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Interleukin-6 trans-signalling in hippocampal CA1 neurones mediates perioperative neurocognitive disorders in mice

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Abstract

Background: Interleukin-6 (IL-6), a pleiotropic cytokine with both degenerative and regenerative properties, is necessary and sufficient to provoke perioperative neurocognitive disorders after aseptic trauma in mice. IL-6 initiates its actions after binding to either membrane-bound IL-6 receptor α (mIL-6Rα) through classical signalling, or soluble IL-6 receptor (IL-6R) through trans-signalling; both signalling pathways require the transducer gp130. We investigated the site and type of IL-6 signalling that pertains in a tibial fracture aseptic trauma model of perioperative neurocognitive disorder.

Methods: Wild-type or genetically altered adult mice that lacked molecules unique to either classical or trans-IL-6 signalling underwent tibial fracture under isoflurane anaesthesia. In separate cohorts, we assessed postoperative memory using a trace fear conditioning paradigm (72 h postoperatively), and post-receptor IL-6 signalling (24 h postoperatively) using phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) in CA1 hippocampal neurones. Fracture healing was assessed at postoperative day 15 after inhibiting either both forms of IL-6 signalling with BE0047 or only trans-signalling with sgp130Fc.

Results: The surgical phenotype of memory decline (decrease in freezing in trace fear conditioning) and upregulated IL-6 signalling (pSTAT3) did not occur after pretreatment before surgery with either BE0047 or sgp130Fc, or after depleting gp130 from CA1 neurones. The surgical phenotype still occurred when IL-6Rα was depleted in either CA1 hippocampal neurones (freezing time, 38.9% [11.5%] *vs* 58.4% [12.3%]; $pSTAT^+$ CA1 neurones, 31.7 [4.9] *vs* 7.0 [3.1]) or microglia (freezing time, 40.1% [13.9%] *vs* 65.2% [12.6%]; $pSTAT^+$ CA1 neurones, 30.1 [5.5] *vs* 7.9 [3.2]). In global IL-6R$\alpha^{-/-}$ mice, hyper-IL-6, the trans-signalling agonist, produced the surgical phenotype when administered i.c.v. (freezing time, 42.4% [8.8%] *vs* 59.7% [10.4%]; $pSTAT^+$ cells, 29.3 [4.3] *vs* 10.0 [4.4]). Bone-fracture healing (% of fracture callus comprised of new collagen) was significantly greater with sgp130Fc than with BE0047 (52.2% [8.3%] *vs* 39.7% [7.9%]).

Conclusions: After orthopaedic trauma, IL-6 produces perioperative neurocognitive disorders through IL-6 trans-signalling in mouse CA1 neurones. Druggable targets of the trans-signalling pathway should be sought to reduce perioperative neurocognitive disorders while allowing the healing properties of classical IL-6 signalling.

Keywords: hippocampus; IL-6 receptor; IL-6 trans-signalling; microglia; perioperative neurocognitive disorder

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Editor's key points

- The cytokine interleukin-6 (IL-6) is necessary and sufficient to model perioperative neurocognitive disorders (PNDs) after aseptic orthopaedic trauma in mice.
- The locus and mechanism of IL-6 signalling involved in a tibial-fracture trauma model of PNDs was investigated using biochemical and genetic models.
- Wild-type or genetically altered mice defective in either classical or trans-IL-6 signalling identified the critical mechanism for surgery-induced memory dysfunction as the IL-6 trans-signalling pathway in hippocampal neurones.
- Inhibition of this pathway provides a druggable target for prevention or treatment of PNDs without deleterious effects on bone healing observed for broader anti-inflammatory therapies.

Perioperative neurocognitive disorders (PNDs) encompass conditions in which new cognitive impairment or deterioration of existing impairment is identified in the period immediately before surgery and concluding 12 months after surgery.[1] PNDs are the most common complication in older surgical patients, affecting an estimated 1.6 to 6.4 million patients in the USA, and results in prolonged hospital stay, loss of independence, higher healthcare costs, and increased morbidity and mortality.[2–4]

A detailed understanding of the pathogenic processes involved will help identify patients at heightened risk and potential targets for prophylactic intervention, therapeutic intervention, or both to combat PNDs. Animal models with face, construct, and pharmacologic validity have revealed hippocampal neuroinflammation as a critical mechanism,[5] and a meta-analysis has associated pro-inflammatory biomarkers with PNDs in surgical patients.[6] Endothelial cells, fibroblasts and monocyte/macrophages are the major sources of interleukin-6 (IL-6).[7] IL-6 has been shown to be both necessary and sufficient to produce cognitive decline in a mouse model of PND[8]; however, neither the site for this action nor the type of IL-6 signal transduction was identified. Dexmedetomidine, a sedative that has been shown to decrease postoperative delirium (the most common PND),[9] is associated with suppression of both IL-6 plasma levels in a clinical trial[10] and hippocampal IL-6 in an animal model.[11]

The pleiotropic actions of IL-6 ranging from energy metabolism to bone healing to immune responses (both pro- and anti-inflammatory) are produced by at least three different signalling mechanisms.[12] Classical signalling occurs when IL-6 binds to membrane-bound IL-6 receptors (mIL-6Rα) that are primarily located on hepatocytes, leucocyte subpopulations (including monocytes, neutrophils, and T and B lymphocytes), and megakaryocytes, and transduces its cellular responses through dimerization of gp130.[13] Trans-signalling refers to a process in which IL-6 binds to soluble IL-6 receptors (sIL-6R) that are produced either through proteolytic cleavage of the exodomain of mIL-6Rα by the adamalysin family of metalloproteinases or by alternative splicing; the IL-6/sIL-6R complex binds to gp130 and can produce its response in $gp130^{+}$/$mIL\text{-}6R\alpha^{-}$ cells.[14] Cluster-signalling occurs when Sirp+ dendritic cells trans-present its own IL-6/mIL-6R to pathogenic TH17 cells[15]; this form of signalling is not further considered as these cells do not contribute to PNDs. In each case the intracellular signalling involves the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway and can be assessed by phosphorylation of signal transducer and activator of transcription 3 (pSTAT3).

Knowledge of the signalling mechanisms that are responsible for a particular action of IL-6 will enable precise targeting to abrogate one set of responses but not others. In this study, we sought to determine the site and type of IL-6 signalling mechanism responsible for surgery-induced cognitive decline in the hippocampus and to determine whether this can be interrupted without interfering with actions of IL-6 on bone healing, which has been shown to require IL-6 classic signalling (mIL-6R), but not IL-6 trans-signalling.[16]

Methods

Animal care

All animal procedures were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at Anhui Medical University (Approval No. LLSC20190061) and adhered to Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Mice were housed in groups of 3–5 at 22–24°C using a 12-h light/12-h dark cycle. Animals had *ad libitum* access to water and food, which was only withdrawn before an experiment that required general anaesthesia.

Mice

Male C57BL/6 mice were obtained from Animal Experiment Center of Anhui Medical University at 4–6 weeks of age. $IL\text{-}6R\alpha^{flox/flox}$ mice (# N1-4309), $CAMKII^{Cre}$ mice (# R5-140), $CX3CR1^{Cre}$ mice (# N5-9555) $gp130^{flox/flox}$ mice (# N1-4310), and $IL\text{-}6R\alpha^{-/-}$ mice (# N5-9556) were purchased from The Shanghai Model Organisms Center, Inc. (Shanghai, China). The hippocampal CA1 neuronal IL-6Rα-deficient, microglial IL-6Rα-deficient, and CA1 neuronal gp130-deficient mice were generated as described in detail in the Supplementary material. All experiments were performed in adult male mice at the indicated ages.

Intracerebroventricular injections

Briefly, 12- to 14-week-old adult mice were anaesthetised and placed into a stereotaxic apparatus. A 26 gauge cannula was implanted into the left lateral ventricle and dental acrylic was used to secure the cannula to the skull surface (see detailed information in the Supplementary material).

