Arc rescues defective synaptic plasticity and cognitive dysfunction in sepsis-associated encephalopathy

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Abstract

Sepsis-associated encephalopathy (SAE) is a major and frequent complication in patients with sepsis resulting in delirium and premature death. Sepsis survivors commonly suffer from long-term cognitive impairment causing immense burden on patients, caregivers, and economic health systems. The underlying pathophysiology of SAE is largely unresolved, thus treatment options are missing. We report that experimental polymicrobial sepsis in mice induces synaptic pathology in the central nervous system underlying defective long-term potentiation and cognitive dysfunction. Analysis of differentially expressed genes revealed severely affected downregulation of genes related to neuronal and synaptic signaling in the brain, e.g. of the activity-regulated cytoskeleton-associated protein (*Arc*), of the transcription-regulatory EGR family, and of the dual-specificity phosphatase 6 (*Dusp6*). On the protein level, ARC expression and mitogen-activated protein (MAP) kinase signaling in the brain was disturbed during SAE. For targeted rescue of dysregulated synaptic signaling and plasticity, we overexpressed ARC in the hippocampus by bilateral *in-vivo* stereotactic microinjection of an adeno-associated virus containing a neuron-specific plasmid of the *Arc* transgene. Hereby, defective synaptic plasticity and signaling in the hippocampus were restored and memory function improved. Accordingly, synaptic plasticity, neuronal spine pathology, and memory dysfunction also improved when post-septic mice were subjected to enriched environment demonstrating the potential for activity-induced recovery of long-term cognitive dysfunction. Together, we identified synaptic pathology of neurocognitive dysfunction after severe systemic infection and provide a proof-of-concept approach to interfere with SAE pathomechanisms leading to cognitive improvement.

Introduction

Sepsis is a world-wide health problem with increasing incidence and high mortality. Current guidelines define sepsis as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Recent epidemiological studies revealed an incidence rate of 437 hospital-treated sepsis patients per 100.000 person-years and a global incidence of 31.5

million sepsis cases with 5.3 million deaths annually (2). Sepsis-associated encephalopathy (SAE) is a frequent complication, often presents as the initial symptom, and was also shown to be an independent risk factor for increased mortality (3). Depending on definition, the frequency of SAE occurs in up to 70% of patients with severe sepsis, however prospective studies provide an accurate estimate of SAE incidence are missing (4, 5). The early phase of SAE during the infectious period manifests with delirium-associated symptoms such as agitation, psychotic experiences, and sleep disturbances. In the later phase patients typically suffer from long-term cognitive deficits comparable to those found in patients with beginning Alzheimer´s dementia (6, 7).

The etiology and pathophysiology of SAE is only incompletely understood and causal therapeutic options are missing. The etiology is likely multifactorial including blood-brain barrier dysfunction, ischemia and hemorrhages, changes in cerebral homeostasis, and dysregulation of cholinergic neurotransmission (5, 7, 8). It is conceivable that severe systemic inflammation leads to activation of cerebral immune cells, e.g. microglia thereby inducing a pathological proinflammatory milieu in the brain (9-12). However, it is unclear how sepsis-induced neuroinflammation leads to severe and persistent neuronal and synaptic dysfunction resulting in neurocognitive defects.

Using a large-scale and multidisciplinary approach, we here present the synaptic pathophysiology of SAE in a mouse model of peritoneal polymicrobial sepsis. By identifying severely impaired pathways of synaptic plasticity, we were able to establish therapeutic interventions leading to rescue of neuronal functionality finally resulting in improvement of disease symptoms. Specifically, targeting defective regulatory mechanisms of synaptic plasticity offers conceptual advances for translational strategies in SAE.

Results and Discussion

We induced severe experimental sepsis in 769 C57BL/6J mice by polymicrobial peritonitis after intraperitoneal injection of a standardized and microbiologically validated human faeces material (peritoneal contamination and infection model, PCI) (13) (Figure 1 A). Mice showed a median disease severity of 12.25 on the 5 day PCI Clinical Severity Score (CSS) (Figure 1 B, Supplemental Table 1) and only surviving mice with a 5 day CSS ≥ 11.5, indicating severe sepsis, were included for further experiments (n = 309 PCI). Concomitant antibiotic rescue therapy with meropenem (weeks 1 and 2) and enrofloxacin from week 3 led to a survival rate of 0.38 after 8 weeks (Figure 1 B). In the late stage 8 weeks after PCI induction, surviving mice displayed delayed learning and defective memory performance as revealed by Morris water maze (MWM) test. Deficits started from day 1 of the learning phase and memory dysfunction persisted in the recall phase on day 8 (Figure 1C). Moreover, mice had increased anxiety-like behavior in the open field (OF) and elevated plus maze test (EPM; Figure 1D). These findings can be specifically attributed to sepsis-induced brain dysfunction and were not caused by post-sepsis related sickness, as the overall phenotype was normal with recovered body weight and regular locomotor activity (Supplemental Figure 1). In order to identify the underlying pathophysiological mechanism of cognitive dysfunction we tested synaptic plasticity by recording local field potentials in the CA1 region of the hippocampus after stimulation of Schaffer collaterals. We found severely affected long-term potentiation (LTP) at late time points after PCI (Figure 1E). Together, these data demonstrate that severe systemic infection results in long-term neurocognitive deficits. Persistent cognitive decline in the late phase after surviving sepsis is substantiated by defective hippocampal LTP.

