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RESEARCH ARTICLE

Astroglial gliotransmitters released via Cx43 hemichannels regulate NMDAR-dependent transmission and short-term fear memory in the basolateral amygdala

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Abstract

Astrocytes release gliotransmitters via connexin 43 (Cx43) hemichannels into neighboring synapses, which can modulate synaptic activity and are necessary for fear memory consolidation. However, the gliotransmitters released, and their mechanisms of action remain elusive. Here, we report that fear conditioning training elevated Cx43 hemichannel activity in astrocytes from the basolateral amygdala (BLA). The selective blockade of Cx43 hemichannels by microinfusion of TAT-Cx43L2 peptide into the BLA induced memory deficits 1 and 24 h after training, without affecting learning. The memory impairments were prevented by the co-injection of glutamate and D-serine, but not by the injection of either alone, suggesting a role for NMDA receptors (NMDAR). The incubation with TAT-Cx43L2 decreased NMDAR-mediated currents in BLA slices, effect that was

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; BLA, basolateral amygdala; CFCT, cued fear conditioning training; CS, conditioned stimulus; Cx, connexin; Etd, ethidium; fEPSP, field excitatory post-synaptic potential; mGluRs, metabotropic glutamate receptor; NMDAR, N-methyl-D-aspartate receptor; US, unconditioned stimulus.

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also prevented by the addition of glutamate and D-serine. NMDARs in primary neuronal cultures were unaffected by TAT-Cx43L2, ruling out direct effects of the peptide on NMDARs. Finally, we show that D-serine permeates through purified Cx43 hemichannels reconstituted in liposomes. We propose that the release of glutamate and D-serine from astrocytes through Cx43 hemichannels is necessary for the activation of post-synaptic NMDARs during training, to allow for the formation of short-term and subsequent long-term memory, but not for learning *per se*.

KEYWORDS

astrocytes, basolateral amygdala, Connexin 43 hemichannels, Cx43, d-serine, fear conditioning, HCs, memory, NMDA receptor, short-term memory

1 | INTRODUCTION

The brain primarily consists of two different cell types: neurons and glia. Among glia, the most abundant are astrocytes (or astroglia), which perform various roles in brain function, including the maintenance of osmotic balance,[1] K^+ homeostasis,[2–4] metabolism and transport of glucose and lactate,[5] recycling of glutamate, and gamma-aminobutyric acid (GABA)—the two most abundant neurotransmitters in the brain[6]—and the release of their own bioactive substances (dubbed gliotransmitters) into the synapses.

Astrocytes are metabolically and electrically coupled through gap junction channels (GJCs),[7,8] which are formed by two hemichannels; one from each adjacent cell.[9] Hemichannels are formed by the oligomerization of six connexin (Cx) subunits around a central pore, with Cx43 being the most abundant isoform in astrocytes.[10] Apart from serving as the building blocks for GJCs, hemichannels are found in non-contacting membranes as well, serving as channels allowing the passage of autocrine and paracrine signaling molecules to the extracellular milieu (e.g., ATP, glutamate, NAD^+, and PGE_2), as well as the influx of small molecules and ions (e.g., glucose, cyclic ADP-ribose (cADPR) and Ca^{2+}).[11–17] Evidence from in vitro and ex vivo studies have indicated that the opening of astroglial Cx43 hemichannels participates in the release of ATP[18] and glutamate,[13,18] among other gliotransmitters (for reviews, see Refs. [19,20]). Evidence from ex vivo studies suggests that the astroglial release of gliotransmitters is required to attain synaptic plasticity,[21] particularly through the release of D-serine,[13,22] or via de release of L-serine which is converted into D-serine by neighboring neurons.[23] Several other molecules are released by astrocytes, including glutamate,[24] glycine,[25] lactate,[26,27] and ATP,[12,24,28] all of which are involved in synaptic plasticity and memory,[22,29–34] although none of the later studies ascertained whether these molecules originate from neurons, astroglia, or both.

It has been shown that the inhibition of Cx43 hemichannels in vivo by microinfusion of TAT-Cx43L2 into the basolateral amygdala (BLA) during fear memory consolidation induces amnesia for auditory fear conditioning.[35] TAT-Cx43L2 is a synthetic cell-permeable mimetic peptide that selectively inhibits Cx43 hemichannels, without affecting Cx43 gap junctional communication or synaptic neurotransmitter release,[35] by mimicking the cytoplasmic L2 loop region (119–144 aa) of Cx43.[36,37] The microinjection of the peptide into the BLA had no effects on learning of a cued fear conditioning paradigm, nor did it induce deficits in locomotion or shock reactivity.[35] Notably, co-infusion of the peptide with a mixture of putative gliotransmitters that included glutamate, glutamine, D-serine, glycine, and ATP prevented the amnesic effects of TAT-Cx43L2. The identity of the specific gliotransmitter or combination of gliotransmitters responsible for memory recovery after blockade of astroglial Cx43 hemichannel activity was not determined. However, all the above gliotransmitters are suitable candidates.

Here, we determined the time dependence for the requirement of astroglial Cx43 hemichannel activity at the BLA by testing the effects of Cx43 hemichannel blockade on learning, short-term memory (1 h post-training), and memory consolidation (24 h post-training) of cued fear memory. Cx43 hemichannel blockade before training inhibited short-term memory, but not learning, and had no effect when administered immediately post-training. We also identified potential gliotransmitters released via Cx43 hemichannels that are necessary for memory and characterized their putative mechanism of action. It was found that fear conditioning increases astroglial Cx43 hemichannel activity 1 h after training, as assessed in BLA slices ex vivo, an increase that was no longer measurable 3 h post-training. In addition, we found that Cx43 hemichannel blockade in BLA slices induced a reduction in post-synaptic NMDA receptor transmission, and the co-administration of the peptide with a

mixture of glutamate and D-serine prevented both the amnesic effects caused by Cx43 hemichannel blockade in vivo and the decrease in NMDAR activity ex vivo. Finally, we have shown the permeation of D-serine through purified Cx43 hemichannels reconstituted in liposomes. Together, these results suggest that Cx43 hemichannels mediate the release of glutamate and D-serine from astrocytes, which is necessary for the activation of post-synaptic NMDAR at the BLA during training for fear conditioning, allowing the formation of short-term and subsequent long-term memory, but not necessary for learning *per se*.

2 | MATERIALS AND METHODS

All procedures involving animals were performed in accordance with NIH guidelines and with the approval of the bioethical committee of the Universidad Andrés Bello (Acta 030-2015). Sprague Dawley male rats (~60-day-old, ~250 g) were caged individually at 22°C under a 12/12 h light/dark cycle. The rats remained in their home cage throughout the study and were removed only briefly for drug micro-infusions and behavioral procedures.

2.1 | Auditory fear conditioning

2.1.1 | Apparatus

All behavioral assays were performed in a sound-attenuating cubicle. Training and testing were conducted in different chambers. For auditory fear conditioning, rats were placed in a Plexiglas chamber with a metal grid floor (40 cm × 40 cm × 40 cm), dimly illuminated by a red light. For testing, rats were placed in a different Plexiglas chamber without the metal grid floor (60 cm × 40 cm × 40 cm), dimly illuminated by a white light bulb. A video camera was mounted atop of each chamber for digital recording throughout the experiments and video analysis was performed offline.

2.1.2 | Behavioral procedures

In all the experiments, rats were habituated to handling during the 7 post-surgical recovery days and were habituated to handling and to the training chamber for 3 days prior to the training day, 5 min each day. On the training day, the animals were left in the chamber for 3 min before training. The training consisted of 3 conditioned stimulus-unconditioned stimulus (CS–US) pairings in 60-s intervals. The CS was an auditory stimulus, 5 kHz, 60 dB, 30-s long, which terminated with a 1.5 mA, 1-s long foot shock (the US). See Figure 1A for a general outline of the experimental procedures used for auditory fear conditioning. In all tests, the total time of freezing (immobility) during CS presentations was measured for each rat and shown as a percentage of the total duration of the tone presentation.

2.2 | Acute coronal brain slices, dye uptake, and confocal microscopy

Dye uptake "snap shot" assays were performed as reported previously,[38] with minor modifications. In brief, naïve

