Rabies virus (RABV) causes fatal encephalitis in more than 59,000 people yearly. Upon the bite of an infected animal, the development of clinical disease can be prevented with post-exposure prophylaxis (PEP), which includes the administration of Rabies immunoglobulin (RIG). However, the high cost and limited availability of serum-derived RIG severely hamper its wide use in resource-limited countries. A safe low-cost alternative is provided by using broadly neutralizing monoclonal antibodies (bnAbs). Here we report the X-ray structure of one of the most potent and most broadly reactive human bnAbs, RVC20, in complex with its target domain III of the RABV glycoprotein (G). The structure reveals that the RVC20 binding determinants reside in a highly conserved surface of G, rationalizing its broad reactivity. We further show that RVC20 blocks the acid-induced conformational change required for membrane fusion. Our results may guide the future development of direct antiviral small molecules for Rabies treatment.
Rabies virus (RABV) belongs to phylogroup I of the Lyssavirus genus within the Rhabdoviridae family of the Mononegavirales order. It is a zoonotic virus found almost ubiquitously worldwide in different animal reservoirs, including domestic and wild canids and bats. Despite significant efforts, most countries face severe difficulties with RABV control, and in fact the virus has been eliminated only from a few developed countries by mass vaccination of wild and domestic canines. Today, an estimated 3 billion people are living at risk of contracting rabies through the bite of infected animals, mainly in Asia and Africa, where half of the victims are children under the age of 15 (refs. 5, 6). Still, 19–50 million people receive post-exposure prophylaxis (PEP) each year. Moreover, rabies disease with equally fatal outcome can also be caused by a number of non-RABV lyssaviruses, many of which use bats as their primary vector.

Following the bite of a potentially infected animal, administration of three doses of vaccine over the first week and one dose of Rabies immunoglobulin (RIG) without delay is recommended in order to eliminate the virus before it enters the nervous system. Recombinant antibody preparations are preferred over a vector.

Epitope mutagenesis. In support of these observations, we found that RVC20 neutralization is relatively robust to single point mutations in the epitope. We selected residues for mutation based on their predicted impact on epitope topology (Fig. 1e, Supplementary Fig. 2). For example, the D190 side chain makes a π-stacking interaction with Y52VH and hydrogen bonds with S54VH and S56VH (Fig. 1d); yet introduction of point mutation D190S by reverse genetics hardly conferred any resistance to RVC20 (Fig. 1e). Similarly, the V230 side chain makes hydrophobic contacts with Y58VH and Y94VK (Fig. 1d); yet the variant V230M, as found in Irkut virus of phylogroup I, or V230K as found in most phylogroup II or III/IV lyssaviruses (Fig. 1c), e), only mildly reduced neutralization by RVC20 (Fig. 1e). We observed the most pronounced but still mild gain in resistance to RVC20 with point mutation K226T, as found in several phylogroup II or III/IV viruses (Fig. 1c, e). The K226 side chain makes contacts with CDR H3, forms a hydrogen bond with T93VK, and participates in the only salt bridge throughout the interface with D92VK, explaining the phenotype of the mutant (Fig. 1d). The above single point mutations have similarly mild effects on mAb binding as on neutralization (Supplementary Fig. 3). These findings highlight the overall high conservation of the epitope, with only a few natural variations in distantly related lyssaviruses such as Lagos bat virus (LBV), a phylogroup II lyssavirus, compromising RVC20 efficacy. Indeed, the G protein of LBV, which was shown to resist RVC20 neutralization, displays 226T and 230K at the epitope. Using a lentivirus pseudotype system, we converted these two residues of LBV G to 226K and 230V and found that RVC20 neutralization was restored, further confirming the importance of these two side chains for antibody–antigen recognition (Fig. 1f, Supplementary Fig. 2).