Next, we investigated synaptic transmission in the hippocampus post-sepsis by whole-cell patch-clamp analysis in dentate gyrus granule cells. We found reduced frequency of quantal excitatory postsynaptic currents (miniature, mEPSCs) and spontaneous EPSCs (sEPSCs) which is indicative for overall reduction of excitatory presynaptic terminals (Figure 1F and G). Unexpectedly, peak amplitudes of mEPSCs, of sEPSCs, and of minimally evoked

EPSCs (eEPSCs) after stimulation of the medial perforant pathway were increased with unchanged kinetics (Figure 1 F to H and Supplemental Figure 2) which might reflect increased recruitment of AMPA receptors to postsynaptic sites, e.g. in a homeostatic scaling mechanism.

In order to obtain mechanistic insight in the molecular pathomechanism of sepsis-induced neuronal dysfunction, we next investigated the transcriptional profile of brain tissue of acute septic mice (day 3 after PCI), of mice at a late stage after surviving sepsis (week 10), and of respective controls. We found differently regulated brain gene expression early after sepsis, whereas after 10 weeks gene expression was rather similar to controls (Supplemental Figure 3 and 4). Gene Ontology (GO) term enrichment analysis of differentially expressed genes (DEGs) at day 3 revealed that transcripts related to neuronal function (e.g., GO terms ‘synapse’, ‘postsynaptic density’, ‘dendrite’) are severely downregulated, whereas transcripts related to inflammation (e.g. GO terms ‘innate immune response’, ‘inflammatory response’) and to gene transcription were upregulated (Figure 2A; Supplemental Figure 5). Against this, in the late stage after sepsis, inflammatory pathways in the brain were no longer disturbed and pathways related to gene transcription were downregulated. Moreover, genes associated to neuronal function (e.g. GO terms ‘neuron projection’, ‘dendrite’) were upregulated, most likely in a compensatory way. The most prominently downregulated gene was *Arc*, encoding activity-regulated cytoskeleton-associated protein ARC/ARG3.1, a master regulator of synaptic plasticity (14) (Figure 2B). Protein interaction network of corresponding DEGs on day 3 after PCI revealed downregulation of genes encoding close ARC interaction partners, thus predicting a major role in dysfunctional synaptic plasticity and signaling (Figure 2B and Supplemental Figure 6). Genes of the early growth response protein (*Egr*) family, transcription regulatory factors involved in neuronal plasticity and direct interactors with *Arc* (15), as well as *Dusp6*, encoding the ERK-directed dual-specificity phosphatase 6, *Nrgn*, encoding Neurogranin/RC3, a calmodulin-binding protein involved in CaMKII dependent synaptic

plasticity (16), and *Mapk11*, encoding p38 Mitogen-activated protein kinase 11, were downregulated (Figure 2C).

On the protein level, we found reduction of ARC and of activated ERK, an upstream mitogen-activated protein (MAP) kinase relevant in transcription of immediate-early genes such as *Arc* (17) after PCI (Figure 2 D and Supplemental Figure 7). Together, these data indicate severe inflammation-induced alterations of synaptic regulatory pathways in early stages together with reactive gene regulation patterns. Impaired neuronal functionality converges on activity-regulated pathways important for synaptic plasticity.

ARC is abundantly expressed in somata and dendrites of CamKII positive excitatory neurons in the CNS (18). It is an essential, bidirectional regulator of long-term synaptic plasticity including LTP, long-term depression (LTD), heterosynaptic LTD, and homeostatic synaptic scaling (14). ARC mediates activity-regulated trapping of AMPA receptors at postsynaptic sites by interaction with the endocytic machinery and PSD-95 complexes (19). In *Arc* knockout (ko) mice and after *Arc* silencing using antisense oligonucleotides, LTP consolidation and memory formation is impaired (20, 21). Furthermore, ARC regulates synaptic AMPA receptor expression dependent on synaptic activity representing the key mechanism of homeostatic plasticity (22, 23). Persistent deficiency of ARC leads to upscaling of AMPA receptor expression (24) which is consistent with increased AMPA receptor mediated EPSC peak amplitude in synaptic transmission observed here in mice after PCI (Figure 1 F to H).

