Sphingolipid biosynthesis induces a conformational change in the murine norovirus receptor and facilitates viral infection

Robert C. Orchard1,2*, Craig B. Wilen1 and Herbert W. Virgin1*

Cellular susceptibility to viral infections is in part determined by the presence of a host cellular receptor. Here we use murine norovirus as a model to uncover an unappreciated connection between an intracellular lipid biosynthetic enzyme and a receptor conformation that is permissive for viral infection. The serine palmitoyltransferase complex is required for de novo sphingolipid biosynthesis and we find that its absence impairs the ability of murine norovirus to bind and enter cells. Although the serine palmitoyltransferase complex is dispensable for the surface expression of the norovirus receptor, CD300lf, serine palmitoyltransferase activity is required for CD300lf to adopt a conformation permissive for viral binding. Addition of extracellular ceramide to serine palmitoyltransferase-deficient cells chemically complements both the conformational changes of CD300lf and the cellular susceptibility to murine norovirus infection. Taken together, these data indicate that intracellular sphingolipid biosynthesis regulates the conformation of the murine norovirus receptor and therefore the tropism of murine norovirus. This indicates that intracellular biosynthetic pathways can regulate viral tropism even when the receptor for a virus is expressed on the target cell surface.

Noroviruses (NoV) are non-enveloped, positive-stranded RNA viruses. Human noroviruses (HNoV) are a leading cause of gastroenteritis worldwide. Although HNoV infections are typically self-limiting in immunocompromised patients, infections can be prolonged, severe and even fatal. There are no approved vaccines or antivirals for NoV therapy at present, largely owing to the difficulty in culturing HNoV in vitro and the strict species tropism of noroviruses that leads to a lack of robust replication of HNoV in small animal models.

One barrier to NoV infection is at the step of viral entry, as direct delivery of viral genomes into cells enables viral replication. Previous work has shown that HNoV binds to histo-blood group antigens (HBGAs) and that susceptibility to specific HNoV strains is correlated with host HBGa status, although HBGAs cannot account for all aspects of HNoV tropism and entry. Unlike HNoV, murine norovirus (MNoV) grows robustly both in vitro and in laboratory strains of mice. We recently leveraged this robust replication to perform a whole-genome CRISPR screen to identify essential host genes required for MNoV replication. We identified the cell surface protein CD300lf as a protease receptor for MNoV. CD300lf mediates the binding of virus to the cell surface and is necessary for viral entry and replication both in vitro and in vivo. Importantly, expression of CD300lf in human cells is sufficient for MNoV to replicate in human cells. These findings indicate that the intracellular replication machinery for NoV is conserved across species. However, it remains unclear what additional cellular factors cooperate with proteinaceous receptors to enable NoV entry.

In our initial screen for genes required for MNoV replication we reported that two genes, Sptlc1 and Sptlc2, were important for MNoV replication. Their encoded proteins are essential members of the serine palmitoyltransferase (SPT) complex, which catalyses the first and rate-limiting step in de novo ceramide and sphingolipid biosynthesis. Ceramide and sphingolipids are important lipid mediators of membrane fluidity and dynamics. Ceramide can also function as a signalling lipid involved in cellular survival, metabolic homeostasis and inflammation. However, the exact function of the SPT complex during NoV replication is unknown.

Here, we define a critical role for sphingolipid biosynthesis in MNoV infection and viral entry. Furthermore, we determine that the step in viral infection at which de novo sphingolipid biosynthesis is required is the binding of the virus to cells, a step also requiring the MNoV receptor CD300lf. Surprisingly, Sptlc2 deficiency did not lead to abnormal trafficking or localization of CD300lf, but altered the conformation of CD300lf so that it was not recognized by MNoV or a conformation dependent antibody. Chemical complementation of the Sptlc2-deficient cells altered the CD300lf conformation and restored MNoV susceptibility. Taken together, these data demonstrate that the lipid composition of host cells is a critical determinant of MNoV entry and that intracellular enzymes can regulate the conformation of viral receptors, providing an additional mechanism to regulate viral tropism.

