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Blocking Kv1.3 potassium channels prevents postoperative neuroinflammation and cognitive decline without impairing wound healing in mice

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Abstract

Background: Postoperative cognitive decline (PCD) requires microglial activation. Voltage-gated Kv1.3 potassium channels are involved in microglial activation. We determined the role of Kv1.3 in PCD and the efficacy and safety of inhibiting Kv1.3 with phenoxyalkoxypsoralen-1 (PAP-1) in preventing PCD in a mouse model.

Methods: After institutional approval, we assessed whether Kv1.3-deficient mice (*Kv1.3*$^{-/-}$) exhibited PCD, evidenced by tibial-fracture surgery-induced decline in aversive freezing behaviour, and whether PAP-1 could prevent PCD and postoperative neuroinflammation in PCD-vulnerable diet-induced obese (DIO) mice. We also evaluated whether PAP-1 altered either postoperative peripheral inflammation or tibial-fracture healing.

Results: Freezing behaviour was unaltered in postoperative *Kv1.3*$^{-/-}$ mice. In DIO mice, PAP-1 prevented postoperative (i) attenuation of freezing behaviour (54 [17.3]% *vs* 33.4 [12.7]%; P=0.03), (ii) hippocampal microglial activation by size (130 [31] pixels *vs* 249 [49]; P<0.001) and fluorescence intensity (12 000 [2260] *vs* 20 800 [5080] absorbance units; P<0.001), and (iii) hippocampal upregulation of interleukin-6 (IL-6) (14.9 [5.7] *vs* 25.6 [10.4] pg mg^{-1}; P=0.011). Phenoxyalkoxypsoralen-1 neither affected surgery-induced upregulation of plasma IL-6 nor cartilage and bone components of the surgical fracture callus.

Conclusions: Microglial-mediated PCD requires Kv1.3 activity, determined by genetic and pharmacological targeting approaches. Phenoxyalkoxypsoralen-1 blockade of Kv1.3 prevented surgery-induced hippocampal microglial activation and neuroinflammation in mice known to be vulnerable to PCD. Regarding perioperative safety, these beneficial effects of PAP-1 treatment occurred without impacting fracture healing. Kv1.3 blockers, currently undergoing clinical trials for other conditions, may represent an effective and safe intervention to prevent PCD.

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Keywords: Kv1.3; microglial activation; neuroinflammation; phenoxyalkoxypsoralen-1; postoperative cognitive decline; potassium channel; wound healing

Editor's key points

- Postoperative cognitive decline (PCD) involves microglial activation, which is regulated by voltage-gated Kv1.3 potassium channels.
- Mouse models were used to determine the role of Kv1.3 in PCD and the efficacy of inhibiting Kv1.3 with phenoxyalkoxypsoralen-1 (PAP-1) in preventing PCD.
- The small-molecule PAP-1 prevented surgery-induced hippocampal microglial activation and neuroinflammation in mice known to be vulnerable to PCD.
- The effects of PAP-1 treatment occurred without affecting fracture healing, a known potential side-effect of inhibition of inflammation.
- Use of Kv1.3 blockers represents a promising potential intervention to prevent PCD.

Perioperative neurocognitive disorders encompass a spectrum from delirium to dementia,[1] and threaten postoperative functional independence and survival. Because perioperative neurocognitive testing is not routinely performed, the true incidence of this surgical complication is not known, but postoperative delirium in unselected surgical populations has been estimated to occur in ~37% of patients.[2] Ageing of the surgical population and increase in worldwide surgeries to >300 million procedures per annum[3] are converging to produce an epidemic of perioperative neurocognitive disorders with high morbidity and mortality.

Prevention, mitigation, or reversal of this surgical complication requires an understanding of the pathophysiological mechanisms that explain age dependency[4] and increased risk in patients with metabolic syndrome[5] and with postoperative infection.[6] In the last decade, we have generated rodent models of postoperative cognitive decline (PCD) that reproduce these features.[7–9] Using these models, we have learned that trauma-released alarmins engage the innate immune system inducing nuclear translocation of the transcription factor nuclear factor kappa light chain enhancer of activated B cells in bone marrow-derived monocytes (BM-DMs) resulting in upregulation and peripheral release of pro-inflammatory cytokines[10] needed for wound healing.[11] These cytokines can also disrupt the blood–brain barrier,[12] allowing entry of BM-DMs into the CNS,[13] where, together with activated microglia,[14] these cells release pro-inflammatory cytokines, such as interleukin (IL)-1β[15] and IL-6,[16] that disrupt long-term potentiation, the neurobiological correlate of learning and memory.[17] Similar inflammatory events,[18] including activation of microglia,[19] also occur in the clinical setting.

In a proof-of-concept study, we reported that depleting microglia before their activation precluded PCD.[14] In searching for a druggable target to prevent PCD without affecting peripheral inflammation and surgical wound healing, we focused on Kv1.3 potassium channels, the opening of which is required for microglial activation.[20] The small-molecule Kv1.3 blocker phenoxyalkoxypsoralen-1 (PAP-1) inhibits lipopolysaccharide-induced microglial activation and synthesis and release of pro-inflammatory cytokines.[21] We hypothesised that inhibiting Kv1.3 channels with PAP-1 prevents the development of postoperative neuroinflammation, and thereby mitigates PCD while maintaining the systemic inflammatory milieu needed for wound healing.[22] Using mice lacking Kv1.3 ($Kv1.3^{-/-}$) and treating mice highly susceptible to PCD with PAP-1, we establish the relevance of Kv1.3 for the development of PCD and the effectiveness of blocking this channel to prevent PCD without affecting tibial-fracture healing. These findings provide laboratory support for translational studies in PCD of Kv1.3 inhibitors, a drug class being developed for autoimmune conditions.[23]