For i.c.v. injection, recombinant mouse IL-6 (400 ng; PeproTech Systems, Rocky Hill, NJ, USA),[17] hyper-IL-6, a fusion protein of sIL-6R and IL-6 that selectively and efficiently stimulates trans-signalling in gp130-expressing cells (40 ng; R&D Systems, Wiesbaden, Germany),[18] sgp130Fc (200 ng; R&D Systems),[17] or BE0047, anti-mouse IL-6R recombinant antibody (20 μg; BioXCell, Lebanon, NH, USA) were used.[19] Sterile NaCl, 0.9% (saline), or phosphate-buffered saline (PBS) 0.1 M, pH 7.5 (Vehicle), was used as the control solution, as indicated. Mice were administered 2 μl of each compound.

Intraperitoneal injections

For i. p. injection, recombinant mouse IL-6 (400 ng or 50 ng g^{-1}; PeproTech Systems), sgp130Fc (0.5 mg kg^{-1}; R&D Systems), or BE0047 (2 mg kg^{-1}; BioXCell) were used.[16] Sterile saline, or PBS 0.1 M, pH 7.5 (Vehicle) was used as the control solution, as indicated. Depending on the compound, volumes of 100 or 200 μl were used for i. p. injections.

Surgical procedure

Twelve-to 14-week-old mice were subjected to an open left tibia fracture with intramedullary fixation under aseptic conditions during general anaesthesia with isoflurane 2% as described[8] (see detailed information in the Supplementary material).

Virus injections

Nine-to 11-week-old $gp130^{flox/flox}$ mice were anaesthetised and stabilised on a stereotaxic apparatus. rAAV-CaMKIIα-EGFP-P2A-CRE-WPRE-hGH pA (Cre-AAV) virus or rAAV-CAMKIIα-EGFP-WPRE-hGH pA (Control-AAV) virus was bilaterally microinjected into CA1 areas of the hippocampus (see detailed information in the Supplementary material). The CA1 region was selected because of its critical importance for recognition memory,[20] and because long-term potentiation, a mechanism of synaptic plasticity required for memory, is disrupted in this region after the same peripheral surgery.[21]

Behavioural tests

Trace fear conditioning (TFC) was used to assess learning and memory as described.[8] Briefly, 12- to 14-week-old animals were trained to associate a conditional stimulus (tone) with an aversive unconditional stimulus (foot shock). Aversive memory is associated with freezing behaviour when the mouse is re-exposed to the same context (see detailed information in the Supplementary material).

Tissue processing

Microglial IL-6Rα and brain albumin were detected by immunobloting. IL-6 and sIL-6R were determined using enzyme-linked immunosorbent assay (ELISA) kit, pSTAT3, NeuN, $Iba1^{+}$, IL-6Rα and gp130 were assessed by immunofluorescence, and bone healing was assessed using Safranin-O/Fast-Green (see detailed information in the Supplementary material).

Statistical analysis

Data are presented as mean (standard deviation [SD]), and individual data points are plotted in the figures. Datasets with only two independent groups were analysed for statistical significance using unpaired, two-tailed Student *t*-test. Datasets with more than two groups were analysed using one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test. Significance was set at $P<0.05$. Data analysis and chart production were performed using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA).

Results

Hippocampal IL-6 signalling is associated with surgery-induced memory decline

Surgery or systemically administered IL-6 (50 ng g^{-1}; ~1500 ng *per* mouse) in C57BL/6 adult mice (Fig 1a) reduced the percentage of time that mice exhibited freezing behaviour in the TFC paradigm (Fig 1b), and increased hippocampal IL-6 (Fig 1c) and sIL-6R in the CSF (Fig 1d). To establish that IL-6 signalling in the CNS was transducing these effects, a 400 ng dose of IL-6 that was ineffective when administered systemically resulted in cognitive decline when administered i.c.v. (Fig 1e); similarly, only when the 400 ng dose of IL-6 was delivered i.c.v. – but not systemically – did sIL-6R in CSF significantly increase (Fig 1f). When IL-6 400 ng was administered systemically, albumin did not cross the blood–brain barrier (Supplementary Fig. S1a) in contrast to what was noted previously with a systemic dose of 50 ng g^{-1} (~1500 ng).[8] A neutralising IL-6R antibody (BE0047) i.c.v. that blocks both classical and trans-signalling, prevented the inhibitory effects of surgery or i.c.v. administered IL-6 on freezing behaviour (Fig 1g). Both surgery and IL-6 i.c.v. upregulated STAT3 phosphorylation in the nucleus of CA1 hippocampal neurones (Fig. 1 h and i).

Hippocampal CA1 neuronal IL-6 signalling mediates surgery-induced cognitive decline

Both classical and trans IL-6 signalling require the presence of the co-receptor gp130. To determine whether the CA1 region of the hippocampus was involved in IL-6 signalling that results in cognitive decline, gp130 was ablated in hippocampal CA1 neurones by infecting $gp130^{flox/flox}$ mice with a hippocampal-delivered Cre- and green fluorescent protein (GFP)-expressing adeno-associated virus (AAV) (Fig. S1b). Efficient bilateral transfection of the virus in the CA1 hippocampal region was confirmed by detection of GFP expression of infected mice (Fig 2a and b); 3 weeks after viral infection, freezing behaviour during training was unaffected (Supplementary Fig. S1c). When CA1 neuronal gp130-deficient mice were exposed to surgery, i.c.v. administered IL-6, or hyper-IL-6 (Fig 2c), freezing behaviour was unaltered (Fig 2d) and there was no increase in pSTAT3 expression in CA1 neurones (Fig 2e and f). These data indicate that the IL-6 signalling needed to produce memory decline occurs in the CA1 neurones of the hippocampus.

Hippocampal CA1 neuronal classical IL-6 signalling is not required for surgery-induced memory decline

As classical IL-6 signalling requires mIL-6Rα, and IL-6 signalling in hippocampal CA1 neurones is required for surgery-induced cognitive decline (above), we reasoned that if it depended on classical IL-6 signalling, cognitive decline should not occur after discretely suppressing expression of mIL-6Rα in those neurones. Successful generation of $IL\text{-}6R\alpha^{flox/flox}$:-$CAMKII^{Cre}$ mice was confirmed by tail genotyping (Supplementary Fig. S2a,b) indicating mIL-6Rα knockdown in Cre recombinase-containing excitatory neurones in the forebrain, including hippocampal CA1 neurones[22]; all mice were viable, and the efficiency and specificity of mIL-6Rα deletion in CA1 neurones was validated by immunohistochemistry of hippocampal sections from 10- to 12-week-old adult mice (Fig 3a and b). Depletion of mIL-6Rα in CA1 neurones did not interfere with hippocampal-dependent freezing behaviour in TFC training sessions (Supplementary Fig. S2c). When the

