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Brain-Defective Insulin Signaling Is Associated to Late Cognitive Impairment in Post-Septic Mice

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Abstract Sepsis survivors frequently develop late cognitive impairment. Because little is known on the mechanisms of post-septic memory deficits, there are no current effective approaches to prevent or treat such symptoms. Here, we subjected mice to severe sepsis induced by cecal ligation and puncture (CLP) and evaluated the sepsis-surviving animals in the open field, novel object recognition (NOR), and step-down inhibitory avoidance (IA) task at different times after surgery. Post-septic mice (30 days post-surgery) failed in the NOR and IA tests but exhibited normal performance when re-evaluated 45 days after surgery. Cognitive impairment in post-septic mice was accompanied by reduced hippocampal levels of proteins involved in synaptic plasticity, including synaptophysin, cAMP response element-binding protein (CREB), CREB phosphorylated at serine residue 133 (CREBpSer^{133}), and GluA1 phosphorylated at serine residue 845 (GluA1pSer^{845}). Expression of tumor necrosis factor α (TNF-α) was increased and brain insulin signaling was disrupted, as indicated by increased hippocampal IRS-1 phosphorylation at serine 636 (IRS-1pSer^{636}) and decreased phosphorylation of IRS-1 at tyrosine 465 (IRS-1pTyr^{465}), in the hippocampus 30 days after CLP. Phosphorylation of Akt at serine 473 (AktpSer^{473}) and of GSK3 at serine 9 (GSK3βpSer^{9}) were also decreased in hippocampi of post-septic animals, further indicating that brain insulin signaling is disrupted by sepsis. We then treated post-septic mice with liraglutide, a GLP-1 receptor agonist with insulinotropic activity, or TDZD-8, a GSK3β inhibitor, which rescued NOR memory. In conclusion, these results establish that hippocampal inflammation and disrupted insulin signaling are induced by sepsis and are linked to late memory impairment in sepsis survivors.

Keywords Sepsis survivors · Cognitive impairment · Insulin receptor substrate-1 (IRS-1) inhibition · Glycogen synthase kinase 3 β (GSK3β) · Liraglutide · TDZD-8 · Late cognitive impairment

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Introduction

Sepsis is defined as a state of high-grade systemic inflammation caused by infection. One of its major symptoms is sepsis-associated encephalopathy (SAE), a form of acute brain dysfunction [1, 2] which initially manifests with confusion, progressing

from mild delirium to coma [1-3]. Sepsis survivors frequently present late cognitive impairment, characterized by deficits in memory and executive functions [4, 5]. Significantly, many patients never fully recover from such deficits [4, 6, 7]. Little is known on the mechanisms underlying long-lasting cognitive impairment after critical illness [1, 5], hampering the development of effective ways to prevent or treat such conditions.

Global cognition scores in sepsis survivors are comparable to scores for patients with mild Alzheimer's disease (AD) [3, 5], and it has been suggested that sepsis survivors present dementia with particular involvement of the frontal lobe and hippocampus [8]. Disrupted brain insulin signaling is a feature of AD and other tauopathies and has been implicated in cognitive deficits in those disorders [9–15]. Because sepsis is associated with severe deregulation of glucose homeostasis and peripheral insulin resistance [16], we hypothesized that inhibition of brain insulin signaling might be associated with cognitive impairment observed in sepsis survivors. Previous studies from our group showed that activation of tumor necrosis factor α (TNF-α)/c-Jun N-terminal kinase (JNK) signaling triggers inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1), a key protein in insulin signaling, centrally implicated in peripheral insulin resistance in diabetes and in brain insulin resistance in dementia [9, 13, 17].

Here, we show that mice surviving sepsis induced by cecal ligation and puncture (CLP) presented cognitive deficits in the novel object recognition (NOR) and step-down inhibitory avoidance (IA) tests, up-regulated hippocampal expression of TNF-α, decreased levels and phosphorylation of cAMP response element-binding protein (CREB), and reduced synaptophysin levels. Of note, CLP also induced hippocampal IRS-1 inhibition, and systemic treatment with an anti-diabetes agent (liraglutide) rescued cognitive function in sepsis-surviving mice. We also demonstrate that hippocampi of sepsis-surviving mice showed aberrant activation of glycogen synthase kinase 3 (GSK3β), a serine kinase that is negatively regulated by insulin signaling, and treatment with the GSK3β inhibitor improved memory performance. Collectively, our results provide the grounds for targeting brain insulin signaling as a potential disease-modifying therapy for late cognitive impairment in sepsis survivors.

Methods

Animals

Two-month-old male Swiss mice, weighing between 25 and 30 g, were used. Animals were housed in groups of five per cage and had free access to food and water, under a 12-h light/dark cycle in a room with controlled temperature and humidity. All procedures followed the Principles of Laboratory Animal Care from the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol no. IBqM 054/2012).

