Release of gliotransmitters through astroglial connexin 43 hemichannels is necessary for fear memory consolidation in the basolateral amygdala

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ABSTRACT **Recent *in vitro* evidence indicates that astrocytes can modulate synaptic plasticity by releasing neuroactive substances (gliotransmitters). However, whether gliotransmitter release from astrocytes is necessary for higher brain function *in vivo*, particularly for memory, as well as the contribution of connexin (Cx) hemichannels to gliotransmitter release, remain elusive. Here, we microinfused into the rat basolateral amygdala (BLA) TAT-Cx43L2, a peptide that selectively inhibits Cx43-hemichannel opening while maintaining synaptic transmission or interastrocyte gap junctional communication. *In vivo* blockade of Cx43 hemichannels during memory consolidation induced amnesia for auditory fear conditioning, as assessed 24 h after training, without affecting short-term memory, locomotion, or shock reactivity. The amnesic effect was transitory, specific for memory consolidation, and was confirmed after microinfusion of Gap27, another Cx43-hemichannel blocker. Learning capacity was recovered after coinfusion of TAT-Cx43L2 and a mixture of putative gliotransmitters (glutamate, glutamine, lactate, D-serine, glycine, and ATP). We propose that gliotransmitter release from astrocytes through Cx43 hemichannels is necessary for fear memory consolidation at the BLA. Thus, the present study is the first to demonstrate a physiological role for astroglial Cx43 hemichannels in brain function, making these channels a novel pharmacological target for the treatment of psychiatric disorders, including post-traumatic stress disorder.—Stehberg, J., Moraga-Amaro, R., Salazar, C., Becerra, A., Echeverría, C., Orellana, J. A., Bultynck, G., Ponsaerts, R., Leybaert, L., Simon, F., Sáez, J. C., Retamal, M. A. Release of gliotransmitters through astroglial connexin 43 hemichannels is necessary for fear memory consolidation in the basolateral amygdala. *FASEB J.* 26, 3649–3657 (2012). www.fasebj.org**

Key Words: astrocyte · amnesia · gliotransmitters · learning

Despite the popular belief that neurons are the main constituents of the human brain, >90% of brain cells are not actually neurons, but star-shaped glial cells known as astrocytes. Their role—until recently—was believed to be neuron sustenance, neurotransmitter recycling, and maintenance of the blood-brain barrier. In recent years, it has become increasingly evident that their role in brain function may be more protagonistic than previously thought.

Hundreds of astrocytes can be connected simultaneously to allow collective metabolic and electric coupling, as well as calcium-wave signaling (1). Such interastrocyte communication is attained by sharing cytoplasmic content through special channels called gap junction channels (2), each formed by 2 hemichannels contributed by each adjacent cell (3). Each hemi-

Abbreviations: ATP, adenosine triphosphate; BLA, basolateral amygdala; CL, cytoplasmic loop; CS, conditioned stimulus; Cx, connexin; Cx43, connexin 43; DCFS, divalent cation-free solution; Etd, ethidium bromide; GFAP, glial fibrillary acidic protein; KO, knockout; LTM, long-term memory; LY, Lucifer yellow; MAP, microtubule-associated protein; STM, short-term memory; TAT-Cx43L2, TAT-associated connexin 43, L2 region mimetic peptide; US, unconditioned stimulus; VAAC, volume-activated anion channel

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channel is composed of 6 protein subunits termed connexins (Cxs), with Cx43 being the most abundantly expressed by protoplasmic astrocytes (4). Interestingly, hemichannels do not always form gap junction channels and may also be found in noncontacting membranes, where they allow intracellular–extracellular communication. Although such "functional hemichannels" show low open probability in cultured cells under resting conditions (5), when opened, they may allow the release of neuroactive substances, such as adenosine triphosphate (ATP) (6) and glutamate (6, 7), now known as "gliotransmitters." *In vivo*, the location of Cx43 hemichannels at the unopposed surface of astrocytes (3, 8) leaves them in a unique position to mediate the release of gliotransmitters into the chemical neuronal synapse.