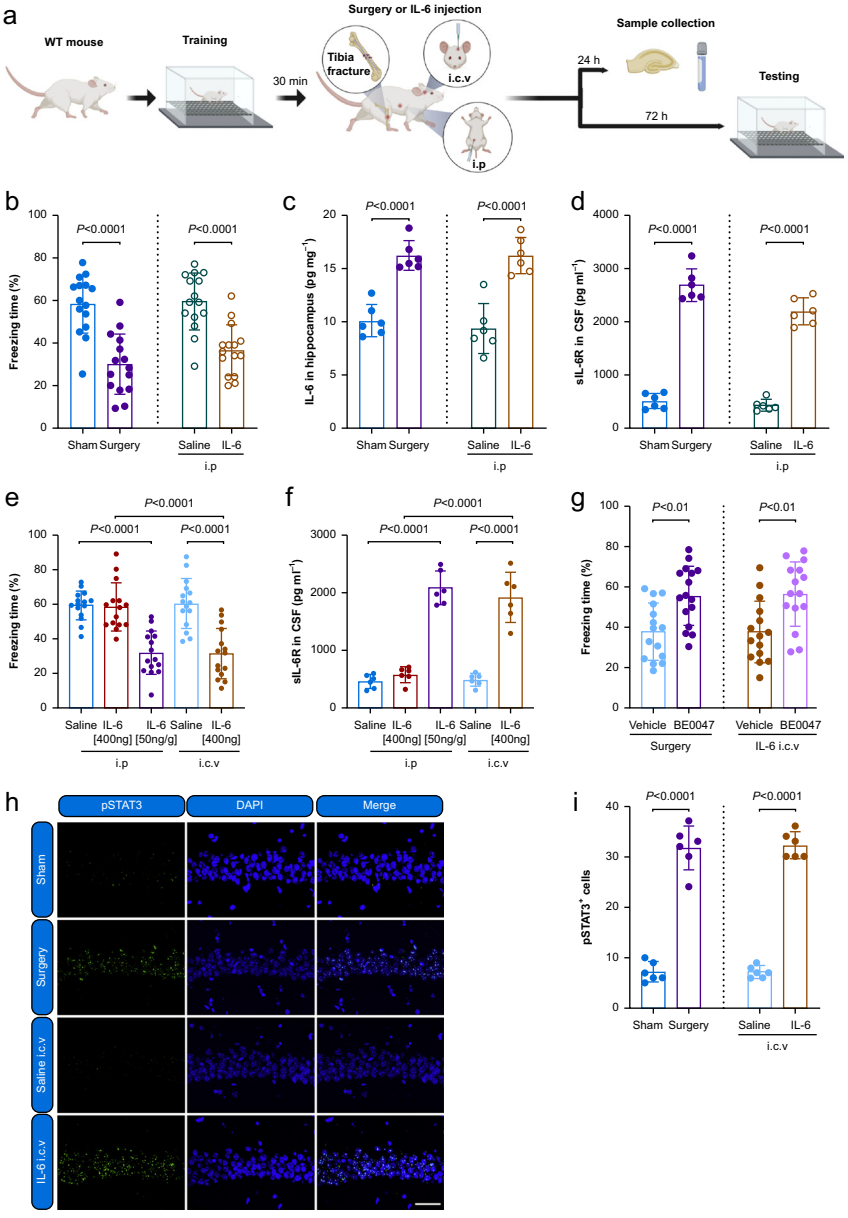

Fig 1. Hippocampal IL-6 signalling is associated with surgery-induced memory decline. (a) Schematic timeline of interventions and assessments in wild-type adult mice. Thirty minutes before the intervention involving tibial fracture under general anaesthesia, a training session for trace fear conditioning (TFC) was performed and contextual testing for freezing behaviour was undertaken 72 h after the intervention (n=15). In another cohort, CSF, blood, and brain samples were harvested 24 h after the intervention (n=6). (b–d) Four groups of wild-type mice were exposed to tibial fracture: surgery, sham surgery, IL-6 50 ng g^{-1} i. p., and saline i. p. (b) Testing for freezing behaviour in the contextual TFC test was undertaken 72 h later (n=15). At 24 h after surgery, mice (n=6) were deeply anaesthetised and the hippocampus and CSF were harvested and assayed for (c) IL-6 and (d) soluble IL-6 receptor (sIL-6R). (e and f) Five groups of wild-type mice were treated with saline i. p., IL-6 400 ng i. p., IL-6 50 ng g^{-1} i. p., saline i.c.v., or IL-6 400 ng i.c.v. (e) Testing for freezing behaviour in the contextual TFC paradigm was undertaken 72 h later (n=15). (f) At 24 h after surgery, mice (n=6) were killed and CSF was collected and assayed for sIL-6Rα. (g) Four groups of wild-type mice were treated with either BE0047 or saline i.c.v. 30 min before surgery or IL-6 400 ng i.c.v. Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h later (n=15). (h and i) Four groups of wild-type mice were treated with surgery, sham surgery, IL-6 400 ng, or saline i.c.v. (i) Representative confocal images of pSTAT3 immunoreactive cells with pSTAT3 in green and corresponding nuclear counterstaining with DAPI in blue; (h) Quantitative comparison of pSTAT3 immunoreactive cells. Scale bars, 100 μm; WT, wild-type; i.c.v., intracerebroventricular; i. p., intraperitoneal; IL-6, interleukin-6; DAPI, 4′,6-diamidino-2-phenylindole; pSTAT3, phosphorylation of signal transducer and activator of transcription 3. Data expressed as mean (standard deviation) and compared using one-way analysis of variance and *post hoc* Tukey test.

effects of surgery or i.c.v. administered IL-6 were tested in IL-6Rα$^{flox/flox}$:CAMKII^{Cre} mice (Fig 3c), both a decrease in freezing behaviour (Fig 3d) and upregulation of CA1 neuronal STAT3 phosphorylation were observed (Fig 3e and f), similar to that seen in wild-type mice (Fig 1h and i), indicating that mIL-6Rα in CA1 neurones is not required for producing IL-6-mediated memory decline. Microglial activation, an absolute requirement for memory impairment,[23] still obtained after both surgery and i.c.v. administered IL-6 in IL-6Rα$^{flox/flox}$:CAMKII^{Cre} mice (Supplementary Fig. S4a–d). From this, we can deduce that classical IL-6 signalling in hippocampal CA1 neurones is dispensable for surgery-induced memory decline. It is well documented that cells which do not express the mIL-6Rα can still be rendered IL-6 responsive via the alternative mechanism of IL-6 trans-signalling if the glycoprotein transducer, gp130, is present[24]; therefore, the possibility that these cells are involved in IL-6 trans-signalling is not excluded by this experiment.

Microglial IL-6R is not required for memory decline induced by surgery

IL-6Rα is highly expressed in microglia,[25,26] the resident tissue macrophage in the CNS, making it a possible source of sIL-6R for IL-6 trans-signalling that is putatively required for surgery-induced cognitive decline. Mice lacking microglial IL-6Rα were generated by crossing CX3CR1^{Cre} mice with IL-6Rα$^{flox/flox}$ to yield IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} mice, which was confirmed by tail genotyping (Supplementary Fig. S3a,b). The efficiency and specificity of mIL-6Rα deletion in microglia was revealed by immunoblotting, indicating a significant reduction in protein levels of mIL-6Rα in microglia of IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} mice (Fig 4a and b). Deletion of mIL-6Rα in microglia did not affect hippocampal-dependent memory in training (Supplementary Fig. S3c). When cohorts of IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} mutant mice were exposed to surgery or IL-6 i.c.v. (Fig 4c), only surgery produced memory decline (Fig 4d) and CA1 neuronal STAT3 phosphorylation (Fig 4e and f). Despite the absence of IL-6R, microglial activation still occurred after surgery but not i.c.v. administered IL-6 (Supplementary Fig. S4e–h), consistent with the observed memory impairment with only surgery (Fig 4d). Surgery, but not IL-6 i.c.v., upregulated sIL-6R in the CSF (Fig 4g) and serum (Fig 4h) and was associated with an increase in albumin translocation into the brain through a permeabilised blood–brain barrier (Fig 4i). Were IL-6 trans-signalling responsible for cognitive decline after surgery, the source of the required sIL-6R cannot be microglia. Given the absolute requirement for bone marrow-derived monocytes (BM-DMs) for surgery-induced memory decline,[27] this is the likely cellular source of sIL-6R for IL-6 trans-signalling after surgery. When IL-6 was administered i.c.v. to mice lacking mIL-6R in microglia (IL-6Rα$^{flox/flox}$:CX3CR1^{Cre}), there was no increase in sIL-6R (Fig 4g). These data suggest that resident microglia are the only source of sIL-6R for this route of IL-6 administration because an extra-CNS source is unavailable as the blood–brain barrier remains intact (Fig 4i and Supplementary Fig. S1a).

