Enhanced astrocytic d-serine underlies synaptic damage after traumatic brain injury

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After traumatic brain injury (TBI), glial cells have both beneficial and deleterious roles in injury progression and recovery. However, few studies have examined the influence of reactive astrocytes in the tripartite synapse following TBI. Here, we have demonstrated that hippocampal synaptic damage caused by controlled cortical impact (CCI) injury in mice results in a switch from neuronal to astrocytic d-serine release. Under nonpathological conditions, d-serine functions as a neurotransmitter and coagonist for NMDA receptors and is involved in mediating synaptic plasticity. The phasic release of neuronal d-serine is important in maintaining synaptic function, and deficiencies lead to reductions in synaptic function and plasticity. Following CCI injury, hippocampal neurons downregulated d-serine levels, while astrocytes enhanced production and release of d-serine. We further determined that this switch in the cellular source of d-serine, together with the release of basal levels of glutamate, contributes to synaptic damage and dysfunction. Astrocyte-specific elimination of the astrocytic d-serine-synthesizing enzyme serine racemase after CCI injury improved synaptic plasticity, brain oscillations, and learning behavior. We conclude that the enhanced tonic release of d-serine from astrocytes after TBI underlies much of the synaptic damage associated with brain injury.

Introduction

Traumatic brain injury (TBI) is a devastating pathological condition that can lead to tissue loss and neurological dysfunction as a result of cell damage or death. Specialized membranes, such as at synapses, are susceptible to unique environmental changes within the injured brain, where astrocytes in the tripartite synapse contribute to synaptic transmission and function (1–3). TBI is known to result in astrocyte reactivity, which has both beneficial and deleterious effects on injury progression and recovery; however, few studies have examined the effects of astrogliosis on synaptic stability in the CNS (4–6).

In addition to direct damage to synaptic membranes, TBI can result in acute cell loss due to excessive release of excitatory amino acids such as glutamate (7–9). Glutamate excitotoxicity occurs when pathological levels of extracellular glutamate lead to enhanced extrasynaptic NMDAR activation, increased influx of calcium, enzymatic activation, and subsequent damage to cell structures (8, 10, 11). NMDAR activation also requires the binding of a coagonist, such as d-serine or glycine. d-serine is considered a more likely binding partner for NMDAR in the hippocampus, since its expression patterns match NMDAR expression (12–16) and it has a higher affinity for the NMDAR glycine-binding site (17).

In the adult mammalian forebrain, d-serine and its synthesizing enzyme, serine racemase (SRR), are expressed by excitatory glutamatergic neurons and subpopulations of GABAergic neurons (12, 18–20). Conversely, recent studies indicate that only a small percentage of astrocytes express a negligible amount of SRR in rodent forebrain tissues (21). Pathological levels of d-serine, similarly to glutamate, have been implicated in mediating acute excitotoxic cell death during pathological conditions (22–26); however, the effect of prolonged increases in d-serine levels on synaptic function remains to be determined.

In the current study, we make use of a murine moderate controlled cortical impact (CCI) injury paradigm in which synaptic damage in the absence of neuronal cell death can model synaptic damage and recovery (27). We take advantage of inducible astrocyte- and neuron-specific Srr-knockout mice to examine the cellular contribution of d-serine on synaptic stability and function in the tripartite synapse. Our findings demonstrate that CCI injury results in a switch in d-serine release from neurons to astrocytes that contributes to synaptic damage and learning dysfunction associated with TBI.

Results

Phasic release of neuronal d-serine mediates synaptic transmission and long-term potentiation. To begin to evaluate the role of d-serine in regulating synaptic transmission, we first examined its expression using immunohistochemical approaches and function using electrophysiology in the naive hippocampus. d-serine was predominantly observed in the neuron-rich pyramidal cell (Figure 1, A and B) and granule cell layers, with low expression lev-
deficiencies are known to have a direct effect on d-serine levels (12, 18). Following the delivery of an in vivo monophasic electrical pulse, input-output (I/O) curves indicated reduced field excitatory postsynaptic potentials (fEPSPs) in nSRKO mice, but only at high stimulating intensities (Figure 1E). These reductions suggest that neurons mainly contribute to the overall resting levels of d-serine in the hippocampus, where activation of NMDAR is predominantly observed at higher stimulating intensities (28). No significant difference was observed in presynaptic function between genotypes using paired pulse facilitation (PPF) (Figure 1F). Potentiation of synaptic transmission following tetanic stimulation (i.e., long-term potentiation [LTP]) was only impaired in nSRKO mice, which showed a significant main effect for group ($P = 0.0137$) and time ($P < 0.001$) using 2-way ANOVA analysis (Figure 1G). Analysis of LTP induction and

eels observed outside this zone. A similar expression profile was observed for the synthesizing enzyme SRR, essential for conversion of L-serine to d-serine (Figure 1, A and C). This expression profile supports previous studies showing that SRR and d-serine are primarily localized to neurons (12, 18–20).