Based on these findings we aimed at specifically rescuing sepsis-induced loss of ARC by constitutive overexpression in the hippocampus of post-septic mice using an adeno-associated virus containing a neuron-specific bicistronic plasmid of the *Arc* and *Venus* transgene under the synapsin promotor (*Arc*-AAV; Figure 3A). Bilateral *in-vivo* injection of *Arc*-AAV in three injection sites of the hippocampus led to robust, constitutively driven and not activity-dependent upregulation of ARC in hippocampal neurons (Figure 3B). According to previous studies showing inverse regulation of synaptic AMPA receptor expression by

synaptic scaling mechanisms after *Arc* overexpression (25-27) we expected affected LTP due to disturbed AMPA receptor mediated signaling in non-septic control mice after *Arc* overexpression. Indeed, we found defective LTP in the Schaffer collateral-CA1 pathway in patch-clamp single cell recordings of non-septic control mice after *Arc*-AAV injection (Supplemental Figure 8).

PCI surviving mice that received injection of the empty control vector expressing only fluorescent Venus showed again learning and memory dysfunction in the Barnes maze (BM) similar to the PCI induced alterations in the MWM (Figure 3C). Importantly, hippocampal *Arc* overexpression in PCI mice induced improvement of spatial learning and memory recall (Figure 3C). As expected, *Arc* overexpression in SHAM mice did not improve cognitive function (Supplemental Figure 9). Fear-related behavior as measured in the EPM was present in PCI mice and did not improve upon *Arc* overexpression in the hippocampus since it is primarily mediated by signaling pathways in the amygdala (Supplemental Figure 10). Corroborating the behavioral findings, hippocampal LTP of PCI mice after *Arc*-AAV injection was rescued to almost normal values when compared to sham mice with injection of *control*-AAV (Figure 3D). These data indicate that increasing Arc protein levels in the hippocampus is sufficient to at least partially reverse sepsis-induced neuronal dysfunction and defective synaptic plasticity mechanisms, which are responsible for persistent cognitive impairment.

These data led us to hypothesize that not only constitutive upregulation of a key regulating synaptic plasticity protein, but also stimulus-induced activation can improve cognitive dysfunction in mice after experimental sepsis. Behavioral tasks are able to induce expression of activity-regulated genes, e.g. *Arc* in neurons (28). We therefore tested if an enriched environment (EE) paradigm, which is known to have beneficial effects in cognitive performance by enhancing synaptic plasticity (29), is capable of rescuing defective synaptic plasticity and memory dysfunction after PCI (Figure 4A). First, we again reproduced PCI-induced long-term cognitive dysfunction in the BM, anxiety-related behavior in the EPM, and defective LTP in mice without special housing conditions (PCI versus SHAM non-enriched

(NE); Figure 4B to D). To substantiate these functional defects we additionally quantified synaptic spines in apical dendrites of hippocampal CA1 neurons providing morphological information on plasticity changes. The overall spine density and, more specifically, mature mushroom spines were reduced corroborating the findings of defective LTP and may reflect defective ARC interaction with actin-binding proteins (Figure 4E). Importantly, when PCI mice were subjected to 6 weeks of EE housing conditions they showed improved performance in the BM test and reduced anxiety-related behavior in the EPM (Figure 4B and C). Again, possible confounders as sickness behavior and locomotor hypoactivity were not present in any of the experimental groups at 8 weeks after PCI (Supplemental Figure 11). Synaptic plasticity as measured by hippocampal LTP was preserved and reduction of synaptic spines was no longer present in PCI mice after EE (Figure 4 D and E).

Together, our findings identify defective regulation of synaptic plasticity as a key pathogenic mechanism of long-term cognitive dysfunction in SAE. Specifically, synaptic expression of ARC and upstream signaling interaction partners are affected. Targeting these pathways offers conceptual advances for translational strategies in SAE. Future therapeutic strategies may therefore comprise general activation, e.g. by personalized cognitive training approaches that need evaluation in controlled clinical trials. The current findings further encourage patient-oriented activation training beginning at early stages in the disease course. Moreover, the identification of impaired neuronal pathways in SAE provides first opportunity for targeted intervention strategies. Increasing ARC levels, e.g. by stimulation of upstream pathways such as neurotrophin BDNF receptor/TrkB activation, ERK signaling, or CREB phosphorylation (25, 30-32), may be promising starting points for target-directed therapies.