IL-6 trans-signalling mediates cognitive decline induced by surgery or i.c.v. administered IL-6

We next determined whether IL-6 trans-signalling in CA1 neurones accounts for the IL-6-dependent regulation of cognition. Wild-type mice were pretreated with the IL-6 trans-signalling inhibitor sgp130Fc i.c.v., before surgery or IL-6 i.c.v. (Fig 5a). The decline in freezing behaviour (Fig 5b), and the upregulation of CA1 neuronal pSTAT3 (Fig 5c and d) induced by surgery or i.c.v. administered IL-6 were reversed by pretreatment with sgp130Fc. For IL-6 trans-signalling to be responsible for surgery-induced cognitive decline, there must be a source of sIL-6R, either from proteolysis of the ectodomain of expressed IL-6Rα or from alternative splicing of the gene transcripts. Therefore, behavioural and signalling effects of surgery or i.c.v. administered IL-6 were studied in IL-6Rα$^{-/-}$ (global knockout) mice (Fig 5e). Neither surgery nor exogenous IL-6 were capable of inducing either memory decline (Fig 5f) or hippocampal pSTAT3 (Fig 5g and h) in global IL-6Rα knockout mice, indicating that a source of sIL-6R is required for surgery-induced cognitive decline. To confirm that the apparatus for trans-signalling was present in the global IL-6Rα$^{-/-}$ knockout mice, hyper-IL-6 was administered i.c.v.; this resulted in a decline in freezing behaviour (Fig 5f) and upregulation of CA1 neuronal pSTAT3 (Fig 5g and h), indicating that the co-receptor, gp130, was unaffected by the global IL-6Rα receptor knockout and permitted IL-6 trans-signalling.

gp130Fc treatment does not impair wound healing after aseptic trauma in wild-type mice

A number of proinflammatory cytokines, including IL-6, are secreted at the local site in the early inflammatory phase after bone fracture. IL-6 serves not only to maintain bone homeostasis by mediating both bone formation by osteoblasts and bone resorption by osteoclasts,[28] but also fracture healing through a biphasic repair process involving early osteoclastogenesis[29] and late bone formation in the fracture callus.[16] Global inhibition of pro-inflammatory cytokines adversely impacts fracture healing in the same manner as anti-inflammatory drugs such as cyclooxygenase-2 inhibitors.[30] To explore the effect of IL-6 signalling during bone healing, either the anti-IL-6R antibody, BE0047, which inhibits both IL-6 classic and trans-signalling, or sgp130Fc, which selectively blocks trans-signalling, was administered i. p. to wild-type mice after surgery (Fig 6a). The safranin O/fast green staining revealed that the newly formed cartilage in the fracture callus area was greater in the saline and sgp130Fc group than in the BE0047 group at 15 days after surgery (Fig 6b and c), indicating that classical IL-6 signalling —but not trans-signalling — is required for bone healing.

Discussion

We have shown that IL-6 is both necessary and sufficient for surgery-induced cognitive decline in a mouse model of perioperative neurocognitive dysfunction.[8] This study shows that surgery induces memory decline through IL-6 trans-signalling in CA1 hippocampal neurones. In order to establish that IL-6 signalling produces memory impairment in the CNS, we used a dose of i.c.v. administered IL-6 that does not permeabilise the blood–brain barrier to show that it both produces IL-6 signalling in the hippocampus and produces memory impairment. That the CA1 region in the hippocampus is the locus for the IL-6 signalling that produces memory impairment is revealed by the selective depletion of the gp130 transducer only in that region, which showed that neither signalling nor memory impairment was produced. We show that classical IL-6 signalling in this region is not involved

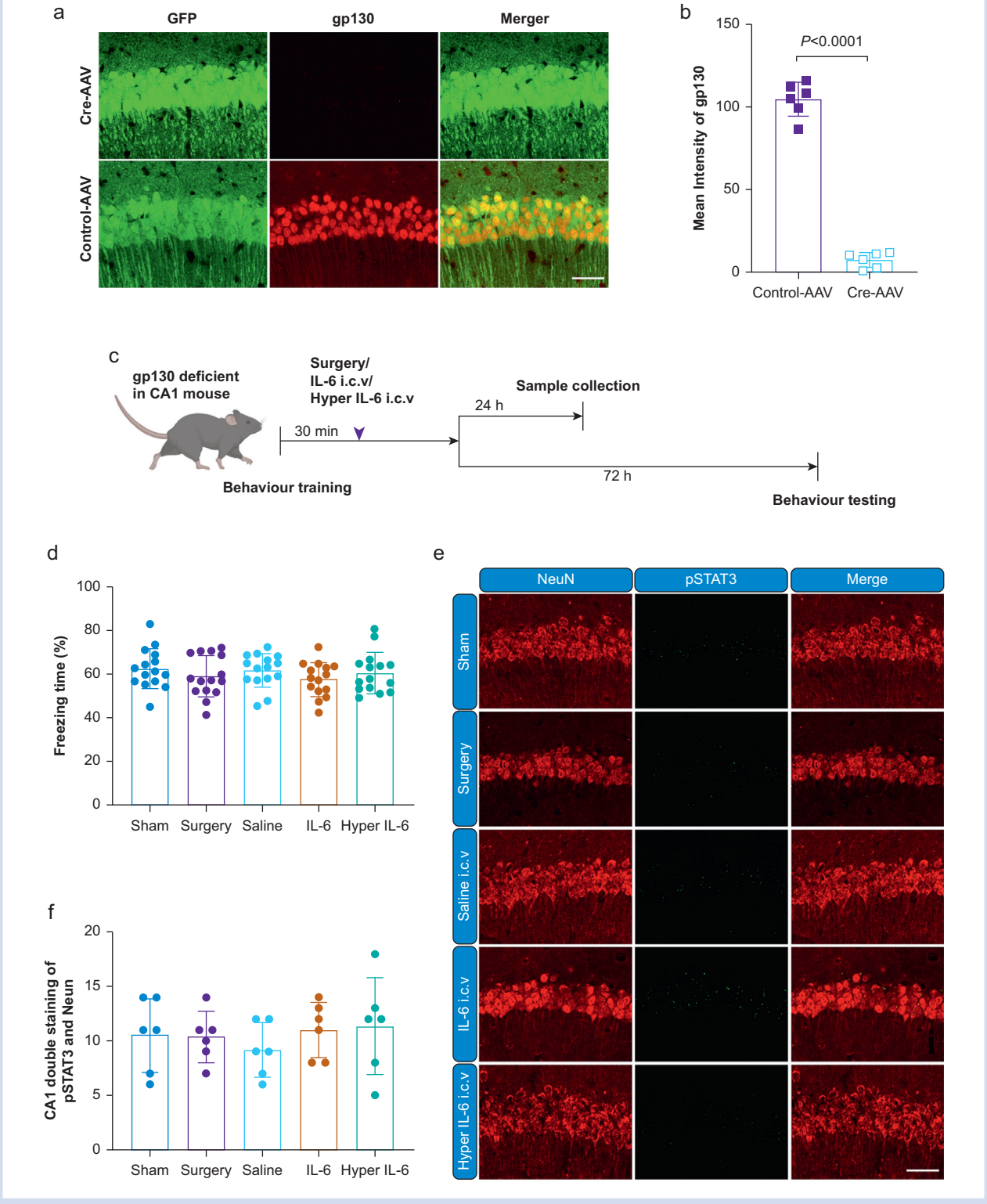

Fig 2. Signalling in hippocampal CA1 neurones mediates surgery-induced cognitive decline. (a, b) Cre-AAV and Control-AAV viruses were stereotaxically injected into the hippocampus of gp130$^{flox/flox}$ mice (n=6), and tissue was harvested 3 weeks later. (a) Representative images of Cre-AAV and Control-AAV infected cells in the CA1 of the hippocampus. (b) Quantification of mean intensity of gp130 expression (Scale bar, 100 μm). (c) Schematic timeline of interventions and assessments in CA1 neuronal gp130-deficient mice. (d–f) Five groups of CA1 neuronal, gp130-deficient mice were exposed to tibia fracture, sham surgery, i.c.v. saline, i.c.v. IL-6 or i.c.v. hyper-IL-6. (d) Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h later (n=15). At 24 h after surgery, separate cohorts of mice (n=6) were killed and brains were collected. (e) Representative confocal images of CA1 double staining of pSTAT3 (green) and NeuN (red). (f) Quantitative comparison of CA1 double staining of pSTAT3 and NeuN. Scale bars, 100 μm; i.c.v., intracerebroventricular; AAV, adeno-associated virus; pSTAT3, phosphorylation of signal transducer and activator of transcription 3. Data expressed as mean (standard deviation) and compared using two-tailed, unpaired Student t-test (b) or one-way analysis of variance and *post hoc* Tukey test (d and f).

