Morphine withdrawal recruits lateral habenula cytokine signaling to reduce synaptic excitation and sociability

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The lateral habenula encodes aversive stimuli contributing to negative emotional states during drug withdrawal. Here we report that morphine withdrawal in mice leads to microglia adaptations and diminishes glutamatergic transmission onto raphe-projecting lateral habenula neurons. Chemogenetic inhibition of this circuit promotes morphine withdrawal-like social deficits. Morphine withdrawal-driven synaptic plasticity and reduced sociability require tumor necrosis factor-α (TNF-α) release and neuronal TNF receptor 1 activation. Hence, habenular cytokines control synaptic and behavioral adaptations during drug withdrawal.

Opiate withdrawal produces negative states, including low mood and reduced sociability, which contribute to relapse during drug abstinence1,2. Dysfunction of the lateral habenula (LHb)—a nucleus that controls monoaminergic systems and processes aversive stimuli—underlies depressive symptoms typical of drug withdrawal3; however, how opiates affect the LHb remains poorly understood1,2.

We subjected mice to naloxone-precipitated morphine withdrawal (MORwd) to examine its repercussions on glutamatergic synapses onto LHb neurons. Indeed, aberrant LHb excitatory transmission underlies negative symptoms in rodent models of depression and addiction1. Spontaneous excitatory postsynaptic current (sEPSC) amplitudes, but not frequencies, were reduced only in LHb neurons located in the medial aspect (MedLHb; Supplementary Fig. 1a–b). Accordingly, MORwd diminished AMPA receptor (AMPAR)/NMDA receptor (NMDAR) ratios solely in the MedLHb (Fig. 1a; Supplementary Fig. 1c) without affecting neurotransmitter release and neuronal TNF receptor 1 activation. Hence, habenular cytokines control synaptic and behavioral adaptations during drug withdrawal.

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To assess whether MORwd affects AMPA conductance or number, we analyzed peak-scaled non-stationary fluctuations of MedLHb-recorded sEPSCs6. While single-channel conductance remained unaffected in MORwd slices, the number of channels opened at the peak was positively correlated with amplitude values (Supplementary Fig. 1f). MORwd failed to alter AMPAR-EPSC rectification (Supplementary Fig. 1g), whereas it reduced glutamate receptor (AMPA)/NMDA receptor (NMDA) ratios solely in the MedLHb (Fig. 1a–b). Accordingly, MORwd diminished AMPA receptor (AMPAR)/NMDA receptor (NMDAR) ratios solely in the MedLHb (Fig. 1a; Supplementary Fig. 1c) without affecting neurotransmitter release and neuronal TNF receptor 1 activation. Hence, habenular cytokines control synaptic and behavioral adaptations during drug withdrawal.

**Fig. 1 | MORwd-driven projection-specific synaptic depression in the LHb.** a. Top: naloxone-precipitated MORwd (NP-MORwd) protocol. Bottom left: traces and AMPAR/NMDAR ratios from MedLHb slices (saline + naloxone (Sal+Nlx; n = 7 mice, 11 cells) versus NP-MORwd (Mor+Nlx; n = 8 mice, 11 cells)). Two-sided t test, \( t_{20} = 0.0548, P = 0.957 \). Bottom right: traces and AMPAR/NMDAR ratios from MedLHb slices (saline + naloxone (n = 7 mice, 12 cells) versus NP-MORwd (n = 8 mice, 13 cells)). Two-sided t test, \( t_{20} = 2.210, P = 0.037 \), i.p., intraperitoneal injection. b. Top: images of retrobeads in raphe and retrogradely labeled LHb neurons. Bottom: Traces and AMPAR/NMDAR ratios from MedLHb slices (saline + naloxone (n = 5 mice, 10 cells) versus NP-MORwd (n = 5 mice, 11 cells)). Two-sided t test, \( t_{19} = 3.153, P = 0.005 \). e. Same as b, but in LHbRaphe neurons (saline + naloxone (n = 2 mice, 6 cells) versus NP-MORwd (n = 4 mice, 7 cells)). Two-sided t test, \( t_{19} = 0.575, P = 0.577 \). PAG, periaqueductal gray; DG, dentate gyrus; MHb, medial habenula; SNr, substantia nigra pars reticulata. Data are presented as box plots, with 10th and 90th percentiles, median and scatter.