Cecal Ligation and Puncture

Animals were subjected to cecal ligation and puncture (CLP) as previously described [18]. Briefly, mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. Under aseptic conditions, a 2-cm midline laparotomy was performed to allow exposure of the cecum and the adjoining intestine. The cecum was ligated with a 4.0-silk suture at its base, below the ileocecal valve, and was perforated two times with an 18-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites, returned to the peritoneal cavity, and the laparotomy was closed with a surgical 9-mm clip. CLP and sham-operated animals received subcutaneous injections of sterile isotonic saline (50 mL/kg) immediately and 12 h after surgery, as well as antibiotic therapy. In the sham-operated (control) group, mice were subjected to all surgical procedures, but the cecum was neither ligated nor perforated. Following sepsis induction, the animals were individually assessed in terms of weight, activity in the homecage, general appearance, behavior, and altered breathing 1, 4, 24, 48 h and 1 week post-surgery. Survival rates were 100% in the sham-operated group and 45% in the sepsis group. All sepsis surviving animals were included in the study.

Drug Treatment

All drugs were prepared fresh on the treatment day and given intraperitoneally (i.p.) or subcutaneously (s.c.) in a final injection volume of 0.1 mL for every 10 g of body weight. After surgery, sham and CLP animals were treated with antibiotics (30 mg/kg ceftriaxone, 25 mg/kg clindamycin, s.c.) every 12 h for 3 days. Treatment with liraglutide (Bachem; 50 nmol/kg/day, s.c., during 10 days) or TDZD-8 (Sigma Aldrich; 5 mg/kg/day, i.p., during 5 days) began 20 and 25 days after CLP, respectively.

Behavioral Experiments

Open-field test: The test was performed in an arena measuring 30 cm × 30 cm surrounded by 50-cm high walls. The floor of the arena was divided into nine squares by lines. During each trial (lasting 300 s), the number of lines crossed on the floor of the arena (number of crossings) and the number of rearings (elevation on rear paws, denoting exploratory behavior) were recorded to determine locomotor/exploratory behavior. Animals were evaluated in the open-field task 10, 15, 25, 30, and 45 days after surgery.

Novel object recognition test (NOR): Thirty days after surgery, when no differences in locomotor and exploratory

behaviors were found between the CLP and sham groups in the open-field test, the animals were assessed in the novel object recognition (NOR) task. Training and test sessions were performed in an arena measuring 30 × 30 × 45 cm. Test objects were made of glass or plastic and had different shapes, colors, sizes, and textures. During sessions, objects were fixed to the box using tape to prevent displacement caused by exploratory activity of the animals. Preliminary tests showed that none of the objects used in our experiments evoked innate preference. Before training, each animal was submitted to a 5-min long habituation session, in which they were allowed to freely explore the empty arena. Training consisted of a 5-min long session during which animals were placed at the center of the arena in the presence of two identical objects. The time spent exploring each object was recorded by a trained researcher. Sniffing and touching the object were considered exploratory behavior. The arena and objects were cleaned thoroughly between trials with 20% ethanol to eliminate olfactory cues. Two hours after training, the animals were again placed in the arena for the test session, when one of the two objects used in the training session was replaced by a new one. Again, the amount of time spent exploring familiar and novel objects was measured. Results were expressed as percentage of time exploring each object during the training or test session and were analyzed using a one-sample Student's *t* test comparing the mean exploration time for each object to the fixed value of 50%. By definition, animals that recognize the familiar object as such (i.e., learn the task) explore the novel object >50% of the total time [13].

Inhibitory avoidance task: For training, mice were placed on a platform measuring 10 cm × 7 cm × 2 cm placed in the center of an inhibitory avoidance box (Insight, São Paulo, Brazil). The box measured 38 cm × 25 cm × 30 cm, and its floor was made of conductive metal grids, spared 1-cm apart, and connected to a power source. When mice stepped down the platform with four paws, they received a 0.7 mA footshock for 2 s. The box and platform were thoroughly cleaned between trials with alcohol 20% to eliminate olfactory cues. Twenty-four hours after the training session, mice were again placed on the platform and time to step down the platform with four paws was recorded. A ceiling time of 300 s was used on test session.

Western immunoblot: Thirty days after surgery, mice were euthanized by decapitation and their hippocampi and frontal cortex were rapidly dissected and frozen in liquid nitrogen. For total protein extraction, the samples were thawed and homogenized in buffer containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40 (Invitrogen), 1% sodium deoxicholate, 0.1% SDS, 5 mM EDTA, 1% Triton X-100, and phosphatase and protease inhibitor cocktails (Thermo Scientific Pierce). Protein concentrations were determined using the BCA kit (Thermo Scientific). Samples containing 30 μg protein were resolved in 4–20% polyacrylamide Tris–glycine gels (Invitrogen) and blotted to nitrocellulose membranes at 300 mA for 1 h. Blots were incubated with 5% nonfat milk in Tween-TBS at room temperature for 2 h and incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. Primary antibodies used were anti-IRS-1pSer^{636} (1:200), anti–IRS-1pTyr^{465} (1:200), anti-IRS-1 (1:500), anti-PSD-95 (1:2000), and anti-tau pSer^{396} (1:2000) (all from Santa Cruz Biotecnology); anti-GSK3βpSer^{9} (1:1000), anti-GSK3β (1:1000), anti-AktpSer^{473} (1:1000), anti-Akt (1:1000), anti-CREBpSer^{133} (1:200), anti-CREB (1:500), anti-JNKpT^{183}/Y^{185} (1:400), and anti-JNK (1:500) (all from Cell Signaling); anti-synaptophysin (1:2000), anti-tau (1:1000), and anti-β-tubulin (1:5000) (all from Abcam); anti-GluA1pSer^{845} (1:300, Invitrogen) and anti-GluA1 (1:500, Millipore). After overnight incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10–50,000; Invitrogen) and IRDye800CW- or IRDye680RD-conjugated secondary antibodies (LI-COR; 1:10,000) at room temperature for 2 h. Chemilumescence was developed using SuperSignal West Femto (Thermo Fisher Scientific). Alternatively, fluorescence intensities were quantified in an Odissey CLx apparatus (LI-COR).