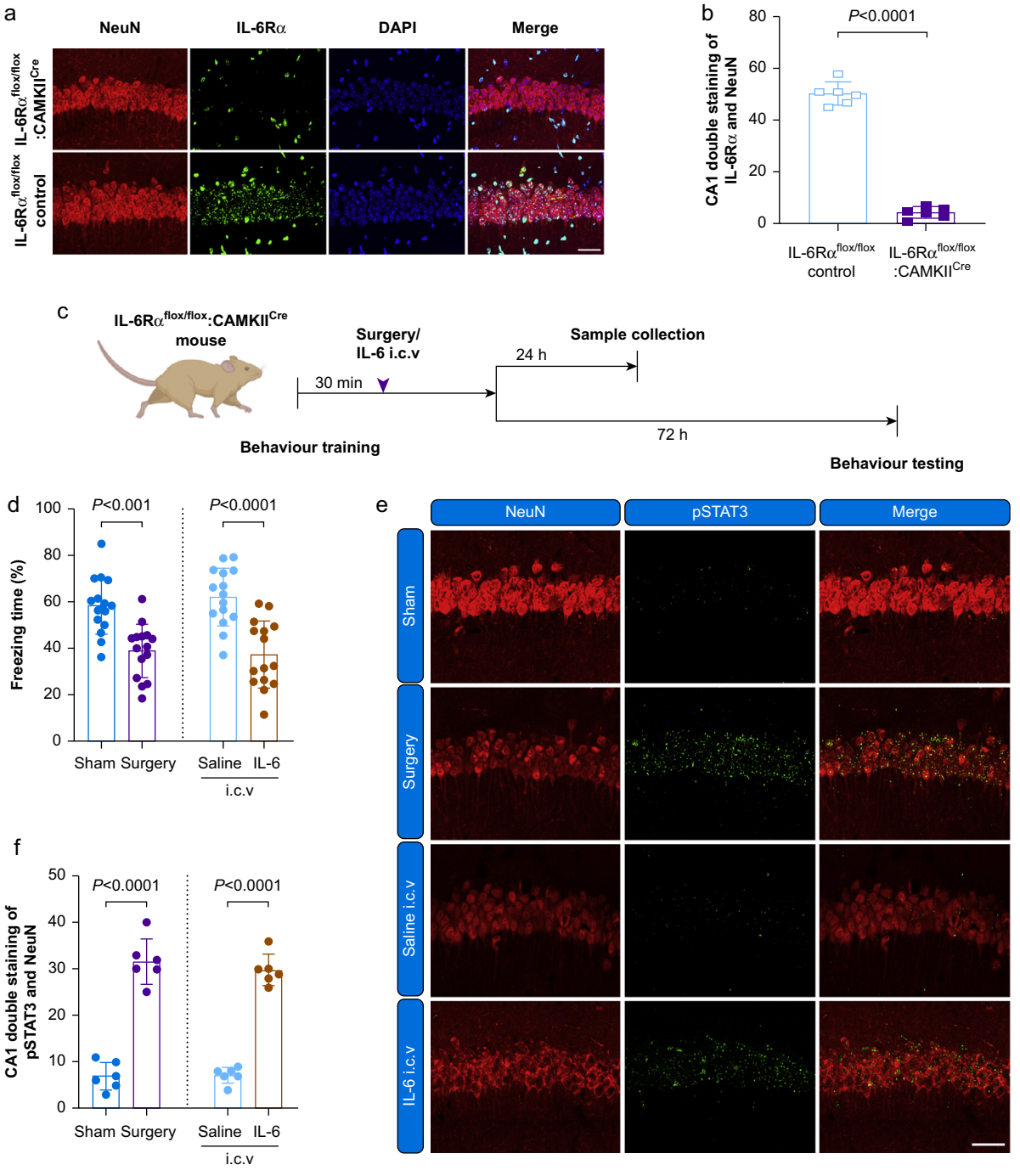

Fig 3. Classical IL-6 signalling in hippocampal neurones is not required for surgery-induced memory decline. (a, b) The efficiency and specificity of mIL-6Rα deletion in hippocampal CA1 sections from adult IL-6Rα$^{flox/flox}$:CAMKII^{Cre} mice. (a) Representative images of mIL-6Rα expression in CA1 area from adult IL-6Rα$^{flox/flox}$:CAMKII^{Cre} (top row) or IL-6Rα$^{flox/flox}$ control (bottom row) mice immunostained for IL-6Rα (green), NeuN (red), and DAP1 (blue). (b) Quantification of CA1 double staining of IL-6Rα and NeuN. (c) Schematic timeline of interventions and assessments in IL-6Rα$^{flox/flox}$:CAMKII^{Cre} mice. Thirty minutes before an intervention, a training session for the trace fear conditioning paradigm was performed; contextual testing for freezing behaviour was undertaken 72 h after the intervention. In another cohort, brain samples were harvested 24 h after the intervention. (d–f) Four groups of IL-6Rα$^{flox/flox}$:CAMKII^{Cre} mice were treated with tibia fracture, sham surgery, IL-6 400 ng, or saline i.c.v. (d) Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h later (n=15). (e) At 24 h after surgery, mice (n=6) were deeply anaesthetised and brains were harvested and immunostained; representative confocal images of CA1 double staining of pSTAT3 (green) and NeuN (red) are provided. (f) Quantitative comparison of CA1 double staining of pSTAT3 and NeuN. Scale bars, 100 μm;i.c.v., intracerebroventricular. Data expressed as mean (standard deviation) and compared using two-tailed, unpaired Student t-test (b) or one-way analysis of variance and *post hoc* Tukey test (d and f).

because both signalling and memory impairment still occurs after mIL-6R has been selectively downregulated. Therefore, it is likely that IL-6 trans-signalling in this region is responsible for postoperative memory decline, although the source of the sIL-6R is unclear.

The putative source of sIL-6R was addressed in mice in which mIL-6R was depleted exclusively in microglia. Under these circumstances, surgery was still able to produce IL-6 signalling and memory impairment because there is likely to be another extra-CNS source of sIL-6R. The selective IL-6 trans-signalling blocker sgp130Fc prevented surgery-induced IL-6 signalling and memory impairment, whereas the IL-6 trans-signalling agonist induced IL-6 signalling and memory impairment even in global mIL-6R knockout mice. Systemic administration of sgp130Fc facilitated normal healing of the surgically induced tibial fracture; conversely, a combined IL-6 classical and trans-signalling blocker adversely affected bone healing.

Hippocampal CA-1 neuronal IL-6 signalling is required to produce a decline in memory

Our previous study[8] established that IL-6, whether administered systemically at a dose of 50 ng g^{-1} (~1500 ng), or through surgery-induced upregulated levels in the circulation and hippocampus, can cause cognitive decline; however, it was not known *where* the IL-6 signalling produced this response. That the critical IL-6 signalling occurs in the CNS is supported by data that established that IL-6 produced memory decline, an increase in CSF sIL6R, and IL-6 signalling in CA1 hippocampal neurones, whereas this same dose was ineffective when delivered systemically, presumably because it could not permeabilise the blood–brain barrier to facilitate translocation of inflammatory mediators into the brain at this systemic dose. Corroboration that the CNS is the site of IL-6 signalling for cognitive decline after surgery is provided by the ability of the i.c.v. administered monoclonal antibody BE0047, which targets both forms of IL-6 signalling, to reverse the surgery-induced decline in freezing behaviour. A caveat to this interpretation is that i.c.v. administered BE0047 does not achieve a sufficiently high plasma level to block extra-CNS IL-6 signalling.