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uncaging-evoked AMPAR/NMDAR ratios, yielding a decrease only in absolute AMPAR currents (Supplementary Fig. 1h). This suggests that MORwd reduces, in a territory-specific fashion, the number of AMPARs without affecting their biophysical properties, NMDAR numbers or presynaptic glutamate release.

MORwd-evoked plasticity occurs onto MedLHb neurons, which innervate downstream structures, including the raphe nucleus and the ventral tegmental area (VTA). MORwd diminished AMPAR/NMDAR ratios solely in retrobead-labeled raphe-projecting (LHbRaphe) but not VTA-projecting LHb (LHb VTA) neurons (Fig. 1b,c). These results point to the specificity of MORwd for discrete habenular circuits.

Which induction mechanism gates MORwd-driven plasticity onto MedLHb neurons? Inflammatory responses and glial cell activation emerge during drug withdrawal1. Indeed, spontaneous MORwd drives microglia adaptations and pro-inflammatory cytokine release (that is, TNF-α). Notably, cocaine also leads to reduced microglia arborization along with TNF-α-dependent AMPAR internalization, which partly underlies drug-mediated behavioral adaptations6. We found that within the MedLHb, MORwd reduced microglial markers, including IBA1 and CD68, and diminished microglial cell volume (Fig. 2a–d). In parallel, naloxone-induced and spontaneous MORwd increased habenular TNF-α immunolabeling (Fig. 2e; Supplementary Fig. 2a–d). Altogether, these findings support the view that there is engagement of inflammatory responses and cytokine signaling within the LHb during MORwd.

We then reasoned that if MORwd promotes TNF-α release, artificially increasing its levels should prove sufficient to recapitulate MORwd-driven synaptic plasticity. Incubating LHb-containing slices obtained from saline-injected mice with exogenous TNF-α reduced AMPAR/NMDAR ratios in the MedLHb. This effect was absent in the lateral aspect of the LHb (LHbL), and occluded by naloxone and spontaneous MORwd (Fig. 2f,g; Supplementary Fig. 3a). TNF-α release may arise from microglial Toll-like receptor 4 (TLR4) signaling10. Systemically activating TLR4 with the agonist monophosphoryl lipid A (MPLA) in morphine-treated mice, instead of naloxone, mimicked MORwd plasticity (Supplementary Fig. 3b). Moreover, MPLA application in slices obtained from morphine-treated animals reduced AMPAR currents in the MedLHb, but not the LHbL (Supplementary Fig. 3c–d). MPLA-driven EPSC reduction did not occur in the presence of a dominant-negative TNF-α (XENP1595; Supplementary Fig. 3e). Furthermore, MORwd occluded MPLA-driven synaptic depression (Supplementary Fig. 3c), and systemic injection of XENP1595 prevented MORwd-induced plasticity (Supplementary Fig. 3f). Altogether, these results support the notion that TLR4 is expressed within the LHb (see the Allen Brain Atlas) and that its effect on AMPARs occurs via TNF-α signaling. Moreover, the results support the necessity and sufficiency of TNF-α for MORwd-driven reduction of LHb glutamatergic transmission.