Recently, an increasing number of studies have supported the notion that synapses between two neurons may require signals released from an astrocyte to be fully functional (8). Further evidence from *in vitro* studies indicates that release of gliotransmitters from astrocytes is required to attain synaptic plasticity (9), including release of D-serine (10, 11), glutamate (7, 12), and ATP (6, 13). Moreover, glutamate release from astrocytes may also affect neurotransmitter release at synapses (14), suggesting that presynaptic efficacy and postsynaptic responses are both modulated by astrocyte release of gliotransmitters. The mechanism by which neuroactive gliotransmitters are released remains unknown, although vesicles, $P2X_7$ channels, transporters (15), and Cx hemichannels (6, 13) have been proposed. However, there is to date no *in vivo* evidence for the release of gliotransmitters from astrocytes, and no studies so far have shown that Cx43 hemichannels are involved in gliotransmitter release *in vivo*. The lack of such evidence can be, at least in part, attributed to a lack of tools that affect gliotransmitter release from astrocytes without affecting neuronal synaptic release or interastrocyte communication.

Here, we explored to what extent the release of astrocyte gliotransmitters is necessary for fear memory consolidation at the basolateral amygdala (BLA) *in vivo*. To this end, we targeted Cx43 hemichannels found in astrocytes and absent in neurons within the adult central nervous system, as shown using both primary cultures of brain cells (16) and immunohistochemistry in whole tissue (17, 18). We induced pharmacological blockade of Cx43 hemichannels during learning by using a synthetic peptide corresponding to the Cx43L2 region (aa 119–144), located in the cytoplasmic loop (CL) of Cx43, known as TAT-associated Cx43, L2 region mimetic peptide (TAT-Cx43L2). This peptide was previously shown to selectively block Cx43 hemichannels by interfering with loop/tail interactions essential for Cx43-hemichannel activity (see ref. 19 for a complete characterization of the peptide, including its Cx43-hemichannel inhibitory properties), without affecting Cx43 gap junction channel communication (19, 20).

The Cx43-peptide blockers were found to affect long-term memory but not short-term memory. The effect of these peptides was only observed when the blocker was applied within the memory consolidation period. In addition, the loss of memory was transitory and could be recovered after coinfusion of putative gliotransmitters known to be released from astrocytes.

MATERIALS AND METHODS

All procedures involving animals were in accordance with the U.S. National Institutes of Health guidelines and with approval of the bioethical committee of the Universidad Andrés Bello. Sprague-Dawley rats (~60 d old, ~250 g) were caged individually at 22°C, 12-h light-dark cycle. The rats remained in their home box throughout the study and were removed only briefly for drug microinfusions.

Drugs

TAT-Cx43L2 (>90% purity; LifeTein, South Plainfield, NJ, USA) was dissolved in PBS to yield final solutions of 1 mM, 100 μM, 10 μM, 1 μM, or 100 nM. Unless stated otherwise, the concentration used for microinfusions of TAT-Cx43L2 was 1 mM. As a control, TAT-Cx43L2$^{H126K/I130N}$ was used at 1 mM. Gap27 (>95% purity; AnaSpec, Fremont, CA, USA) and Gap27 scramble were dissolved to a final concentration of 1 mM. For the recovery cocktail, 100 mM glutamate (Sigma, St. Louis, MO, USA), D-serine (200 nM), glutamine (100 mM), ATP (100 μM), lactate (10 mM), and glycine (100 nM) were diluted in sterile saline.

Apparatus

All behavioral assays were performed in a sound-attenuating cubicle. Conditioning and tone testing were conducted in different chambers. For conditioning, rats were placed in a Plexiglas chamber with a metal grid floor (40×40×40 cm). The chamber was dimly illuminated by a red light. For testing, rats were placed in a different Plexiglas chamber without the metal grid (60×40 cm) dimly illuminated by a white light bulb. A video camera was mounted at the top of each chamber to allow digital recording throughout the experiments.

Surgery and histology

Under ketamine/xylazine anesthesia (0.02 and 0.33 μl/kg, respectively), rats were stereotaxically implanted with bilateral 22-gauge stainless-steel cannulas aimed 1.0 mm above the BLA (3.0 mm posterior to bregma, 5.3 mm lateral to the midline, and 8.0 mm ventral to the skull surface; ref. 21). The cannulas were fixed with acrylic dental cement and secured by 4 skull screws. A stylus was placed inside the guide cannula to prevent clogging. Rats were given ≥7 d to recover before experimental procedures began.