To establish where in the CNS IL-6 signalling occurs to produce memory decline, we focused on the CA1 region of the hippocampus because it exhibits synaptic plasticity required for long-term potentiation, a neurobiologic correlate of learning and memory.[31] To establish that IL-6 signalling in the CA1 region of the dorsal hippocampus was needed to produce memory decline, these neurones were stereotaxically infected with a virus ('Cre-AAV') that successfully eliminated the co-receptor gp130, which is required for both forms of IL-6 signalling. Neither surgery nor i.c.v. administered IL-6 or hyper-IL-6 (a specific IL-6 trans-signalling agonist) induced memory decline or hippocampal IL-6 signalling in mice deficient in gp130 in CA1 hippocampal neurones. From these studies, the site (i.e. hippocampal CA1 neurones), but not the mechanism of IL-6 signalling, that results in surgery-induced memory decline was established.

Hippocampal CA1 neuronal classical IL-6 signalling is not required for surgery-induced memory decline

Neuronal mIL-6Rα is expressed in the hippocampus,[32] and therefore capable of transducing classical IL-6 signalling. To determine whether classical signalling at this site mediates the surgical phenotype, mice were generated that lacked mIL-6Rα, and therefore the capacity to transduce classical IL-6R signalling, in CA1 neurones. In this conditional knockout of mIL-6Rα, surgery still induced memory decline and IL-6 signalling in that site. Therefore, classical IL-6 signalling in CA1 neurones is not required for surgery-induced cognitive decline.

Components of IL-6 trans-signalling are required to produce surgery-induced cognitive decline

For IL-6 trans-signalling, there is an absolute requirement for both IL-6 and sIL-6R[33]; the putative cellular sources of these components after surgery include bone marrow-derived monocytes[34] and microglia.[35] The soluble form of the receptor (sIL-6R) is derived either from proteolysis of the ectodomain of mIL-6Rα[36] or through alternative splicing.[37] The initial experiments indicated a requirement for sIL-6R in the CSF to produce cognitive decline, but non-definitively and without revealing the possible cellular source. Because we had shown that microglia are essential for surgery-induced cognitive decline,[38] we determined whether these cells were the source of sIL-6R. Mice that lacked microglial IL-6Rα (IL-6Rα$^{flox/flox}$:CX3CR1^{Cre}) exposed to aseptic surgical trauma exhibited cognitive decline and increases both of IL-6 signalling in the hippocampus and of sIL-6R in the CSF, indicating that microglial mIL-6Rα is dispensable. Interestingly, i.c.v. administered IL-6 no longer exhibited these features presumably because the cytokine requires a CNS source of sIL-6R to form the agonist (IL-6/sIL-6R) for trans-signalling. We favour bone marrow-derived monocytes as the source of sIL-6R for putative IL-6 trans-signalling mediated cognitive decline after surgery because they are required for surgery-induced cognitive decline.[27] Furthermore, bone marrow-derived monocytes penetrate the permeabilised blood–brain barrier after surgery,[39] and could be the source for the increase in sIL-6R in the CSF after surgery but not after i.c.v. administered IL-6 at a dose that does not permeabilise the blood–brain barrier.

IL-6 trans-signalling produces surgery-induced cognitive decline

To address whether IL-6 trans-signalling mediates surgery-induced cognitive decline, wild-type mice were treated before surgery with sgp130Fc, a specific inhibitor of IL-6 trans-signalling. This pretreatment prevented surgery-induced IL-6 signalling and cognitive decline. Global IL-6Rα$^{-/-}$ knockout mice should be incapable of transducing either IL-6 classical or trans-signalling, unless there is an alternative source of sIL-6R for trans-signalling. In global IL-6Rα$^{-/-}$ knockout mice, neither surgery nor i.c.v. administered IL-6 produced IL-6 hippocampal signalling or cognitive decline, ruling out the possibility that classical IL-6 signalling is required to produce cognitive decline from either surgery or exogenously administered IL-6. Hyper-IL-6 contains IL-6 bound to a dimer of sIL-6R, the two molecules required to generate IL-6 trans-signalling.[18] In the global IL-6Rα$^{-/-}$ knockout, in which there is neither message for alternative splicing nor the ectodomain of IL-6Rα for shedding, hyper IL-6 still generates signalling and memory decline. Using approaches that produced both a loss (sgp130Fc) and a gain (hyper-IL-6) of function, we have shown an absolute dependence on IL-6 trans-signalling for cognitive decline produced by surgery.