TNF-α triggers its central effects partly through TNF receptor 1 (TNF-R1; encoded by Tnfr1 (also known as Tnfrsfla))11. We employed 129-Tnfrslatm3GK1 (referred to as Tnfr1fl/fl) mice in which Tnfr1 expression in LHb neurons was knocked down via adenovirus (AAV)-Cre (Fig. 3a–c). After viral injection, 4/4 mice (3 mice, 8 cells) showed a decrease in AMPAR/NMDAR ratios (Fig. 3d), and basal AMPAR/NMDAR ratios were highly correlated with ASH neuron total dendritic ARS (Fig. 3e). These results suggest that TNF-R1 is required for AMPAR/NMDAR ratio reduction following hippocampal AAV-Tnfr1fl/fl injection. Thus, TNF-R1 is a key regulator of AMPAR/NMDAR ratios and synaptic plasticity in the LHb.
MORwd drives negative symptoms, including social detachment\(^1\). Similarly, LHb dysfunction contributes to the negative states emerging in addiction; however, the implications of LHb dysfunction with respect to sociability remains poorly addressed. We examined the contribution of the LHb-to-raphe pathway, the locus of MORwd plasticity, in social behavior. We employed an intersectional chemogenetic approach to reduce the efficiency of the LHb-to-raphe projection. This involved the combined retrograde expression of Cre-recombinase (HSV-Cre) in the dorsal raphe with the Cre-dependent projection. This involved the combined retrograde expression of Cre-recombinase (HSV-Cre) in the dorsal raphe with the Cre-dependent expression of hM4Di (rAAV-hM4Di-mCherry, DREADD) in the LHb (Fig. 3c). Reducing LHb-to-raphe efficiency with clozapine-N-oxide diminished social preference (Fig. 3d); this result supports the concept that the LHb contributes to social behaviors.

Next, we recapitulated MORwd-driven reduction in social preference in C57BL/6 mice (Fig. 3g; Supplementary Fig. 4a–d). Slices obtained from mice subjected to MORwd and exhibiting low or high sociability scores showed that MedLHb AMPAR/NMDAR ratios were positively correlated with the social score (Fig. 3h). This indicates that reduced synaptic strength in the LHb predicts opiate-withdrawal-driven sociability deficits.

Notably, microglia and TNF-\(\alpha\) signaling also contributes to social behaviors\(^2\). Accordingly, MORwd-driven sociability deficits were absent after Cre-dependent LHb \(\text{Tnfr}\) knockout (Fig. 3i; Supplementary Fig. 4e–i). This genetic intervention did not affect locomotion (Supplementary Fig. 4j).

We found that MORwd-driven TNF-\(\alpha\) release requires neuronal TNF-R1 to reduce AMPAR transmission onto raphe-projecting, medially located, LHb neurons. This ultimately gates MORwd-driven social impairment, a negative symptom typical of opiate withdrawal.

Together with sociability deficits, MORwd also leads to anxiety and hyperalgesia\(^1\). Since the contribution of the LHb on these two behavioral aspects remains elusive, we cannot rule out that MORwd-driven habenular plasticity is specific for withdrawal-mediated sociability defects.

The TNF-\(\alpha\)–TNF-R1 engagement within the LHb represents a previously unidentified mechanism underlying precise cellular and behavioral aspects of MORwd. However, this is consistent with the following data: (1) drugs and drug-withdrawal-mediated modulation of AMPAR transmission partly rely on cytokine signaling\(^3\); (2) inhibition of TLR4 attenuates MORwd symptoms\(^4\); and (3) TNFRs contribute to social behaviors\(^5\). Notably, in pyramidal neurons of the hippocampus and cortex, TNF-\(\alpha\) regulates AMPAR surface expression\(^6,7\). This phenomenon is opposite at striatal synapses\(^8\). Importantly, both AMPAR and NMDAR expression partly, consistent with previous findings\(^9\), yet it remains correlational with respect to TNF-\(\alpha\) levels. This heightens the need to fill the gap in understanding regarding microglia function and its relationship with TNF-\(\alpha\) within the habenula. Overall, while pharmacotherapies targeting pro-inflammatory pathways in substance abuse are missing, our data support cytokine signaling as a cellular pillar for aspects of drug addiction.
MORwd-driven TNF-α-dependent depression of AMPAR transmission occurs at LHbRaphe neurons. From a circuit standpoint, this may provide an ‘antisocial’ signal that is likely produced through reduced actions onto raphe neuronal populations. This is consistent with the evidence reported here that chemogenetic manipulation of the LHb-to-raphe projection diminishes sociability. In addition, dopamine-containing and serotonin-containing raphe neurons monosynaptically connect to the latter17–19. Understanding the repercussions of LHb activity onto raphe neuronal subtypes during MORwd remains an important aspect for future investigation.

In conclusion, our data support the concept that cytokine-mediated plasticity participates in opiate-evoked negative symptoms, a mechanism by which the LHb ultimately contributes to the addiction spiral.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0421-4.

