to evaluate the expression of GLT-1 on spinal cord astrocytes. Therefore, spinal cords were harvested at 10 DPI and GLT-1 expression was evaluated by staining with GLT-1 antibodies. The astrocytic receptor GLT-1 is responsible for 90% of glutamate recapture. Consequently, as glutamate excitotoxicity may be due to a problem in this recapture, we decided to evaluate the expression of GLT-1 on spinal cord astrocytes. Therefore, spinal cords were harvested at 10 DPI and GLT-1 expression was evaluated by staining with GLT-1 antibodies. The astrocytic receptor GLT-1 is responsible for 90% of glutamate recapture.

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immunofluorescence and Western blotting analysis. Infection by both viruses led to significant astrocyte activation compared to sham-infected mice treated with vehicle (Fig. 5A, B (### P<0.001)). Even though a greater number of GFAP-positive astrocytes were detected following infection, glial GLT-1 expression was significantly down-regulated in mice infected by rOC/U5241 (P<0.01) as revealed by immunofluorescence analysis (Fig. 5A) and confirmed by Western blotting analysis (Fig. 5B) compared to mice infected by rOC/ATCC and sham-infected mice. However, treatment with GYKI-52466 led to partial up-regulation of GLT-1 expression in mice infected by rOC/U5241 (** P<0.01), as shown by immunofluorescence and Western blotting analysis (Fig. 5A, B).

Treatment with AMPA receptor antagonist reduces microglial activation.

Microglia are often activated during glutamate excitotoxicity. Therefore, microglial activation was evaluated in the spinal cords of infected mice by immunohistochemistry and Western blotting analysis at 10 DPI. Mice infected by both viruses presented a significant activation of microglia/macrophages in the spinal cord as compared to sham-infected mice, where these cells were not activated under normal physiological conditions. Moreover, rOC/U5241 infection led to a drastically significant increase (* P<0.05) in the activation of microglial cells, compared to rOC/ATCC as revealed by quantitation of Mac-2 expression on Western blots (Fig. 6A, B). Treatment with GYKI-52466 reduced activation of microglia/macrophages (Mac-2 expression) in mice infected with rOC/U5241 (* P<0.05) but the level of microglial activation did not return to basal level of sham-infected animals.
We have previously shown that four mutations in the spike (S) glycoprotein of human coronavirus OC43 (D24Y, S83T, H183R and Y241H) modulate disease in infected BALB/c mice from an encephalitis to a flaccid paralysis and eventual demyelination (24). The aim of the present study was to determine whether only one of these four mutations was sufficient to induce hind-limb paralysis in mice and to determine the mechanism underlying this virus-induced neuropathology. Making use of a series of recombinant viruses produced with our infectious clone pBAC-OC43FL (47), we were able to identify a single point mutation (Y241H) in the HCoV-OC43 S glycoprotein that is necessary and sufficient for induction of hind-limb paralysis in infected BALB/c mice, without affecting viral neurovirulence or the kinetics of viral replication in brain, when compared to rOC/ATCC.

Even though in some viral model of CNS infection, blockade of excitotoxicity results in decrease of mortality of infected (21), we showed that treatment with an AMPA antagonist neither affected the survival rate of mice nor the viral replication in mice infected by both HCoV-OC43 variants (rOC/ATCC or rOC/U5241). However, the number of mice presenting motor dysfunctions or severe paralysis induced by the rOC/U5241 variant decreased in the presence of the AMPA antagonist GYKI-52466 and mice recovered more rapidly from motor disability compared to infected mice treated with vehicle alone. These results strongly suggest that glutamate excitotoxicity is involved in pathological process leading to motor dysfunction without any direct association with viral replication, which is not modified by the treatment with the AMPA antagonist.
Mice infected by rOC/U5241 or rOC/ATCC showed abnormal loss of soma nonphosphorylated NF-H in the grey matter (GM) and this was more important following rOC/U5241 infection. Moreover, spinal cord white matter (WM) of mice infected by rOC/U5241 or rOC/ATCC presented abnormal axonal nonphosphorylated NF-H, which was also more important following rOC/U5241 infection, even leading to axonal beading and swelling. It has already been reported that modifications in the phosphorylation state of neurofilaments contribute to motor neuron disease, as seen in Amyotrophic Lateral Sclerosis (ALS) and Multiple Sclerosis (MS) (9, 43). This abnormal disruption of NF-H phosphorylation state in axon and cell body observed following infection could lead to neuronal dysfunction, disruption of axonal transport with perturbations in neuronal transmission, which was demonstrated to account for motor disabilities (36). Treatment with GYKI-52466 significantly reduced the imbalance of NF-H phosphorylation state and also improved motor dysfunctions. This is consistent with the fact that glutamate excitotoxicity causes damages to the cytoskeleton of axons in vivo and that the administration of AMPA antagonist attenuated axonal damages (18). Moreover, glutamate excitotoxicity was shown to be involved in the slowing of axonal transport (1, 22, 31) that could account for axonal swelling observed in axons. As neurons are the main target of HCoV-OC43 infection, viral replication in neurons may induce neuronal stress that can lead to a dysregulation of glutamate metabolism and homeostasis (glutamate synthesis, release, recycling) as already shown in other neurotropic viral model (26, 33, 51). Indeed, it was reported that human immunodeficiency virus (HIV)-1 can upregulate glutaminase expression in infected macrophages (15), that the HIV-1 viral Tat protein increases neuronal glutamate exocytosis (32). Moreover, other studies have shown that the astrocytic
glutamate transporter GLT-1 was also down-regulated in viral models involving Sindbis virus or HIV (11, 53). Furthermore, excessive stimulation of glutamate on its specific receptors is known to induce degeneration of neural cells and to lead to neurodegenerative diseases (13).