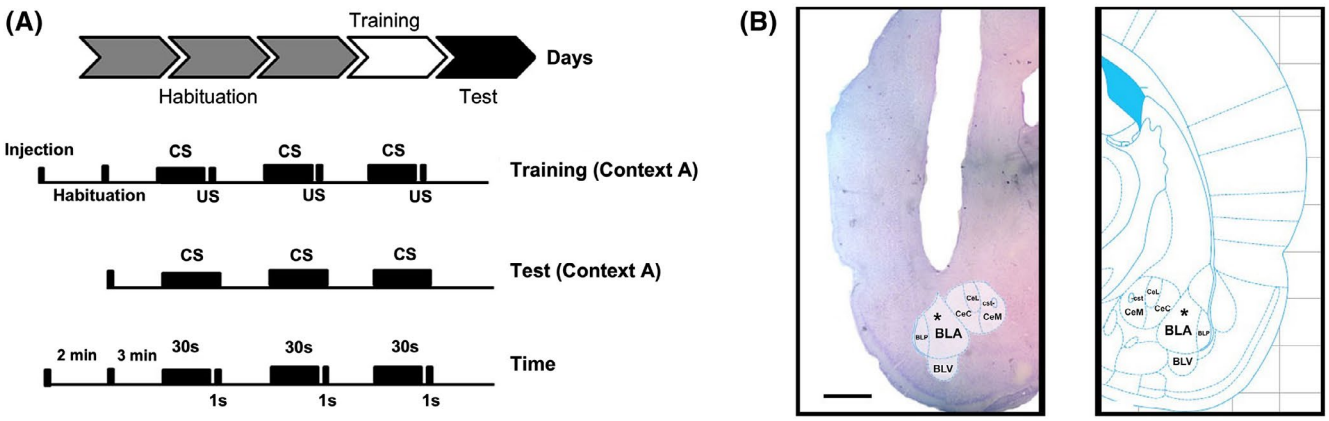


FIGURE 1 Experimental design and histology. (A) General experimental design for cued fear conditioning. Each chevron in the scale at the top indicates a day. The conditioned stimulus (CS) corresponds to a 5-kHz, 30-s tone that terminates with a 1.5 mA 1-s foot shock as the unconditioned stimulus (US). The training protocol is shown in more detail in the second row from the top. The behavioral test is shown on the third row from the top, while the time courses are depicted in the bottom row. Note the change in context between training and test. (B) Representative photomicrograph of a Nissl-stained brain slice after microinjection. The location of the injection cannula tip 1 mm below the end of the guide cannula is shown with an asterisk (*). The basolateral amygdala (BLA), lateral amygdala (LA), and central amygdala (CeA) borders are shown to ease viewing (dashed lines) opposed to a corresponding scheme of implant location. Scale bar: 1 mm

(control) rats and rats that underwent cued fear conditioning training (CFCT) were anesthetized under isoflurane 1 or 3 h after the end of context exposure or CFCT, respectively. They were decapitated and their brains were extracted and cut into coronal slices (300 μm) in an ice-cold slicing solution containing (in mM): sucrose (222); KCl (2.6); $NaHCO_3$ (27); $NaHPO_4$ (1.5); glucose (10); $MgSO_4$ (7); $CaCl_2$ (0.5) and ascorbate (0.1), bubbled with 95% O_2/5% CO_2, using a vibratome (Leica, VT1000GS; Leica, Germany). Then, the slices were transferred to a holding chamber filled with artificial cerebral spinal fluid (ACSF-1), at room temperature (20–22°C), containing (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$, bubbled with 95% O_2/5% CO_2, pH 7.4, for a stabilization period of 60 min before dye uptake experiments.

For dye uptake and ex vivo "snapshot" experiments, the slices were incubated with 5 μM ethidium (Etd) for 10 min in a chamber oxygenated by bubbling 95% O_2/5% CO_2 into ACSF-1. To ascertain that the dye-uptake was mediated by Cx43 hemichannels, some of the brain slices were incubated with 100 μM of the synthetic mimetic peptide TAT-Cx43L2 (YGRKKRRQRRRDGANVDMHLKQI EIKKFKYGIEEHGK) for 15 min before the addition of Etd. TAT-Cx43L2 selectively blocks Cx43 hemichannels,[36,37] so incubation with TAT-Cx43L2 was used to rule out any non-Cx43 hemichannel mediated dye-uptake. Following the 10-min exposure to Etd, the slices were washed three times (5-min each) with ACSF-1 and were fixed at room temperature with 4% paraformaldehyde for 60 min, rinsed once for 5 min with 0.1 mM glycine in PBS, and then twice with PBS for 10 min with gentle agitation. The slices were incubated two times for 30 min each with a blocking solution (PBS, gelatin 0.2%, Triton-X 100 1%) at room temperature and then incubated overnight at 4°C with cell-specific antibodies to identify astrocytes (anti-GFAP monoclonal antibody; Sigma-Aldrich), neurons (anti-NeuN monoclonal antibody; Cell Signaling), and microglia (anti-Iba-1 monoclonal antibody, WAKO). After the incubation with the antibodies diluted 1:1000 in the blocking solution the slices were washed 3 times (10-min washes) with blocking solution. The sections were incubated for 2 h at room temperature with goat anti-mouse Alexa Fluor 488 antibody (1:1.000, Abcam) and after three washes (10-min each) the slices were mounted in Fluoromount, cover-slipped, and examined in a confocal laser-scanning microscope (Olympus Fluoview FV1000). Stacks of consecutive confocal images were taken with a 60× objective at 1-μm intervals, acquired sequentially with two lasers (argon 488 nm and helium/neon 543 nm), and *Z* stack projections were reconstructed using ImageJ software (National Institute of Health, USA). Dye uptake was calculated as: corrected total cell Etd fluorescence intensity (as arbitrary units [AU]) = integrated density − ([area of selected cell] × [mean fluorescence of background readings]). At least six cells per field were selected from at least three fields in each brain slice. The background fluorescence was set as the average value of intensity in three regions of interest (ROI) void of any fluorescence. The laser was set to constant output, assuring that the excitation energy and detector sensitivity were always the same. The fluorescence intensity was always calibrated first in the control slices so that labeled cells remained visible but at low intensity to avoid saturation in the event of an increase in Etd uptake, and the rest of the microphotographs for all experimental groups were taken maintaining the same setting. Imaging results are presented as Etd fluorescence intensity for the 10-min uptake period, comparing the intensity of controls to that of experimental groups.

2.3 | Intracerebral microinjections

The protocol used for intra-BLA microinjections was similar to the one previously reported,[35] with slight modifications. The targeted region (BLA) can be seen in a scheme in Figure 1B.

2.3.1 | Surgery and histology

Rats under ketamine/xylazine/acepromazine anesthesia (60.6 mg/kg; 0.6 and 6.67 mg/kg, respectively) were stereotaxically implanted with bilateral 21-gauge stainless steel cannulas aimed 1.0 mm above the BLA (3.0 mm posterior to Bregma, 4.8 mm lateral to the midline, and 7.5 mm ventral to the skull surface[39]). The cannulas were fixed with acrylic dental cement and secured by 4 skull screws. A 25-gauge stylus was placed inside the guide cannula to prevent clogging. Rats were given 7 days to recover before experimental procedures began. In all experiments, the stylus in the guide cannula was replaced with a 25-gauge injection cannula that extended 1.0 mm beyond its tip, into the BLA. Drugs were simultaneously infused in a volume of 0.3 μl into each hemisphere, at a rate of 0.2 μl/min, through the injection cannula connected via PE20 tubing to Hamilton micro-syringes, driven by a micro-infusion pump. Following drug infusion, injection cannulas were left in place for 1 min to allow drug diffusion away from the cannula tip. At the end of the experiments, animals were anesthetized as above, perfused intracardially with a saline solution, and then with 4% paraformaldehyde buffered in phosphate-buffered saline (PBS). The brains were extracted and post-fixed in 30% sucrose until their density equaled that of the sucrose solution. The brains were sectioned using a cryostat, Nissl stained with Cresyl Violet,

and examined by light microscopy for cannula placement and assessment of histological lesions (tissue damage or gliosis). Animals with histological lesions beyond the size of the cannula tip or guide cannula diameter, as well as animals that did not show implanted cannulas in the correct location were excluded from the analysis. All experiments were performed blinded to the experimenter and blinding was opened once histological assessments were concluded.

2.3.2 | Drugs

TAT-Cx43L2 (Biomatik, Cambridge, ON, Canada, >95% purity) or a mutated version (TAT-Cx43L2$^{H126K/I130N}$) with a similar sequence except for the replacement of histidine 126 with lysine (H126K) and isoleucine 130 with asparagine (I130N), known as TAT-Cx43L2$^{H126K/I130N}$, which renders the peptide inactive,[35,36] were dissolved in PBS to 10 nM. In all experimental series, glutamate (100 mM; SIGMA), D-serine (200 nM, SIGMA), glutamine (100 mM, SIGMA), glycine (100 nM, WINKLER), and ATP (100 μM, SIGMA) were dissolved in sterile saline. All concentrations of gliotransmitters were used in a previous report.[35] For experimental series 1 (individual gliotransmitters), TAT-Cx43L2 was either injected alone, or combined with glutamate, D-serine, glutamine, ATP, or glycine. For experimental series 2 (combinations), the TAT-Cx43L2 peptide was co-infused with a combination of glutamate, D-serine, and glycine, or with glutamine and ATP. For experimental series 3 (combinations with D-serine or glycine), TAT-Cx43L2 was microinjected either alone, or combined with only glutamate, glutamate + D-serine, or glutamate + glycine. For experimental series 4 (short-term memory), TAT-Cx43L2 was microinjected either alone, or combined with D-serine. For experimental series 5 (before or after training), TAT-Cx43L2 was microinjected alone.