Affinity maturation of RVC20. RVC20 shares 94% sequence identity in its variable domains with the inferred unmutated ancestor (UA) (Fig. 2a). Somatic hypermutation (SHM) has introduced at least 14 amino acid changes, only two of which, N92DVK and S93TVK, map directly to the paratope (Fig. 2b). As discussed above, these two residues interact with K226 of the antigen via a salt bridge and two hydrogen bonds. We found that only the D92NVK reversion, which breaks the salt bridge, but not the T93SVK reversion, which leaves the interactions unchanged, had a mild negative effect on neutralization (Fig. 2c). Likewise, the S98YVH reversion, positioned within CDR H3 but not in direct contact with the antigen, did not affect neutralization (Fig. 2c). Nevertheless, the UA neutralized RABV 20-fold less efficiently than the mature RVC20, a difference that cannot be attributed to the SHM N92DVK at the paratope alone (Fig. 2c).
Neutralization of wild-type and mutant Lagos bat virus G-pseudotyped lentiviruses with mAb RVC20 on BHK-21 cells 72 h after infection. LOD, limit of detection; +/-, isolate-dependent; nd, not determined. a Domain organization of RABV G (top row) as inferred by homology to VSV G\(^{10}\), and design of the recombinant domain III construct for structure determination (bottom row). Hatched fields in the construct denote unresolved regions in the X-ray structure. β strands are shown as arrows labeled in lower case in accordance with the VSV G structure\(^{10}\). TM transmembrane region, ST Strep tag. b Crystal structure of the complex between RABV G domain III and the RVC20 scFv. The variable domain of the heavy chain (VH) is shown in white and the antigen is shown in orange. The CDRs of the mAb and the antigen are labeled. c Sequence conservation of the tripartite epitope. The RABV G sequence is displayed as a sequence logo, indicating the conservation per residue across 1412 unique full-length RABV G sequences in GenBank (details are listed in Table 2). The color-coded bar chart shows the BSA per RABV G residue with a sixfold increase in neutralization efficiency relative to the wild-type. d Detail of the interaction interface. Residues on both sides of the interface are labeled and are shown as sticks with oxygen atoms in red and nitrogen atoms in blue. Secondary-structure elements and disulfide bonds are labeled. e Neutralization of recombinant RABV mutants with mAb RVC20 on BSR cells 48 h after infection; n = 3 independent experiments. f Neutralization of wild-type and mutant Lagos bat virus G-pseudotyped lentiviruses with mAb RVC20 on BHK-21 cells 72 h after infection. LOD, limit of detection; n = 2 independent experiments. Data are displayed as means ± s.d. Statistical analysis was performed using Tukey's test with α = 0.05. ***P < 0.0001; **P < 0.001; *P < 0.05; ns, not significant (P > 0.05). Source data are provided as a Source Data file.

is thus instructive that four SHMs near the VH/VK interface, Y35SVH, S35bNVH, F100fLVH and Y87FVK, together contributed with a sixfold increase in neutralization efficiency relative to the UA. These changes likely affect the paratope indirectly through improved relative orientations of the two chains (Fig. 2b, c). Indeed, we observed that the SHMs on both the heavy and light chains contributed similarly to affinity maturation (Fig. 2c). The neutralization capacity of all tested RVC20 variants correlated with their affinity (Supplementary Fig. 3). Overall, the small number of SHMs and the intrinsic neutralization activity of the inferred UA suggest that RVC20-like antibodies utilizing the same germline gene segments may frequently be selected in response to vaccination or infection.