The SPT complex consists of two proteins, Sptlc1 and Sptlc2. The Sptlc2 protein is the catalytic subunit whereas Sptlc1 is required for the stability of Sptlc2 in cells. Therefore, we generated Sptlc2-deficient BV2 cells (BV2ΔSptlc2) using CRISPR–Cas9 technology (Supplementary Figure 1). We then tested the ability of MNoV strains that cause acute, systemic infection (MNoVCRW3) or persistent, enteric infection (MNoVCm2) to replicate in Sptlc2-deficient cells. Whereas wild-type BV2 cells produced high titres of MNoVCRW3 and MNoVCm2 strains of virus, BV2ΔSptlc2 cells were deficient in MNoV production both after a single cycle (12 h) or multiple cycles (24 h) of replication (Fig. 1a). Importantly, the growth of both strains of MNoV was restored on expression of Sptlc2 cDNA (Fig. 1b).

A mutation that abolished Sptlc2 catalytic activity, Sptlc2E207G, was unable to rescue MNoV replication in BV2ΔSptlc2 cells, indicating that the SPT complex must be enzymatically active in order to support MNoV replication (Fig. 1b). Treatment of BV2 cells with myriocin, a potent inhibitor of the SPT enzyme, also reduced the number of MNoV infected cells (Fig. 1c).

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Fig. 1 | De novo ceramide biosynthesis is required for efficient MNoV infection, binding and entry. a, BV2 or BV2ΔSptlc2 cells were challenged with the MNoV strains, MNoV<sup>CV3</sup> (top) or MNoV<sup>CV6</sup> (bottom), at a multiplicity of infection (MOI) of 0.05. Viral production was measured using plaque assays (PFU; plaque forming units) at indicated time points. Time point 0 represents input inoculum. Data are shown as mean ± s.e.m. from three independent experiments. **P < 0.01, ****P < 0.0001, unpaired two-sided t-test. A representative western blot for indicated proteins is shown. The arrow indicates expected molecular weight and yellow circles denote non-specific bands. b, Wild-type BV2 or BV2ΔSptlc2 cells expressing an empty vector, Sptlc2, or Sptlc2<sup>R507A</sup>, were challenged with the strains, MNoV<sup>CV3</sup> (top) or MNoV<sup>CV6</sup> (bottom), at an MOI of 0.05. Viral production was measured using plaque assays at 12 h post-infection. Results shown are mean ± s.e.m. from three independent experiments. **P < 0.01, ***P < 0.001, ns, not significant; one-way ANOVA with Tukey’s multiple comparison test. A representative western blot for indicated proteins is shown. The arrow indicates expected molecular weight and yellow circles denote non-specific bands. c, MNoV<sup>CV3</sup> infection of BV2 cells treated with vehicle (methanol) or myriocin (25 μM) 24 h before challenge. Infection was measured by FACS for intracellular production of VP1. Data are shown as mean ± s.e.m. from four independent experiments and data were analysed by unpaired two-sided t-test. d, BV2 or BV2ΔSptlc2 cells were transfected with viral RNA from MNoV<sup>CV3</sup> and harvested 12 h post-transfection. Viral production was measured by plaque assay. Data are shown as mean ± s.e.m. from three independent experiments and data were analysed by unpaired two-sided t-test; ns, not significant. e, Indicated cell lines were assayed for MNoV<sup>CV3</sup> binding using quantitative polymerase chain reaction and normalized to the median of BV2 + vector for each experiment. Data are shown as mean ± s.e.m. from three independent experiments. *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparison test.