Methods

See Supplemental Methods for detailed methods.

Animal studies. All animal experiments were approved by the state authorities of Thuringia (UKJ-02-085-14) and were performed in accordance with animal welfare and the

ARRIVE guidelines (33). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Statistics. Data presented represent the median or the mean ± SEM as indicated in the figure legends. An unpaired, 2-sided Student's t test or a 2-way ANOVA (> 2 groups) was used to evaluate statistical significance using P less than 0.05 as the cutoff.

Microarray data: The raw and preprocessed data have been deposited in NCBI GEO DataSets with the accession number GSE167610.

Author contributions

CG and BG conceived and designed the experiments. BG, HH, HR, NHE, LG, MB and LS conducted in vitro experiments. BG, JW, HR, RAC performed the *in-vivo* experiments. BG, NHA, FH, H-YC, ASS, HR, AU, and RK analyzed the data. BG and NHA prepared the figures and CG wrote the manuscript. All authors have read and approved the final manuscript.

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Figures and figure legends

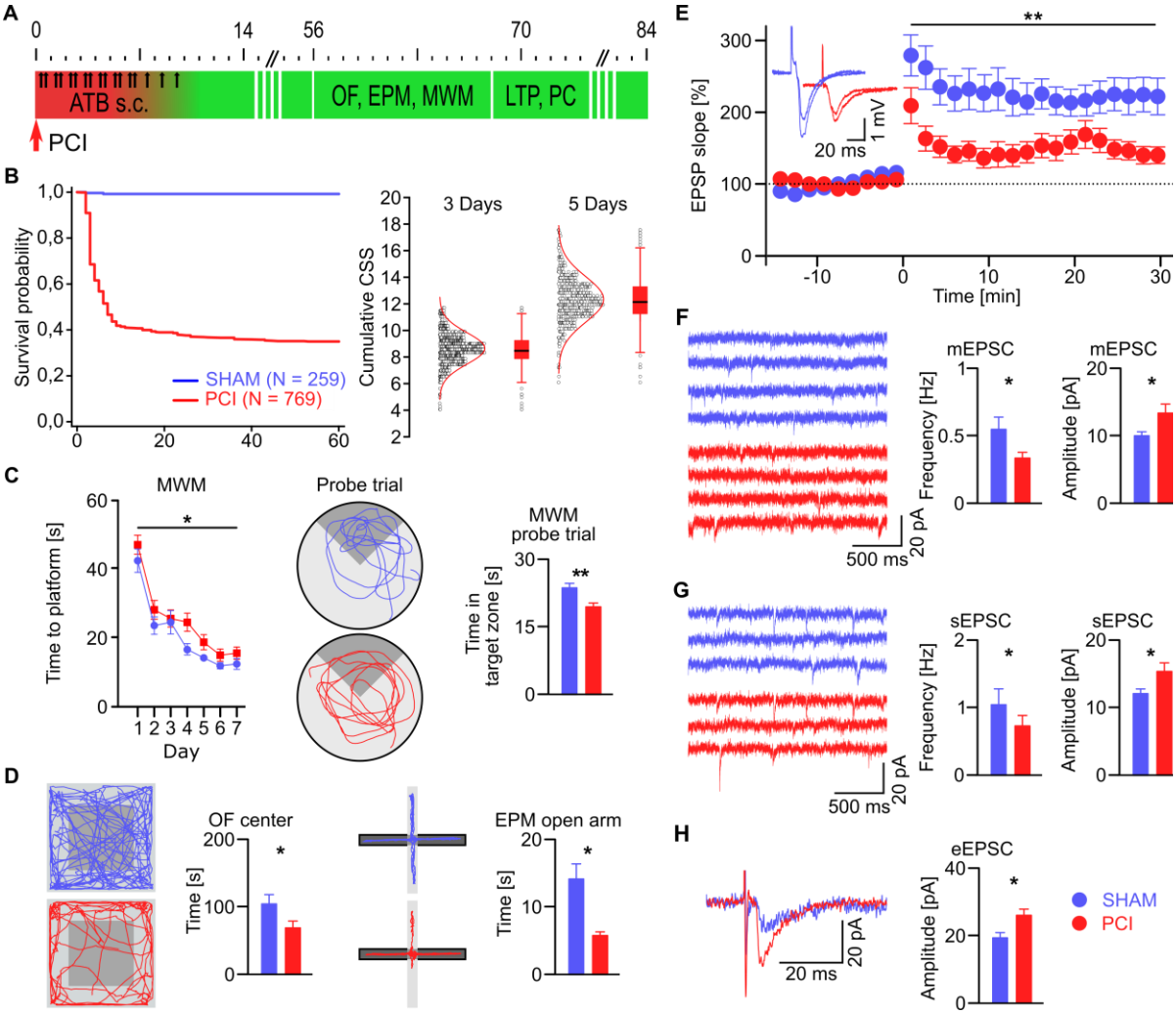

Figure 1. Experimental sepsis causes neurocognitive dysfunction, defective long-term potentiation, and synaptic scaling in excitatory synapses. (**A**) Experimental schematic: acute phase of PCI is marked in red. Antibiotics (ATB) are delivered i.p. until day 10 and behavioral testing (open field, OF; elevated plus maze, EPM; Morris water