2.4 | BLA slice preparation and electrophysiology

Transverse slices (400 μm) from the BLA were cut under cold (~4°C) ACSF-2 using a Vibratome (BSK microslicer DTK-1500E) and incubated in ACSF-2 for 1 h at room temperature. The ACSF-2 used here was different from the ACSF-1 used in the previous experiment and contained (in mM): 124 NaCl, 2.6 $NaHCO_3$, 10 D-glucose, 2.69 KCl, 1.25 KH_2PO_4 2.5 $CaCl_2$, and 2.60 Na_2HPO_4. Slices were transferred to an experimental chamber (2 ml), superfused (3 ml/min, at room temperature) with ACSF-2 bubbled with 5% CO_2 in O_2. The ACSF-2 used for recording was supplemented with 10 μM picrotoxin (PTX, TOCRIS) to suppress inhibitory GABAA transmission, 20 μM AMPAR antagonist NBQX (TOCRIS) to decrease AMPAR-dependent activity, and no Mg^{2+} to unblock NMDAR currents. Slices were visualized by trans-illumination with a binocular microscope (Amscope). Bipolar concentric electrodes (Tungsten, 125 μm OD diameter, Microprobes) connected to an isolation unit (Isoflex, AMPI, Jerusalem, Israel) were used to evoke field excitatory post-synaptic potentials (fEPSPs). The stimulation was performed in fibers identified visually within 100–200 μm from the recording site at the BLA. Recordings were filtered at 2.0–3.0 kHz, sampled at 4.0 kHz using an A/D converter (National Instruments), stored, and analyzed using the WinLTP program.[40,41] Basal excitatory synaptic transmission was measured using an input/output curve protocol, which consisted of 8 stimuli ranging from 200 to 900 μA, with a 10-s interval between stimuli. Solutions of TAT-Cx43L2 (50 nM) with or without glutamate or D-serine were added to the ACSF-2 in the recording chamber. Data were collected and analyzed offline with pClamp 10 (Molecular Devices). To control for potential combinatorial ordinal position effects, the slices were first incubated with TAT-Cx43L2, and then incubated either with glutamate and then D-serine, or D-serine and then glutamate.

2.5 | Primary cortical neuronal cultures

Cortical neuronal cultures were prepared as described elsewhere.[42] Briefly, cerebral cortices from mice pups at P0-1 were dissected in Petri dishes filled with ice-cold PBS. The dissected cortices were disaggregated and digested with 20 U/ml papain for 15 min at 37°C (PAPAIN-022, Worthington). After the incubation, the disaggregated tissue was washed with pre-warmed media and triturated with flame-polished pipettes of progressively smaller bores. Approximately 250 000 cells were used for plating onto each 12-mm diameter round coverslip. After 1 h, the cells were fed with 2 ml of pre-warmed neurobasal medium (Gibco, 21103049) supplemented with B27 (Gibco, A3582801) and kept in an incubator.

2.6 | Calcium imaging

Cortical neurons at 4 DIV were transformed for 72 h using the calcium sensor GCaMP6s under control of the hSyn promoter, delivered through an AAV1/2 virus. Calcium changes were recorded in an epifluorescence microscope (Nikon TE2000e, 20× objective, and Andor Zyla 5.5 camera). Images were taken every 50 ms for 1 min using an

excitation wavelength of 480 nm and emission at 510 nm. For quantification of the frequency of calcium events, the soma of an isolated cortical neuron was selected and analyzed using the "Z profiler" plugin (https://imagej.nih.gov/ij/plugins/z-profiler.html) in ImageJ Fiji software. Each intensity peak was counted manually.

2.7 | Determination of Cx43 expression in the basolateral amygdala

Animals were anesthetized under isoflurane for 1 h after the end of CFCT. They were decapitated and their brains were extracted and placed in ice-cold PBS. The brains were dissected, and the amygdala was extracted under a magnifying glass (KL 1500 LCD) using Dumont No.5 forceps. Expression of Cx43 in amygdalae of naïve (control) rats and rats that underwent CFCT (trained) was determined by immunoblotting. The amygdalae were homogenized for 10 min in ice-cold RIPA buffer (0.1% SDS, 150 mM NaCl, 1% Triton X-100, 1.5% sodium deoxycholate) with protease and phosphatase inhibitor cocktails (Roche, Germany) and proteosome inhibitor MG132 (Sigma–Aldrich, USA) using sterile syringes. The lysates were centrifuged at 10 000 *g* for 30 min at 4°C, insoluble pellets were discarded, and the supernatants were centrifuged again at 10 000 *g* for 30 min at 4°C. Finally, protein concentration was determined in lysate supernatants by the bicinchoninic acid (BCA) method (Santa Cruz Biotechnology, USA). Proteins (10 μg per sample) were resolved on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Thermo Scientific, USA). Cx43 was detected with a primary rabbit polyclonal anti-Cx43 antibody (dilution 1:10 000; Abcam, USA) followed by a secondary HRP-conjugated anti-rabbit IgG antibody (dilution 1:5.000; Invitrogen, USA), and detection with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, USA). Membranes were stripped and re-probed with mouse monoclonal anti-α-tubulin antibody (dilution 1:2000; Millipore, USA) followed by a secondary HRP-conjugated anti-mouse antibody (dilution 1:5.000; Invitrogen, USA) as a control for sample loading. Protein bands were quantified and normalized relative to the loading control band with ImageJ software.

2.8 | Purification and reconstitution of recombinant Cx43 hemichannels

Rat Cx43 followed by a poly-His tag (six His) at its C-terminal was expressed using a baculovirus/insect cell system. Details on the expression and purification of connexin hemichannels have been published previously.[17,43,44] Cx43 was expressed in Sf9 cells, and crude membranes were first alkali-extracted, and then solubilized with 2.5% n-dodecyl-β-D-maltoside (DDM), in 2 M NaCl, 2 mM EGTA, 2 mM DTT, 10% glycerol, 1 mM PMSF and 10 mM glycine/NaOH, pH 8, at a protein concentration <2 mg/ml. The suspension was sonicated with a probe sonicator and then incubated for 2 h with gentle rotation. After ultracentrifugation at 100 000 *g* for 30 min, the solubilized material was diluted with 8 volumes of 5 mM imidazole, 10% glycerol, 1 mM PMSF, 0.03% DDM, and 50 mM HEPES/NaOH, pH 8.0. The diluted samples were loaded onto a Talon Co^{2+} column (Talon Superflow, Clontech) pre-equilibrated with the dilution buffer, washed with 10 column volumes of 10 mM KCl, 10% glycerol, 20 mM imidazole, 0.03% DDM, and 10 mM HEPES/KOH, pH 7.4, and eluted with the same buffer, but increasing imidazole to 250 mM. Purified Cx43 was reconstituted in a mixture of phosphatidylcholine/phosphatidylserine at a 2/1 ratio (w/w) as described previously.[17,45] Briefly, lipids and purified Cx43 protein in detergent were mixed at a protein/lipid ratio of 1/30 (w/w), and the mixture was run through a size-exclusion column (Zeba, Thermo Fisher Scientific, Waltham, MA) pre-equilibrated with 50 mM KCl, 0.05 mM EGTA, and 25 mM Tris/HCl, pH 7.6. The samples were then extruded through a 0.1 μM-pore polycarbonate membrane (Mini-Extruder, Avanti Polar Lipids, Alabaster, AL).

2.9 | Permeation assays of reconstituted Cx43 hemichannels

For the D-serine permeability studies, liposomes and Cx43 proteoliposomes were loaded with 5 mM D-serine (Alfa Aesar, Thermo Fisher Scientific) by 4 freeze-thaw cycles. In each cycle, the samples were placed for 1 min in liquid N_2 followed by 5 min in a bath at 27°C. After loading, extra-liposomal D-serine was removed by gel filtration on a PD10 column (GE Healthcare Life Sciences, Marlborough, MA), and liposomes were extracted once with a methanol/chloroform mixture (1/2; v/v). The upper phase was dried overnight and resuspended in 50 mM KCl, 0.05 mM EGTA, and 25 mM Tris/HCl, pH 7.6. The amount of D-serine on the samples was determined by liquid chromatography-mass spectrometry analysis on a Q-EXACTIVE HF-Orbitrap mass spectrometer, Vanquish UHPLC system (Thermo Fisher Scientific) with an Unison UK-Amino column (2 × 250 mm, 3 μm particle size) at the Center of Biotechnology and Genomics of Texas Tech University (Lubbock, TX). Permeation of the fluorescent probes Alexa Fluor 350 hydrazide (AF350) and Alexa Fluor 647 hydrazide (AF647) was assayed as described elsewhere.[17,43] Liposomes and proteoliposomes

were loaded with 250-μM AF350 (Mr 349) and 125-μM AF647 (Mr 1200) as permeation controls, following the procedure described above for D-Ser loading. The probes associated with liposomes and proteoliposomes were determined by fluorescence detection (1200 Series Fluorescence Detector, Agilent Technologies, Santa Clara, CA) during size-exclusion chromatography of the samples (SRT-C SEC-300, 4.6 × 150 mm, Sepax Technologies, Newark, DE) using excitation and emission wavelengths of 345 and 445 nm for AF350, and 650 and 670 nm for AF647. For each experiment, the probe retained in Cx43-proteoliposomes was normalized to the value obtained from liposomes.

2.10 | Data analysis

Data are expressed as means ± SE. All data sets were tested for normality. A *t*-test was used for comparisons between two groups. For multiple comparisons, statistical differences were assessed by a two-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test. Differences were considered significant when $p < .05$, and *p*-values in the text are written as either $^{***}p < .001$, $^{**}p < .01$, or $^{*}p < .05$.

3 | RESULTS

For a general outline of the experimental procedures used for auditory fear conditioning see Figure 1A and a scheme of the targeted region (BLA) can be seen in Figure 1B.