To examine the contributions of neuronal and astrocytic d-serine, we examined local in vivo field potentials in the CA1 stratum radiatum in response to electrical stimulation of Schaffer collaterals in mice deficient for neuronal SRR (nSRKO; Thy1-CreERT2 Srrfl/fl mice) or astrocytic SRR (aSRKO; Gfap-CreERT2 Srrfl/fl mice, where Gfap indicates glial fibrillary acidic protein) (Figure 1D and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI92300DS1) as compared with control Srrfl/fl mice (cSR) that did not express Cre recombinase. SRR deficiencies are known to have a direct effect on d-serine levels (12, 18). Following the delivery of an in vivo monophasic electrical pulse, input-output (I/O) curves indicated reduced field excitatory postsynaptic potentials (fEPSPs) in nSRKO mice, but only at high stimulating intensities (Figure 1E). These reductions suggest that neurons mainly contribute to the overall resting levels of d-serine in the hippocampus, where activation of NMDAR is predominantly observed at higher stimulating intensities (28). No significant difference was observed in presynaptic function between genotypes using paired pulse facilitation (PPF) (Figure 1F). Potentiation of synaptic transmission following tetanic stimulation (i.e., long-term potentiation [LTP]) was only impaired in nSRKO mice, which showed a significant main effect for group ($P = 0.0137$) and time ($P < 0.001$) using 2-way ANOVA analysis (Figure 1G). Analysis of LTP induction and
Figure 2. SRR is downregulated in neurons and upregulated in astrocytes after CCI injury. (A) CLARITY image of GFAP-labeled astrocytes (red) and GFP-expressing neurons (green) in Thy1-GFP transgenic mice shows close association of astrocytes and CA1 dendrites in the stratum lucidum/radiatum of the hippocampus. High-magnification images (B) and astrocyte-neuron membrane association (yellow; arrow) in stratum lucidum/radiatum (C). Scale bar: 25 μm. (D) SRR expression using Western blot analysis at 24 hpi is not altered in the hippocampus. (E) D-serine levels using chemiluminescence analysis show increased levels at 4 and 24 hpi. (F) Immunohistochemical expression of SRR was reduced in pyramidal cell layer at 3 (G) and 7 (H) dpi, but increased in cells outside that region as compared with sham-treated controls (F). Scale bar: 25 μm. SRR-labeled cells outside the pyramidal cell layer (K) colabel with the astrocytic marker (J) GFAP. No crossreactivity observed in secondary only controls (L and M). Scale bar: 25 μm. (I) SRR levels were reduced at 3 dpi and returned to basal levels by 7 dpi using Western blot analysis. (N) Purification of hippocampal astrocytes from GFAP-GFP mice showed GFP-positive cells were only observed in isolated GLAST+ isolated cells. (O) Isolated GLAST+ astrocytes express increased levels of SRR at 7 dpi using Western blot analysis. (P) SRR was reduced in the aSRKO hippocampus at 7 dpi using Western blot analysis. (Q) SRR was increased in the nSRKO hippocampus at 7 dpi using Western blot analysis. Data represent mean ± SEM. (D, E, I, O–Q) n = 4–6/group. Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001, compared with sham-treated controls. §§P < 0.01, compared with 3 dpi. For Western blot images, lanes were run on the same gel, but lanes separated by a line were noncontiguous (see Supplemental Figure 2).
maintenance showed significant reduction in both early (P < 0.001) and late-phase (P < 0.001) LTP in nSRKO mice as compared with cSR and aSRKO sham-treated mice (Figure 1, H and I). These in vivo observations strongly support a predominant role for phasic release of neuronal D-serine in synaptic plasticity.

CCI injury leads to a cellular switch in SRR expression in the hippocampus. Astrocytosis is known to result in a spectrum of morphological and molecular changes that can influence tissue pathology; however, the effects of reactive astrocytes on synapse function after TBI remain poorly resolved. In the hippocampal tripartite synapse, CLARITY imaging showed close interactions of astrocytes with synaptic membranes originating from CA1 pyramidal neurons (CA1) (Figure 2, A–C), CA3 pyramidal neurons (CA3), and dentate granule (DG) cells. To examine the role of D-serine in the traumatic injured brain, we had recently developed a CCI injury model that resulted in synaptic damage in the absence of hippocampal neuron cell death (27). This model enables us to examine the mechanisms of synaptic damage independently of neuronal loss.