Methods

Animals

Experimental procedures involving animals were approved by the Institutional Animal Care Use Committee of the University of California, San Francisco (San Francisco, CA, USA), and were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male mice 12–14 weeks old on a C57bl/6j background (Jackson Laboratory, Sacramento, CA, USA) were group-housed mice (five per cage) with 12 h light/dark cycles in a temperature-controlled environment with *ad libitum* access to standard rodent chow and water. Before the experiments, the mice were randomly assigned into groups. The $Kv1.3^{-/-}$ mice were a gift from Leonard Kaczmarek (Yale University, New Haven, CT, USA), and have been rederived onto the C57bl/6j background to homozygosity.[24] Five-week-old C57bl/6j mice were fed either a standard chow diet or a high-fat (42% of calories fat derived) Western diet (TD.09682; Envigo-Teklad, Livermore, CA, USA) for 8 weeks to create diet-induced obesity (DIO) mice,[14] which exhibit exaggerated neuroinflammation after aseptic peripheral trauma.[14]

Surgical procedure

Aseptic tibial surgical trauma under isoflurane anaesthesia and buprenorphine analgesia was performed as reported.[16] Just before surgery, mice in the intervention groups received either i.p. PAP-1 (40 mg kg^{-1}), a dose that inhibits Kv1.3 channel signalling,[25] or the diluent MIGLYOL® (Spectrum Chemicals, Gardena, CA, USA) in a volume of 5 μl g^{-1} body weight (control). Dosing with PAP-1 or MIGLYOL was repeated at 12-hourly intervals until use (Fig. 1). PAP-1 was synthesised at the Department of Medical Pharmacology and Toxicology at the University of California, Davis (Davis, CA, USA) as described.[26]

Cognitive assessment

Trace fear conditioning (TFC) was used to assess hippocampus-dependent memory in rodents, as described.[16]

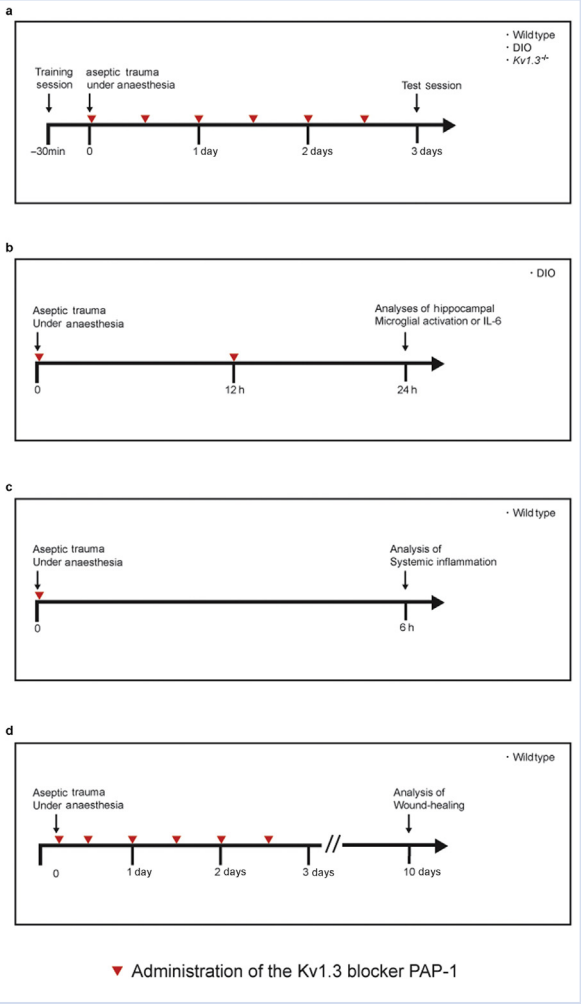

Fig 1. Timeline of studies. (a) Behavioural assessment: mice (wild-type, with diet-induced obesity [DIO] or Kv1.3 deficient [$Kv1.3^{-/-}$]) were trained in the trace fear-conditioning paradigm 30 min before induction of general anaesthesia with isoflurane. During general anaesthesia, surgery was performed on the tibia of the left hindlimb. Before surgical incision, wild-type and DIO mice received either i.p. phenoxyalkoxypsoralen-1 (PAP-1) 40 mg kg^{-1} or vehicle (MIGLYOL) repeated every 12 h thereafter. After 3 days, freezing behaviour was tested in the same context as the training was performed. (b) Hippocampal neuro-inflammation assessments: mice with DIO underwent tibial surgery during general anaesthesia. At the end of surgery, the mice received either PAP-1 or vehicle (MIGLYOL) i.p., and repeated 12 h later. At 24 h, the mice were killed and prepared for assessment of either hippocampal microglial activation by immunohistochemistry or hippocampal interleukin-6 (IL-6) by enzyme-linked immunosorbent assay. (c) Systemic inflammation assessment: wild-type mice underwent tibial fracture and internal fixation surgery during general anaesthesia. At the end of surgery, the mice received either PAP-1 or vehicle (MIGLYOL) i.p. At 6 h, the mice were killed and blood collected for analysis of plasma IL-6. (d) Wound-healing assessment: wild-type mice underwent tibial surgery during general anaesthesia. At the end of surgery, the mice received either PAP-1 or vehicle (MIGLYOL) i.p., and repeated every 12 h, thereafter until the third day. On the 10th day, the mice were killed and the left hindlimbs were removed for assessment of fracture callus.