RNA extraction and quantitative real-time PCR analysis: Hippocampal tissue was homogenized in 1 mL Trizol (Invitrogen) and RNA extraction was performed according to the manufacturer's instructions. Purity and integrity of RNA were determined by the 260/280 nm absorbance ratio and by agarose gel electrophoresis. Only preparations with ratios >1.8 and no signs of RNA degradation were used. One microgram of RNA was used for cDNA synthesis using the Super-Strand III Reverse Transcriptase kit (Invitrogen). Expression of genes of interest was analyzed by qPCR on an Applied Biosystems 7500 RT-PCR system using the Power SYBR kit (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was used as endogenous controls. TNF-α and CREB were amplified using the following primers: for TNF-α, forward: 5′-CCCT CACACTCAGATCATCTTCT-3′, reverse: 5′-GCTA CGACGTGGGCTACAG-3′; for CREB, forward: 5′-CCAG CCATCAGTTATCCAGTCTC-3′, reverse: 5′-GAAT CAGTTACACTATCCACAGACTCCT-3′. Cycle threshold (Ct) values were used to calculate fold changes in gene expression using the 2^{-DCt} method. In all cases, reactions were performed in 15 μl reaction volumes.

Data analysis: Data from the open-field task and molecular analyses are expressed as means ± SEM, and statistical significances were determined by paired samples Student's *t* test, one-way ANOVA followed by Bonferroni, or Mann-Whitney, as indicated in figure legends. Results from the NOR test are expressed as percentage of time spent exploring each object (novel or familiar) during the training or test session and were analyzed using a one-sample *t* test comparing the mean

exploration time for each object with the fixed value of 50%. Animals that recognize the familiar object as such (i.e., learn the task) explore the novel object >50% of the total time.

Results

Sepsis Survivors Show Late Aversive and Recognition Cognitive Impairment

We initially examined locomotor and exploratory behaviors in post-septic mice by testing them in open-field sessions at different time points after CLP. Locomotor and exploratory behaviors were impaired for up to 25 days after CLP and were fully recovered after 30 days (Fig. 1a, b). Thirty days after CLP, when animals had fully recovered from sepsis-induced locomotor deficit, they were tested in the NOR paradigm. Sepsis-surviving mice failed in the NOR test when evaluated late after the acute clinical signals of sepsis (30 days after surgery) (Fig. 1c). Sepsis survivors recovered the ability to learn the NOR task when retrained 45 days after surgery (Fig. 1d). As expected, sham-operated mice had no locomotor and exploratory deficits and learned the NOR task at both 30 and 45 days post-surgery (Fig. 1c, d). In addition, when evaluated in an aversive and hippocampal-dependent task, post-septic mice also showed late long-term memory impairment (Fig. 1e).

Cognitive Impairment in Post-Septic Mice Is Accompanied by Synapse Damage

We next investigated markers of synapse structure and function in the hippocampus of sepsis survivors. We found that mRNA (Fig. 2a) and protein (Fig. 2b) levels of CREB, a transcription factor that plays key roles in synaptic plasticity, learning, and memory [19], were markedly decreased in sepsis-surviving mice 30 days after CLP. Further, levels of CREB phosphorylated at Ser^{133} were reduced in the hippocampus of post-septic mice (Fig. 2c). We also analyzed levels of synaptic markers in sepsis-surviving mice 30 days after surgery. We found CLP induced a decrease in hippocampal levels of synaptophysin, a pre-synaptic marker, but had no effect on levels of PSD-95, a post-synaptic scaffolding protein (Fig. 2d, e). AMPA receptors (AMPARs) are central to glutamatergic synaptic plasticity in the hippocampus [20], and trafficking of AMPARs into post-synaptic densities is dependent on phosphorylation of the GluA1 subunit [21]. Compared to control mice, GluA1pSer^{845} levels were significantly decreased in the hippocampus of sepsis survivors (Fig. 2f). Results suggest that hippocampal synapse integrity and