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Author contributions
K.V., A.T. and M.M. performed and analyzed the ex vivo recordings and behavior experiments. A.L.L. and J.A.C. contributed to the ex vivo recordings. M.T., I.M. and L.M. performed the molecular biology experiments. C.B. and S.T. provided support for the behavioral experiments. A.M. and R.C.P. analyzed the microglia morphology. A.V. provided conceptual and experimental input related to TNF-α signaling and the Tnfr1fl/fl mice. K.V. and M.M. conceptualized, designed the study and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Methods

Animals and morphine treatment. C57BL/6J wild-type mice (male) and 129-TnsrflsA1tm3Gkl mice (male and female, referred to as Tnfrfls(−/−)) aged 4–10 weeks were group-housed (three to five per cage) on a 12–12 light cycle (lights on at 7:00) with food and water ad libitum. All procedures aimed to fulfill the criteria of the 3Rs and were approved by the Veterinary Offices of Vaud (Switzerland; license VD3172). Part of the current study was carried out at the Institut du Fer a Moulin, Paris, France, and experiments were performed in accordance with the guidelines of the French Agriculture and Forestry Ministry. MORwd was either precipitated with naloxone or was induced naturally after the criterion of the 3Rs and were approved by the Veterinary Offices of Vaud (Switzerland).

Spontaneous EPSCs were recorded either in the LatLHb or MedLHb (AP: −4.4 mm; dorsal–ventral (DV): −2.9 mm; lateral–medial (ML): −1.35 mm; ML: −0.45 mm; DV: −3.00 mm). In another set of experiments, C57BL/6 mice were injected with a herpessimplex virus derivative hEF1α-Cre vector (MGA Gene Delivery Technology Core) in the raphe nucleus and with rAAV-DJ-EF1α-Flex-hM4D(Gi)-mCherry (Gene Vector and Virus Core, Stanford Medicine) in the LHb. Animals were allowed to recover for about 5–7 days after injection of the retrobeads or 5 weeks after viral infusion before being treated with morphine or saline. The injection sites were carefully examined during all electrophysiology experiments, and only animals with correct injections were used for recordings. Similarly, for behavioral studies, only animals with correctly placed observation sites were included in the analyses. Brain slices obtained from mice injected with retrobeads or viruses were directly examined under an epifluorescence microscope.

Ex vivo electrophysiology. Animals aged 5 weeks were anesthetized with an injection of ketamine (150 mg/kg) xyazine (100 mg/kg; veterinary office the University of Lausanne) and were placed on a stereotactic frame (Kopf). Bilateral injections of 200–400 nl were performed through a glass needle at a rate of approximately 100 nl/min. The injection pipette was withdrawn from the brain 10 min after the infusion. Retrobeads (Luminafluor) were infused into the dorsal raphe nucleus (anterior–posterior (AP): −3.5 mm; medial–lateral (ML): 0 mm; dorsal–ventral (DV): −3.8 mm) or the VTA (AP: −2.4 mm; ML: ± 0.65 mm; DV: −4.9 mm) of C57BL/6 mice. Tnfrfls(−/−) mice were injected with either rAAV2-kshn-GFP or rAAV2-kshn or CMV-Cre-eGFP into the LHb (AP: −1.35 mm; ML: ± 0.45 mm; DV: −3.00 mm). Some experiments were performed in LHb-containing slices incubated for a minimum of 1 h with exogenous TNF-α (100 ng/ml). To test the effect of MPLA on AMPAR transmission, neurons were patched either in the sLHb or the tLHb, and sEPSCs were evoked with extracellular stimulation. Following a 10-min baseline, MPA (1 μg/ml) was added to the recording solution and sEPSCs were recorded for a minimum of 40 min. Some experiments were performed in the presence of the TNF-α dominant negative peptide XENP1595 (6 μg/ml; Xencor) in the recording solution.