Freezing behaviour, a manifestation of memory for the aversive event, was recorded and quantified by Video Freeze® software (Med Associates Inc., St Albans, VT, USA). Evaluation was performed by a reviewer (XF) blinded to experimental conditions.

Systemic inflammatory response

At 6 h after surgery, blood was drawn from the inferior vena cava of wild-type (WT) mice under terminal isoflurane anaesthesia in heparin-coated syringes. Plasma, collected after centrifugation of the blood at 2000× *g* for 20 min at 4°C, was stored at −80°C for later analysis. Because of the mediating role of IL-6 in the development of PCD,[16] the systemic inflammatory state was assessed by measuring concentrations of this pro-inflammatory cytokine using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's manual that lists the lower detection limits at 1.6 pg ml^{-1} (R&D Systems, Minnneapolis, MN, USA). Evaluation was performed by a reviewer (IKL) blinded to experimental conditions.

Hippocampal inflammatory response

The hippocampus, involved in context-sensitive memory in rodents,[27] was removed 24 h after surgery in DIO mice and homogenised in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethanesulphonyl fluoride (Cell Signaling Technology). Protein concentration was measured with a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Hippocampal IL-6 concentrations were assayed by ELISA as for plasma. Evaluation was performed by a reviewer (IKL) blinded to experimental conditions.

Microglial activation

At 24 h after surgery, DIO mice were systemically perfused with ice-cold saline followed by paraformaldehyde 4% in 100 mM phosphate buffer, and brains were removed and post-fixed in the same fixative overnight (4°C), and then dehydrated in sucrose 30% (w/v) for at least 48 h until completely submerged. The hippocampus was embedded in optimal cutting temperature compound, immediately frozen on dry ice, and stored (−80°C). Coronal sections (35 μm thick) from the dentate gyrus region were cut on a cryostat, mounted on glass slides using VECTASHIELD® antifade mounting media (Vector Labs, Burlingame, CA, USA), blocked for 1 h with bovine serum albumin 5% (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline containing Triton™ X-100 0.1% (v/v) (Fisher Scientific, Pittsburgh, PA, USA), and incubated with anti-ionised calcium binding adaptor molecule 1 (Iba1) antibody (1:1000 rabbit polyclonal; Wako Chemicals, Richmond, VA, USA) to detect activated microglia. Immunofluorescence was performed with Alexa Fluor® 488-labelled anti-rabbit (Fisher Scientific, South San Francisco, CA, USA). DAPI VECTASHIELD solution (Vector Labs) was used to identify cell nuclei.

Images were acquired using a confocal laser-scanning microscopy (Leica TCS SP5, Buffalo Grove, IL, USA) with 20× magnification. Confocal images were collected in z-stacks with a distance of 0.4 mm. Manual morphological analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The Iba1+ cells were counted

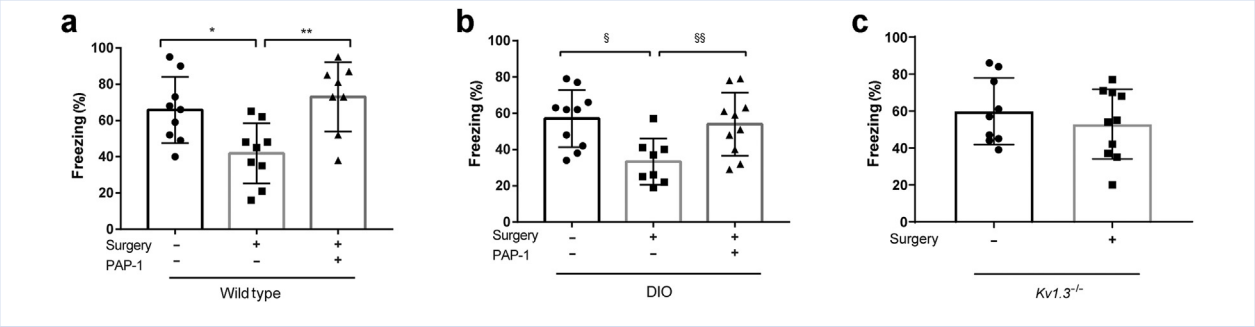

Fig 2. Role of Kv1.3 in postoperative cognitive decline in the trace fear-conditioning (TFC) paradigm. (a–c) Cohorts of mice were trained in a TFC paradigm immediately before surgery and tested for freezing behaviour 3 days after surgery. (a) Wild-type mice (12–14 weeks old C57bl/6j) were randomised to three groups (n=8–9 per group) that received no surgery/vehicle, surgery/vehicle, or surgery/phenoxyalkoxypsoralen-1 (PAP-1). (b) Wild-type mice with diet-induced obesity (DIO) were randomised to three groups (n=8–10 per group) that received no surgery+vehicle, surgery+vehicle, or surgery+PAP-1. (c) Mice deficient in Kv1.3 ($Kv1.3^{-/-}$) were randomised to two groups (n=9–10 per group) that received either sham (no surgery) or surgery. After wound closure and every 12 h thereafter, the mice randomised to PAP-1 received 40 mg kg^{-1} i.p. vehicle consisted of MIGLYOL in the same volume as PAP-1. On the third day, freezing behaviour was tested in the same context as the training. (a) and (b) Analysed by one-way analysis of variance followed by Bonferroni *post hoc* test. (c) Analysed by unpaired *t*-test. *P=0.029; **P=0.005; §P=0.011; §§P=0.030.

manually by visual inspection in hippocampal sections of anatomically matched photomicrographs, with an average taken from four sequential sections per mouse. To analyse microglial cell-body size, each section was processed in a systematic way to create a binary image by applying a previously established threshold. The Iba1 fluorescence intensity was similarly measured with ImageJ software from images captured using identical exposure times that also avoided saturating the pixel intensities. Evaluation was performed by a reviewer (MV) blinded to experimental conditions.