In physiological conditions, glutamate homeostasis is in large part regulated by glial glutamate transporter 1 (GLT-1), mainly expressed on astrocytes, which is responsible for up to 90% of the total glutamate clearance in adult CNS (2). We showed a significant activation of astrocytes following infection by both viruses. However, the expression of GLT-1 did not correlate with this activation associated with an increased number of astrocytes expressing high levels of GFAP. Indeed, the results presented in Fig. 5 show that GLT-1 transporter expression was significantly down-regulated following rOC/US241 infection compared to mice infected by rOC/ATCC or sham-infected mice. Interestingly, treatment with GYKI-52466 led to a partial restoration of expression of the GLT-1 transporter, which however always remained inferior to basal levels. This incomplete restoration may account for the remaining motor dysfunctions observed. It was demonstrated that knockout mice for GLT-1 undergo seizures and death as a result of excitotoxicity caused by too elevated extracellular glutamate concentration (50). Moreover, decreased expression of this transporter was reported in several neurological diseases, as well as in viral models (7, 17).

We have previously reported that infection of mice by HCoV-OC43 led to the release of several pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) with a significant increase of IL-6 in mice infected with an S-mutant virus (24). These cytokines are known to down-regulate glutamate transporter GLT-1 expression (11, 34, 38). In the present study, we...
show that the mutant virus rOC/US241 (which harbors a single S point mutation Y241H) led to significant microglia/macrophage activation, compared to what is observed with the reference virus, and that treatment with the AMPA antagonist partially reduced microglial cell activation. Moreover, our results (Fig. 6) also indicate that the increased microglial/macrophage activation correlated with the GLT-1 down-regulated expression. Previous studies have demonstrated that microglia/macrophages can sense extracellular glutamate concentration via AMPA receptors on their membrane, and that glutamate acts as a chemokine for these cells (29). Furthermore, activated microglia/macrophages are a significant source of glutamate that can induce excitotoxic neuronal cell death (6). These results suggest that, in our model, the role of astrocytes is to rescue neurons from excitotoxicity by regulating glutamate homeostasis whereas activation of microglia/macrophages may exert excitotoxicity by down regulating GLT-1 expression via the release of some cytokines or by their capacity to release glutamate, as previously described (28). This is consistent with a previous study that showed that microglia/macrophages can release glutamate and that blockade of glutamate release from activated microglia attenuates experimental autoimmune encephalomyelitis in mice (45).

In summary, our study suggests that persistence-acquired mutations in the S glycoprotein of a human coronavirus (HCoV-OC43), which represents a possible viral adaptation within the CNS, could be involved in the development of neurological disorders in humans, and that a single such point mutation is sufficient to modulate virus-induced neuropathology from an encephalitis to an MS-like neuropathology that involves glutamate excitotoxicity in a mouse model. Deciphering the underlying mechanisms of HCoV-OC43 interaction with the central nervous system should lead to a better understanding of
potentially pathological consequences of this infection, as well as the importance of viral determinants in the process. Future studies will investigate the mechanism of action of both viruses (rOC/ATCC and rOC/U3241) regarding glutamate synthesis, release and uptake in order to better understand the exact role of excitotoxicity and its pathological consequences following HCoV-OC43 infection.
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REFERENCES