In the CCI-injured hippocampus, Western blot analysis showed little change in total SRR levels at 24 hours post CCI injury (hpi) as compared with those in the sham-treated controls (Figure 2D and Supplemental Figure 2), but chemiluminescence analysis of total D-serine levels showed a significant increase at 4 and 24 hpi (Figure 2E). This increase in D-serine likely represents an acute injury–induced conversion of L- to D-serine in neurons. At 3 and 7 days post-CCI injury (dpi), SRR expression patterns and levels changed dramatically (Figure 2, F–H). SRR expression was visibly decreased, by more than 50%, in the hippocampal pyramidal cell layer (Figure 2, G and H; arrowheads) as compared with sham-treated controls (Figure 2F). Western blot analysis showed an approximately 25% decrease (P < 0.01) in total SRR levels at 3 dpi (Figure 2I). This discrepancy most likely is the result of increased SRR expression in astrocytes outside the pyramidal cell layer, including regions of the stratum oriens and lucidum/radiatum (Figure 2G; arrows). At 7 dpi, expression in the pyramidal cell layer remained low (Figure 2H; arrowheads), while greater numbers of glial cells expressed SRR (Figure 2H; arrows).

Western blot analysis showed that SRR expression returned to preinjury levels by 7 dpi, representing a significant increase (P < 0.01) from 3 dpi (Figure 2I). Confocal imaging demonstrated that the majority of SRR-positive cells outside the pyramidal cell layer colocalized with GFAP-positive astrocytes (Figure 2, J and K), which was not observed in controls using only the secondary antibody (Figure 2, L and M).

To confirm that SRR protein levels were increased specifically in astrocytes after CCI injury, we column purified (>98% pure) astrocytes using glutamate-aspartate transporter (GLAST) antibodies (Figure 2N and Supplemental Figure 4) followed by Western blot analysis for SRR. We observed a 21% increase (P < 0.001) in SRR levels at 7 dpi in astrocytes isolated from the CCI-injured hippocampus as compared with sham-treated controls (Figure 2O). SRR levels at 7 dpi in the aSRKO whole hippocampus were reduced (P < 0.001) compared with those in sham-treated controls (Figure 2P), lending greater support for switch in the cellular expression pattern of SRR after CCI injury. In whole hippocampal tissues isolated from nSRKO mice (Figure 2Q), where SRR levels had been shown to be reduced in neurons at 7 dpi (Figure 2H), we observed an increase similar to that observed in WT mice (Figure 2O). Taken together, these findings support our conclusion that TBI results in a downregulation of neuronal SRR and upregulation of astrocytic SRR levels within the first week after injury.

CCI injury increases astrocyte-derived D-serine levels. To demonstrate that increased astrocytic SRR expression leads to an increase in D-serine synthesis, we examined hippocampal D-serine in the presence and absence of astrocyte-derived SRR (Figure 3). Similarly to what occurred with SRR levels, we observed a significant reduction in D-serine levels at 3 dpi in WT mice using HPLC (0.108 ± 0.018 μmol/g tissue; P < 0.05) as compared with sham-treated controls (0.184 ± 0.025 μmol/g tissue) (Figure 3A). Chemiluminescent assay of D-serine supported our HPLC observations at 3 dpi,
and by 7 dpi, d-serine levels were significantly increased \( (P < 0.001) \) as compared with those at 3 dpi (Figure 3B). Examination of isolated astrocytes from WT mice showed a 20% increase \( (P < 0.05) \) in d-serine levels at 7 dpi (Figure 3C), which was similar to whole hippocampal d-serine levels \( (P < 0.01) \) observed in CCI-injured nSRKO mice compared with sham-treated controls (Figure 3D). We next compared aSRKO mice with cSR control mice (Figure 2B), where both received tamoxifen (TAM) treatment 2 weeks prior to injury. In cSR mice, HPLC analysis showed a 2-fold increase \( (0.363 \pm 0.015 \mu \text{mol/g tissue}; \ P < 0.001) \) in hippocampal d-serine levels at 7 dpi as compared with cSR sham-treated \( (0.176 \pm 0.011 \mu \text{mol/g tissue}) \) mice (Figure 3E). In aSRKO mice, no significant differences were observed between sham-treated \( (0.206 \pm 0.026 \mu \text{mol/g tissue}) \) and CCI-injured \( (0.253 \pm 0.026 \mu \text{mol/g tissue}) \) mice, whereas CCI-injured aSRKO mouse d-serine levels were significantly \( (P < 0.01) \) less than levels of CCI-injured cSR mice (Figure 3E). This was further supported by examination of \( Srr \) mRNA in aSRKO tissues, where \( Srr \) mRNA levels were similar between sham-treated and CCI-injured mice in 7 dpi WT tissues, but reduced in CCI-injured aSRKO mice (Supplemental Figure 3). No significant changes were observed in amino acid oxidase mRNA levels, such as D-amino acid oxidase (DAO) and amine oxidase, copper-containing 1 (AOC1) enzymes, between WT and aSRKO sham-treated mice and 7 dpi tissues (Supplemental Figure 3), suggesting that changes in SRR and not large reductions in d-serine–metabolizing enzymes underlie CCI injury–induced increases in d-serine. Taken together, our observations confirm that CCI injury results in a switch in SRR (Figure 2) and d-serine (Figure 3) expression in the hippocampus from neurons to astrocytes over the first week after injury.