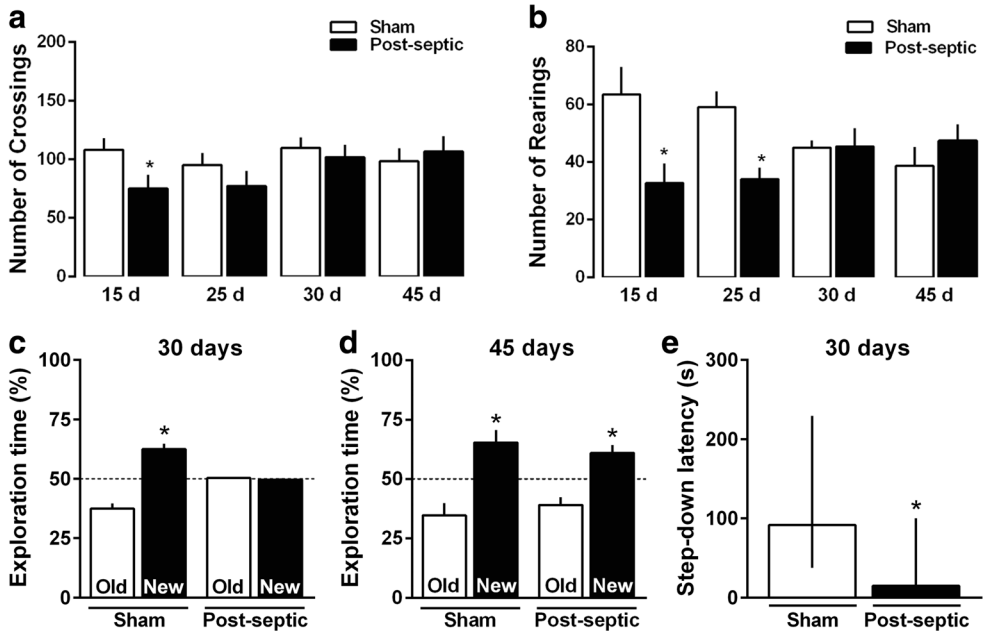

Fig. 1 Post-septic mice show impaired aversive and recognition memory. Two-month-old male Swiss mice were subjected to cecal ligation and puncture (post-septic) or were sham operated (Sham). Animals were tested in an open-field arena 15, 25, 30, and 45 days after surgery and the numbers of crossings (**a**) and rearings (**b**) were measured. At days 30 (**c**) and 45 (**d**) post-sepsis, mice were trained and tested in the novel object recognition (NOR) task. The percentages of time spent exploring the familiar (old) or the novel (new) objects in the test session were measured. **e** A different group of mice was trained in the step-down inhibitory avoidance task and, 24 h after, step-down latencies were recorded in a test session. In **a**–**d**, bars represent means ± S.E.M. $*p < 0.05$, one-sample Student's t test. In **e**, bars represent median ± interquartile range, $*p < 0.05$ in Mann-Whitney test (n = 10 mice/group)