3.1 | CFCT triggers an increase in astroglial Cx43 HC activity at the basolateral amygdala 1 h, but not 3 h post-training

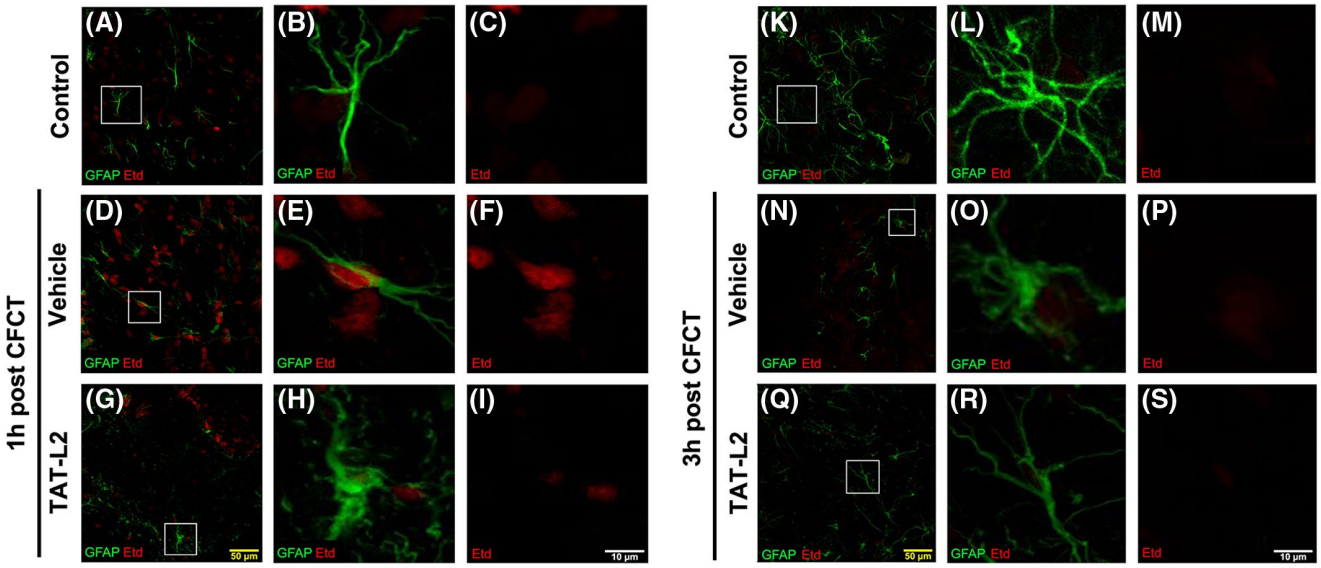
Previous studies have shown that blockade of Cx43 hemichannels in the BLA induces amnesia for auditory fear conditioning,[35] suggesting that Cx43 hemichannel activity, likely in astrocytes, is crucial for fear memory consolidation. However, it is still unknown whether training for auditory fear conditioning induces the activation of astroglial Cx43 hemichannels in the BLA. To address this issue, we evaluated hemichannel activity in GFAP-positive astrocytes at the BLA by performing Etd uptake "snapshot" experiments in brain slices 1 and 3 h after CFCT. In live cells, Etd uptake has been used for decades to measure the activity of hemichannels. Hence, when brain slices are incubated with the tracer, Etd uptake occurs in different brain cells. As astrocytes abundantly express Cx43[10] and Etd uptake in GFAP-positive astrocytes is prevented by Cx43 hemichannels blockers, it is used as a surrogate for astroglial Cx43 hemichannel activity.[38] In the present study, to ensure that the uptake was indeed Cx43 hemichannel-dependent, we preincubated the BLA slices with the TAT-Cx43L2 peptide, and the Etd uptake was prevented. Tracer uptake snapshot experiments have been previously used to determine the effects of acute and chronic stress on hippocampal Cx43 hemichannel activity,[38] astroglial hemichannel-dependent neuronal death evoked by Aβ-peptide,[46] effects of experimental brain abscess[47] and metabolic inhibition[48] on astroglial hemichannel activity, among many other studies.

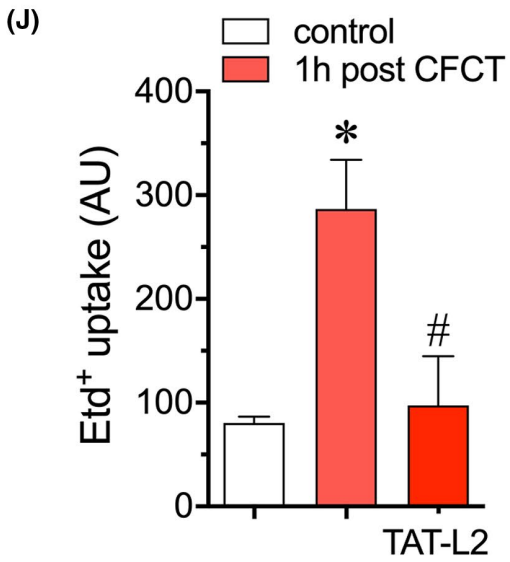
Using this method, we found that CFCT triggered a threefold increase of Etd fluorescence intensity in GFAP-positive astrocytes in the BLA, compared to control (untrained) rats, an effect that was observed 1 h, but not 3 h post-training (Figure 2). The intensity of Etd fluorescence (as measured in arbitrary units, AU) was significantly increased in GFAP positive astrocytes from the BLA in trained animals 1 h after CFCT (Figure 2D–F,J, 1 h post-CFCT; 287 ± 47, $n = 3$) when compared to control untrained rats (Figure 2A–C,J, control; 81 ± 6, $n = 3$). This increase in hemichannel activity was completely blunted by incubation with the TAT-Cx43L2 peptide (see Figure 2G–I,J, 1 h post-CFCT + TAT-L2; 97.5 ± 47.5, $n = 3$), suggesting that the increased Etd uptake was mediated by Cx43 hemichannels (Figure 2J). In contrast, there were no differences in Etd uptake between the 3 h post-training (Figure 2N–P,T; 3 h post-CFCT; 107 ± 25, $n = 10$) and the untrained (Figure 2K–M,T, control; 91 ± 25, $n = 10$) groups. Incubation with TAT-Cx43L2 in both untrained rats and 3 h after training had no effect on Etd uptake (Figure 2Q–S,T, 3 h post-CFCT + TAT-L2; 79 ± 18, $n = 9$). For the data and statistics see Table S1.

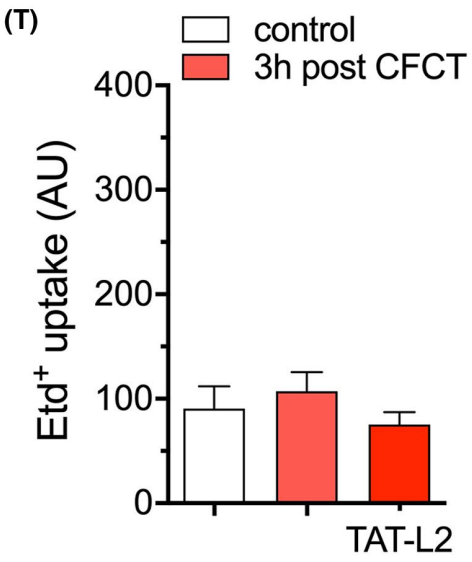
To determine if the increase in Etd uptake was accompanied by a change in Cx43 expression, the amount of Cx43 protein in the BLA was determined through Western blot analysis. As can be observed from Figure 2U,V, CFCT had no effect on Cx43 expression in the BLA 1 h post-training. This suggests that the increase in Cx43 hemichannel activity seen 1 h post-training in the Etd uptake experiments is likely due to an increase in the activity of existing Cx43 hemichannels and not caused by an increment in the number of Cx43 hemichannels. For a summary of the results, see Table S1.

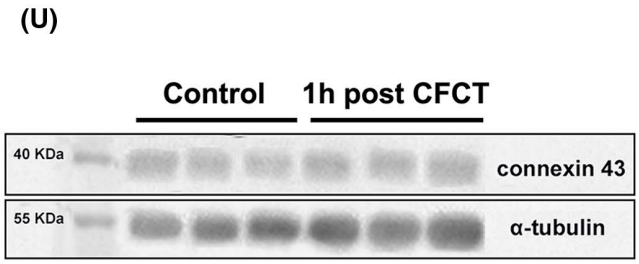
Although we focused on measuring Cx43 hemichannel activity in the BLA in GFAP-labeled astrocytes, we also used NeuN and Iba-1 as markers to identify neurons or microglia respectively, which may show changes in Etd uptake. In general, 85% of the cells that showed Etd uptake

under normal (untrained) conditions and 1 h after CFCT, were GFAP labeled astrocytes. In untrained animals, there was no Etd uptake in neither neurons nor microglia, and 1 h after CFCT, 7% of cells showing Etd uptake were NeuN-positive, equivalent to 2% of NeuN-labeled neurons, suggesting that CFCT can trigger Etd uptake from a low percentage of neurons (for a summary of the data, see Table S2). In the experimental group (1 h after