Since glutamate serves as the NMDAR agonist, glutamate levels were examined in CCI-injured mice to determine whether its expression was altered in aSRKO mice. HPLC analysis showed no significant differences in glutamate levels between groups or after CCI injury \( (\text{cSR sham, } 10.96 \pm 0.31 \mu \text{mol/g tissue}; \text{cSR CCI, } 13.42 \pm 0.71 \mu \text{mol/g tissue}; \text{aSRKO sham, } 11.41 \pm 0.67 \mu \text{mol/g tissue}; \text{aSRKO CCI, } 12.87 \pm 0.93 \mu \text{mol/g tissue}) \) (Figure 3F), which is similar to what we have previously reported for WT mice at periods between 4 hpi and 7 dpi (27). Although our findings suggest that CCI injury or alterations in d-serine expression do not change total glutamate levels, we cannot exclude the possibility that glutamate homeostasis may be affected during the first week after injury.

Reducing astrocytic d-serine spares the hippocampus from CCI injury–induced dysfunction. We next determined whether inhibiting astrocytes from producing d-serine (i.e., aSRKO mice) altered synaptic transmission and plasticity after CCI injury when reactive astrocytes appeared. Consistent with our observations using WT mice (27), CCI injury in cSR control mice led to a significant downward shift in fEPSP amplitude \( (P < 0.01) \) at higher stimulus intensities \( (40–60 \mu \text{A}) \) as compared with cSR and aSRKO sham-treated controls (Figure 4A). These previous studies had shown that synaptic damage and losses and not neuronal loss from CCI injury underlie the observed deficits in synaptic transmission and plasticity (27). Similar trends were also observed in PPF between groups, where the PPF ratio was attenuated in CCI-injured cSR mice at shorter interstimulus intervals as compared with cSR and aSRKO controls (Figure 4B). Interestingly, we did not observe a significant reduction in the I/O curve or PPF in CCI-injured aSRKO mice, although there was a downward shift in PPF ratio curve compared with sham-treated controls.

We next evaluated synaptic plasticity in the presence and absence of astrocytic d-serine. Two-way repeated measures (RM) ANOVA of normalized fEPSP after high-frequency stimulation (HFS) showed significant interaction \( (P < 0.001) \) as well as a significant main effect for animal groups \( (P = 0.0203) \) and time \( (P < 0.001) \). In vivo HFS of sham-treated cSR mice resulted in LTP that was not observed in CCI-injured cSR mice (Figure 4C). Conversely, aSRKO sham-treated and CCI-injured mice both had a significant synaptic potentiation following in vivo tetanic stimulation (Figure 4C), where CCI-injured aSRKO mice had significantly greater potentiation \( (P < 0.05) \) than CCI-injured cSR mice. Our results suggest that astrocyte-derived d-serine plays a substantial role in the synaptic dysfunction observed following CCI injury. Analysis of LTP induction and maintenance in CCI-injured cSR mice showed a large reduction in both early \( (P < 0.001) \) and late-phase \( (P < 0.001) \) LTP as compared with sham-treated cSR mice (Figure 4, D and E). A smaller reduction in early phase LTP was observed in sham-treated and CCI-injured aSRKO mice; however, this was not maintained in the late LTP phase. Comparison of late-phase LTP between CCI-injured cSR and aSRKO mice showed significantly higher potentiation \( (P < 0.001) \) in mice deficient in astrocyte-derived SRR. Together, these findings demonstrate that the release of d-serine from astrocytes underlies TBI-induced synaptic dysfunction and that blocking this d-serine source is neuroprotective.