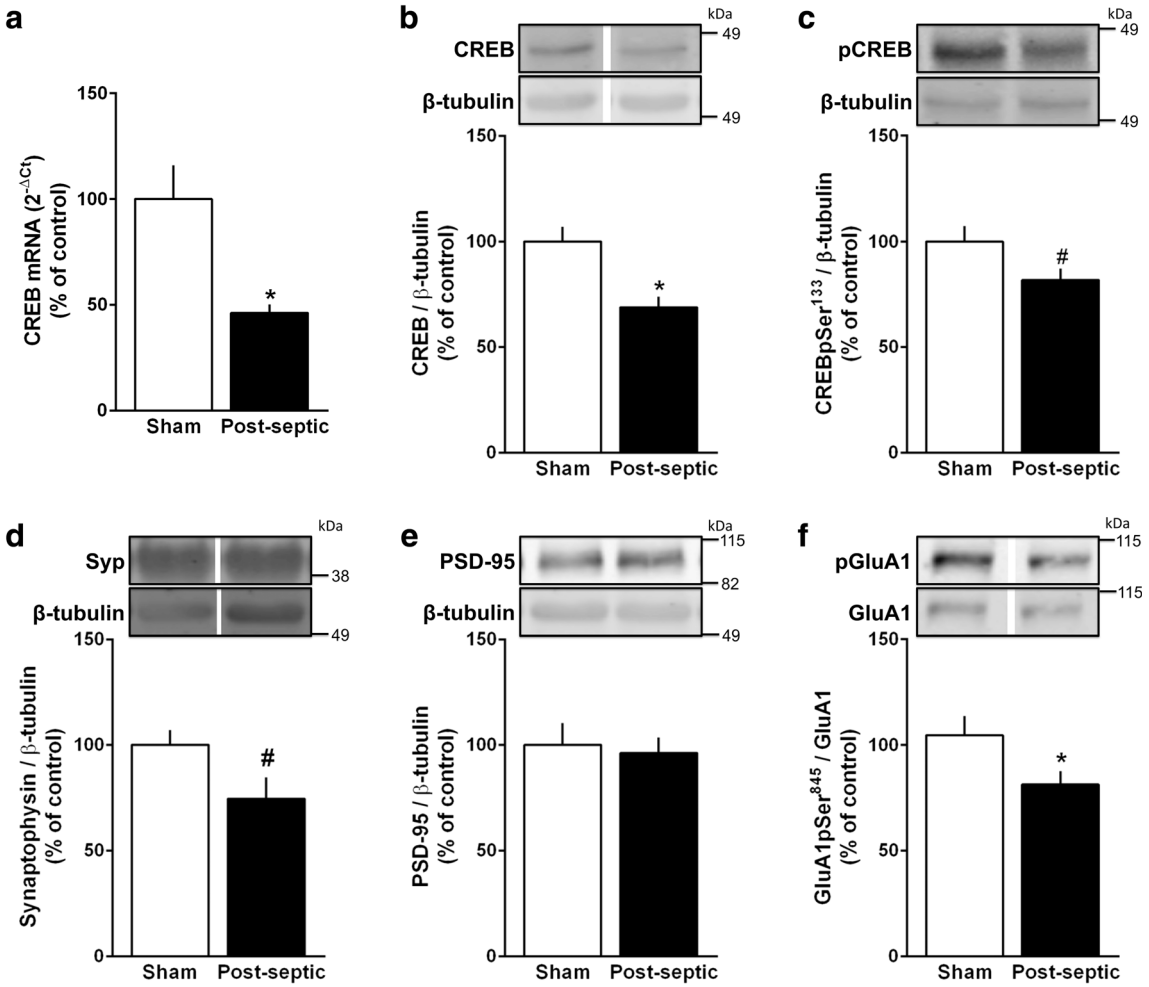

Fig. 2 Cognitive impairment is accompanied by synapse damage in post-septic mice. Two-month-old male Swiss mice were subjected to cecal ligation and puncture (post-septic) or were sham operated (Sham). qPCR or Western blot analysis were performed in hippocampal extracts 30 days after CLP. **a** CREB mRNA levels analyzed by qPCR; **b** total CREB; **c** pCREB ($pSer^{133}$); **d** synaptophysin (SYP); **e** PSD-95; **f** $GluA1pSer^{845}$ normalized by total GluA1. Graphs show densitometric data normalized by β-tubulin (**e**–**e**). Bars represent means ± S.E.M.; $*p < 0.05$; $\#p = 0.0850$ (**c**) or $p = 0.0816$ (**d**); Student's *t* test (n = 5–10 animals/group). Lanes were run in the same gel but were non-contiguous

function are impaired in sepsis-surviving mice, which could contribute to cognitive impairment.

Sepsis-Surviving Mice Show Hippocampal Inflammation and Impaired Insulin Signaling

Because the hippocampus plays a major role in memory processing and TNF-α is a key inflammatory mediator implicated in hippocampus-dependent memory deficits in dementia [9, 13], we evaluated hippocampal expression of TNF-α 30 days after CLP. TNF-α mRNA levels were significantly increased in the hippocampus of sepsis-surviving animals (Fig. 3a), showing that local production of TNF-α is up-regulated in post-septic brains late after the acute clinical signals of sepsis. Increased TNF-α levels can lead to activation of stress kinases such as JNK and IKK, which in turn cause serine phosphorylation and inhibition of IRS-1 [9, 13]. Indeed, we found that JNK was activated in the hippocampus of CLP mice 30 days after surgery (Fig. 3b). Because serine phosphorylation of IRS-1 has been implicated in brain insulin resistance in dementia, we examined IRS-1pSer levels in the hippocampi of sepsis-surviving mice. Hippocampal levels of $IRS\text{-}1pSer^{636}$ were increased in post-septic mice compared to sham-operated mice (Fig. 3c). Further, hippocampi from sepsis-surviving mice showed decreased phosphorylation of IRS-1 at tyrosine residue 465 ($IRS\text{-}1pTyr^{465}$), an essential step in physiological insulin signaling (Fig. 3d).

Insulin signaling activates Akt through a well-defined mechanism mediated by the IRS1/PI3K pathway. Activated Akt phosphorylates glycogen synthase kinase 3β (GSK3β) at serine residue 9 ($GSK3\beta pSer^{9}$), inhibiting its activity. GSK3β is a serine kinase that plays key roles in a number of physiological processes as well as in the pathogenesis of dementia [22] and in insulin resistance [23]. Thus, we investigated the impact of sepsis on hippocampal phosphorylation of Akt and GSK3β. Levels of Akt phosphorylated at serine residue 473