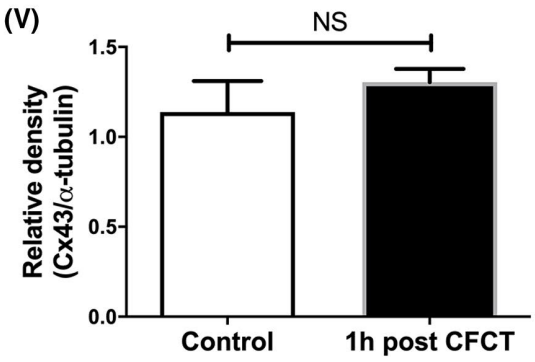


FIGURE 2 Fear conditioning increases ethidium uptake in astrocytes from the basolateral amygdala 1 h, but not 3 h post-training and does not affect Cx43 expression. (A–I, K–S) Representative confocal images of ethidium (Etd) uptake (red) in GFAP positive (green) astrocytes from the basolateral amygdala (BLA) after training. Brain slices correspond to untrained control rats 1 h (A–C) and 3 h (K–M) after context exposure, and rats 1 h (D–F) or 3 h (N–P) after training for cued fear conditioning (CFCT). Slices were incubated with the TAT-Cx43L2 peptide (100 μM), a selective inhibitor of Cx43 hemichannels for 1 h (G–I) or 3 h (Q–S). Zoomed images of BLA astrocytes from the white squares shown in the panels. White arrows point to Etd labeling of the cell nuclei. (J, T) Means ± SE of Etd fluorescence quantified from experiments shown in the confocal images on the left (1 h post-CFCT) (J) and right (3 h post-CFCT) (T). Slices from the 1 h post-training group of animals showed a significant increase in Etd uptake compared to controls (*$p < .05$ for control versus 1 h post-CFCT or 3 h post-CFCT), which was prevented by TAT-Cx43L2 ($^{\#}p < .05$: trained versus 1 h post-CFCT; 3-h post-CFCT + TAT-L2), $N = 3$ per group. The 3 h post-training group showed no significant differences in Etd uptake compared to controls or slices incubated with TAT-Cx43L2; $N = 10$ (control & 3 h post-CFCT) and $N = 9$ (3 h post-CFCT + TAT-L2). Calibration bars: yellow bar = 50 μm; white bar = 10 μm. (U) Example of a Western blot of protein samples from the BLA probed with anti-connexin 43 (Cx43) and anti- α-tubulin antibodies. (V) Summary of the quantification of Cx43 protein expression normalized to the house-keeping protein α-tubulin. Data are means ± SE; NS: $p > .05$; *$p < .05$; $N = 3$ per group

CFCT), given the lack of Etd uptake in microglia (0%), the low uptake in neurons (7%), and the high percentage of GFAP-labeled astrocytes that showed Etd uptake, it is likely that the 8% of unlabeled cells showing Etd uptake are non-GFAP-labeled astrocytes, although a contribution from oligodendrocytes cannot be ruled out. Note that we were not able to determine the identity of the protein that mediates neuronal Etd uptake, as they express different connexins (Cx36, Cx45, and Cx57) and pannexins 1 and 2 (reviewed in Ref. [49]), any of which could mediate their Etd uptake.

3.2 | Co-administration of glutamate and D-serine prevents the amnesic effects of TAT-Cx43L2 on long- (24 h) and short-term (1 h) memory

Cx43 hemichannels are considered one of the main pathways for the release of gliotransmitters from astrocytes (reviewed in Ref. [20]). Consequently, an increase in astroglial Cx43 hemichannel activity in response to CFCT supports the notion that gliotransmitters released via astroglial Cx43 hemichannels are necessary for fear memory consolidation.[35] In that study, we reported that the selective Cx43 hemichannel blocker TAT-Cx43L2 reduced Cx43 hemichannel opening in astrocytes, while not affecting the neuronal release of glutamate or ATP or gap junction mediated inter-astrocyte communication.[35] Furthermore, in the same study, we showed that the infusion of TAT-Cx43L2 into the BLA before training for fear conditioning induced memory deficits 24 h post-training, but did not affect learning, using the same training protocol as used here. The microinjection of TAT-Cx43L2 into the BLA had no effects on locomotion or shock reactivity and had no amnesic effects when microinjected 6 h post-training, suggesting that memory consolidation was affected. Moreover, microinjected rats recovered their capacity to consolidate on subsequent training, tested 64 h after the original training. Several controls were used; the Gap27 peptide, which decreases both Cx43 gap junctional and hemichannel activity, also showed amnesic effects, while a scrambled peptide and a mutated version of the TAT-Cx43L2 peptide that has two mutated residues that prevent its interaction with Cx43 and renders it inactive (TAT-Cx43L2$^{H126K/I130N}$ peptide), had no effects on memory. Then the lowest concentration of TAT-Cx43L2 with amnesic effects was co-injected with a mixture of gliotransmitters, including glutamate, D-serine, glycine, glutamine, lactate, and ATP, which prevented the amnesic effects of TAT-Cx43L2.[35]

In the present study, we decided to identify the specific gliotransmitters that could prevent the amnesic effects of TAT-Cx43L2 from the mixture of gliotransmitters used in Ref. [35]. Hence, we co-infused TAT-Cx43L2 with the putative gliotransmitters via microinjection into the BLA before CFCT, and memory was evaluated 24 h after training (Figure 3A). All animals included in the analysis exhibited successful bilateral implants into the BLA and showed no histological lesions beyond the diameter of the guide and injection cannulas (see Figure 1B for a representative microphotograph and a scheme of the target region). Consistent with what we had previously reported,[35] intra-BLA microinjection of TAT-Cx43L2 (TAT-L2 in the figures) induced amnesia when memory was tested 24 h post-training (Figure 3A), but did not affect learning (Figure S1B). Unexpectedly, none of the putative gliotransmitters co-injected with TAT-Cx43L2 individually (D-serine, glutamine, glycine, ATP, or glutamate) prevented the amnesic effect of the peptide (Figure 3A).

As no individual gliotransmitter could prevent the amnesic effects of TAT-Cx43L2, we decided to determine whether a combination was required. We microinjected TAT-Cx43L2 with two different combinations of putative gliotransmitters: glutamate, D-serine, and glycine, or