Examination of hippocampal field potential oscillations in vivo in CCI-injured mice showed a significant interaction between frequencies and experimental groups using 2-way ANOVA analysis \( (P = 0.002) \). Examination of \( \theta \) oscillations at the 4- to 5-Hz frequency range showed significantly greater \( (P < 0.001) \) power in CCI-injured cSR mice as compared with that in all the other groups (Figure 4F). Additionally, power at the 4- to 5-Hz range in CCI-injured aSRKO mice was substantially reduced as compared with that in CCI-injured cSR mice at 7 dpi. Our results suggest that CCI injury leads to an enhancement in \( \theta \) rhythms, which likely involves d-serine release and activation of NMDARs.

To verify whether our observed physiological effects translate into behavioral responses, we examined hippocampal-associated learning and memory using a fear-conditioning paradigm (Figure 4, G and H). Following CCI injury, all groups were habituated at 5 dpi, trained at 6 dpi, and tested at 7 dpi. No significant differences were observed in freezing behavior during the training periods, while significant interactions and main effects were observed among groups \( (P = 0.0084) \) and on test days compared with training days \( (P < 0.001) \). CCI injury resulted in an approximately 50% reduction in contextual (Figure 4G) and cue (Figure 4H) freezing behavior in CCI-injured cSR mice as compared with in sham-treated cSR mice. Contextual freezing behavior was significantly increased \( (P < 0.05) \) in sham-treated aSRKO mice as compared with that in sham-treated cSR mice, and although the basis for this enhanced freezing response is unclear, it may result from other limbic structures not examined in our analysis. What is clear is that CCI-injured aSRKO mice were not significantly different from sham-treated aSRKO mice (Figure 4G).
of CCI-injured aSRKO mice with CCI-injured cSR mice showed significantly higher ($P < 0.001$) contextual freezing behavior in the former. Similar but nonsignificant trends ($P = 0.0678$) between CCI-injured cSR and aSRKO mice were observed in cued freezing behavior. These behavioral studies support our observations of synaptic plasticity and together suggest that astrocyte-derived d-serine likely underlies the synaptic dysfunction and/or damage associated with reduced synaptic plasticity.
Increasing d-serine can reverse the enhanced synaptic potentiation observed in aSRKO mice after CCI injury. To validate our conclusion that CCI injury–induced synaptic dysfunction is aggravated by increased d-serine levels observed in the CCI-injured aSRKO mice, we examined CA3-CA1 hippocampal fEPSPs in vivo in CCI-injured aSRKO mice after 7-day infusion of vehicle or d-serine (840 μg/kg/d) into the contralateral lateral ventricle (Figure 5). d-serine infusion into CCI-injured aSRKO mice resulted in reduced basal synaptic transmission as revealed by a downward shifted I/O curve at stimulation intensities of 30 μA or higher compared with that in vehicle-infused controls (Figure 5A). No significant differences were observed in PPF between groups (not shown); however, d-serine infusion reversed the significant potentiation after HFS observed in aSRKO CCI-injured mice (Figure 5B) to levels similar to those observed in CCI-injured cSR mice (Figure 4C). These findings demonstrate that high levels of d-serine in the injured hippocampus are detrimental to synaptic transmission and plasticity.

Discussion

TBI is a dynamic and progressive disorder that can lead to the disruption of brain circuitry that underlies many of the functional deficits observed in TBI patients. One component prevalent in all brain injuries, ranging from mild to severe, is synaptic damage. In regions where synaptic plasticity is important for cognitive function, glial cells play critical roles in modulating synaptic transmission and plasticity. In fact, several proteins have been implicated in glial regulation of synaptic functions after TBI, such as matrix metalloproteinases, astrocytic signal transducers and activators of transcription, and the calcineurin-dependent transcription factor nuclear factor of activated T cells (NFAT) (4–6). Here, we show that astrocytes in the tripartite synapse are active participants in promoting synaptic damage following CCI injury, where enhanced d-serine release leads to slow excitotoxic synaptic damage in the hippocampus. In nonpathological conditions, d-serine is produced and released by neurons to regulate the resting state of NMDAR activation. In these conditions, a phasic release of d-serine by neurons elicits LTP. CCI injury results in reduced synaptic transmission, plasticity, synchrony, and memory as a result of increased astrocytic d-serine release. Astrocyte-specific gene-targeted deletion of Srr restored synaptic responses and learning, which can be reverted back to injury states by d-serine administration.