Having established a step in sphingolipid biosynthesis as required for efficient MNoV replication, we next identified the stage of the viral life cycle that is impaired in BV2ΔSptlc2 cells. Equivalent levels of infectious virus were produced after transfection of MNoVCW3 viral RNA into wild type and BV2ΔSptlc2 cells, indicating that sphingolipid biosynthesis is required for MNoV entry (Fig. 1d). We then tested the ability of MNoV<sup>CV3</sup> to bind to cells defective in sphingolipid biosynthesis. Wild-type BV2 cells had significantly enhanced binding compared to both BV2ΔCD300lf and BV2ΔSptlc2 cells (Fig. 1e). This binding defect in BV2ΔSptlc2 cells was rescued by expression of a wild-type Sptlc2 construct but not a catalytically inactive Sptlc2 (Fig. 1e). These findings are consistent with a model in which Sptlc2 is required for efficient binding and entry of cells while post-entry replication of MNoV is not affected.

As CD300lf expression is essential for MNoV binding, we next tested the hypothesis that Sptlc2 is required for cell surface expression of this viral receptor. The amount of CD300lf on the surface of BV2ΔSptlc2 cells was equivalent to wild type and complemented
Fig. 2 | Sptlc2 is not required for CD300lf surface localization. a, Histogram of a representative FACS experiment from three independent experiments assessing CD300lf surface levels using AF647-anti-CD300lf antibody 3F6 with indicated cell lines. b, Quantification of mean fluorescence intensity (MFI) of anti-CD300lf antibody 3F6. Data are shown as mean ± s.e.m. from three independent experiments. *P < 0.05, ns, not significant, one-way ANOVA with Tukey’s multiple comparison test. c, A representative western blot of the indicated cell lysates with CD300lf antibodies 3F6 (4 µg ml⁻¹, top) and TX70 (4 µg ml⁻¹, bottom). Beneath each lane, the total protein measured relative to BV2 + vector is listed. TX70 is unable to recognize CD300lf, when overexpressed, by western blot analysis but is able to recognize CD300lf by FACS, thus indicating that the molecule is conformation dependent. Data is representative of three independent experiments.

Having established that genetic disruption of the SPT complex altered MNoV infection and changed the conformation of CD300lf at the cell surface, we tested the hypothesis that this effect is due to the lack of sphingolipids and thus examined whether we could chemically complement the genetic deficiency in BV2ΔSptlc2 cells. We chose to use a synthetic, soluble ceramide, C2 ceramide, to deliver to cells due to the synthesis of ceramide requiring SPT activity and the solubility of the C2 ceramide, which can traverse the lipid bilayers of cells. Addition of C2 ceramide to BV2ΔSptlc2 cells restored MNoV infection and changed the conformation of CD300lf (Fig. 4a). Treatment with C2 ceramide treatment also restored the ability of the conformation-specific antibody TX70 to recognize a CD300lf-Flag transgene in BV2ΔSptlc2 cells (Fig. 4b, c). Taken together, our genetic and chemical data indicate an essential role for sphingolipid biosynthesis, which occurs intracellularly, in regulating the conformation of CD300lf, which probably contributes to MNoV binding and cellular tropism.