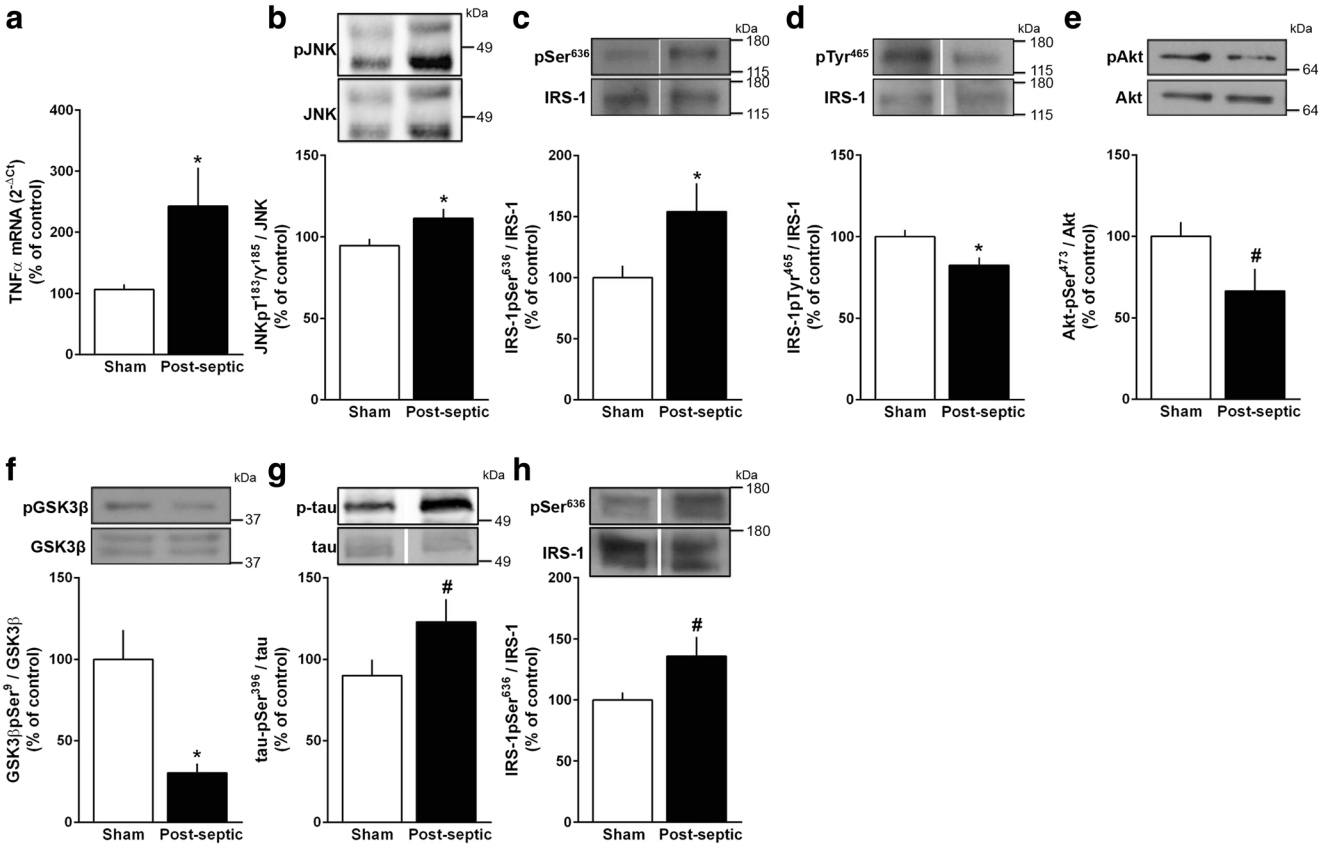

Fig. 3 Cognitive impairment is accompanied by hippocampal inflammation and impaired insulin signaling in post-septic mice. Two-month-old male Swiss mice were subjected to cecal ligation and puncture (post-septic) or were sham operated (Sham). qPCR or Western blot analysis were performed in hippocampal (**a**–**g**) or cortical (**h**) extracts 30 days after CLP. **a** TNF-α mRNA levels analyzed by qPCR; **b** JNKpT^{183}/Y^{185} normalized by total JNK; **c** IRS-1pSer^{636} normalized by total IRS-1; **d** IRS-1pTyr^{465} normalized by total IRS-1; **e** Akt-pSer^{473} normalized by total Akt; **f** GSK3βpSer^{9} normalized by total GSk3β; **g** tau-pSer^{396} normalized by total tau; **h** IRS-1pSer^{636} normalized by total IRS-1 in cortical tissue of mice. Bars represent means ± S.E.M.; *$p < 0.05$; #$p = 0.0615$ (**e**), $p = 0.0748$ (**g**), #$p = 0.0513$; Student's t test (n = 5–9 animals/group). Lanes were run in the same gel but were non-contiguous

(AktpSer^{473}) and of GSK3β phosphorylated at serine residue 9 (GSK3βpSer^{9}) were both decreased in the hippocampi of post-septic animals compared to control mice (Fig. 3e, f). An important target of GSK3β in the brain is tau, a microtubule-stabilizing protein whose hyperphosphorylation has been implicated in several neurodegenerative diseases collectively known as tauopathies [24]. In agreement with an over-activation of GSK3β (revealed by reduced GSK3βpSer^{9} levels), increased levels of tau phosphorylated at serine 396 (tau-pSer^{396}), a residue that is hyperphosphorylated in Alzheimer's disease and other tauopathies, were found in the hippocampi of sepsis-surviving mice (Fig. 3g). In addition to the hippocampus, other brain regions play essential roles in memory processing. We therefore examined levels of IRS-1pSer^{636} in the cortex of post-septic mice, as a representative protein of insulin signaling disruption. We also found increased levels of IRS-1pSer^{636} in the cortex of sepsis-surviving mice when compared to sham-operated animals (Fig. 3h). Collectively, these findings support the notion that impaired brain insulin signaling, increased GSK3β activity, and tau hyperphosphorylation accompany late memory impairment in sepsis.