In nonpathological conditions, glutamate release can activate both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and NMDARs, where NMDARs play a regulatory role in basal synaptic transmission (29, 30). Unlike AMPARs, NMDARs require multiple inputs, including binding of the coagonist d-serine and Ca^{2+} permeation, which are essential components of long-lasting alterations in synaptic strength. A monophasic electrical pulse can activate AMPARs at low intensities; however, high intensities are known to activate both AMPARs and NMDARs (28). HFS might selectively increase d-serine levels by stimulating release from the neuronal pool, which is reduced in neuronal SRR–deficient mice, leading to deficits in synaptic plasticity. These findings are supported by studies in which stimulating d-serine release from neurons enhanced LTP in mouse and rat hippocampal slices (31) and neuron-specific deletion of the Srr gene led to reduced spine density and dendritic arborization (32) as well as LTP deficits (14). To date, several groups have confirmed that SRR is expressed by inhibitory and excitatory neurons throughout the mammalian brain (12, 19, 33), where neuronal d-serine is mainly released in response to depolarization (20).
An important component of our study was to employ a murine model of synaptic damage in the absence of neuronal loss in order to directly evaluate the mechanisms that regulate synaptic dynamics within the complex and evolving TBI environment. Our CCI injury model produces a moderate cortical injury with only mild damage to subcortical tissues, such as the hippocampus, where synaptic damage and loss within the hippocampus underlie learning and memory deficits (27). This enabled us to examine discrete and reversible synaptic responses while maintaining the overall environmental characteristics associated with TBI, including distal tissue losses (34), reactive gliosis (27), a compromised blood-brain barrier, and inflammatory responses (35). The presence of astrocytosis in the CCI-injured hippocampus allowed us to examine how gliotransmitters from reactive astrocytes may regulate synaptic function and/or dysfunction. It has been well documented that astrocytes release glutamate minutes following TBI (8, 36), which leads to an acute toxic injury and cell death in severely compromised tissues (i.e., glutamate excitotoxicity). NMDARs are also involved in slow excitotoxicity that occurs over hours and days after injury and have been associated with enhanced D-serine levels (37, 38). While we cannot exclude the possibility that D-serine contributes to excitotoxic cell death in regions of severe injury, our studies do support a direct role for astrocytic D-serine in slow excitotoxic synaptic damage in the CCI-injured hippocampus. What is less clear is whether increased astrocytic D-serine is sufficient to induce synaptic damage independently of neuronal D-serine reductions that occur after CCI injury. Importantly, our findings in astrocytic SRR-deficient mice, where synaptic plasticity and learning behaviors are restored to preinjury levels, support this possibility. We also cannot exclude the possibility that glutamate participates in D-serine–mediated slow excitotoxicity. Total hippocampal glutamate levels are not altered between 4 hours and 7 dpi (27), but potential alterations in glutamate release and reuptake may participate in our observed delayed synaptic damage. Overall, it is clear that astrocytic D-serine release is a key player in mediating synaptic damage after CCI injury.

Learning involves an intricate interplay of a multitude of neural circuits in response to an experience. Within the hippocampus, θ oscillations are known to facilitate memory processes, although the exact mechanisms are not completely understood. TBI can result in a decrease in power in the θ frequency band (39, 40). However, changes in θ frequency after TBI are dependent on the severity of the injury (41). It is important to note that hippocampal θ rhythms occur at different frequencies dependent on the state of wakefulness of the animal. The higher frequency rhythm (7–9 Hz) occurs when animals are exploring and has been termed type 1, while the slower type 2 rhythm (3–5 Hz) occurs when animals are in a quiescent state of sensory processing (42). Only type 2 θ is observed in animals under urethane anesthesia (43), which was used in the present study. Thus, the increase in hippocampal field oscillation power observed here is the first report, to our knowledge, of the effect of TBI on type 2 θ, as other studies of TBI were conducted in awake, freely moving animals. Curiously, progressive cognitive dysfunction, such as in Alzheimer’s disease (AD) or Parkinson’s disease (PD) models, has been associated with an increase in hippocampal θ power at rest as opposed to a decrease in θ power after severe TBI (44, 45). In this regard, our mild-to-moderate CCI model involving synaptic damage without neuronal losses is more akin to models of progressive synaptic dysfunction, such as AD models, where injury results in a marked increase in θ power associated with increased astrocytic D-serine levels. Alternatively, impaired NMDAR activation on parvalbumin-positive interneurons can result in the changes observed (44).

The role of synaptic and extrasynaptic receptors in neuronal cell death is currently a matter of intense debate, where excitotoxicity is thought to involve extrasynaptic NMDARs (36, 49–51). We did not determine whether reactive astrocytic D-serine activates synaptic and/or extrasynaptic NMDARs after CCI injury, but we have shown that the NMDAR GluN2B subunit is elevated in our CCI injury model (27). GluN2B subunits are known to segregate outside the synapses, whereas GluN2A subunits are confined to the postsynaptic membrane (11, 25, 52, 53). Previously, D-serine has been shown to preferentially bind GluN1/GluN2A-containing NMDARs in synaptic sites, whereas glycine may preferentially bind GluN1/GluN2B-containing receptors in the extrasynapse (54). However, coagonist binding with a particular NMDAR subunit is not a strict rule. NMDAR heterotrimers can also exist, containing GluN1 and both GluN2A and GluN2B subunits (30, 55). At this time, the synaptic segregation of NMDAR subunits in our CCI injury model is unclear and additional studies are needed to determine whether enhanced astrocytic D-serine activates GluN2B-containing extrasynaptic NMDARs.