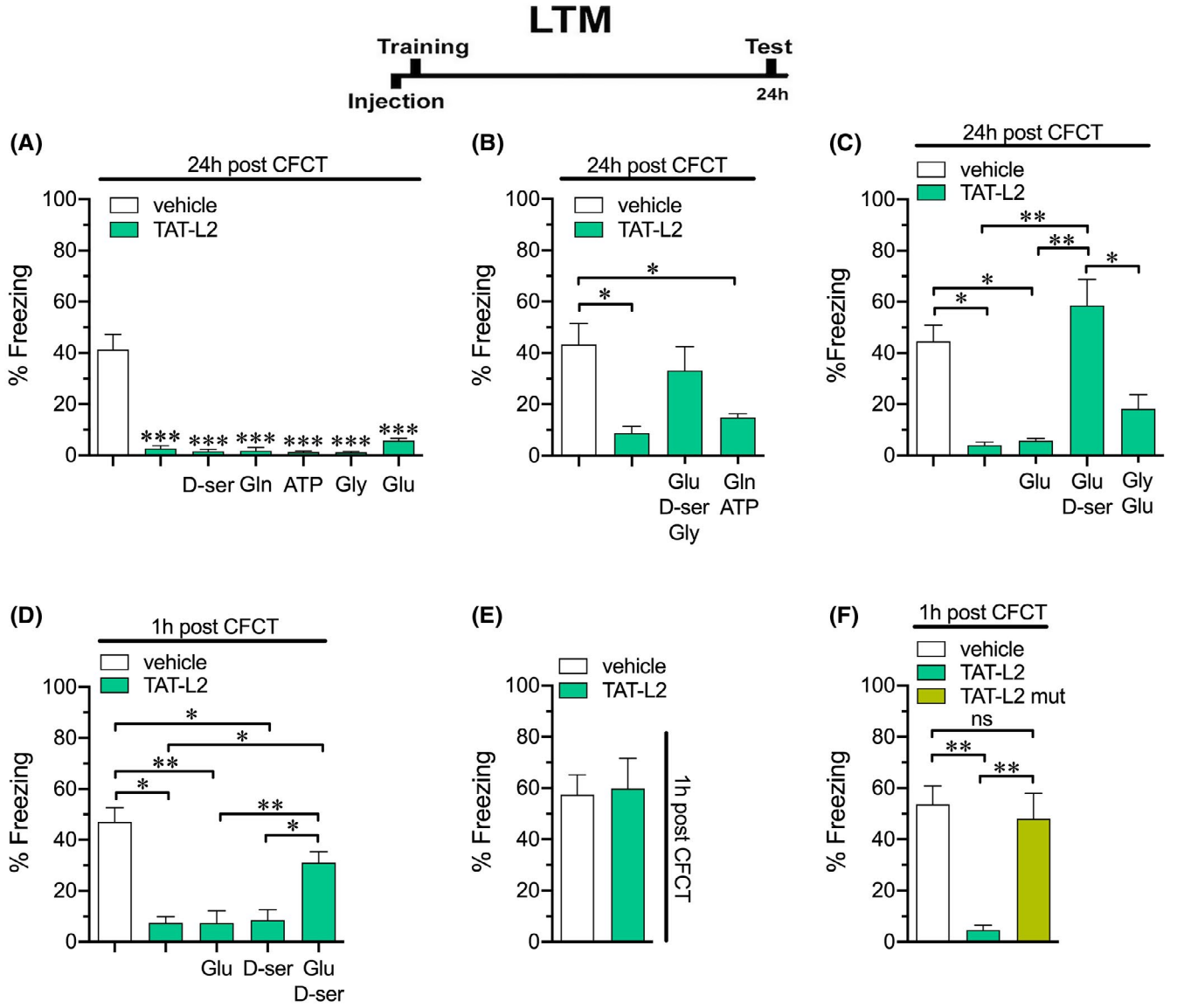


FIGURE 3 A combination of glutamate and D-serine co-injected into the basolateral amygdala prevents TAT-Cx43L2-induced impairments in short- (STM) and long-term (LTM) fear memory. (A) Percentage of freezing time during tone presentation at the test 24 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 20$), TAT-Cx43L2 alone (TAT-L2; $n = 5$) or combined with different gliotransmitters: D-serine (TAT-L2 + D-ser; $n = 7$), glutamine (TAT-L2 + Gln; $n = 5$), glycine (TAT-L2 + Gly; $n = 5$), ATP (TAT-L2 + ATP; $n = 4$) or glutamate (TAT-L2 + Glu; $n = 5$) before training. (B) Percentage of freezing to the tone obtained at the test 24 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 10$), TAT-Cx43L2 alone (TAT-L2; $n = 4$) or with glutamate, D-serine, and glycine (TAT-L2 + Glu + D-ser + Gly; $n = 8$) or glutamine and ATP (TAT-L2 + Gln + ATP; $n = 7$) before training. Note that only the combination of glutamate, D-serine, and glycine prevented the amnesic effects of TAT-Cx43L2. (C) Percentage of freezing to the tone obtained 24 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 15$), TAT-Cx43L2 alone (TAT-L2; $n = 5$) or with glutamate (Glu; $n = 5$), glutamate, and D-serine (Glu + D-ser; $n = 8$) or glutamate and glycine (Glu + Gly; $n = 5$). Note that only the combination of glutamate and D-serine prevented the amnesic effects of TAT-Cx43L2. (D) Percentage of freezing to the tone obtained 1 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 4$), TAT-Cx43L2 alone (TAT-L2; $n = 4$) or combined with glutamate (TAT-L2 + Glu; $n = 4$), D-serine (TAT-L2 + D-ser; $n = 7$) or glutamate and D-serine (TAT-L2 + Glu + D-ser; $n = 7$). Note that the combination of glutamate and D-serine prevented the short-term memory disruption induced by TAT-Cx43L2. (E) Percentage of freezing to the tone obtained 1 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 6$) or TAT-Cx43L2 (TAT-L2; $n = 7$) after cued fear conditioning training. Note that TAT-Cx43L2 had no effects on short-term fear memory. (F) Percentage of freezing to the tone obtained 1 h after training in rats microinfused into the BLA with saline as a vehicle (Veh; $n = 5$), TAT-Cx43L2 (TAT-L2; $n = 5$) or mutated TAT-Cx43L2 (TAT-Cx43L2$^{H126K/I130N}$; TAT-L2 mut; $n = 5$) before cued fear conditioning training. Note that TAT-Cx43L2$^{H126K/I130N}$ had no effects on short-term fear memory. Data are shown as means ± SE; *$p < .05$; **$p < .01$; ***$p < .001$

glutamine and ATP. The amnesic effect of TAT-Cx43L2 was prevented when the peptide was co-infused with glutamate, glycine, and D-serine (Figure 3B), suggesting that these gliotransmitters are sufficient to prevent the amnesic effects of intra-BLA Cx43 hemichannel blockade. In contrast, the combination of glutamine and ATP had no effect (Figure 3B). Again, none of the combinations co-injected into the BLA with TAT-Cx43L2 had effects on learning (Figure S2A–D).

Glutamate can activate multiple receptors, including AMPAR, kainate, and mGluRs,[50–52] but it did not affect TAT-Cx43L2-induced amnesia when co-injected with the peptide by itself (Figure 3A), suggesting that none of those receptors are critically involved. The combination of glutamate, D-serine, and glycine did prevent the amnesic effects of TAT-Cx43L2 (Figure 3B). Hence, involvement of NMDARs is likely, since D-serine and glycine are both NMDAR co-agonists.[53,54] To identify the specific co-agonist involved, we microinjected TAT-Cx43L2 alone or in combination with glutamate and D-serine, or glutamate and glycine. The mixture of glutamate and D-serine, but not that of glutamate and glycine, prevented the amnesic effects of TAT-Cx43L2 (Figure 3C).

Given that blockade of BLA astroglial Cx43 hemichannels by TAT-Cx43L2 impaired fear memory consolidation and that this effect was prevented by co-injection with glutamate- and D-serine, we decided to determine the effects of TAT-Cx43L2 on short-term fear memory. To this end, we microinjected TAT-Cx43L2 into the BLA before training, but tested memory 1 h post-training. Notably, TAT-Cx43L2 induced a total disruption of short-term memory when tested 1 h after training (Figure 3D), again without affecting the learning curve (Figure S3B). To determine whether the mixture of glutamate and D-serine could prevent the impairment in short-term memory induced by TAT-Cx43L2 microinjection, TAT-Cx43L2 was microinjected into the BLA prior to training, alone or in combination with glutamate, D-serine, or a mixture of both. Only the mixture of glutamate and D-serine prevented the amnesic effects of TAT-Cx43L2 (Figure 3D). As with all previous experiments, TAT-Cx43L2 alone or with glutamate, D-serine or their combination had no effects on the learning curve, showing an increase in freezing to the tone (CS) after subsequent CS–US pairings (Figure S3D–F).

To determine whether Cx43 hemichannel activity was required during or after the training, TAT-Cx43L2 was injected in a group of animals immediately after training. Figure 3E shows that Cx43 hemichannel blockade after training had no effects on short-term memory 1 h post-training. These results suggest that the astroglial release of gliotransmitters via Cx43 hemichannels takes place during training and is critical for short-term memory and subsequent memory consolidation, but not necessary for learning per se.

To assess the specificity of the effects of TAT-Cx43L2, we used a control peptide, TAT-Cx43L2$^{H126K/I130N}$, in which two amino acids that are critical for the interaction of the peptide with Cx43 were replaced (H126K/I130N), rendering the peptide inactive.[35,36] In our previous study, the intra-BLA administration of TAT-Cx43L2$^{H126K/I130N}$ had no effect on memory consolidation.[35] Likewise, in the present study, the administration of TAT-Cx43L2$^{H126K/I130N}$ had no effect on short-term memory (Figure 3F) or learning (Figure S3C), suggesting that the effects induced by the peptide are specific for Cx43 hemichannels. To see a summary of the data from Figure 3, see Table S3.

Given that glutamate and D-serine co-activate NMDARs, we tested whether the amnesic effects of TAT-Cx43L2 were associated with a decrease in NMDAR-mediated currents. Accordingly, we determined the effects of Cx43 hemichannel blockade on post-synaptic NMDAR-mediated currents in BLA slices from naïve rats (see scheme in Figure 4A). To unmask NMDAR-dependent activity, rat BLA slices were incubated with picrotoxin (PTX; to suppress inhibitory GABAA transmission), the AMPAR antagonist NBQX (to decrease AMPAR-dependent transmission), and low Mg^{2+} solution (to unblock NMDAR currents). After treatment with TAT-Cx43L2, BLA slices were incubated with either glutamate or D-serine, followed by an additional exposure to either D-serine or glutamate, respectively (see scheme in Figure 4B). TAT-Cx43L2 induced a significant decrease in NMDAR-mediated currents, an effect that was reversed by the combination of D-serine and glutamate, and to a lesser extent by glutamate alone (Figure 4C). The analysis of the fEPSP slope in response to stimulation showed a significant decrease in NMDAR currents after incubation with TAT-Cx43L2, an effect that was reversed by D-serine and glutamate together, but not by each separately (Figure 4D). See Figure S4A for a representative scheme of NMDAR activity recorded under each condition. The recordings were performed in BLA slices which contained neurons and astrocytes, and the data suggest that astroglial Cx43 hemichannel blockade induces a decrease in synaptic D-serine and glutamate that leads to a reduction in post-synaptic NMDAR activity. This interpretation assumes that there is no effect of TAT-Cx43L2 on neuronal NMDARs. To test this, primary cultures of mice cortical neurons were incubated with TAT-Cx43L2 alone or in the presence of NMDA. TAT-Cx43L2 had no effect on basal NMDAR activity or on NMDAR activity elicited by NMDA, as measured by intracellular calcium transients (Figure 4E). As expected, the increase in NMDAR activity induced by NMDA was completely blocked by incubation with the specific non-competitive NMDAR antagonist AP5 (Figure 4E). These results show that TAT-Cx43L2 has