A key factor in determining the cells, tissues and species a virus can infect, is the ability of viral proteins to engage cellular receptors. Here we demonstrate that the essential and rate-limiting enzyme for de novo ceramide and sphingolipid biosynthesis, SPT, is required for efficient MNoV entry and binding, at least in part through controlling the conformation of a protein receptor. As norovirus entry is a limiting factor for in vitro replication, these findings may have implications for the development of efficient HNoV culture systems. Extensive efforts to identify cellular models that enable HNoV replication have focused on potential receptors including HBGAs; however, our data shows that intracellular factors may contribute to the susceptibility of cells to norovirus infection. As HBGAs alone are not sufficient to explain HNoV cellular tropism, it is tempting to speculate that unappreciated intracellular pathways that regulate HBGA accessibility, or the conformation of unidentified protein receptors, regulate HNoV tropism and underlie the difficulty in establishing robust and tractable HNoV cell culture systems. Most investigations into the relationship between viral entry and sphingolipids have identified a direct interaction between viruses and host lipids or describe an endosomal escape mechanism leveraging the unique geometry of ceramide in lipid bilayers. We now...
provide evidence to support a new model in which the intracellular production of sphingolipids is required for a functional conformation of a viral receptor. Our data indicates that CD300lf is expressed equivalently on the surface of wild type and Sptlc2-deficient cells (Fig. 2a–c). Importantly, we and others have previously demonstrated that even minimal surface expression of CD300lf renders cells susceptible to MNov infection29,30. Rather surprisingly, here we uncover an unappreciated connection between the conformation of CD300lf and sphingolipid production that is associated with recognition by both the virus for binding to cells and a conformation specific anti-receptor antibody. The molecular details underlying the Sptlc2-dependent conformation of CD300lf are currently unknown. We previously mapped the critical regions of MNov receptor activity of CD300lf to the amino acids that compose the CD300lf lipid binding pocket (Nelson et al., manuscript in preparation and ref.31). Taken together these findings suggest the possibility that ceramide or a sphingolpid, controls the conformation of CD300lf through its interactions with this ligand binding pocket. However, our data does not exclude the possibility that sphingolipids interact specifically with the transmembrane domain, as observed for other proteins32. It is also possible that the ceramide-dependent conformation of CD300lf is a function of ceramide and/or sphingolipid molecules regulating the formation of membrane microdomains, such as lipid rafts, which may facilitate CD300lf clustering or controlling interactions with additional proteins at the cell surface33.

Previous work has shown that post-translational modifications of viral receptors by intracellular enzymes can regulate cellular susceptibility26,27. Here, however, we report an intracellular biosynthetic enzyme that alters the conformation of a cell surface viral receptor. More broadly, these findings suggest that the blockade in binding and establishing infection for certain viruses is not the absence of a receptor but an intracellular deficiency that renders the receptor non-permissive for viral engagement.

Methods

Cells. BV2 cells previously karyotyped and confirmed to replicate MNovV and 293T cells (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 10 mM HEPEs. Puromycin (2.5 µg ml⁻¹; Sigma Aldrich) and blasticidin (5 µg ml⁻¹; Invitrogen) were added as indicated.

BV2ΔCD300lf cells have been described previously30. BV2ΔSptlc2 cells were generated at the Genome Engineering and iPSC Center at Washington University School of Medicine. BV2 cells were nucleofected with Cas9 and Sptlc2-specific sgRNA (GATATCTTCGAGATTTCTTGAGG). Cells were then single-cell sorted and genomic DNA was extracted. DNA was amplified
Fig. 4 | Chemical complementation restores MNoV infection and a CD300lf conformation in Sptlc2-deficient cells. a. MNoV<sup>CWR</sup> infection of indicated cell lines treated with either DMSO (−) or C2 ceramide (+). Infection was measured by FACS for intracellular production of VP1. Data are shown as mean ± s.e.m. from three independent experiments. b. Histogram of a representative FACS experiment from three independent experiments looking at the CD300lf conformation-dependent TJX70 antibody ability's to stain indicated cells treated with either DMSO (−) or C2 ceramide (+). c. Quantification of MFI of anti-CD300lf antibody TJX70 from three independent experiments. MFI within an experiment was normalized to BV2 + DMSO. Data are shown as mean ± s.e.m. from three independent experiments. * P<0.05, ** P<0.001, ns, not significant; one-way ANOVA with Tukey’s multiple comparison test.

Antibodies, flow cytometry and western blotting. The following antibodies were used for flow cytometry or western blotting as indicated: rabbit anti-CD300lf (Proteintech), mouse anti-GAPDH-HRP (Sigma), rat anti-CD300lf-PE clone TX70 (BioLegend), armenian hamster anti-CD300lf clone 3F6 (Genentech), rat anti-Flag-PE (BioLegend), rat anti-Flag-APC (BioLegend) and mouse anti-VP1-FITC (Ab6.2 monoclonal antibody conjugated to FITC, ref. 5). Cells were placed on ice, washed once with PBS and then lysed in cold RIPA Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% SDS) with HALT protease and phosphatase inhibitor cocktail (Sigma). Lysates were clarified by centrifugation before resolving on SDS-PAGE Stain-Free gels (BioRad) and transferring to PVDF membranes. Where indicated, total protein was quantified and used for normalizing using a ChemiDoc MP Imaging System (BioRad).