In summary, our findings demonstrate that excessive release of the gliotransmitter D-serine from reactive astrocytes may regulate slow excitotoxic damage to the brain’s circuitry that underlies some of the cognitive dysfunction associated with TBI. Further advances on the role of gliotransmission in regulating synaptic stability, function, and regeneration will provide the foundation for developing therapeutic strategies to prevent TBI neuronal damage and enhance TBI patient recovery.

Methods

Animals. The generation of mutant mice involved breeding mice in which the first coding exon of SRR had been flanked with loxP sites (Srrfl/fl) as previously described (18, 56), bred with either Thy1-CreERT2 mice (JAX Mice, Jackson Laboratory, 012708; Tg[Thy1-cre/ERT2, EYFP]HGfng/PyngJ) to eliminate SRR in neurons (i.e., nSRKO) or human GFAP (Gfap-CreERT2 mice to eliminate SRR in astrocytes, i.e., aSRKO). Cre recombinase was induced between 2 and 4 weeks prior to experimentation through i.p. injections of 120 mg/kg TAM (Sigma-Aldrich) at a volume of 2 ml/kg for 10 consecutive days. TAM was dissolved in a vehicle of 90% sunflower oil and 10% ethanol. All animals received TAM treatment at least 2 weeks prior to any experimental procedure unless otherwise stated, including control Srrfl/fl.
mice without Cre-recombinase (cSR). All mice were maintained on a C57BL/6 background, group-housed under a 12-hour light/12-hour dark cycle, and given free access to food and water.

**CCI injury and perfusion.** All surgical procedures were performed using an i.p. injection of ketamine-xylazine cocktail (100 mg/kg/10 mg/kg, respectively) to anesthetize the mice, and animals were maintained on a heating pad for the duration of the procedure. A small skin incision was made to expose the skull, and mice were placed in a stereotaxic frame to provide accurate coordinates for CCI injury. Mice were subjected to a moderate CCI injury at a velocity of 4 m/s, 0.50 mm depth, and 150 ms duration using an eCCI-6.0 device (Custom Design & Fabrication), where a hemispherical (rounded) 1.3-mm tip piston was placed over the right parieto-temporal cortex. Coordinates of the injury epicenter were ~2.0 mm from bregma and lateral ~2.5 mm, and a 5-mm craniotomy was performed using a small drill bit without disturbing the dura tissue layer. Skin was closed using 5-0 Vicryl synthetic absorbent sutures; mice were placed on a heating pad and monitored bidaily for 1 week after injury. At the time of harvesting, mice were intracardially perfused with 0.1 M phosphate buffer solution (PBS) (pH 7.4) followed by either 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.4) for immunohistochemical analysis or a glutaraldehyde-PFA fixing solution (3% glutaraldehyde, 1% PFA, 0.2% sodium metabisulfite, and heparin).

**Immunohistochemistry.** Immunohistochemistry was performed as previously described (12). Briefly, 20-μm free-floating brain sections were treated with Schiff’s Reagent (Sigma-Aldrich), followed by washing with 0.1 M hydrochloric acid containing 0.5% sodium metabisulfite for 10 minutes. Sections were then washed 4 times with 0.2% sodium metabisulfite and 1.0% sodium borohydride, followed by blocking solution. Immunostaining with rabbit anti-SRR antibody (1:200, Santa Cruz Biotechnology Inc.) was performed by diluting primary antibodies in blocking solution. For astrocyte colocalization, sections were incubated with biotinylated secondary antibody (ACSA-1) Biotin Antibody (Miltenyi Biotec). Solution was mixed well, incubated at 2–8°C for 10 minutes, and washed by adding 2 ml of buffer and centrifuging at 300 g for 10 minutes. The supernatant was aspirated and 20 μl of Anti-Biotin MicroBeads (Miltenyi Biotec) were added, mixed well, and incubated for 15 minutes at 2–8°C. Cells were washed by adding 2 ml of buffer and centrifuged at 300 g for 10 minutes; supernatant was aspirated, and cells were resuspended in 500 μl of buffer.