In all experiments, the stylus was removed from the guide cannula, and a 28-gauge injection cannula was inserted through the guide cannula, extending 1.0 mm beyond its tip into the BLA. Drugs were infused slowly *via* the injection cannula, connected by PE-20 tubing to Hamilton microsyringes (Hamilton, Reno, NV, USA) driven by a microinfusion pump. Infusions were of 0.25 μl per hemisphere at a rate of 0.32 μl/min. Following drug infusion, injecting cannulas were left in place for 10 min to allow drug diffusion away from

the cannula tip. Cannula placement and maximal diffusion were verified by infusing 0.5 μl of India ink in a group of 5 rats. The maximal diffusion spread observed included only lateral and basal amygdala nuclei. At the end of all experiments, animals were anesthetized as above and perfused intracardially with saline and 4% buffered paraformaldehyde. Brains were extracted and postfixed in 30% sucrose until density equaled that of sucrose. The brains were sectioned in a cryostat, Nissl-stained (cresyl violet), and examined with light microscopy for cannula placement and assessment of histological lesions, as seen by tissue damage or gliosis. Animals with histological lesions beyond the size of the cannula tip and guide cannula diameter were excluded from the analysis.

Behavioral procedures

The conditioning is based on the animal learning that a previously neutral stimulus (a conditioned stimulus, CS; *e.g.*, an innocuous sound) becomes predictive of a stressful stimulus (unconditioned stimulus, US; *e.g.*, footshock). In all of experiments, rats were habituated to handling during the 7 recovery days and also habituated to the training and testing chambers for 3 d, 10 min/d. On the conditioning day, the animals were left in the chamber for 3 min before the beginning of the training. Each training session consisted of 3 CS-US pairings in 30-s intervals. The CS was an auditory stimulus, 5-kHz, 60-dB, 10-s tone that terminated with a 1.5-mA, 1-s foot shock (the US). To measure the effect of TAT-Cx43L2 on locomotor activity, entire body movements were measured on the training chamber before training began, which were counted as the number of transitions along virtual square subdivisions of 10 cm^2 measured offline from the screen of digital video recordings.

To study whether TAT-Cx43L2 microinfusions disrupt shock reactivity, rats' activity bursts displayed during the 2 s of shock were compared to the activity found 2 s before the shock and were measured as velocity (cm/s). To measure distance, entire body movements were analyzed from recordings of the first 2 s from the first footshock of the first training session over virtual subdivisions of 10 cm^2 with a chronometer. Then values were converted to real distance in centimeters using known landmark distances from the chamber. Distance was then converted into velocity by dividing distance by time (cm/s). Short-term memory (STM) was assessed as an increase in freezing to the tone between the 3 consecutive pairings during training. Long-term memory (LTM) was tested 24 h after training. To control for nonspecific effects of the drug and to rule out permanent damage to the BLA, rats were retrained 48 h after the LTM test and tested again 24 h later. At the tests, rats received the same 10-s tone presentations in the testing chamber, in the same manner as the trainings (5 kHz, 60 dB, 10 s, and every 30 s). In all tests, total seconds of freezing during the CS presentations (immobility) were calculated for each rat and shown as a percentage of freezing during the total duration of the tone presentation.

Immunohistochemistry

Brain slices previously fixed in buffered 4% paraformaldehyde and maintained in 30% sucrose were mounted in gelatin-coated glass slides. After rinsing with PBS, slices were blocked and permeabilized with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA), and 3% Triton X-100 in PBS for 1 h at room temperature. After rinsing again, sections were incubated overnight at 4°C with the primary anti-Cx43 antibody (Sigma). Afterward, sections were rinsed with PBS and incubated for 1 h at 37°C with Alexa Fluor 488-conjugated secondary antibody. To identify neurons and astrocytes, sections were incubated overnight at 4°C with either monoclonal anti-microtubule-associated protein 2 (MAP2; M 4402; Sigma-Aldrich) for neurons or polyclonal anti-glial fibrillary acidic protein (GFAP; Sigma-Aldrich) for astrocytes. Sections were washed and incubated for 1 h at 37°C with Alexa Fluor 568-conjugated secondary antibodies. Finally, slides were washed and permanently mounted using ProLong (Invitrogen). Staining specificity was assayed by incubation of sections in the absence of the primary antibody. Samples were examined using laser-scanning confocal microscopy on a Fluoview FV1000 (Olympus, Tokyo, Japan).