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LEGEND TO FIGURES

Figure 1. Schematic representation of the point mutations introduced within the viral spike glycoprotein and associated neurovirulence in mice of the different recombinant viruses. (A) Schematic representation of the main structural domains of the S protein of human coronavirus OC43, and the approximate locations of the four point mutations D24Y, S83T, H183R, Y241H. These mutations were introduced into the viral genome to generate rOC/U\textsubscript{S24-241} (24). Alternatively, only pairs of mutations were introduced to generate rOC/U\textsubscript{S24-43} and rOC/U\textsubscript{S183-241}. Finally, one single mutation was introduced at a time to generate rOC/U\textsubscript{S183} and rOC/U\textsubscript{S241}. * indicates recombinant viruses which induced a paralytic disease following infection. RBD, putative cell receptor - binding domain ; HVR, hypervariable region; HR, heptad repeat regions (B) Survival curves following intracerebral inoculation of the different recombinant HCoV-OC43 harboring various mutations in the S protein compared to reference rOC/ATCC virus. Whereas infection of mice with the reference virus rOC/ATCC induced 15% mortality, mice infected with rOC/U\textsubscript{S24-241} showed a mortality rate of 80%. The relative survival rates of mice infected by different viruses containing one, two or four mutations in the spike protein suggest a synergic effect of these mutations on mortality rate. Results are representative of three independent experiments.
Figure 2. Infectious virus production measured in the CNS of infected mice. The kinetics of infectious virus production in brains and spinal cords was evaluated every two days for 22 DPI. Both viruses replicated to similar levels with the same kinetics in brains and the highest levels of infectious virions were found at 10 DPI in brains as well as in spinal cords. Recombinant virus rOC/U3241 was cleared less rapidly from the spinal cord as infectious virus was repeatedly detected for up to 15 DPI. Results are representative of two independent experiments.
Figure 3. AMPA receptor antagonist GYKI-52466 treatment attenuates motor dysfunctions in infected mice without modifying neurovirulence or viral replication (A) Survival curves of mice infected by rOC/U5241 or rOC/ATCC and treated with GYKI-52466 or vehicle. GYKI-52466 treatment did not change the rate of survival of mice following infection by both viruses. Sham-infected mice treated with GYKI-52466 alone illustrated that AMPA treatment was not toxic under the condition used. Results are representative of two independent experiments. (B) Effect of GYKI-52466 treatment on motor clinical scores (CS) in mice infected by rOC/U5241 and treated with GYKI-52466 or vehicle. GYKI-52466 treatment attenuated clinical scores related to mild (CS 1.5-2) or severe paralysis (CS 2.5-3.5) of mice infected by rOC/U5241 compared to mice treated only with vehicle. Twenty to 30% of mice infected and treated with vehicle presented CS of severe paralysis (2.5-3.5) whereas only 5-10% of mice treated with GYKI-52466 fell in this category. CS of motor dysfunction completely disappeared at 18 DPI in GYKI-52466 treated mice whereas 20% of mice infected and treated with vehicle only were still presenting severe paralysis (CS 2.5-3.5) at 21 DPI. Fewer mice presented mild paralysis (CS 1.5-2) were fewer and recovered more rapidly when treated with GYKI-52466. Results are representative of three independent experiments. (C) Infectious virus titers in the CNS of mice infected by rOC/U5241 or rOC/ATCC and treated with GYKI-52466 or vehicle. For both recombinant viruses, viral replication (kinetics and total amount of infectious virus) was not affected following GYKI-52466 treatment. Results are representative of two independent experiments.
Figure 4. AMPA receptor antagonist treatment reduces neuronal dysfunction as observed by the phosphorylation state of heavy neurofilaments in mice infected with rOC/US241. Immunohistochemistry of lumbar spinal cord grey and white matter (GM, WM, respectively) segments of mice infected with rOC/US241 or rOC/ATCC and treated with GYKI-52466 or vehicle at 10 DPI. In normal spinal cord (a), SMI 311 stained neurofilament of neuronal soma and dendrites. Following infection with rOC/ATCC or rOC/US241 (b and d, respectively), GM presented less SMI 311 neuronal soma staining, illustrating a loss of normal nonphosphorylated NF in GM compared to sham-infected mice (a) which was more pronounced following rOC/US241 infection (d) compared to rOC/ATCC (white arrows). Interestingly, spinal cord white matter (WM) of mice infected with rOC/ATCC (b) or rOC/US241 (d) showed higher level of abnormal axonal nonphosphorylated NF-H with abnormal axonal swelling, which was more pronounced following infection by rOC/US241 compared to sham-infected mice (a) (black arrows). Staining with SMI-312 showed a low level of phosphorylated NF-H in WM axons following infection by rOC/US241 compared to rOC/ATCC or sham-infected mice. Treatment with GYKI-52466 of mice infected by rOC/ATCC (c) or rOC/US241 (e) partially restored the physiological NF-H phosphorylation state. a: sham-infected mice + vehicle; b: rOC/ATCC + vehicle; c: rOC/ATCC + GYKI-52466; d: rOC/US241 + vehicle; e: rOC/US241 + GYKI-52466. (Magnification x400). Results are representative of two independent experiments with three mice per group.
Figure 5. Expression of glutamate transporter 1 (GLT-1) is down-regulated in mice infected with rOC/U5241 and partially restored following treatment with AMPA receptor antagonist. (A) Immunofluorescence analysis of lumbar spinal cord segments of grey matter from mice infected by rOC/ATCC or rOC/U5241 and treated with vehicle (b and d) or GYKI-52466 (c and e) at 10 DPI. Infected mice (b and d) presented an increased number of activated astrocytes compared to sham-infected mice (a) and a down-regulation of GLT-1 staining which was more pronounced following rOC/U5241 infection compared to rOC/ATCC (d). Treatment with GYKI-52466 led to partial restoration of GLT-1 expression levels (c and e) (Magnification x1000). Results are representative of two independent experiments with three mice per group. (B) Western blotting analysis strengthened histological data. Mice infected by both recombinants showed significant (###P<0.001) GFAP expression compared to sham-infected mice. GLT-1 expression was significantly down-regulated in mice infected by rOC/U5241 (d) (P<0.001) compared to mice infected by rOC/ATCC (b) or control (a). Treatment with GYKI-52466 led to significant upregulation of GLT-1 expression in mice infected by rOC/U5241 (**P<0.01) compared to mice infected by rOC/ATCC or control. Note that following infection of mice with HCoV-OC43, an additional band of GFAP is detected at 45 kDa, which is suggested to represent a proteolytic fragment derived from the 50 kDa band. Results are expressed as percentage of control (sham-infected mice + vehicle (a)) and data are represented as a mean ± SEM. (n=3). **P<0.01 (Dukey’s test) for comparison between groups of infected mice. ###P<0.001 for comparison to sham-infected mice. a: Sham-infected mice + vehicle; b: rOC/ATCC + vehicle; c: rOC/ATCC + GYKI-52466; d: rOC/U5241 + vehicle; e: rOC/U5241 + GYKI-52466. Results are representative of three independent experiments.
Figure 6. Treatment with AMPA receptor antagonist reduced microglial activation