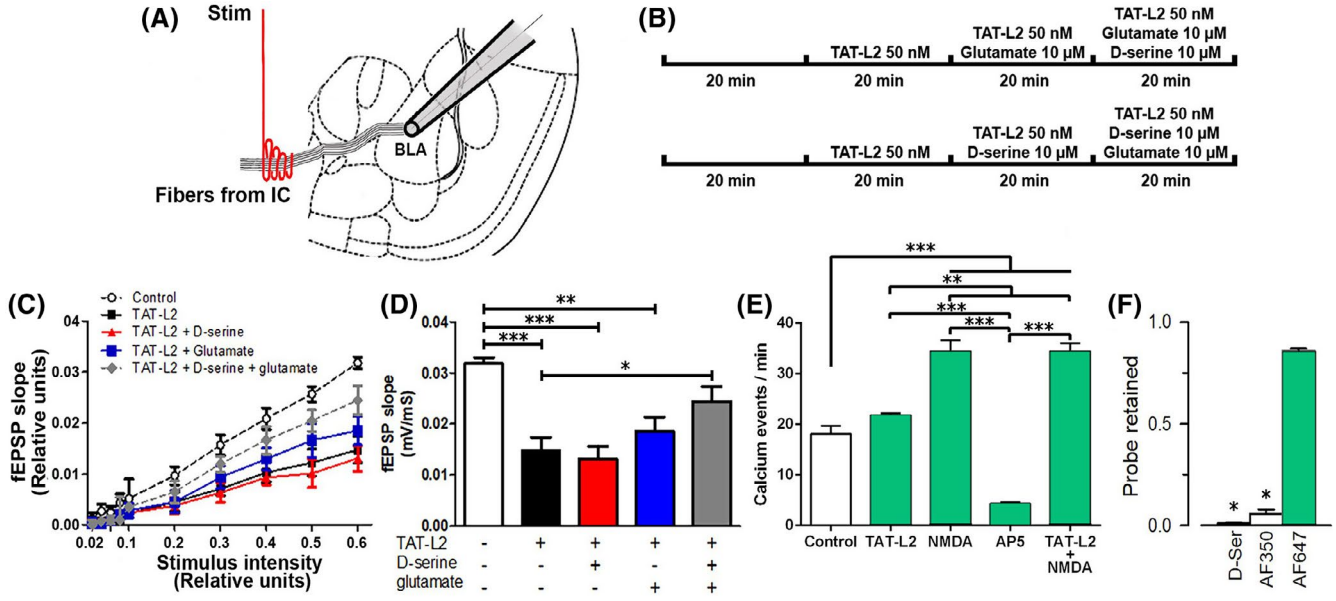


FIGURE 4 TAT-Cx43L2 induces a D-serine- and glutamate-dependent reduction in NMDAR-mediated currents in BLA slices, but has no effect on NMDAR responses to NMDA in primary cortical neuronal cultures. (A) Schematic representation of the location of the recording and stimulation electrodes. (B) Experimental design of the pharmacological treatments of BLA slices. (C) Input-output curves showing the relationship between fEPSP slope and stimulus intensity in BLA slices before (control) and during the application of 50 nM TAT-Cx43L2 alone or in combination with D-serine, glutamate, or glutamate plus D-serine. (D) Quantification of evoked BLA fEPSPs recorded before (control) and during the application of 50 nM TAT-Cx43L2 alone or in combination with glutamate, D-serine, or glutamate plus D-serine. (E) Analysis of the frequency of calcium transients in cortical neurons incubated with TAT-Cx43L2 (TAT-L2) compared to controls (control), and incubation with the NMDAR agonist NMDA (NMDA), NMDA and the NMDAR antagonist AP5 (AP5), and NMDA and TAT-Cx43L2 (TAT-L2 + NMDA). Note that TAT-Cx43L2 has no effects of NMDAR responses to NMDA. (F) Permeation of D-serine (D-Ser) and the fluorescent probes Alexa Fluor 350 (AF350) and 647 (AF647) through purified Cx43 hemichannels reconstituted in liposomes. Liposomes without protein and proteoliposomes containing Cx43 hemichannels were loaded with 250 µM AF350, 125 µM AF647, and 5 mM D-Ser during formation. Retention of the probes inside the liposomes and proteoliposomes was performed after separation from the free probes by size-exclusion chromatography followed by fluorescence (AF350 and AF647) or mass spectrometry (D-Ser) quantification. The probe retained in Cx43-proteoliposomes was normalized to the value in liposomes. Note that the probe with Cx43 hemichannels shows a loss of D-serine compared to the liposomes that lacked Cx43 hemichannels. Data are shown as means ± SE; $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$

no direct effects on NMDAR activity. See Figure S4B for a representative scheme of intracellular calcium transients recorded under each condition.

3.3 | Direct evidence for permeation of D-serine through Cx43 hemichannels

Based on the above results, it is possible that astrocyte Cx43 hemichannel-dependent release of glutamate and D-serine is necessary for the activation of post-synaptic NMDARs in the BLA. The release of glutamate via Cx43 hemichannels[13,18] and D-serine via astrocytes pannexin channels[55] have been reported. D-serine is expected to permeate through Cx43 hemichannels based on their estimated pore size, but there is no direct evidence for the release of D-serine through Cx43 hemichannels. This is relevant to our present results since astroglia can release glutamate and D-serine directly via Cx43 hemichannels, or alternatively, the release of ATP via Cx43 hemichannels[18] could activate astroglial P2X7 channels, triggering the release of D-serine via pannexin channels.[55] It is also possible that L-serine is released via Cx43 hemichannels, which can be converted into D-serine by neurons.[23] To determine whether D-serine permeates through Cx43 hemichannels, we studied D-serine transport through purified Cx43 hemichannels reconstituted in liposomes. For these studies, we trapped D-serine into the proteoliposomes and verified the release of the amino acid into the extraliposomal medium. The fluorescent probes AF350 and AF647 were used as higher- and lower-permeability controls, respectively. The results in Figure 4F show that purified Cx43 hemichannels reconstituted in liposomes are permeable to D-serine, and that the permeability of these hemichannels to AF350 and D-serine is much higher than that to AF647 (D-serine: 0.0102 ± 0.0036; AF350: 0.0582 ± 0.0210; AF647: 0.8572 ± 0.0130; $N = 3$, 5 and 3, respectively). The limited but measurable permeability of

AF647 through Cx43 hemichannels (~15% of the probe left the proteoliposomes during the size-exclusion chromatography) contrasts with the absence of permeability through purified Cx26 hemichannels reported elsewhere[17] and is consistent with the higher molecular weight cut-off of Cx43 versus Cx26 hemichannels.[56] The data demonstrate that D-serine can permeate through Cx43 hemichannels and supports the idea that D-serine could be released via Cx43 hemichannels in astrocytes. Glutamate has been previously reported to be released via Cx43 hemichannels in astrocytes,[13,18] and based on the present results in vitro using proteoliposomes, it is likely that both glutamate and D-serine are released by astrocytes via Cx43 hemichannels during learning, regulating the post-synaptic NMDAR activity required for short-term memory and subsequent memory consolidation. To see the data and statistics from Figure 4, see Table S4.

4 | DISCUSSION

This study is a continuation of a previous study, in which we reported that the infusion of TAT-Cx43L2 into the BLA before training for fear conditioning induced memory deficits 24 h post-training, but did not affect learning, using the same training protocol as used here. The amnesic effect of TAT-Cx43L2 affected memory consolidation, as the microinjection of the peptide had no effect on memory when injected 6 h post-training. Here, we demonstrate that astroglial Cx43 hemichannel activity in the BLA is required for short-term cued fear conditioning memory, but not for learning. Additionally, we show that the amnesic action of the selective Cx43 hemichannel inhibitor TAT-Cx43L2[35] is prevented by co-injection with a mixture of glutamate and D-serine, two well-established agonists/co-agonists of NMDARs.[22,53,54] Since we found that D-serine can permeate through Cx43 hemichannels and previous studies have shown that glutamate is released from astrocytes via Cx43 hemichannels,[13,18] our results suggest that D-serine and glutamate are likely the gliotransmitters released from astrocytes via Cx43 hemichannels that are necessary for BLA-dependent fear memory. The present results, however, do not exclude the possibility that rather than D-serine, it is L-serine which is released through astroglial Cx43 hemichannels and then converted into D-serine by neurons.[23]

Neither TAT-Cx43L2 microinfused alone or together with any of the putative gliotransmitters caused learning alterations. This implies that gliotransmission via Cx43 hemichannels is not necessary for learning since rats microinjected with TAT-Cx43L2 increased their freezing to the tone after subsequent training sessions. Notably, TAT-Cx43L2 intra-BLA injection before training impaired memory when tested 1 h after training, but not when the microinjection took place right after training. This may imply that Cx43 hemichannel activity is required during training to form a short-term memory but is not necessary for learning *per se*. This is particularly interesting because our training consisted of 3 CS/US sessions, 2 minutes apart; therefore, TAT-Cx43L2-treated animals increased their freezing over time in response to subsequent tone presentations, which implies a short-term retention of a memory (of around 10 min), which was lost 1 h post-training. So does TAT-Cx43L2 microinjection into the BLA affect short-term memory after all? The answer to this question is complex, and there are many options that should be considered. The most simple and parsimonious explanation is based on the fact that classic amnesic agents (e.g., protein synthesis inhibitors), which impair memory consolidation when injected pre-training, also induce memory impairments 1-h post-training (reviewed in Ref. [57]). Hence, it is possible that TAT-Cx43L2 is acting as a consolidation blocker and is not affecting short-term memory, but shows memory effects 1 h post-training, like other amnesic agents. Another option is that training itself may include a short-span memory that is not dependent on gliotransmitters or NMDAR-mediated synaptic plasticity. A relevant series of studies delved in more detail into this issue and found that in context fear conditioning there is a short-term memory component of around three minutes that is insensitive to intraventricular NMDAR competitive antagonist APV, suggesting that there may be a NMDAR-independent short-term memory during early training, followed by an APV-sensitive period.[58,59] Moreover, they also reported that APV affected memory only if administered during training and not post-training.[58] In consequence, we could speculate that astroglial release of glutamate and D-serine via Cx43 hemichannels may be required for the NMDAR-dependent later short-term memory, but not for the NMDAR-insensitive initial short-term memory during training. Hence, Cx43 hemichannel contribution to NMDAR activity would match the periods in which NMDAR antagonist APV affects memory. It must be noted, however, that while studies have reported that microinjection of APV into the BLA induces learning deficits in context fear conditioning,[60] and in tone-cued fear conditioning,[61] a study reported that intraventricular administration of APV impaired learning for context but not tone-cued fear conditioning.[62] Hence, care should be taken when comparing results from intraventricular or peripheral administration of APV and intra-BLA microinjections. More research into the differential mechanisms associated with learning and short-term memory is crucial to properly interpret the current results.