Wound healing

Assessment of tibia fracture healing 10 days after surgery was performed as described.[28] Sections of the callus were mounted on slides and stained using Hall–Brunt's quadruple stain to visualise bone tissue (red) and cartilage tissue (blue). Tissue volume and composition of the callus were evaluated using a stereology system (BX51 microscope and CAST software; Olympus Inc., Center Valley, PA, USA) and image analysis software (VIS version 6.10; Visiopharm A/S, Hoersholm, Denmark) according to established stereological methods.[29] Boundary outlines of the callus were acquired at 2× magnification, and bone and cartilage tissue were individually identified by standard histological staining patterns and morphology at 20× magnification. Composition measurements were calculated as each tissue volume divided by total fracture callus volume. Evaluation was performed by a reviewer (KM) blinded to experimental conditions.

Statistical analysis

Statistical analyses were performed using Prism versions 7.0 and 8.0 (GraphPad Software, San Diego, CA, USA) and data expressed as mean (standard deviation [SD]). Statistical comparisons were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test, unpaired *t*-test, or two-way ANOVA followed by Sidak's *post hoc* test. Statistical significance was set at $P<0.05$.

Results

PAP-1 treatment attenuates surgery-induced loss of fear-conditioned memory

Adult C57bl/6j (WT) mice that had experienced the relationship between a tone and foot shock in a classical TFC paradigm (Fig. 1a) were documented to have a robust freezing response in the training session that was unaffected by PAP-1. These mice subsequently underwent a stereotypical tibial fracture and 3 days later were assessed for their ability to recall prior fear conditioning when placed into the same TFC spatial context. Surgery induced a reduction in the percentage of time that mice exhibited freezing behaviour from 65.8 (18.3)% to 41.9 (16.6)% (one-way ANOVA F=7.12; P=0.004; *post hoc* P=0.029; Fig. 2a). Pretreating the mice with PAP-1 before surgery restored postoperative memory of prior TFC learning, as indicated by a freezing time percentage of 73.0 (19.1)% (*post hoc* P=0.005; Fig. 2a), levels akin to those seen in the non-surgical sham group. The PAP-1 treatment on its own had no impact on TFC memory when measured in the context of sham-operated mice, and it did not alter the ability of the mice to learn the TFC stimulus–response relationship during training (not shown). These data suggest that inhibition of microglial activation by Kv1.3 channel blockade is sufficient to prevent surgery-induced loss of fear-conditioned memory in WT mice.

We next tested the effectiveness of preoperative PAP-1 treatment in preserving postoperative TFC memory in mice with DIO, a model that has increased vulnerability to PCD in a TFC paradigm.[14] Mice with DIO that had previously learned the TFC relationship had a reduction in the percentage of time spent freezing 3 days after surgery (33.4 [12.7]%) when compared with sham-operated mice (57.1 [15.8]%; one-way ANOVA F=5.87; P=0.008; *post hoc* P=0.011; Fig. 2b). Pretreatment with PAP-1 was sufficient to restore postoperative freezing behaviour (54 [17.3]%; *post hoc* P=0.03; Fig. 2b) to levels similar to those seen in the control group, indicating that this treatment was effective in mitigating this early