maze, WMW) and ex-vivo electrophysiological recordings (long-term potentiation, LTP, and patch-clamp recordings, PC) are performed at the indicated time points. (**B**) Kaplan-Meier survival curve of experimental animals (left). Mice intentionally leaving the study e.g. for tissue harvesting were included up to the time when they are censored. Mean and distribution of sepsis severity score (CSS) on day 3 and on day 5 after PCI (right; each

circle represents an individual mouse after PCI). (**C**) In the MWM PCI mice (n = 12, red) show affected learning (left; 2-way ANOVA) and reduced memory recall in the probe trial (right; example trackings are shown in the center, target quadrant in dark grey; SHAM mice n = 18, blue). (**D**) In the OF and the EPM PCI mice spent reduced time the OF center (dark grey) or in the EPM open arms (light grey). (**E**) LTP in the hippocampal Schaffer collateral – CA1 pathway is severely impaired in PCI mice (n = 7; SHAM: n = 12; 2-way ANOVA). Inset shows average traces of example recordings before and after theta burst stimulation. (**F** to **H**) Patch-clamp recordings of dentate gyrus granule cells indicates reduced frequency of miniature (m) and spontaneous (s)EPSC, and increased peak amplitude of minimally evoked (eEPSC) and mEPSC (PCI: n = 11/13/13, SHAM: n = 11/13/16, respectively). Example traces in (F) and (G), average traces of minimal evoked eEPSC in (H). Bar graphs show mean ± SEM; 2-tailed Student's t test, unless otherwise indicated. *P < 0.05.