For fluorescence-activated cell sorting (FACS) analysis, cells were isolated and probed for extracellular CD300lf expression previous to being fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences). Cells were stained with indicated antibodies intracellularly. Cells were then washed and analysed on a FACS Calibur flow cytometer (BD). At least 20,000 events were collected per condition. Each experiment was performed in triplicate in each of three independent experiments.

Cell surface biotinylation. For cell surface biotinylation experiments, the Pierce Cell Surface Protein Isolation Kit (ThermoFisher) was used following the manufacturer’s guidelines. Briefly, 12 × 10<sup>5</sup> BV2 or BV2ΔSptlc2 cells expressing CD300lf-Flag were seeded overnight, washed and Sulfo-NHS-SS-Biotin was added to cells in PBS on ice for 30 min. The labelling reaction was quenched before collecting and lysing cells. A sample of the clarified lysate was kept for western blot analysis and the remainder was added to streptavidin columns for the isolation of biotinylated proteins.

Microscopy. BV2 or BV2ΔSptlc2 cells expressing CD300lf-Flag-eGFP were seeded overnight onto glass coverslips in six-well dishes. Cells were fixed, permeabilized with 0.5% Triton X-10 and stained with AlexaFlour555-Phalloidin (ThermoFisher). Coverslips were mounted onto slides with Prolong Gold Antifade with DAPI (ThermoFisher), which were imaged on a Zeiss LSM 880 Confocal Laser Scanning Microscope.

Chemical inhibitors and complementation. Myriocin was purchased from Sigma and dissolved in methanol at 1.25 mM. For inhibition studies, cells were seeded overnight and the following day the media were changed to contain either methanol or myriocin (25μM). Twenty-four hours later, cells were either processed for FACS (Figs. 3e and 4f) or infected with MNoV<sup>CWR</sup> (Fig. 1c) at an MOI of 5. Cells were collected for FACS 16–18h after infection and stained intracellularly for MNoV capsid production.

C2 ceramide (d18:1/2:0; Avanti Lipids) was resuspended in dimethylsulphoxide (DMSO). Cells were seeded overnight in a six-well plate (2 x 10<sup>5</sup> cells per well). Media were removed and cells were subsequently washed with PBS. For infection...
assays, cells were incubated with either 500μM C2 ceramide or DMSO with 1 x 10^6 PFU in a total volume of 500μl complete media, at room temperature for one hour with gentle rocking. Media was removed, cells were washed with PBS and fresh media were added to the cells. After 16–18h, cells were collected for FACS and stained intracellularly for MNoV capsid production. A similar experimental setup was used for chemical complementation of the conformational dependent antibody recognition, except no virus was added and cells were only incubated for 45 min at room temperature before washing.

Data availability. The data that support the findings of this study are available from the corresponding authors upon request.

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Author contributions
R.C.O. designed, performed and analysed the experiments and wrote the manuscript. C.W.B. and H.W.V. designed and analysed the experiments. All authors read, discussed and edited the manuscript.

Competing interests
Washington University School of Medicine holds patents on several aspects of murine norovirus. These have been licensed, generating income for the University and the inventors, including H.W.V.