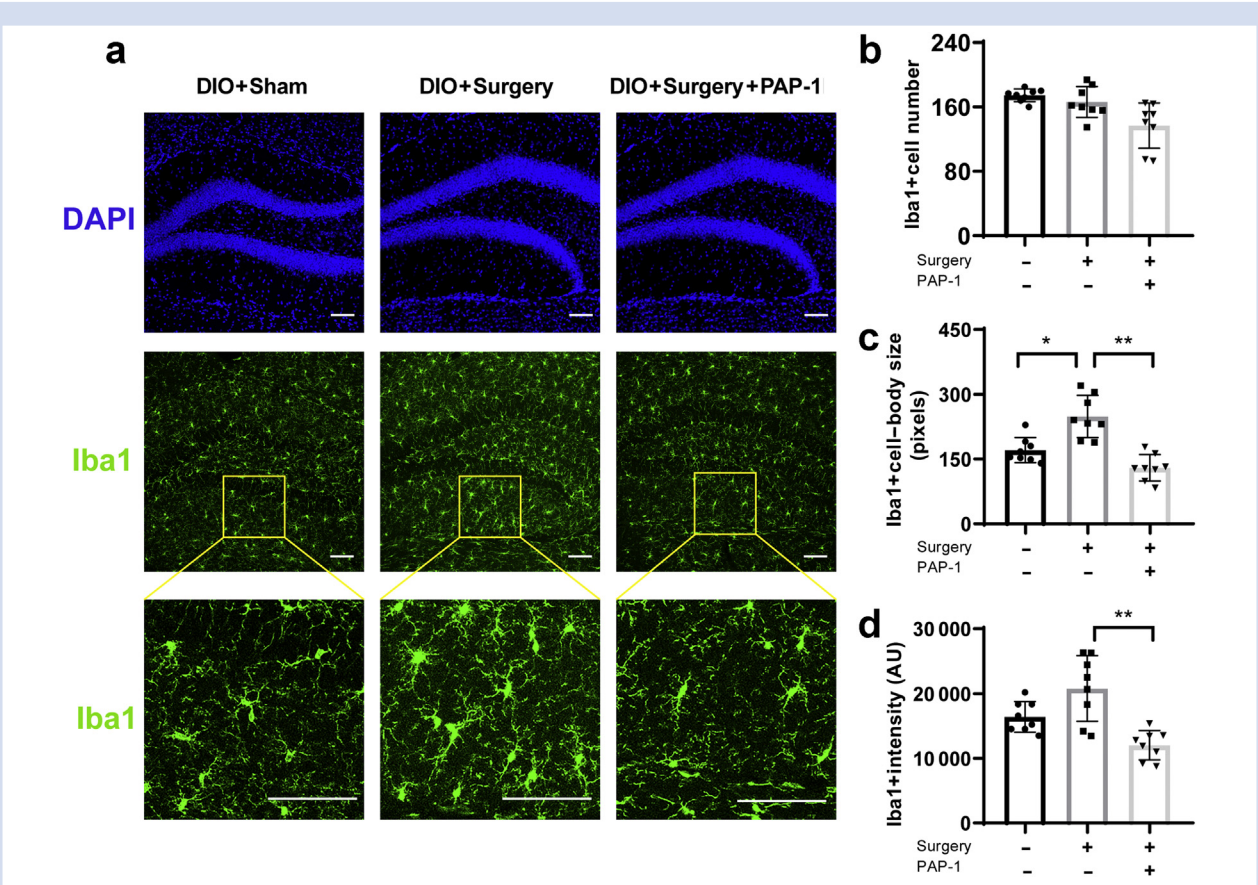

Fig 3. Phenoxyalkoxypsoralen-1 (PAP-1) attenuates surgery-induced microglial activation. Mice with diet-induced obesity (DIO) were randomised to three groups (n=8 per group) that received neither surgery nor PAP-1 (DIO+sham), surgery alone with no PAP-1 (DIO+surgery), or surgery together with PAP-1 (DIO+surgery+PAP-1). After surgery, mice not randomised to receive PAP-1 (DIO+sham; DIO+surgery) were administered MIGLYOL, and mice randomised to PAP-1 received doses intraoperatively and 12 h postoperatively. At 24 h after surgery, mice were killed, and brains were perfused, fixed, and sectioned. Coronal sections (35 μm thick) from the dentate gyrus were stained with anti-Iba1 antibody. Immunofluorescence was performed with Alexa Fluor 488-labelled anti-rabbit antibody. (a) Representative photomicrographs from each of the groups. The upper panel represents DAPI staining, the middle panel staining for Iba1, and the lower panel a higher magnification. The internal scale marker represents 50 μm. (b) Iba1+ cells were counted manually by visual inspection of hippocampal sections of anatomically matched photomicrographs, with an average taken from four sequential sections per mouse. (c) To analyse microglial cell-body size (in pixels), each section was processed in a systematic way to create a binary image by applying a previously established threshold. (d) Iba1 fluorescence intensity (absorbance unit [AU]) was similarly measured with ImageJ from images captured using identical exposure times that also avoided saturating pixel intensities. Data (mean [standard deviation]) were analysed by one-way analysis of variance followed by Bonferroni *post hoc* test. *P=0.0012; **P<0.001.

indicator of PCD even in a model with heightened susceptibility.

Kv1.3$^{-/-}$ mice retain TFC memory after surgery

We examined the possibility that results obtained in WT mice using PAP-1 treatment could have resulted from an off-target drug effect (e.g. on a channel other than Kv1.3 or on a non-channel target); we examined *Kv1.3*$^{-/-}$ mice that lack Kv1.3 attributable to deletion of the gene. As opposed to WT mice, *Kv1.3*$^{-/-}$ mice that had previously undergone TFC learning exhibited similar freezing behaviour whether they had undergone surgery (52.9 [18.9]%) or not (59.9 [18.1]%) (unpaired *t*-test *P*=0.422; Fig. 2c). Coupled with the findings obtained using PAP-1 treatment, these findings implicate Kv1.3 as an essential mediator of behaviours indicative of PCD-linked memory loss after peripheral surgery in mice.

PAP-1 treatment attenuates surgery-induced activation of hippocampal microglia in mice with diet-induced obesity

Clinical translation for the prevention of PCD would likely occur in a setting, in which PAP-1 is given coincident with surgery in patients at high risk for a perioperative neurocognitive disorder, such as those with the metabolic syndrome as modelled in mice with DIO.[5] Prevention of fear-conditioned memory loss by PAP-1 treatment was associated with a reduction in surgery-induced activation of hippocampal microglia 24 h post-surgery (Fig. 3a). The absolute number of microglia (mean [SD]), marked by their expression of Iba1, in hippocampal sections from mice with DIO was similar whether assessed under basal conditions (175 [8]), after surgery (166 [19]), or in the context of surgery combined with PAP-1 pretreatment (137 [28]) (Fig. 3b). In contrast, microglial size, as quantified by pixel number, was larger in the post-