Regarding controls, in our previous study, we reported that intra-BLA injection of TAT-Cx43 had no effects on the locomotion of shock reactivity.[35] We also showed that TAT-Cx43L2 reduced Cx43 hemichannel opening in astrocytes, while not affecting the neuronal release of glutamate or ATP, or gap junction mediated inter-astrocyte communication.[35] We also reported that the peptide had no amnesic effects on memory when microinjected 6 h post-training, suggesting that it affected fear memory consolidation, as it had no effects outside the consolidation window. Moreover, the microinjected rats recovered their capacity to consolidate on subsequent training, tested 64 h after the original training, suggesting that the amnesic effects were temporal.[35] Several controls were also used to assess the specificity of the effects of the peptide. The Gap27 peptide, which decreases both Cx43 gap junctional and hemichannel activity, also showed amnesic effects, which proved that the effects of TAT-Cx43L2 were Cx43-dependent. A scrambled peptide and a mutated version of the TAT-Cx43L2 peptide that is inactive (TAT-Cx43L2$^{H126K/I130N}$), both had no effects on memory.[35] In the present study, we also used the mutated peptide to corroborate the specificity of the TAT-Cx43 peptide in its short-term memory impairments.

Etd uptake snapshot experiments showed that Cx43 hemichannels in astrocytes are open 1 h post-training and closed 3 h after training. The Cx43 hemichannel blockade was able to block short-term memory when it took place before but not after learning. Hence, it can be proposed that the formation of short-term memory requires Cx43 hemichannel activity during learning.

Whereas the mixture of glutamate and D-serine prevented the amnesic effects of TAT-Cx43L2, glutamate, D-serine, glycine, glutamine, or ATP, individually, did not. Because glutamate alone failed to ameliorate the amnesic effects of Cx43 hemichannel blockade, gliotransmission may involve the synaptic activation of NMDARs rather than kainate receptors,[51] presynaptic mGluRs,[50] or post-synaptic AMPARs.[52] NMDAR stimulation requires not only glutamate, but also the co-agonists D-serine or glycine, depending on their localization.[53,54] Our recordings from BLA slices show that TAT-Cx43L2 incubation reduces post-synaptic NMDAR-mediated currents, an effect that was prevented by the combination of glutamate and D-serine, but not each separately. These findings are similar to the pharmacological combination of gliotransmitters needed for preventing TAT-Cx43L2 amnesic effects in fear memory.

Although NMDARs can be found both synaptically and extrasynaptically,[63] it has been suggested that glycine acts as a co-agonist on extrasynaptic NMDARs, whereas D-serine may act on synaptic NMDARs.[53] Hence, given that the combination of glutamate and D-serine, but not the combination of glutamate and glycine, was able to prevent the amnesic effects of TAT-Cx43L2, our results support the participation of synaptic rather than extrasynaptic NMDARs in fear memory.

Our results show that TAT-Cx43L2 does not affect NMDARs directly, as assessed from the Ca^{2+} transients in response to NMDA from primary cultures of cortical neurons void of astrocytes. This is important, as the NMDAR activity reduction elicited by TAT-Cx43L2 is expected to arise from the lack of gliotransmitters at the synapse and not from a direct effect of the peptide on NMDARs.

It must be noted that in a previous study, we incubated primary hippocampal neurons void of astrocytes with TAT-Cx43L2 and found that the peptide does not affect the neuronal release of neurotransmitters like glutamate and ATP.[35] Hence, the effect of TAT-Cx43L2 is mediated specifically by astrocytes since the peptide does not directly affect post-synaptic neuronal NMDAR activity or presynaptic neurotransmitter release, and its effects appear only when astrocytes are present.

Our findings are in accordance with the study of Meunier and colleagues, which showed that clamping intracellular calcium in astrocytes in slices of the prefrontal cortex decreases NMDAR-mediated currents and NMDAR-dependent long-term potentiation, a decrease that could be prevented by adding extracellular D-serine. In that same study, a similar reduction in NMDAR-mediated currents was obtained by pharmacological and genetic inhibition of Cx43 hemichannel activity, or the inhibition of D-serine synthesis, all of which could be prevented by the addition of D-serine.[64] D-serine has been shown to be critical for NMDAR-dependent synaptic plasticity in a number of previous studies[22,31,33] and is released from astrocytes.[22,22,54,65] In the present study, we obtained the first in vivo evidence that astrocytes control the NMDAR-mediated currents required for short-term fear memory through the release of glutamate and D-serine via Cx43 hemichannels.

There is in vitro evidence for the release of glutamate via astroglial Cx43 hemichannels,[13] and in the present study, we have reported the first direct evidence that D-serine can permeate via Cx43 hemichannels. Hence, our study is the first to directly show the permeation of D-serine through Cx43 hemichannels in vitro, and present evidence, albeit indirect, that D-serine may be released from astrocytes via Cx43 hemichannels. It is still plausible, however, that inhibition of Cx43 hemichannels by TAT-Cx43L2 affects the release of D-serine indirectly. One possibility is that TAT-Cx43 is blocking ATP release through Cx43 hemichannels, which has been reported to be necessary for the pannexin-mediated release of D-serine through purinergic $P2X_7$ receptors.[55] Moreover, serine racemase, the enzyme that converts L- to D-serine, is also

expressed in neurons,[66] with some studies suggesting that astrocytes release L-serine, which is then shuttled to neurons, where it is converted to D-serine.[23] In consequence, it is also possible that L-serine rather than D-serine is released by astrocytes through Cx43 hemichannels, which would, in turn, be converted into D-serine in presynaptic neurons and then released onto synapses to activate postsynaptic NMDARs. In this case, blocking Cx43 hemichannels would decrease L-serine, which is the substrate for neuronal D-serine production, thus, reducing the release of neuronal D-serine. This decrease in synaptic D-serine would then diminish NMDAR activity, which could be restored by the exogenous addition of D-serine. Hence, the possibility that TAT-Cx43L2 induces the blockade of D-serine release indirectly cannot readily be ruled out, although the present evidence supports the notion that D-serine may be released directly via Cx43 hemichannels.

An unexpected finding of the present study is that exogenous D-serine alone was not sufficient to prevent the amnesic effects of TAT-Cx43L2. This is intriguing because glutamate is released not only by astrocytes, but by presynaptic neurons as well. Thus, when astroglial Cx43 hemichannels are blocked by TAT-Cx43L2, the presynaptic glutamate released at the synapse would be expected to combine with the exogenous D-serine to restore NMDAR-dependent currents, and hence prevent the amnesic effects of TAT-Cx43L2. One possible, yet unlikely explanation for this would be that the glutamate released by the presynaptic neuron is not sufficient to activate NMDARs. Another, a more likely possibility is that the presynaptic glutamate release may be affected by the lack of astroglial gliotransmitters (possibly glutamate) in response to TAT-Cx43L2. Notably, in a previous study using hippocampal slices from stressed mice, we showed that extracellular glutamate is almost completely depleted after Cx43 hemichannel blockade, or upon inhibition of NMDARs or purinergic $P2X_7$ receptors.[38] These observations suggest that under physiological conditions, either the glutamate released via Cx43 hemichannels is larger than the amount released by presynaptic neurons, and/or that presynaptic glutamate release depends on gliotransmitters such as glutamate, D-serine or ATP, or even astroglial NMDARs.[67] There is evidence supporting the notion that astrocytes modulate basal presynaptic glutamate release, based on studies that have shown that Cx43 hemichannel activity can modulate basal glutamatergic transmission,[68] and that astroglial glutamate can affect glutamate release probability at hippocampal synapses via activation of presynaptic mGluRs.[69,70] Clearly, the interplay between neurons and astroglia is an exciting field that requires further research.

Ours is also the first study to show "snap shots" of hemichannel-mediated uptake in the BLA after fear memory training. The CFCT training induced an increase in the uptake of GFAP-positive cells during the first-hour post-training but did not affect the total number of GFAP cells.

Altogether, the present results show that the astroglial release of glutamate and D-serine via Cx43 hemichannels at the BLA during CFCT is critical for short-term memory and subsequent memory consolidation, but not for learning, and that the mechanism is via modulation of NMDAR-mediated currents. Thus, astrocytes and their Cx43 hemichannels appear to be critical for memory, opening new possibilities for these hemichannels as targets for drugs aimed at improving memory or treating memory-related disorders.

5 | CONCLUSION

Astroglial release of glutamate and D-serine via Cx43 hemichannels at the BLA during fear memory training is critical for short-term memory and subsequent memory consolidation, but not for learning, via regulation of post-synaptic NMDAR activity.

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DISCLOSURES

There are no competing interests to disclose.

AUTHOR CONTRIBUTIONS

Sergio Linsambarth performed pharmacological, snapshot experiments, and analyzed data; Francisco J. Carvajal, Juan A. Orellana and Ivanka Jimenez performed snapshot experiments; Rodrigo Moraga-Amaro, Luis Mendez, Giovanni Tamburini, and Daniel Antonio Verdugo contributed with microinjections, behavioral experiments, and histology; Gonzalo I. Gómez, Nur Jury, and Brigitte van Zundert contributed with calcium recordings in primary hippocampal neurons; Guillermo A. Altenberg and Mariana C. Fiori contributed with the proteoliposome permeation assays; Waldo Cerpa and Pablo Martínez contributed with electrophysiological recordings in BLA slices. Jimmy Stehberg, Lorena Varela-Nallar, Mauricio A.

Retamal, and Claire Martin contributed to experimental design and manuscript writing. All authors read and corrected the final Manuscript.

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