Neutralization mechanism. Rhabdovirus G is a class III membrane fusion protein that induces fusion of the viral envelope with endosomal membranes in order to deliver the viral genome into the cytoplasm\(^{15}\). Fusion requires G to undergo a specific conformational change upon exposure to the acidic environment of the endosome. This rearrangement is known to be reversible in vitro\(^{16}\), and the structures of both the alkaline-pH prefusion conformation and the acidic-pH postfusion conformation have been described for the VSV G ectodomain\(^{10,11}\). Comparison of our immune complex to these previously determined structures suggested that the RVC20 epitope should be accessible only in the low-pH conformation pre-dominates (Fig. 3c). Yet, dissociation remains slow and is largely unaffected by pH (Fig. 3c). As suggested by the comparison to VSV G, the pH-dependency of the association step appeared to be
Table 1 Crystallographic data collection and refinement statistics.

| Data collection and processing |
|--------------------------------|
| Space group | P 4; 2, 2 |
| Cell dimensions | α, β, γ (Å) 81.95, 81.95, 155.93 |
| Resolution range (Å) | 46.51–2.59 (2.72–2.59) |
| Ellipsoidal highest resolution (Å) | 2.58/σ* |
| Number of unique reflections | 15,457 (321) |
| Rwork / Rfree | 0.19 / 0.22 (0.36 / 0.39) |
| Number of atoms | Protein 2323 |
| Lygands/ions | 4 |
| Water | 303 |
| Average B-factor (Å²) | Protein 106 |
| Lygands/ions | 124 |
| Water | 93 |
| R.m.s. deviations | Bond lengths (Å) 0.003 |
| Bond angles (°) | 0.62 |

.aValues in parentheses are for the highest-resolution shell.

.bThe datasets were anisotropically truncated using the STARANISO web server. An ellipsoid was fitted to the anisotropic cut-off surface to provide approximate resolution limits along three directions in reciprocal space. The real cut-off surface is only approximately ellipsoidal and the directions of the worst and best resolution limits may not correspond with the reciprocal axes.

Table 2 Percent conservation and frequency of amino acids per position of the RVC20 epitope across 1412 unique full-length RABV G sequences in GenBank.

| Position | Consensus | BSA a (Å²) | Conservation (%) | Observed amino acid counts |
|----------|-----------|------------|------------------|---------------------------|
| 42       | S         | 27         | 99.9             | S-1411, P-1               |
| 44       | M         | 41         | 99.1             | M-1399, I-7, L-4, V-2     |
| 47       | K         | 10         | 99.9             | M-1410, R-2               |
| 186      | G         | 43         | 99.1             | G-1399, R-10, E-3         |
| 187      | T         | 33         | 99.3             | T-1402, M-8, K-1, A-1     |
| 188      | S         | 50         | 99.4             | S-1404, P-5, Y-2, F-1     |
| 189      | C         | 67         | 99.9             | C-1411, R-1               |
| 190      | D         | 79         | 98.1             | D-1385, N-27              |
| 191      | I         | 23         | 99.9             | I-1411, T-1               |
| 192      | F         | 22         | 99.7             | F-1408, L-3, S-1          |
| 194      | N         | 47         | 87.8             | N-1240, T-77, S-61, Y-33, K-1 |
| 225      | L         | 12         | 99.1             | L-1399, M-10, V-2, I-1   |
| 226      | K         | 74         | 99.6             | K-1406, M-3, R-3          |
| 227      | L         | 28         | 100.0            | L-1412                     |
| 228      | C         | 49         | 99.9             | C-1411, S-1               |
| 229      | G         | 67         | 100.0            | G-1412                     |
| 230      | V         | 38         | 99.8             | V-1409, I-3               |
| 231      | L         | 16         | 76.4             | L-1078, P-192, S-140, H-1, T-1 |

.aBuried surface area, as determined by the PDBePISA web server.