Histology and immunofluorescence. Mice were injected daily with saline or morphine (20 mg per kg, i.p.) for 6 days. Some mice were left to develop spontaneous withdrawal, while others received naloxone (2 mg per kg, i.p.) injection 30 min after the last saline or morphine injection on day 6. After 10–13 days of spontaneous withdrawal or 30 min after naloxone injection on day 6, mice were anesthetized and perfused with cold 4% paraformaldehyde (PFA) in PBS. The brains were extracted, post-fixed in 4% PFA in PBS and then incubated in 30% sucrose in PBS until they sank. Slices (30 μm) were cut using a cryostat and stored in PBS containing 0.02% NaN3 for future analyses. For immunofluorescence, the slices were incubated for 2 h in blocking buffer (5% normal goat serum (NGS) and 0.3% Triton X in PBS). Slices were washed 3 times and blocked for 24 h at 4 °C with the primary antibody solution (mouse anti-TNF-α antibody, 1:100 in blocking buffer; ab1793, Abcam). After extensive rinses, the secondary antibody was applied (goat anti-mouse IgG-conjugated Alexa 488, Invitrogen, 1:400 in blocking buffer, 24 h at 4 °C). The slices were then incubated in 4,6-diamidino-2-phenylindole (DAPI) (1:400 solution in PBS), extensively rinsed, mounted on glass slides with Pro-Long Gold Antifade Reagent (Invitrogen) and coverslipped. Images were acquired with an epifluorescence microscope with a x20 objective (AxioVision, Zeiss) using the same parameters for all the samples. The images were analyzed and processed using the software ImageJ. Optical density was measured on the whole LHb area and normalized against the neighboring thalamus using the following equation: LHb – thalamus/ thalamus). A total of three to six slices distributed in the rostrocaudal axis were analyzed per animal (eight morphine-treated, seven saline-treated).

Microglia analysis. Mice were anesthetized and perfused with cold 4% PFA in PBS. The brains were extracted, post-fixed in 4% PFA in PBS and then incubated in 30% sucrose in PBS until they sank. Slices (30 μm) were cut using a cryostat and stored in PBS containing 0.02% NaN3, for future analyses. Brain sections were permeabilized at room temperature in 0.5% Triton X-100 (Sigma) for 1 h at room temperature, followed by blocking in 2% BSA 0.5% Triton X-100 for 1 h at room temperature, followed by overnight incubation with the primary antibody solution (1:1,000 (cat. no. 019–19741, Wako Chemicals) and CD68, 1:100 (cat. no. MCA1957, Bio)) at 4 °C. After washing, sections were incubated for 2 h at room temperature with Alexa-fluorophore-conjugated secondary antibodies (Invitrogen) and counterstained with DAPI (Invitrogen).
Confocal microscopy was performed using a TCS-SP5 (Leica) Laser Scanning System with a ×20 dry objective. Images were processed and analyzed using the software Fiji or Imaris (Bitplane) as appropriate. Imaris was used for three-dimensional (3D) rendering of confocal images for quantification of volumes.

For density analyses, for each acquisition, the DAPI channel was max-projected and the MedLHb and LatLHb were manually drawn as regions of interest. Then, stacks ranging from 15 μm to 20 μm in thickness, with a z-step size of 1 μm, were processed as follows: IBA1 and DAPI channels were thresholded in Fiji and multiplied to each other for each stack, with the image calculator function. The resulting thresholded stack was max-projected, and the microglia nuclei were counted using the function Analyze Particle.

For cell soma size and IBA1 intensity analyses, each acquisition was max-projected, and the contour of cell somata in the MedLHb were manually drawn based on the immunoreactivity of IBA1 and then analyzed per size in μm² and intensity.

3D imaging analysis was performed using Imaris and applying recorded algorithms (fixed thresholds for signal intensity) to all the images of the same experiment to produce unbiased signal quantification. In each experiment, one brain slice per animal (n = 4) per group was acquired. The microglial cell volume and the volume of phagocytic structures were reconstructed based on the absolute intensity of IBA1 and CD68 signals, respectively. The volume of CD68 was then normalized to the IBA1 volume to take account of the cell size.