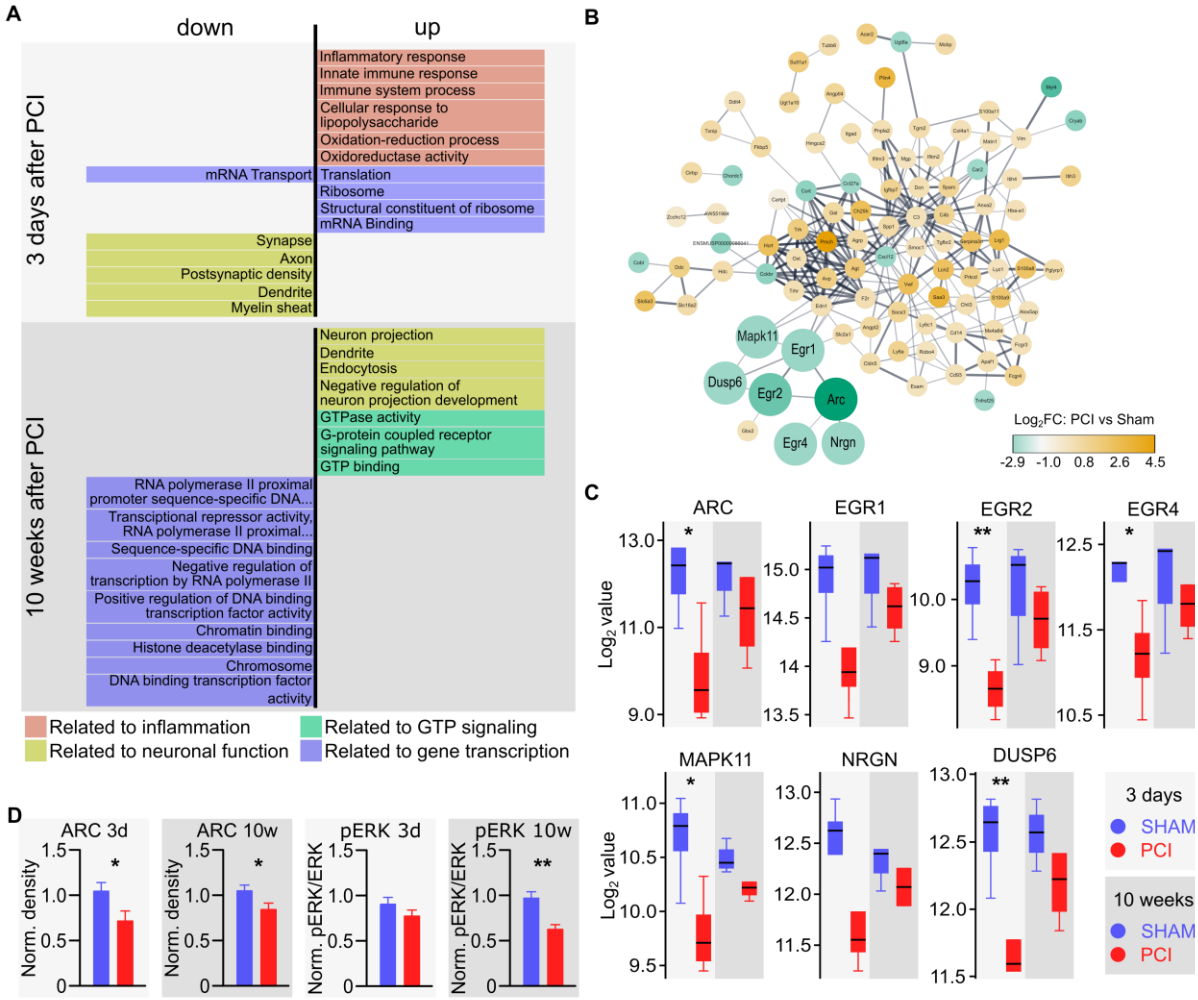

Figure 2. PCI induces downregulation of neuronal pathways mediating synaptic plasticity. (**A**) GO terms enriched in differently expressed genes (144 DEGs; $Log_2FC > 1$) found by microarray analysis of brain tissue at 3 days and 10 weeks after PCI. GO terms related to downregulated DEGs are facing to the left and GO terms related to upregulated DEGs to the right. (**B**) Interaction network of proteins corresponding to DEGs on day 3 after PCI. The node color depicts the respective Log_2FC from microarray data. Proteins related to ARC synaptic signaling are highlighted by size. (**C**) Expression of seven selected DEGs that are related to ARC signaling and synaptic function (highlighted in B). 3 days SHAM and PCI and 10 weeks PCI n = 4, 10 weeks SHAM n = 3. Boxplots show median and 75th to 25th interquartile range, whiskers show respective maximum or minimum values within 1.5 times of the interquartile range; 2-tailed Student's t test. (**D**) Quantitative analysis of hippocampal Arc protein and pErk/Erk protein ratio (n = 8, each; normalized to SHAM

Arc or SHAM pERK/ERK ratio, respectively) shows downregulation of signaling pathways in the network shown in (B). 2-tailed Student's t test. *P < 0.05, **P < 0.01.