Magnetic separation was performed using LS Columns (130-042-401, Miltenyi Biotec) on a MidiMACS Separator (130-042-302, Miltenyi Biotec). Cell suspension was applied to prepared columns, columns were washed with buffer (3 × 3 ml), and flow-through containing unlabeled cells was collected. LS Columns were removed from separator, and 5 ml of buffer was pipetted onto the column and immediately flushed out onto a collection tube by pushing a plunger into the column. Astrocytes from GFAP-Rg reporter mice were magnetically sorted, and the purity of the positive (i.e., labeled) and negative (i.e., unlabeled flow-through) fractions was verified using a Beckman Coulter CytoFLEX S Flow Cytometer with CytExpert software 1.2. The resulting FCS files were analyzed using Beckman Coulter Kaluza 1.3 software.

**Western blot analyses.** Hippocampal ipsilateral tissues from sham-treated and CCI-injured mice were homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (1:100, P8340, Sigma-Aldrich), phosphatase inhibitor cocktail 2 (1:100, P5726, Sigma-Aldrich), and benzamidine nuclease (1:1000, 70664, Millipore Corp.) and allowed to mix in a 4°C rocker for at least 15 minutes.
Tissues were then centrifuged for 20 minutes at 4°C, supernatant was recovered, and samples were diluted and standardized to protein concentrations, with 10–25 μg of total protein per well. Protein samples were separated on an 8%–10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% milk or 5% BSA in 0.1 M PB with 0.1% Tween-20 for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: anti-β-tubulin (1:30000, Sigma-Aldrich), anti-GFAP (1:2000, BD Biosciences), and anti-SSR antibodies (1:800, Santa Cruz Biotechnology Inc.). Membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories; 1:20000, 1:5000, and 1:800 for β-tubulin [catalog T-8535], GFAP [catalog 556327], and SSR [catalog sc-5751], respectively). Bands were visualized using SuperSignal substrate (Thermo Fisher Scientific). Densitometric analysis was performed using ImageJ (NIH). Protein measurements were standardized to β-tubulin and normalized to average WT or control sham signals.

**Ventricular infusion of d-serine.** Alzet pumps (1007D, Alzet) were preloaded to deliver 840 μg/kg/day of d-serine or vehicle for 7 days after implantation. Loaded pumps were connected to a brain infusion catheter and placed in 0.1 M PB at 37°C overnight (27). Immediately after CCI, the infusion device was lowered using a stereotactic holder until the tip of the cannula reached the lateral ventricle (−2.0 mm from dura). The infusion device was secured to the skull without disturbing the incision. A FLUOstar Omega Microplate Reader (BMG Labtech) was used to record chemiluminescence kinetics at 30-second tone (85 dB, 2 kHz) with a 0.7-mA foot shock for the last 2 seconds. At 24 hours after habituation, the training period involved placing the mouse back in the apparatus for 120 seconds followed by a 30-second tone (85 dB, 2 kHz) with a 0.7-mA foot shock for the last 2 seconds. As a control, a 30×21 cm, Coulbourn Instruments) with an electric grid floor (0.8-cm spacing, 4.8-mm diameter rods) for habituation for 600 seconds. After habituation, the training period involved placing the mouse back in the apparatus for 120 seconds followed by a 30-second tone (85 dB, 2 kHz) with a 0.7-mA foot shock for the last 2 seconds.
seconds of the tone. After the shock, mice were maintained in the box for an additional 60 seconds (total duration of 210 seconds). At 24 hours after training, mice were returned to the apparatus and freezing behavior was measured for 300 seconds to assess contextual fear conditioning. Cue fear conditioning was measured 1 hour after contextual fear assessment by measuring freezing behavior for 360 seconds during which the tone (85 dB, 2 kHz) was delivered for the last 180 seconds. The wall coloration, lighting, and scent were changed between contextual and cue assessments, and the box was cleaned with 70% EtOH and an enzymatic cleaner between animals. Freezing behavior was quantified using video-based analysis software (FreezeFrame, Coulburn Instruments).

Statistics. Data were analyzed using Student’s 2-tailed t test for comparison of 2 experimental groups, or 1-way or 2-way ANOVA for comparison of more than 2 groups with Bonferroni’s post-hoc correction. For hippocampal electrophysiological recordings, 2-way ANOVA and repeated measures ANOVA with Bonferroni’s post-hoc correction were used. Data are represented as mean ± SEM.

Study approval. All procedures were performed on male mice 2 to 4 months of age and approved by the University of Miami Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011).

Author contributions
EJP and DJL designed research studies. EJP conducted experiments. EJP, SAT, ZBL, DTB, and TJS acquired data. EJP, SAT, TJS, and DJL analyzed data. JTC and DJL provided reagents and data interpretation. EJP and DJL wrote the manuscript, and SAT, ZBL, DTB, TJS, JTC provided feedback.

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