Boosting Insulin Signaling Prevents Cognitive Impairment in Sepsis Survivors

In order to investigate whether drugs that target the insulin signaling pathway could ameliorate sepsis-associated cognitive impairment, CLP-mice were treated with liraglutide, a GLP-1 receptor agonist known to up-regulate insulin-triggered pathways [25]. Remarkably, s.c. treatment with liraglutide for 10 days beginning on day 20 post-CLP rescued cognitive impairment in post-septic mice (Fig. 4a). To further determine if hippocampal over-activation of GSK3β was involved in the development of sepsis-associated cognitive defects, a different group of mice was treated with daily i.p. injections of the selective GSK3β inhibitor, TDZD-8, which rescued performance in the NOR task (Fig. 4b). These results support the notion that disruption of brain insulin signaling in post-septic mice is an important factor underlying late

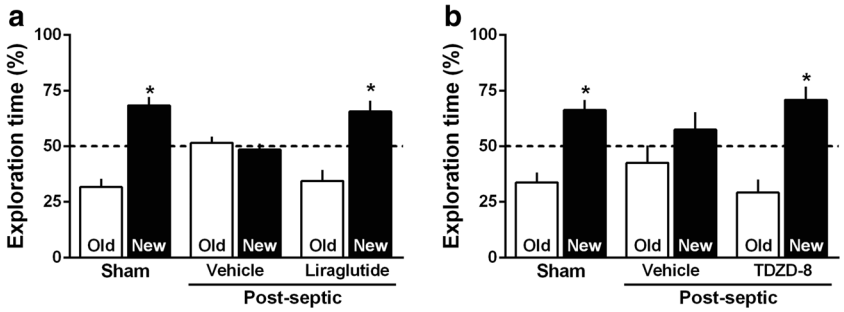

Fig. 4 Cognitive impairment is rescued by normalizing insulin signaling in post-septic mice. Two-month-old male Swiss mice were subjected to cecal ligation and puncture (post-septic) or were sham operated (Sham). Beginning on days 20 and 25 after surgery, respectively, mice were treated with **a** liraglutide (50 nmol/kg/day, for 10 days, s.c.) or **b** TDZD-8 (5 mg/kg/day, for 5 days, i.p.) or vehicle. At day 30 post-sepsis, mice were trained in the novel object recognition (NOR) task. The percentages of time spent exploring the familiar (old) or the novel (new) objects in the test session were measured. Bars represent means ± S.E.M. $*p < 0.05$, one-sample Student's *t* test (n = 10 mice/group)

cognitive impairment and that boosting insulin signaling could be further investigated as a therapeutic alternative for this devastating condition.

Discussion

Sepsis-induced brain dysfunction has received increasing attention in recent years, as convincing evidence shows that infectious diseases and systemic inflammation are risk factors for delirium, cognitive impairment, and neurodegenerative diseases [1, 3–6, 26, 27]. Even though sepsis survivors frequently show cognitive deficits that persist for months or years, seriously compromising patient recovery and independence, the mechanisms underlying this memory dysfunction are unclear.

Early cognitive impairment has been described in animal models of sepsis [18, 28, 29]. Barichello and colleagues evaluated cognitive function of sepsis-surviving mice 10 days after CLP surgery, using an aversive behavior paradigm [18]. The model described here significantly extends those findings by recapitulating late cognitive impairment seen in post-septic patients, after acute inflammation has ceased, which has translational importance for this condition. In agreement with previous studies, we found that sepsis-surviving mice show impaired locomotor/exploratory activities in an open-field task, which lasts for up to 25 days after CLP. Most experimental studies to date have assessed sepsis-induced cognitive deficits within this time frame and therefore are susceptible to interference from locomotor deficit [18, 30–32]. Moreover, the majority of studies have evaluated fear memories, which is not the type of memory mainly affected in sepsis-surviving patients [18, 30, 32]. Here, we followed post-septic mice until they no longer showed impaired locomotor/exploratory activities and trained them in an aversive memory task and also in the NOR test, evaluating memories that require integrity of the hippocampus and other brain regions [33, 34]. Post-septic mice showed impaired recognition and aversive memory 30 days after CLP. Remarkably, this is the first animal model of sepsis with cognition-related translational impact, since it recapitulates late cognitive effects seen in sepsis-surviving humans.

Even though some authors suggest that many sepsis survivors never fully recover cognitive functions [4, 7], some studies consider this impairment to be at least partially reversible [6]. Of note, it is not known whether sepsis induces or worsens a pre-existing neurodegenerative process, as information concerning the previous cognitive status of patients admitted to intensive care units is most often lacking. It is possible that previous cognitive status of patients may account substantially for the link between sepsis and long-lasting cognitive impairment. Under our conditions, recognition memory deficit seen late after CLP was transient, as mice had normal performance in the NOR test when re-trained 45 days after induction of sepsis. Results show that CLP in mice followed by cognitive evaluation in the novel object recognition task provides a suitable model to study the late, reversible cognitive impairment caused by sepsis, as seen in humans.

Synapse loss has been found to be a better correlate of memory impairment than other neurodegeneration markers in some forms of dementia, including Alzheimer's disease [35]. Our previous studies have shown that synaptophysin levels serve as a suitable marker of synapse damage and correlates well with cognitive impairment in mice [36, 37]. A recent study showed decreased levels of synaptophysin and PSD-95 in the hippocampus and cortex of septic mice 24 h and 3 days after CLP [31]. Weberpals and colleagues also showed that a single intraperitoneal injection of bacterial lipopolysaccharide (LPS) (5 mg/kg) induces long-term cognitive deficits and changes in synaptic key proteins (PSD-95 and synaptophysin) in the brain of wild-type mice, at least 2 months after sepsis induction [29]. Herein, we found that CLP still exerted a deleterious impact on hippocampal presynaptic terminals 30 days after surgery, as demonstrated by

decreased synaptophysin levels compared to control animals. On the other hand, hippocampal levels of PSD-95, a major scaffolding protein in the post-synaptic density, were comparable in sepsis-surviving mice and in sham-operated animals 30 days after surgery.