Additional information
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- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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Software and code

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| Data collection       | No software was used |
|-----------------------|----------------------|
| Data analysis         | No software was used |

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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No predetermined sample size calculations were performed. Based on previous experiences three independent experiments done in technical duplicate or triplicate have provided enough rigor and power to make firm conclusions given that virus growth is logarithmic. |
| Data exclusions | No data was excluded |
| Replication | All replications repeated the experimental findings |
| Randomization | Cellular samples were pooled together prior to separating into individual groups. No randomization was performed or needed. |
| Blinding | During data acquisition for plaque assays and FACS samples were given a numerical value and during data collection we were blinded to the grouping of the samples. Only after after data acquisition were data analyzed and their identity revealed. |

Materials & experimental systems

Policy information about availability of materials

| n/a | Involved in the study |
| --- | --- |
| ☐ | ☒ Unique materials |
| ☐ | ☒ Antibodies |
| ☐ | ☒ Eukaryotic cell lines |
| ☐ | ☒ Research animals |
| ☐ | ☒ Human research participants |

Unique materials

Obtaining unique materials

All materials are available under an MTA

Antibodies

Antibodies used

The following antibodies were used for flow cytometry or western blotting as indicated: rabbit α-Sptlc2 (Proteintech), mouse α-GAPDH-HRP (Sigma), rat α-CD300lf-PE clone TX70 (Biolegend), armenian hamster α-CD300lf clone 3F6 (Genentech), rat α-FLAG-PE (Biolegend), rat α-FLAG-APC (Biolegend), mouse anti-VP1-FITC (A6.2 monoclonal antibody conjugated to FITC; (Wobus et al., 2004)).

Validation

All antibodies were tested and titrated on control antigen positive and antigen negative control cell lines for both flow cyometry and western blot.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

293T cells were acquired from ATCC. BV2 cells were originally isolated by Blasi et al and have since been acquired and maintained at Washington University where they have been previously karyotyped (Orchard et al Science).

Authentication

Cell lines were not validated

Mycoplasma contamination

All cell lines tested negative for mycoplasma

Commonly misidentified lines

(See ICLAC register) N/A
Research animals

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Animals/animal-derived materials	N/A

Human research participants

Policy information about studies involving human research participants

Population characteristics	N/A

Method-specific reporting

| n/a | Involved in the study |
|-----|-----------------------|
| X   | ChIP-seq              |
| X   | Flow cytometry        |
| X   | Magnetic resonance imaging |

ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
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Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells were isolated and probed for extracellular CD300lf expression prior to being fixed and permeabilized with Cytofix/Cytoperm (BD biosciences). Cells were stained with indicated antibodies intracellularly.
Instrument FACSCalibur (BD biosciences)

Software Data were analyzed using FlowJo

Cell population abundance No sorting was performed and all flow cytometry was done on single populations of BV2 cells and derivatives.

Gating strategy Gates were set based on the staining of antigen negative cells for each individual experiment

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

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### Magnetic resonance imaging

#### Experimental design

**Design type**
- Indicate task or resting state; event-related or block design.

**Design specifications**
- Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

**Behavioral performance measures**
- State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

#### Acquisition

**Imaging type(s)**
- Specify: functional, structural, diffusion, perfusion.

**Field strength**
- Specify in Tesla

**Sequence & imaging parameters**
- Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

**Area of acquisition**
- State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

**Diffusion MRI**
- Used

#### Preprocessing

**Preprocessing software**
- Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

**Normalization**
- If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

**Normalization template**
- Describe the template used for normalization/ transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

**Noise and artifact removal**
- Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).

**Volume censoring**
- Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

#### Statistical modeling & inference

**Model type and settings**
- Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

**Effect(s) tested**
- Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

**Specify type of analysis:**
- Whole brain
- ROI-based
- Both

**Statistic type for inference**
- Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

(See Eklund et al. 2016)

**Correction**
- Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).
Models & analysis

n/a Involved in the study

☑ Functional and/or effective connectivity
☑ Graph analysis
☑ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.

Behavioural & social sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | N/A |
|-------------------|-----|
| Research sample   | N/A |
| Sampling strategy | N/A |
| Data collection   | N/A |
| Timing            | N/A |
| Data exclusions   | N/A |
| Non-participation | N/A |
| Randomization     | N/A |