The high stability of the immune complex even at pH 5.5 suggests that RVC20 efficiently locks G in its prefusion state or in an early intermediate conformation, preventing the structural rearrangements that drive membrane fusion. Indeed, we showed that RVC20 completely inhibited G-mediated syncytia formation at a concentration of 800 ng mL⁻¹ in a cell–cell fusion assay (Fig. 3e). The high degree of conservation of the RVC20 epitope across RABV strains and related lyssaviruses is probably linked to its involvement in the interaction between domains III and IV during fusion (Fig. 3a, b), which likely limits the mutation rate at this interface. Targeting this epitope and thereby blocking membrane fusion, which is an essential and universal step of the viral life cycle, is a safer approach than the inhibition of cell-type-specific receptor binding, which may protect only a subset of target cells from infection. Our study has thus characterized a vulnerable site on Lyssavirus G, which could be targeted not only for mAb-based prophylaxis, but also for future therapeutic applications in cases where the virus has already entered the nervous system. High-throughput screening of small molecules competing with RVC20 or a structure-based design of short peptide mimics of the bnAb’s CDRs could be employed in a first step of drug development, as recently demonstrated for the influenza virus fusion protein. Our results therefore also provide the groundwork for the design of low-molecular-weight fusion inhibitors capable of crossing the blood–brain barrier to extend the narrow time window for countermeasures against lyssavirus infection beyond what is currently feasible with PEP.
Differences to the most closely matching germline sequences as determined by IGMT/V-QUEST are indicated below each sequence, deconvention) are highlighted in gray background.

Fig. 2 RVC20 sequence analysis. a Annotation of the RVC20 variable heavy (VH) and variable kappa light (VK) sequences using the Kabat numbering. Differences to the most closely matching germline sequences as determined by IGMT/V-QUEST are indicated below each sequence, defining the inferred unmutated ancestor (UA) sequences. Arrows denote strands. The color-coded bar chart indicates the BSA per residue in contacts with the light chain, the heavy chain, or the antigen in white, green or orange, respectively; h, hydrogen bond involved; s, salt bridge involved. The CDRs (IMGT convention) are highlighted in gray background. b Positions of the inferred SHMs on the structure of the RVC20 scFv. RABV G residue K226 interacting with D92VK and T93VK is shown in orange. c Neutralization of RABV with RVC20 variants or with poorly neutralizing RVG68 on BSR cells 48 h after infection; n = 3 independent experiments. Data are displayed as means ± s.d. Statistical analysis was performed using Tukey’s test with α = 0.05. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant (P > 0.05). Source data are provided as a Source Data file.

Methods

Mammalian cell culture. HEK293-T clone 17 cells (ATCC CRL-11268), BHK-21 clone 13 cells (ATCC CCL-10) and BSR cells (a BHK-21 clone, kindly provided by Monique Lafon, Institut Pasteur, Paris) were cultured at 37 °C, 5% CO2, in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). The BSR-T7 cells (kindly provided by Karl-Klaus Conzelmann, Max von Pettenkofer Institute and Gene Center, Munich) for reverse genetics were cultured in Glasgow medium supplemented with 10% FCS, 2% (0.59 g L−1) tryptophase, 1% non-essential amino acids and 0.1% (50 µg mL−1) geneticin.

Recombinant protein preparation for crystallization. Domain III of RABV G (strain 9147FRA, GenBank: AF401286) and the RVC20 scFv were produced in Drosophila S2 cells (Gibco) expressing codon-optimized synthetic genes (Invitrogen). Domain III was constructed joining codons for E31-V56 and N182-D262 with a short linker of three glycine codons. The RVC20 scFv was constructed joining the VH and VK coding regions with a glycine-serine linker of 20 codons (sequence: GGGGS GGGGS GGGGS GGGGS). All cloning primers are listed in Supplementary Table 1.

S2 cells were grown at 28 °C in HyClone SFM4Insect medium with 1-glutamine (GE Healthcare) supplemented with 25 U mL−1 penicillin/streptomycin (Gibco). Expression plasmids were co-transfected with the selection plasmid pCoPURO at a mass ratio of 20:1 using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. Polyclonal stable S2 cell lines were established by selection with 7.5 µg mL−1 puromycin (Invivogen), which was added to the medium 48 h after transfection. Cultures were expanded to 1 L of 106 cells mL−1 in Erlenmeyer flasks shaking at 100 rpm and at 28 °C. Recombinant protein expression was then induced with 500 µM CuSO4. Cell supernatants were harvested 1 week after induction, concentrated to 50 mL on a 10-kDa MWCO PES Vivaspin centrifugal concentrator (Sartorius). The complex was purified by gel permeation chromatography on a HiLoad Superdex 200 pg column (GE Healthcare) in 20 mM Tris-Cl pH 8.0, 150 mM NaCl, and were subsequently mixed at equal molar ratio. The complex was purified by gel permeation chromatography. The final sample was concentrated to a protein concentration of 18 mg mL−1 in a 10-kDa MWCO PES Vivaspin centrifugal concentrator (Sartorius).