(A) Immunohistochemical staining of activated microglia using Mac-2 antibody in mice infected with rOC/U5241 or rOC/ATCC and treated with GYKI-52466 or vehicle at 10 DPI. Spinal cord grey matter ventral horn from infected mice demonstrated a significant activation of microglia/macrophages following infection by both viruses. Treatment with GYKI-52466 reduced microglia/macrophage activation in mice infected by rOC/U5241. (Magnification x400). Results are representative of two independent experiments with three mice per group. (B) Western blotting analysis of spinal cord proteins confirmed the histological finding. Infection of mice by rOC/U5241 led to significant increased (*P<0.05) activation of microglial cells (Mac-2 staining) compared to mice infected by rOC/ATCC, and GYKI-52466 treatment of mice attenuated microglial cells activation in mice infected by rOC/U5241 (*P<0.05). These results are expressed as percentage of reference, as 100% represented mice infected by rOC/ATCC and treated with vehicle (b), as microglial cells activation was undetectable in sham-infected animals (a). Data are represented as a mean ± SEM. (n=3) *P<0.05 (Dukey’s test). a: Sham-infected mice + vehicle; b: rOC/ATCC + vehicle; c: rOC/ATCC + GYKI-52466; d: rOC/U5241 + vehicle; e: rOC/U5241 + GYKI-52466. Results are representative of three independent experiments.
A

Survival curve

% of survival

Days post-infection

B

Vehicle

GYKI-52466

% of mice

Days post-infection

C

Infectious virus in brains

Infectious virus in spinal cords

TCID$_{50}$/g of tissue (log$_{10}$)

Days post-infection

rOC/ATCC + vehicle

rOC/ATCC + GYKI-52466

rOC/U5241 + vehicle

rOC/U5241 + GYKI-52466
A

GFAP (x200)

GFAP (X1000)

GLT-1 (X1000)

merge

B

GLT-1

GFAP

GAPDH

a: Sham-infected + vehicle
b: rOC/ATCC + vehicle
c: rOC/ATCC + GYKI-52466
d: rOC/Us241 + vehicle
e: rOC/Us241 + GYKI-52466

GFAP expression

GLT-1 expression

GFAP/GAPDH ratio (% of normal)

GLT-1/GAPDH ratio (% of normal)
A

Mac-2

b: rOC/ATCC + vehicle
c: rOC/ATCC + GYKI-52466
d: rOC/US241 + vehicle
e: rOC/US241 + GYKI-52466

B

Mac-2
GAPDH

Mac-2 expression