surgical group (249 [49] pixels) than in the non-surgical group (170 [29] pixels) (one-way ANOVA $F=20.88$; $P<0.001$; *post hoc* $P=0.0012$; Fig. 3c). Treatment of mice with PAP-1 was sufficient to prevent enlargement of hippocampal microglia otherwise induced by surgery (130 [31] pixels) (*post hoc* $P<0.001$; Fig. 3c). As increasing size is an indicator of microglial activation, these data suggest that PAP-1 treatment prevents hippocampal microglial activation after tibial fracture in vulnerable mice with DIO. Along with microglial size, the intensity of Iba1 staining is also a histological indicator of inflammatory activation. The Iba1 staining intensity within the hippocampus (mean [SD]; absorbance unit [AU]) was not significantly greater after surgery (20 800 [5080] AU) than in the non-surgical control group (16 400 [2340] AU) (one-way ANOVA $F=12.66$; $P<0.001$; *post hoc* $P=0.06$; Fig. 3d). Pretreating mice with PAP-1 decreased the staining intensity of microglia in the hippocampus (12 000 [2260] AU; *post hoc* $P<0.001$). These data support the concept that microglia require activation of Kv1.3 channels to undergo inflammatory activation after surgery.

PAP-1 treatment attenuates surgery-induced hippocampal inflammation in mice with diet-induced obesity

Hippocampal IL-6 is a pivotal factor in the development of surgery-induced PCD in mice.[16] Given this, we measured hippocampal IL-6 by ELISA as a marker of neuroinflammation 24 h post-surgery. Focusing on the vulnerable DIO mouse model, we found that surgery upregulated concentrations of IL-6 (mean [SD]) within the hippocampus, from a control value of 10.9 (2.2) to 25.6 (10.4) pg mg^{-1} (one-way ANOVA $F=9.592$; $P<0.001$; *post hoc* $P<0.001$; Fig. 4). However, PAP-1 treatment prevented upregulation of hippocampal IL-6 concentrations (14.9 [5.7] pg mg^{-1}), which were no different from those seen in non-surgical control mice and sharply reduced with respect to untreated mice that underwent surgery (*post hoc* $P=0.011$; Fig. 4). Thus, PAP-1 treatment prevents surgery-associated hippocampal neuroinflammation.

PAP-1 treatment does not prevent surgery-induced peripheral inflammation in WT mice

To assess the extent to which PAP-1 treatment might attenuate systemic inflammation in the context of tibial fracture and surgical repair, we measured circulating concentrations of IL-6 in plasma samples from mice that had undergone either surgery or a non-surgical sham control procedure 6 h previously. Whereas surgery increased plasma IL-6 concentrations (mean [SD]) *vs* control (one-way ANOVA $F=127.1$; $P<0.001$; *post hoc* $P<0.001$), these concentrations were not affected by PAP-1 treatment, either under control conditions or before surgery. Plasma IL-6 concentrations after surgery were 210 (23) pg ml^{-1} in mice receiving only vehicle prior, and 210 (23) pg ml^{-1} in mice receiving PAP-1 before surgery. This suggests that PAP-1 treatment does not alter the systemic inflammatory response to surgery, and supports the concept that PAP-1 is unlikely to exert its effects on memory as an indirect consequence of an action on cell types located either in the circulation or peripheral tissues (Fig. 5).

PAP-1 treatment does not impair wound healing after aseptic trauma in WT mice

In WT mice, in which surgery-induced upregulation of peripheral inflammation was not attenuated, PAP-1 did not alter wound healing (Fig. 6a and b). Bone and cartilage comprising the calluses of treated and control animals were stereologically quantified throughout the entire fracture callus. No significant differences in either the volumes (Fig. 6c) or proportions (Fig. 6d) of bone and cartilage were observed in treated and control animals.

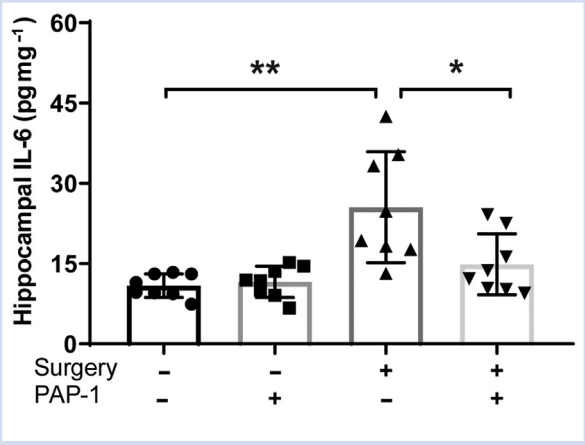

Fig 4. Phenoxyalkoxypsoralen-1 (PAP-1) prevents surgery-induced hippocampal inflammation. Mice with diet-induced obesity were randomised to four groups ($n=8$ per group) that received either no surgery or surgery, and either PAP-1 or vehicle. At 24 h after surgery, the animals were killed and hippocampi were removed and assayed for interleukin-6 (IL-6) by enzyme-linked immunosorbent assay. Data were analysed by one-way analysis of variance followed by Bonferroni *post hoc* test. *$P<0.001$; **$P=0.011$.

Discussion

Our findings suggest that attenuating surgery-induced microglial activation with the Kv1.3 blocker PAP-1 prevents postoperative cognitive dysfunction and hippocampal inflammation without disrupting wound healing.