Cryoprotection before cryocasting the complex was achieved by diluting 1 part into 3 parts of a mixture of 18% glycerol, 35% PEG4000, 20% water. Crystals were then cryo-protected in 80 mM Tris-Cl pH 8.0, 150 mM NaCl, and were subsequently mixed at equal molar ratio. The complex was purified by gel permeation chromatography. The final sample was concentrated to a protein concentration of 18 mg mL−1 in a 10-kDa MWCO PES Vivaspin centrifugal concentrator (Sartorius).

Crystallographic data collection and structure determination. X-ray diffraction data were recorded on synchrotron beamline PX2 at SOLEIL in St Aubin, France, with an EIGER X 9M detector (Table 1). The wavelength was set to 0.9801 Å. Data were processed using XDS and the STARANISO web server (Global Phasing Ltd.). Initial phases were obtained by molecular replacement in Phenix.MR using a model of the RVC20 scFv generated with the Phyre2 web server for 3D homology

Crystallization of the RABV G domain III with the RVC20 scFv. Optimal crystals were obtained by the sitting-drop vapor diffusion method. A total of 0.7 µL of 18 mg mL−1 complex in 20 mM Tris-Cl pH 8.0, 150 mM NaCl were added to 0.7 µL of reservoir solution containing 100 mM Tris-Cl pH 8.0, 300 mM CaCl2, 22% v/v PEG4000. The drops were equilibrated against reservoir solution for 2 weeks at 18 °C. Crystals were then cryo-protected in 80 mM Tris-Cl pH 8.0, 240 mM CaCl2, 17.6% v/v PEG4000, 20% v/v glycerol prior to conservation in liquid nitrogen.

Crystallographic data collection and structure determination. X-ray diffraction data were recorded on synchrotron beamline PX2 at SOLEIL in St Aubin, France, with an EIGER X 9M detector (Table 1). The wavelength was set to 0.9801 Å. Data were processed using XDS and the STARANISO web server (Global Phasing Ltd.). Initial phases were obtained by molecular replacement in Phenix.MR using a model of the RVC20 scFv generated with the Phyre2 web server for 3D homology.
Fig. 3 RVC20 locks RABV G in its prefusion state. a Structure of the trimeric VSV G in its alkaline-pH prefusion (left) and the acidic-pH postfusion conformation (right) color-coded according to domains, as labeled. The RVC20 scFv/RABV G domain III complex is superposed onto VSV G domain III, and only the RVC20 moiety is shown. For clarity, the scFv is not shown on the front protomer, in which a circle marks the location of its epitope. The right panel shows that the epitope should become occluded by domain IV after the acidic-pH-triggered conformational change of G. b Detail of the VSV G region corresponding to the epitope (orange) superposed onto RABV G domain III (gray). The CDRs making the RVC20 paratope (green and white) are shown on the left panel. The right panel shows that the epitope is not accessible at acid pH, as domain IV buries a substantial part of the epitope area. The distribution (histograms) is shown for n = 3 independent experiments (bottom). e Fusion inhibition by RVC20 in a GFP-split cell–cell fusion assay. Fusion of RABV G-expressing HEK 293T cells was determined qualitatively by biolayer interferometry (BLI). d Binding of RVC20 (left) or poorly neutralizing RVC68 (right) to RABV G-expressing HEK 293T cells in suspension. Association (As.) and washing (Dis.) was performed at the indicated pH values, and binding was assessed by flow cytometry in comparison to an isotype control mAb (top). The mean fluorescence intensity of mAb-bound cells (shaded area in histograms) is shown for n = 3 independent experiments (bottom).  