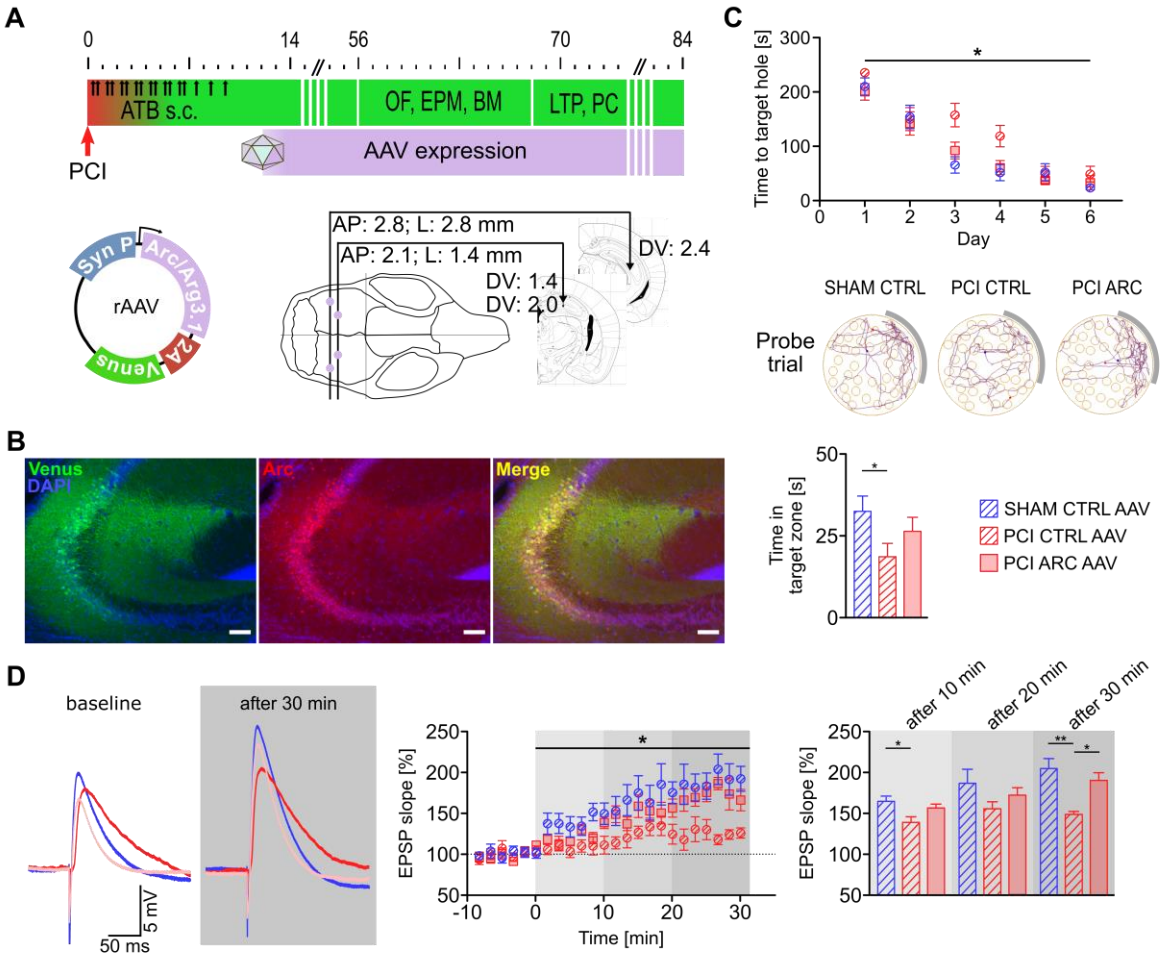

Figure 3. Hippocampal overexpression of Arc rescues sepsis-induced cognitive dysfunction and defective long-term potentiation (**A**) Experimental schedule indicating Arc overexpression in the hippocampus from day 10 after PCI. Stereotactic intrahippocampal microinjection of an adeno-associated virus (AAV) containing the bicistronic vector for Arc overexpression and Venus fluorescence under the synapsin promotor at the indicated coordinates. (**B**) Representative example of constitutive Arc overexpression in the CA3 region of the hippocampus. Scale bars: 40 µm. (**C**) PCI mice receiving control AAV (PCI CTRL AAV; n = 17) show learning deficits in the Barnes Maze compared to SHAM CTRL AAV (n = 18) as indicated by prolonged time to the target hole (upper panel) and reduced time in the target zone in the probe trail (day 7, lower panel). Arc overexpression in PCI mice (PCI ARC AAV; n = 18) results in rescue of learning and memory dysfunction. Example traces indicate tracks of individual mice during the probe

trial; the target quadrant containing the open hole is marked in grey. (**D**) LTP as measured by slope of excitatory postsynaptic potential (EPSP) in single-cell whole cell recordings in CA1 neurons after stimulation of Schaffer collaterals. Paired stimulation results in LTP in SHAM CTRL AAV (n = 11) and PCI ARC AAV (n = 8) mice whereas PCI mice with control virus injection (PCI CTRL AAV; n = 9) show reduced LTP. Left panel: average traces (blue: SHAM CTRL AAV; red: PCI CTRL AAV; light red: PCI ARC AAV). Middle panel: LTP time course. Right panel: comparison of LTP at the indicated time points after theta burst stimulation. 2-way ANOVA with Tukey's post hoc test. *$P < 0.05$, **$P < 0.01$.