Microglial activation and Kv1.3 potassium channels

We recently reported that microglial activation is a pivotal step in the pathway to neuroinflammation that is required for the development of PCD.[14] It is pertinent that lipopolysaccharide-induced microglial activation, which also causes cognitive decline,[30] has been shown to require participation of microglial voltage-gated Kv1.3 potassium channels.[20] The Kv1.3 ion channel both (i) sets the resting membrane potential for tissue macrophages, including microglia[31]; and (ii) is required for activation to the pro-inflammatory state.[20] Disabling Kv1.3 prevents activation of downstream effectors of the inflammatory response, including pro-inflammatory cytokine production in the context of inflammatory stimuli.[32] Microglia in the brains of $Kv1.3^{-/-}$ mice fail to activate after intracerebroventricular injection of lipopolysaccharide, confirming that Kv1.3 is important for microglial activation and downstream pro-inflammatory mediator expression and production both *in vitro* and *in vivo*.[20,32]

Kv1.3 in immune responses

Kv1.3 was first described in human T-cells,[33] where it plays an important role in regulating membrane potential and Ca^{2+} signalling by providing counterbalancing K^+ efflux for the Ca^{2+} influx necessary for T-cell activation.[34] Other immune cell types that express Kv1.3 under specific conditions include B cells, macrophages, and dendritic cells.[34] It is notable that chemotaxis of monocytes across a model of the blood–brain barrier is enhanced by activation of Kv1.3. Therefore, it is not clear whether the beneficial effects of PAP-1 on PCD and neuroinflammation are attributable to its effects on BM-DMs, microglia, or on another pool of immunocytes. However, the fact that PAP-1 prevented hippocampal microglia from adopting a pro-inflammatory morphology and reduced hippocampal concentrations of IL-6 after surgery without altering the postoperative increase in circulating IL-6 concentrations supports the concept that at least some of the beneficial effects of PAP-1 treatment in this context are attributable to a specific effect on microglial function. Our data align with others, indicating that blocking Kv1.3 prevents brain injury initiated by activation of innate immune cells.[35]

Kv1.3 blockade in models of disease

Kv1.3 inhibitors are classed as immunomodulators rather than immunosuppressants[36] based on the observation that Kv1.3 blockers do not affect the ability of rodents to clear influenza and *Chlamydia* infections or of primates to respond to vaccination,[37] although interferon-γ and IL-17 production by effector memory T-cells are both reduced.[38] PAP-1, although potently able to prevent inflammatory activation of hippocampal microglia and neuro-inflammatory changes more broadly (e.g. increased concentrations of hippocampal IL-6), did not suppress the peripheral inflammatory responses after surgery. A key aspect of the immunomodulatory effects of Kv1.3 blockade may be a preference for counteracting CNS inflammation; however, such anatomical specificity will require further studies with a blood–brain barrier impenetrant blocker of Kv1.3.[23]

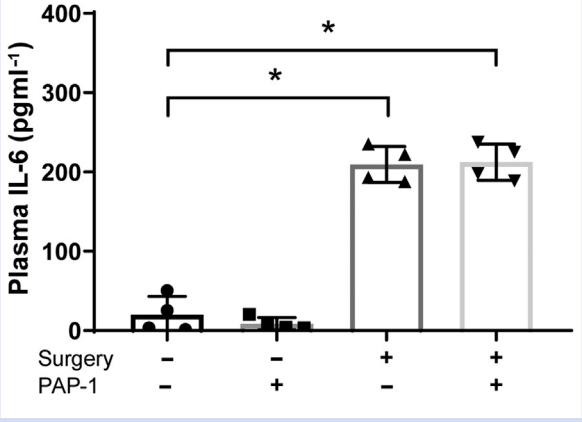

Fig 5. Phenoxyalkoxypsoralen-1 (PAP-1) does not affect surgery-induced peripheral inflammation. Wild-type mice were randomised to four groups (n=4 per group) that received either no surgery+vehicle, no surgery+PAP-1, surgery+vehicle, or surgery+PAP-1. At 6 h after surgery, the animals were killed and blood removed for measurement of plasma interleukin-6 (IL-6) by enzyme-linked immunosorbent assay. Data were analysed by one-way analysis of variance followed by Bonferroni *post hoc* test. *$P<0.001$.

Increased microglial Kv1.3 expression has been observed in both humans and animal models of ischaemic stroke[39] and Alzheimer's disease,[40] suggesting that targeting Kv1.3 may be an effective strategy for reducing neuroinflammation in the context of a variety of acute and chronic CNS insults. The therapeutic benefit of Kv1.3 blockade, using the brain-penetrant small-molecule PAP-1,[26] was tested in a rodent model of ischaemic stroke and in which it reduced microglial activation and infarct size and improved neurological deficit.[39] Similarly, PAP-1 treatment reduced neuroinflammation, decreased cerebral amyloid load, enhanced hippocampal neuronal plasticity, and improved behavioural deficits in mouse models of Alzheimer's disease.[40] Kv1.3 inhibitors have also been shown to ameliorate disease severity in rodent models of multiple sclerosis,[41] prevent autoimmune diabetes mellitus and rheumatoid arthritis,[38] and suppress inflammatory skin diseases such as contact dermatitis and psoriasis in animal models.[42] The peptidic Kv1.3 blocker dalazatide was shown to reduce the severity of plaque psoriasis in a small Phase-Ib study.[23] Possibly, the specific T-cell subsets involved in these autoimmune conditions express Kv1.3 in a manner that makes them amenable to treatment with a specific channel blocker. However, such peripheral immune cell populations are unlikely to have a significant role in the acute response to peripheral surgery as PAP-1 treatment did not appear to reduce peripheral inflammation or impact wound healing in this model.