Behavior. Social preference test. A three-chambered social preference test was used. The arena was a rectangular Plexiglas (60 × 40 × 22 cm) (Ugo Basile) divided into three chambers. The walls of the center chamber had doors to allow free access to all compartments. The luminosity was around 10 lux. Thirty minutes after naloxone injection, each mouse was placed in the arena for a habituation period of 10 min and was allowed to freely explore the whole empty arena. The social preference test was performed immediately after the end of the habituation period: two enclosures with vertical bars were placed in the middle of the two lateral compartments, while the central chamber remained empty. One enclosure was empty (serving as an inanimate object), whereas the other contained a social stimulus (an unfamiliar juvenile mouse 25 ± 1 days old). The enclosures allowed visual, auditory, olfactory and tactile contact between the experimental mice and the social stimuli mice.

The juvenile mice in the enclosures were habituated to the apparatus and the enclosures for 3 days before the experiment, and each one of them served as a social stimulus for no more than 2 experimental mice (at least 6 weeks old). The test lasted 10 min, whereby the experimental mice were allowed to freely explore the apparatus and the enclosures. The position of the empty and juvenile-containing enclosures alternated and was counterbalanced for each trial to avoid any bias effects. Every session was video-tracked and recorded using Ethovision XT (Noldus) or AnyMaze (Stoelting), which provided an automated recording of the entries and time spent in the compartments, the distance moved and the velocity. The time spent in each chamber was assessed and then used to determine the preference score for the social compartment as compared to the object compartment (social/(social + object)). The arena was cleaned with 1% acetic acid solution and dried between trials.

Analyses and statistics. Animals were randomly assigned to experimental groups. Compiled data are reported and presented as whisker box plots (the upper and lower whiskers representing the 90th and 10th percentiles, respectively, and the upper and lower boxes representing the 75th and 25th percentiles, respectively, and the horizontal line representing the median) or the mean ± s.e.m., with single data points plotted (single cell for electrophysiology and single animal for behavioral experiments). Animals or data points were not excluded unless stated, and a normality test was applied. Data collection and analyses were not performed blinded to the conditions of the experiments. When applicable, statistical tests were paired or unpaired t-test and one-way or two-way analysis of variance (ANOVA). Significance for correlations was obtained applying Pearson’s estimates. Testing was always performed two-tailed with α = 0.05. More information on the methods and analyses can be found in the Nature Research Reporting Summary.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.
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| [x] [ ] Human research participants | | |
| [x] [ ] Clinical data | | |

Antibodies

| Antibodies used | Validation |
|-----------------|------------|
| mouse anti-TNFα antibody, ab1793, clone number 52883, Lot#GR66942 Abcam, 1:100 in blocking buffer | mouse anti-TNFα antibody validation was based on published results from Lewitus et al., 2016. This is now stated in the manuscript, methods section. |
| goat anti-mouse IgG-conjugated Alexa 488, Catalog # A28175, Lot#1874804 (No clone# available), Invitrogen, 1:400 in blocking buffer, 24h at 4°C | Iba1 1:1000, Wako Chemicals, Cat. 019-1974 Clone NCNP24, Lot. PTN5930 CD68 1:400, Bio-Rad Cat. MCA1957, Clone FA-11, Lot. 1807 |
| Iba1 1:1000, Wako Chemicals, Cat. 019-1974 Clone NCNP24, Lot. PTN5930 CD68 1:400, Bio-Rad Cat. MCA1957, Clone FA-11, Lot. 1807 | Iba1 and CD68 validation was based on previous publication including Lewitus et al., 2016 and Paolicelli et al., 2011. Dr. Paolicelli is an authors of this manuscript therefore the validation of these antibodies were based on her experience in the field. The entire validation reported here is valid for the species used in this study which is mice. |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | C57Bl/6j wild-type (male) and 129-Tnfrsf1atm3Gki (male and female, referred as TNF-R1fl/fl) mice of 4–10 weeks were used |
|--------------------|----------------------------------------------------------------------------------------------------------|
| Wild animals | This study did not involve the use of wild animals |
| Field-collected samples | This study did not involve the use of field collected samples |
| Ethics oversight | All procedures were approved by the Veterinary Offices of Vaud (Switzerland; License VD3172) |

Note that full information on the approval of the study protocol must also be provided in the manuscript.