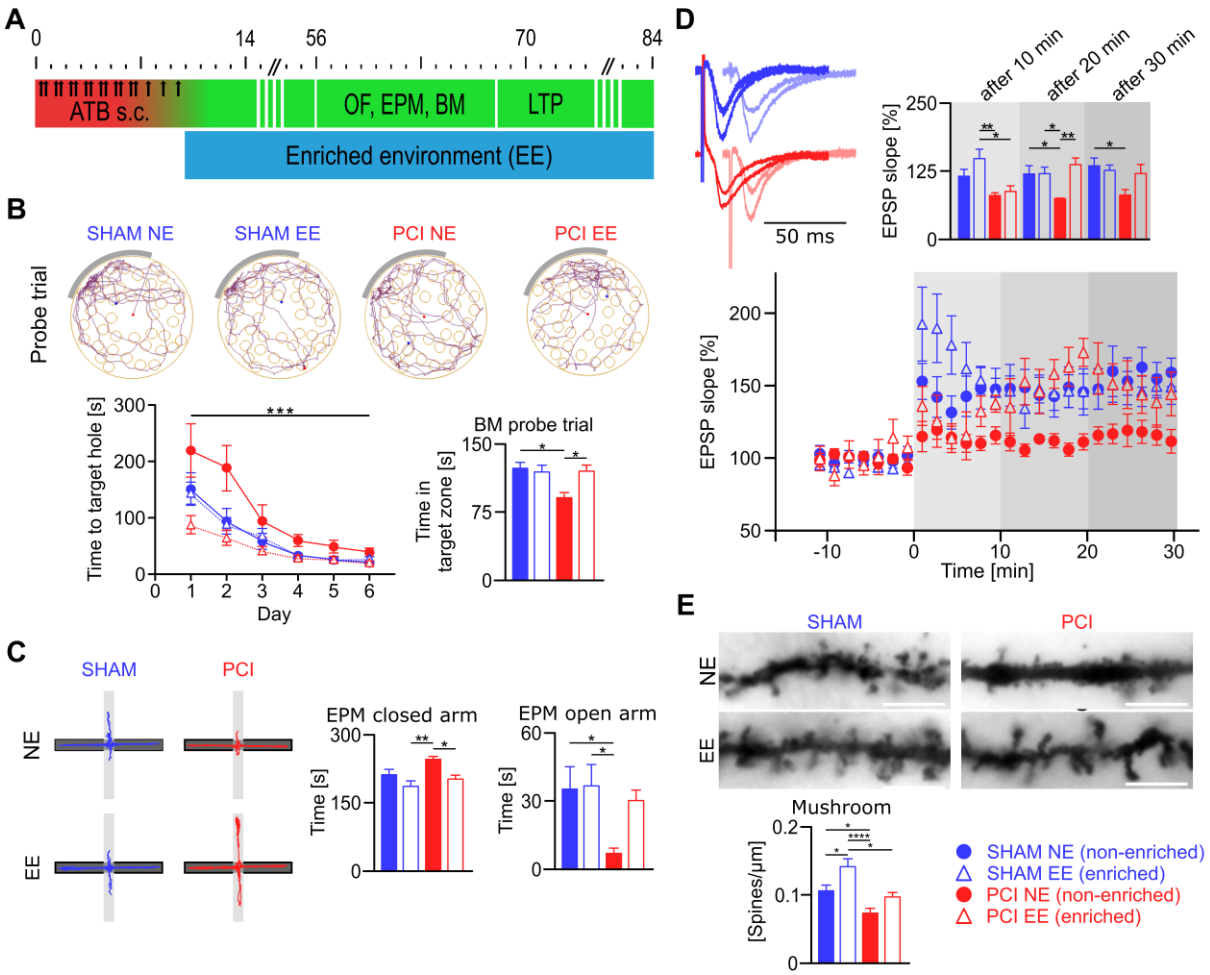

Figure 4. Enriched environment leads to improvement of synaptic pathology and cognitive dysfunction after PCI. (**A**) Mice were subjected to enriched environment (EE) or standard housing (NE) from day 10 after PCI. (**B**) EE improves cognitive dysfunction in the BM after PCI (PCI EE) as indicated by reduced time to target hole in the training phase (left) and by increased time in the BM target zone during the probe trial compared to PCI mice in standard care (PCI NE). Example traces indicate tracks of individual mice during the probe trial; target quadrant is marked in grey. (**C**) EE leads to reduced anxiety-related behavior in the EPM after PCI as shown by reduced time in the closed and increased time in the open arms; n = 14 / 18 / 13 / 16; SHAM NE / SHAM EE / PCI NE / PCI EE in B and C). (**D**) LTP as evaluated by field potential recording in CA1 is improved in PCI mice after EE. Left panel: average traces before and after theta burst stimulation (SHAM EE light blue; PCI EE light red; amplitudes of the baseline traces of each group were scaled to the amplitude of the SHAM NE trace (dark blue).

Right panel: comparison of LTP at the indicated time points after theta burst stimulation; bottom panel: LTP time course. (**E**) Quantification of synaptic mushroom spines in apical dendrites of CA1 neurons. Example images are provided (scale bar: 5 µm). PCI leads to reduction of mature mushroom spine density (PCI NE) which is rescued to basal levels (SHAM NE) after enriched environment (PCI EE); n = 42 / 21 / 33 / 36; SHAM NE / SHAM EE / PCI NE / PCI EE. 2-way ANOVA with Tukey`s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.