Epitope conservation analysis across RABV isolates. RABV G sequences were downloaded from NCBI (2019/04/10) by taxon classification (taxon:11292)25. Sequences with “cell culture” in the “host” field of the GenBank record (n = 129) were excluded. Full-length G sequences with country source and collection date information (n = 2875) were collapsed to a non-redundant set of 1412 sequences for analysis.

To determine amino acid identity and coverage at epitope residues, each putative G sequence was aligned pairwise against the reference G sequence of the CVS-11 isolate (GenBank: ACA57830). Alignments were performed with the pairwiseAlignment method from the R BioStrings package in local alignment mode (Smith-Waterman) using the BLOSUM80 amino acid substitution matrix. The sequence motif was generated using the ggseqlogo package in R26.

Preparation of the RABV G ectodomain for BLI. The ectodomain construct comprising residues K1–S403 (strain 9147FRA, GenBank: AF401286) with a C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. Primers for cloning and mutagenesis are listed in Supplementary Table 1. In order to improve secretion into the culture supernatant of stably transfected S2 cells, point mutations F74H and W121H were introduced into the C-terminal Strep tag (sequence: GGWSHPQFEK) and its mutants D190S, K226T, V230M and V230K were prepared in the same way as domain III for crystallization. 

Biolayer interferometry. Biolayer interferometry (BLI) was carried out on an Opti RED384 instrument (Fortebio). Recombinant RVC20 wild-type or variants at a concentration of 10 µg mL⁻¹ were immobilized on anti-human IgGFc Capture (AHFc) biosensors (Fortebio). Running buffers were either 50 mM Tris-Cl pH 8.0, 50 mM MES pH 7.0 or 50 mM MES pH 5.5, each in 150 mM NaCl 10% v/v glycerol—a condition, which was found to destabilize the interaction sufficiently for separation. The final sample was diluted to a protein concentration of 0.5 mg mL⁻¹ in 50 mM Tris-Cl pH 8.0, 150 mM NaCl 10% v/v glycerol prior to analysis.
Recombinant IgG production. UA sequences were determined with reference to the IMGT database and produced by gene synthesis (Genscript). VH and VK sequences of RCV20 antibody and derived variants were cloned into human IgC1 and IgK expression vectors and recombinant mAbs were produced by transient transfection of ExpiCHO cells (Thermo Fisher Scientific, Cat# A29127), purified by Protein A chromatography (GE Healthcare) and desalted against PBS.

Reverse genetics and virus titration. The GA-GFP recombinant virus is based on the wild isolate 8743THA, EVAg collection, Ref-SKU: 2014-020160, isolated from a human bitten by a dog in 1983 in Thailand. Mutations D190S, K226T, V230M and V230P were introduced into the G gene using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific) according to the manufacturer’s instructions. All mutagenesis primers are listed in Supplementary Table 2. Recombinant RABV wild-type or mutants were rescued by transfection of BSR-T7 cells with the complete genome (2.5 µg) together with plasmids pN-PIT (2.5 µg), pL-PIT (1.25 µg) and L-PIT (1.25 µg). At 6 days post-transfection, the cells were serially passage every 3 days. When 100% of the cells were infected, the supernatant was harvested and titrated on BSR cells. The infection was monitored by immunofluorescence using a FITC-conjugated anti-RABV nucleoprotein antibody (Bio-Rad, Cat #3572112) according to the manufacturer’s instructions. Neutralization tests were performed on BSR cells by the fluorescent focus method. A total of 20 µl of serial dilutions (1 to 5) of virus were inoculated in duplicates on 5 × 10^3 BSR cells and incubated at 37 °C. At 40 h post-infection, the medium was removed, the cells were fixed with 80% acetone and incubated with the FITC-conjugated recombinant RABV nucleoprotein antibody (Bio-Rad, Cat #3572112) according to the manufacturer’s instructions. The number of fluorescent foci was determined under a fluorescence microscope and the titer was calculated in fluorescent focus units per milliliter (FFU mL^-1). To determine growth curves for the recombinant viruses, BSR cells were inoculated with each virus at MOI = 0.1 and supernatants were recovered at 24 h, 48 h and 72 h post-infection for titration on BSR cells.