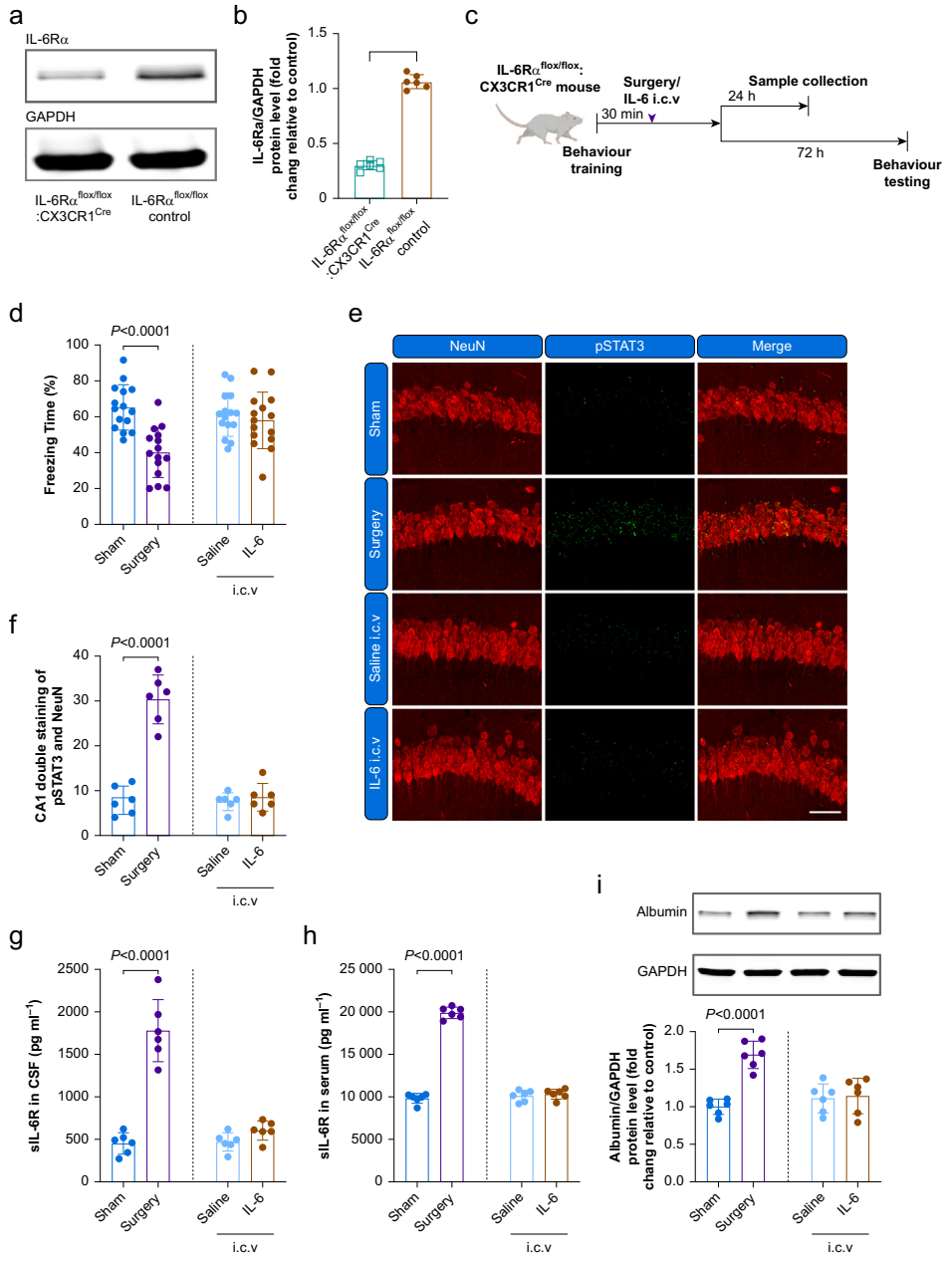

Fig 4. Microglial mIL-6Rα is not required for memory decline induced by surgery. (a, b) The efficiency and specificity of mIL-6Rα deletion in microglia from adult mice (IL-6Rα$^{flox/flox}$:CX3CR1^{Cre}) is reflected by (a) representative immunoblot and (b) quantitative analysis showing IL-6Rα expression in isolated microglia of IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} *vs* IL-6Rα$^{flox/flox}$ control mice (n=6). (c) Schematic timeline of interventions and assessments in IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} mice. Thirty minutes before intervention, a training session for TFC was performed and contextual testing for freezing behaviour was undertaken 72 h after the intervention. In another cohort, brain samples were harvested 24 h after the intervention. (d–f) Four groups of IL-6Rα$^{flox/flox}$:CX3CR1^{Cre} mice were exposed to tibia fracture, sham surgery, IL-6 400 ng, or saline i.c.v. (d) Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h later (n=15). (e) At 24 h after surgery, mice (n=6) brains were harvested for immunostaining and representative confocal images of CA1 double staining of pSTAT3 (green) and NeuN (red) are shown. (f) Quantitative comparison of CA1 double staining of pSTAT3 and NeuN. (g) CSF obtained 24 h after the intervention was assayed for sIL-6R. (h) Serum obtained 24 h after the intervention was assayed for sIL-6R. (i) Brains were assayed for albumin. Scale bars, 100 μm; i.c.v., intracerebroventricular; TFC, trace fear conditioning; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data expressed as mean (standard deviation) and compared using two-tailed, unpaired Student *t*-test (b) or one-way analysis of variance and *post hoc* Tukey test (d, f, and g–i).

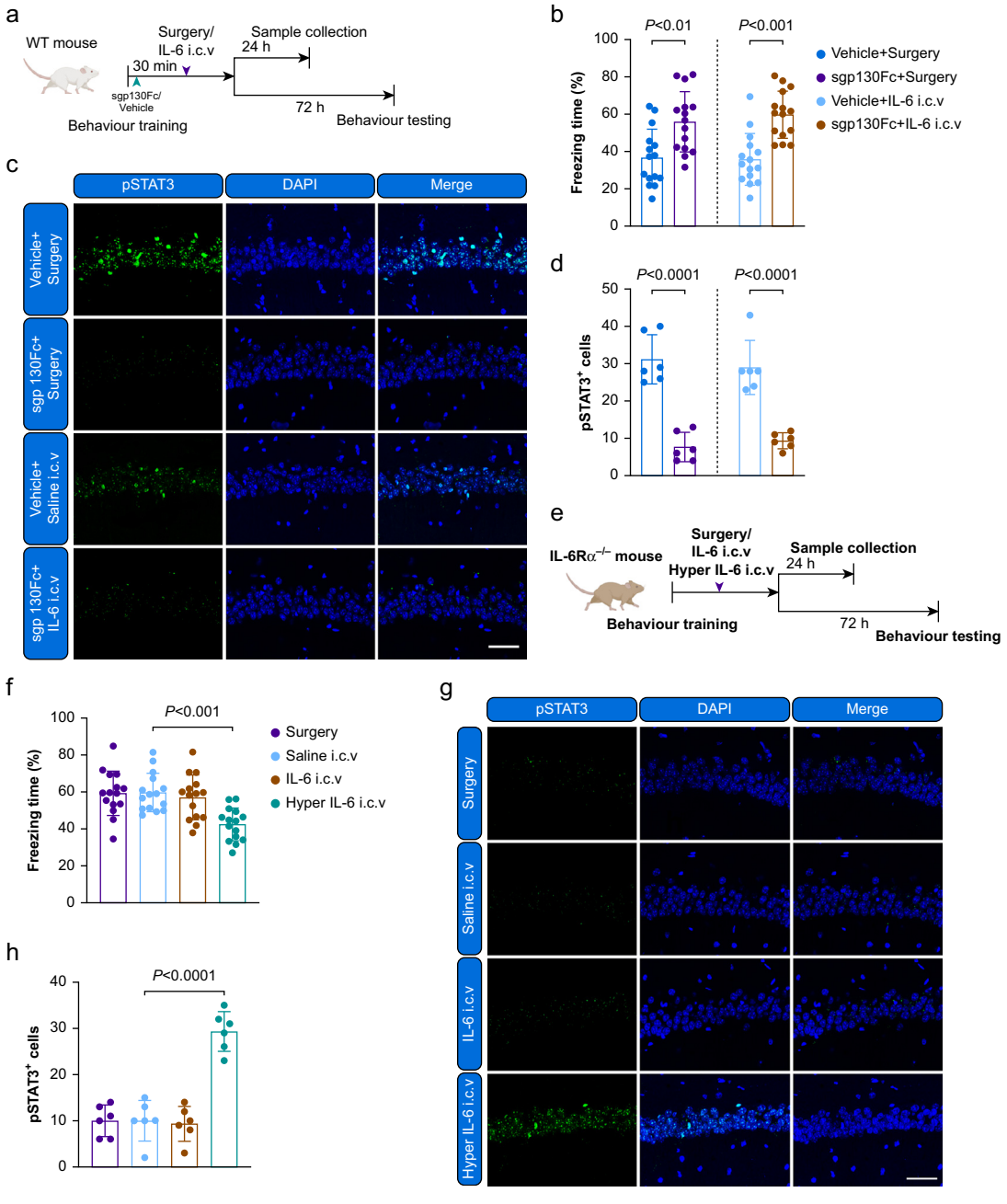

Fig 5. IL-6 trans-signalling mediates cognitive decline induced by surgery or i.c.v. administered IL-6. (a) Schematic timeline of interventions and assessments in wild-type mice. (b–d) Four groups of wild-type mice exposed to tibia fracture with pretreatment with either sgp130Fc or PBS, or IL-6 400 ng i.c.v. with either sgp130Fc or vehicle; pretreatments were given 30 min before the intervention. (b) Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h after the intervention (n=15). (c) At 24 h after the intervention four other cohorts of mice (n=6) brains were harvested and immunostained; representative confocal images of pSTAT3 immunoreactive cells (pSTAT3, green) and corresponding nuclear counterstaining (DAPI, blue) are provided. (d) Quantitative comparison of pSTAT3 immunoreactive cells. (e) Schematic timeline of interventions and assessments in IL-6Rα$^{-/-}$ mice. (f–h) Four groups of IL-6Rα$^{-/-}$ mice were treated with tibia fracture, or i.c.v. administered IL-6 400 ng, hyper IL-6, or saline. (f) Testing for freezing behaviour in the contextual fear conditioning paradigm was undertaken 72 h after the intervention (n=15). (g) At 24 h after the intervention, four other cohorts of mice (n=6) brains were harvested and immunostained, and representative confocal images of pSTAT3 immunoreactive cells (pSTAT3, green) and corresponding nuclear counterstaining (DAPI, blue) are presented. (h) Quantitative comparison of pSTAT3 immunoreactive cells. Scale bars, 100 μm; i.c.v., intracerebroventricular; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; pSTAT3, phosphorylation of signal transducer and activator of transcription 3. Data are expressed as mean (standard deviation) and compared using one-way analysis of variance and *post hoc* Tukey test.