Peripheral inflammation and wound healing

An influx of circulating immunocytes, attracted by upregulation of pro-inflammatory cytokines in the fracture haematoma,[43] initiate orderly bone healing. A number of pro-inflammatory cytokines, including IL-6, are secreted at the local site in the early inflammatory phase after fracture. IL-6 serves not only to maintain bone homeostasis by mediating both bone formation via osteoblasts and bone resorption via osteoclasts,[44] but also fracture healing through a biphasic repair process involving early osteoclastogenesis[45] and late bone formation in the fracture callus.[46] Global inhibition of pro-inflammatory cytokines adversely impacts fracture healing in the same manner as anti-inflammatory drugs, such as cyclooxygenase-2 inhibitors.[47]

An intriguing finding is that the elevation of IL-6 concentrations in the circulation induced by surgery was not diminished by PAP-1. Indeed, wound healing by morphometric assessment of cellular components and fracture callus formation was grossly intact in the face of Kv1.3 blockade. Thus, Kv1.3 blockade may be able to avoid at least one key obstacle, namely, altered wound healing, that might otherwise thwart therapeutic development of neurocognitive protective strategies in the setting of surgery.

Limitations

Some of the studies presented here were performed using WT mice fed a standard chow diet (Fig. 2a), whereas other studies (Figs 2b, 3, and 4) involved mice with DIO, which are vulnerable to PCD. We decided not to use mice with DIO in the studies that determined the effect of PAP-1 on peripheral inflammation and wound healing because fracture healing is already impaired in diabetes mellitus.[48]

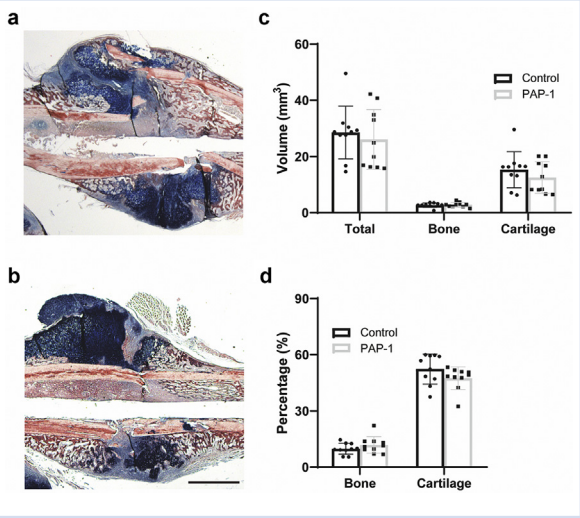

Fig 6. Phenoxyalkoxypsoralen-1 (PAP-1) does not affect fracture healing. Wild-type mice were randomised to two groups (n=10 per group) that received either PAP-1 or vehicle (control) administered intraoperatively and at 12 h intervals for 3 days. At 10 days after surgery, the mice were killed and the left tibiae were removed for stereological evaluation of fracture healing. (a) Vehicle control and (b) PAP-1 treated are representative histological images of fracture callus stained with Hall–Brunt's quadruple stain that illustrates bone tissue (red) and cartilage tissue (blue). The scale bar is 1 mm. (c) Volume of bone and cartilage tissue in the total fracture callus volume. (d) Percentage composition of bone and cartilage tissue in the fracture callus. Data were analysed by two-way analysis of variance followed by Sidak's *post hoc* test.

The inflammatory milieu that affects bone healing is in the haematoma surrounding the fracture site. In lieu of sampling the haematoma, we sampled cytokines in the peripheral circulation, which may not reflect the site of interest. Furthermore, we chose to focus on the cytokine IL-6 as a marker of systemic and neuroinflammation because our previous studies showed the relevance of this pro-inflammatory cytokine for PCD[16] and because this cytokine promotes fracture healing.[45]

In a series of patch-clamp electrophysiological experiments on a cell line that expresses Kv1.3 channels, isoflurane directly affected conductance through the Kv1.3 channels in a biphasic manner (potentiation at sub-anaesthetic and inhibition at supra-anaesthetic concentrations).[49] Therefore, it is possible that the ameliorative effect of PAP-1 on PCD may be attributable to its interaction with isoflurane on Kv1.3 channel gating kinetics. However, general anaesthetics do not influence the development of postoperative cognitive dysfunction in clinical studies.[50]

The mice were exposed to PAP-1 at the initiation of aseptic peripheral trauma; whether this perturbant can still be effective if administered *after* surgery remains unknown.

Finally, relatively young male mice were used throughout, whereas perioperative neurocognitive disorders tend to occur in older surgical patients of both sexes. We believe that the age-related criticism is largely rebutted by our use of mice with DIO, in which all the precocious ageing features of diabetes mellitus are present.[51] Confirmation in other species, females, and other models of PCD are required to demonstrate the overall effectiveness of Kv1.3 blockade.

Conclusions

Targeting the Kv1.3 channel represents a possible strategy to prevent development of perioperative neurocognitive disorders without affecting surgical wound healing. Additional studies are required to further probe the molecular mechanisms underlying this effect and to determine the feasibility of PAP-1 for clinical translation of these preclinical findings.

Authors' contributions

Study conception/design: JP, HW, MM, SK, RM
Data acquisition/analysis: IKL, MV, KM, XF, SS, RL, YU, AL, WL
Drafting figures: IKL, MV, KM, XF
Drafting of manuscript: JP, HW, MM

Declarations of interest

MM serves on the Board of Directors of the Foundation for Anesthesia Education and Research. HW is an inventor of a 2005 University of California-issued patent claiming phenoxyalkoxypsoralen-1 and related Kv1.3 blockers as immunosuppressants. The patent was licensed to Airmid, which is no longer an operating company. Patent rights were returned to the University of California in 2017. The international filings have been abandoned by the University of California. No other author has pertinent interests.

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