Virus neutralization test. A total of 2 x 10^5 FFU of RABV wild-type or mutants were incubated with different concentrations of RVC20 (mature or variants) in DMEM with 10% fetal bovine serum for 1 h at 37 °C in 96-well plates (Greiner Bio-One, #655090); 1 x 10^4 BSR cells were then added to each well and the plates were incubated at 37 °C. At 48 h, the cells were fixed with 4% paraformaldehyde, washed and the nuclei were counterstained with 20 µM Hoechst 33342. Image acquisitions of 16 fields per well (total 26.6 mm² per well) were performed on the automated confocal microscope O王晓 Phenix (Perkin Elmer) using the 10x objective. The data were transferred to the Columbus Image Data Storage and Analysis System (Perkin Elmer) and the percentage of GFP-positive cells was determined. IC50 values were determined by nonlinear regression analysis (GraphPad Prism) from three independent experiments.

Pseudotype neutralization test. Lentiviral pseudotypes were produced in HEK293T clone 17 cells. Neutralization assays were undertaken on BHK-21 clone 13 cells. In a 96-well white plate, pseudotyped virus that resulted in an output of 30–70 × 10^3 relative light units (RLU) was incubated with dilutions of RVC20 for 1 h at 37 °C and 5% CO2 before the addition of 10,000 BHK-21 cells. These were incubated for an additional 4 h, after which supernatant was removed and fixed with 4% PFA prior to staining with secondary Goat anti-Human IgG, Alexa Fluor 568 (Invitrogen, #A21090) diluted 1:500 in PBS + 1% bovine serum albumin. Cells were washed and fixed for 10 min in 4% PFA prior to fluorescence measurement on an Attune NaïF Flow Cytometer (Thermo Fisher). The gating strategy is exemplified in Supplementary Fig. 5.

GFP-Split fusion assay. Cell–cell fusion experiments were performed using the HEK 293T GFP split system, GFP10 and GFP11 expressing HEK 293T cells were mixed at a 1:1 ratio. A total of 6 × 10^4 cells per well were suspended in 96-well plates (mClear, #655090) and were transfected with 5 ng of phCMV-8743 expressing a GFP variant mixed with 95 ng of DNA with pQCXIP-empty using Lipofectamine 2000 (Thermo Fisher). MAbs were added to the medium at the indicated concentrations on the day of transfection; 18 h after transfection, 1/2 of the medium was exchanged for 60 mM MES + 100 mM NaCl at several pH values (7.0, 6.0, 5.5 or 5.0). Following incubation for 15 min at 37 °C, the medium was again exchanged for DMEM 10% FBS at pH 7 and the cells were left to fuse for an additional 90 min. Thirteen images per well corresponding to 90% of the well surface were acquired on an Opera Phenix High-Content Screening System (PerkinElmer) and the surface area covered with GFP-positive cells was determined.

Statistical analysis. Statistical analysis was performed in GraphPad Prism 6 using one-way ANOVA followed by Tukey’s multiple comparisons test with α = 0.05, except for the data presented in Fig. 3e, where two-way ANOVA was applied.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and the Supplementary Information. The crystal structure of the RCV20 scFv/RABV G domain III complex from this study is available in the PDB with the accession code 6TOU (https://doi.org/10.2210/pdb6TOU/pdb). The source data underlying Figs. 1e, H, 2c, 3d, 3e and Supplementary Fig. 3 are provided as a Source Data file.

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