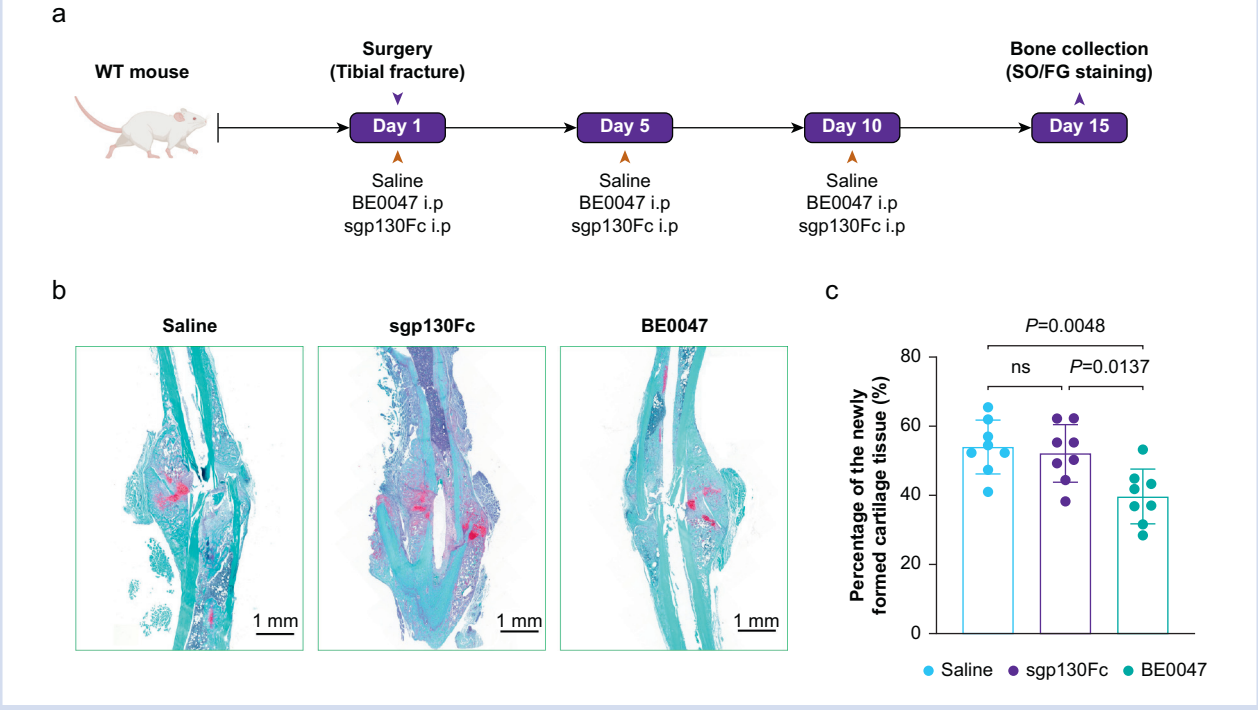

Fig 6. BE0047 but not sgp130Fc impairs bone fracture healing. (a) Schematic timeline of interventions and assessments in three cohorts (n=8) of wild-type mice. (b) Representative histological images of fracture callus stained with SO/FG that reflects cortical bone tissue (green) and newly formed cartilage tissue (red) in mice administered BE0047, sgp130Fc, or saline i. p. (c) Percentage of newly formed cartilage tissue in the fracture callus area. Scale bars 1 μm; i. p., intraperitoneal; SO/FG, safranin O/fast green. Data expressed as mean (standard deviation) and compared using one-way analysis of variance and *post hoc* Tukey test.

Appropriate bone healing can occur when IL-6 trans-signalling is interrupted

Preservation of classical IL-6 signalling with sgp130Fc, the selective IL-6 trans-signalling blocker, resulted in improved bone healing compared with blockade of both IL-6 classical and trans-signalling with BE0047. These data support the goal of preserving classical IL-6 signalling to sustain appropriate wound healing.

Caveats

PNDs typically occur with advanced age, and it has been advocated that preclinical models of PND should use aged animals. Interestingly, all our previous preclinical data that led to the conclusion that neuroinflammation was required for PND were acquired from experiments performed in adult male (12–16 weeks) but not aged mice; notwithstanding the younger age in our animal model, the same requirements for development of PNDs in mice have also been shown to obtain in surgical patients.[40–42]

The gp130 co-receptor is also responsible for signalling from another cytokine, IL-11.[43] Therefore, the observation that depletion of gp130 in hippocampal neurones prevents surgery-induced cognitive decline does not alone prove that the process requires IL-6 signalling. Because BE0047, a monoclonal antibody directed at IL-6 and not IL-11 signalling, prevented surgery-induced cognitive decline, the gp130 co-receptor depletion experiments establish that prevention of cognitive decline is through IL-6 signalling.

Druggable targets to prevent perioperative neurocognitive disorders

Interventions that interrupt IL-6 trans-signalling while leaving classical signalling unaffected are needed to prevent surgery-induced cognitive decline. Monoclonal antibodies that target the IL-6R, such as tocilizumab[8] and BE0047,[44] although effective at abrogating surgery-induced cognitive decline, also mitigate the beneficial effects of IL-6 classical signalling directed against invading microbial pathogens and thereby increase the likelihood of infections that would complicate recovery in the perioperative period.[45] Similarly, monoclonal antibody therapy directed against the cytokine IL-6, such as sirukumab, is also associated with increased risk of infections.[46] Therefore, more focused interventions directed against IL-6 trans-signalling should be considered. As sgp130Fc is efficacious in preventing surgery-induced cognitive decline without interfering with bone healing, and as olamkicept, its commercial successor, is now being studied in clinical trials to prevent inflammatory bowel diseases[47] that encompass conditions that depend upon IL-6 trans-signalling,[48] olamkicept is a candidate intervention for either prevention or reversal of PNDs. As we have now shown that IL-6 trans-signalling in hippocampal CA1 neurones produces surgery-induced cognitive decline, systemically administered olamkicept will need to penetrate the blood–brain barrier to produce an effect; however, sgp130Fc has a molecular weight of 186 kDa,[49] a relatively large peptide that is unlikely to penetrate the intact blood–brain barrier. Postoperatively, the blood–brain barrier is permeabilised[41] facilitating translocation of molecules as large as fibrinogen

(~340 kDa)[39]; therefore, it is possible that olamkicept can penetrate the blood–brain barrier in the perioperative period. Based on risk factors such as advanced age and existing cognitive impairment, olamkicept could be administered at the beginning of surgery as a prophylaxis against PNDs in high-risk patients. Alternately, once exaggerated IL-6 trans-signalling has been identified postoperatively, possibly by circulating biomarkers such as sIL-6R (upregulation) or sgp130 (down-regulation), olamkicept could be administered to reverse the deleterious processes.

Another possible approach to diminish IL-6 trans-signalling is to limit the amount of sIL-6R generated. Metalloproteinases of the adamalysin class, including ADAM 17 (also known as TNF-α convertase enzyme [TACE]) and ADAM 10, are the principal proteolytic enzymes involved in shedding of sIL-6Rs, which can increase with aging[50] and in inflammatory states, both of which increase the risk of surgery-induced cognitive decline. It was not possible to test the absolute requirement for ADAM 17, ADAM 10, or both for surgery-induced cognitive decline because these knockouts are embryologically lethal, and there are no selective small molecule inhibitors that do not affect other proteases. Therefore, until we can show that the sIL-6R for surgery-induced memory decline is generated from shedding and not by alternative splicing, use of metalloproteinase inhibitors cannot be considered.

Conclusions

We show that, after orthopaedic trauma, IL-6 produces perioperative neurocognitive disorders in mice through hippocampal IL-6 trans-signalling. Druggable targets of the trans-signalling pathway should be sought to prevent perioperative neurocognitive disorders while preserving the healing properties of classical IL-6 signalling.

Authors' contributions

Project conception: JH, YZ, MM
Study design: JH, YZ, MM
Performance of experiments: JH, YZ, CH, SH
Initial data collection and analysis: JH, YZ, CH, SH, XF
Final data analysis: JH, YZ, XF, MM
Writing of paper: JH, YZ, MM
Critical revision of paper: MM

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Declarations of interest

MM is a co-inventor on a patent for the use of dexmedetomidine for sedation; he has not and will not receive royalty payments for sales of dexmedetomidine and since 2005 has not received any support from companies selling dexmedetomidine. MM consulted for Masimo in 2010.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2022.08.019.

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