Several studies have established the relevance of cAMP-responsive element binding protein (CREB) in hippocampal memory-related processes [19]. During memory consolidation, CREB undergoes phosphorylation at serine residue 133 and binds to the CRE promotor region as a consequence of calcium influx in the post-synaptic terminal [38]. Here, we show that post-septic mice show decreased hippocampal expression of CREB and lower levels of CREBpSer^{133} compared to control animals. AMPAR phosphorylation/trafficking is another important consequence of glutamatergic post-synaptic activation in the hippocampus. Phosphorylation at specific sites following neuronal activation determines AMPAR translocation to synapses [20]. Here, we found decreased levels of GluA1pSer^{845} in the hippocampus of mice 30 days after CLP. Altogether, these findings suggest that sepsis compromises plasticity mechanisms in hippocampal post-synaptic terminals.

Previous studies have shown increased levels of IL-1β and other pro-inflammatory cytokines in the hippocampus of rodents following sepsis [28, 31, 39–41]. When evaluated at longer periods after sepsis, increased levels of pro-inflammatory cytokines have been found in cerebrospinal fluid or in whole-brain homogenates of CLP-induced sepsis animals [42, 43]. In addition, wild-type mice or rats submitted to LPS-induced sepsis also show a prolonged increase in pro-inflammatory cytokines in the hippocampus [29, 41]. Current results demonstrate increased hippocampal expression of TNF-α 30 days after CLP, suggesting that even after the acute phase of peripheral inflammation has ceased, production of inflammatory mediators persists in post-septic brains. This local production of pro-inflammatory mediators may contribute to the persistent cognitive impairment seen in our model.

Recent findings suggest that several types of dementia, including AD, are characterized by defective brain insulin signaling [15], which is apparently linked to cognitive dysfunction [10–12]. Additional studies have implicated increased brain TNF-α levels in impaired insulin signaling and memory deficits [9, 13]. We thus hypothesized that insulin signaling could be disrupted in the brains of sepsis-surviving mice and could underlie sepsis-induced cognitive impairment. Indeed, we found that hippocampi of post-septic mice show over-activation of TNF-α/JNK signaling, increased levels of IRS-1pSer^{636} and decreased levels of IRS-1pTyr^{465}, hallmarks of insulin resistance in both periphery and CNS [10, 11, 44].

In the insulin signaling pathway, Akt activation occurs as a key step downstream from IRS-1 activation [45–47]. Under physiological conditions, activation of Akt by phosphorylation at serine and threonine residues leads to inactivation of GSK3β via serine phosphorylation [23, 48]. In type 2 diabetes (T2D), for example, defective insulin signaling leads to reduced levels of AktpSer^{473} and consequent over-activation of GSK3β. Increasing evidence suggests that GSK3β plays a key role not only in diabetes but also in dementia [49, 50]. Consistent with defective insulin signaling following CLP, levels of AktpSer^{473} and GSK3βpSer^{9} were decreased in the hippocampus of post-septic mice. We also found that the hippocampus of post-septic mice had elevated levels of phosphorylated tau protein, a major target of GSK3β. Our results also suggest that disrupted insulin signaling is not restricted to the hippocampus, since findings on IRS-1pSer^{636} levels were reproduced in the cortex of post-septic mice. Collectively, these findings support the notion that sepsis-surviving mice show impaired brain insulin signaling, similar to AD brains and other dementias.

Previous studies have shown that liraglutide and exenatide, GLP-1 receptor agonists used to treat T2D patients, prevent cognitive impairment related to impaired brain insulin signaling in several models [9, 13, 51–53]. Significantly, clinical trials are under way to determine the clinical relevance of these drugs in AD. To determine whether normalizing insulin signaling could prevent sepsis-induced cognitive impairment, mice submitted to CLP were systemically treated with liraglutide or TDZD-8, a GSK3β inhibitor. Interestingly, treatment with liraglutide or TDZD-8 recovered cognition in sepsis-surviving mice 30 days after CLP. These findings improve our understanding of the mechanisms underlying late cognitive impairment in sepsis survivors, and provide new targets for the development of treatments for this condition.

The current study highlights the impact of sepsis on hippocampal insulin signaling, synapse structure and function and demonstrates that late cognitive impairment in sepsis-surviving mice is associated with up-regulation of brain TNF-α, disruption of insulin signaling cascades, and plasticity-associated molecules. Moreover, late cognitive impairment after sepsis was reversed when insulin signaling was pharmacologically boosted in mice. Our results suggest that stimulation of brain insulin signaling could provide a novel neuroprotective approach to prevent cognitive impairment in sepsis survivors. We note that liraglutide is a drug already approved for treatment of T2D, and that identifying a similar pathogenic mechanism of insulin resistance contributing to sepsis-induced cognitive impairment may open new avenues for rapid implementation of clinically approved anti-diabetic drugs as therapeutics in post-septic patients.

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