Primary cell cultures

Astrocyte cultures:

Primary astrocyte cultures were prepared from hippocampus of newborn (PN1) rats. Briefly, the brains were removed, meninges were carefully peeled off, and the hippocampus was dissected. Cells were seeded on glass coverslips (Gassalem, Limeil-Brevannes, France) placed inside 16-mm diameter 24-well plastic plates (NunClon; Nunc, Roskilde, Denmark) at a density of 1×10^5 cells/well in DMEM supplemented with penicillin (5 U/ml), streptomycin (5 μg/ml), and 10% FCS. After 8–10 d, when cells had reached confluence, 1 μM of cytosine-arabinoside was added to the culture medium every day for 3 d to eliminate proliferating microglia.

Neuronal cultures

Hippocampal neurons were obtained from hippocampus of E18 rats. Briefly, hippocampi were dissected as mentioned above for astrocyte cultures. Cells were seeded directly on poly-L-ornithine-coated coverslips (1×10^5 cells/coverslip) in neurobasal medium supplemented with penicillin (5 U/ml), streptomycin (5 μg/ml), B27 supplement, and glutamax. Partial medium changes (1/4) were done 2×/wk.

Dye uptake

For single image visualization of dye uptake, astrocytes were bathed in recording solution (148 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.4) containing 5 μM ethidium bromide (Etd; Sigma-Aldrich), and fluorescence intensity was recorded for 10 min in selected cells. In some experiments, astrocytes were exposed to recording solution, but with no added Ca^{2+} and Mg^{2+}, and supplemented with 10 mM EGTA [divalent cation-free solution (DCFS)] to increase hemichannel opening probability. In all experiments, astrocytes were preincubated for 10 min with TAT-Cx43L2 peptide (100 μM) before dye uptake measurements. Images were captured every 30 s (exposure time 30 ms, gain 0.5) using a Q Imaging model Retiga 13001 fast-cooled monochromatic digital camera (12-bit; Q Imaging, Burnaby, BC, Canada). Metafluor 6.2R5 software (Universal Imaging, Downingtown, PA, USA) was used for offline image analysis and fluorescence quantification.

For data representation, the average of 2 independent background fluorescence (F_B) intensity measurements (expressed as arbitrary units) was subtracted from the fluorescence intensity in each cell (F_1). Results of this calculation (F_1-F_B) in 20 cells were averaged and plotted *vs.* time (expressed in minutes). Slopes of dye uptake were calculated using Microsoft Excel software (Microsoft, Redmond, WA, USA) and expressed as arbitrary units per minute. Micro-

scope and camera settings remained the same in all experiments.

Measurement of ATP and glutamate release induced by TAT-Cx43L2

Neurons were plated in multiwell culture trays (10^6 cells/well/0.5 ml), and 48 h later, they were used for experiments. Extracellular ATP was measured by luciferin/luciferase bioluminescence assay kit (Sigma-Aldrich). Levels of extracellular glutamate were determined using an enzyme-linked fluorimetric assay, as described by Genever and Skerry (22). In the presence of glutamate dehydrogenase (GDH) and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), glutamate is oxidized to α-ketoglutarate, yielding NADPH, which can be determined fluorometrically (excitation and emission wavelengths of 355 and 460 nm, respectively) to provide an indirect quantification of glutamate concentration.

For each assay, standard curves were constructed by using known ATP or glutamate concentrations. The concentrations of ATP and glutamate in samples of extracellular medium were calculated from standard curves and referred to 10^6 cells). The fraction of ATP or glutamate released by cells to the extracellular milieu was estimated by the difference between the concentration detected in the medium of cells under resting conditions and the concentration measured after stimulation in the presence or absence of hemichannel inhibitors.

Dye coupling

Astrocytes plated on glass coverslips were bathed with recording medium (HCO_3^--free F-12 medium buffered with 10 mM HEPES, pH 7.2), and intercellular communication mediated by gap junctions was tested by evaluating the transfer to neighboring cells of Lucifer yellow (LY; Sigma-Aldrich) microinjected into one cell. The cultures were observed on an inverted microscope equipped with xenon arc lamp illumination and a Nikon B filter (excitation wavelength 450–490 nm; emission wavelength >520 nm). LY (10 mM in 150 mM LiCl) was microinjected through a glass microelectrode by brief overcompensation of the negative capacitance circuit in the amplifier to cause oscillations until the impaled cell was brightly fluorescent. At 3 min after dye injection, cells were observed to determine whether dye transfer occurred. The incidence of coupling was scored as the percentage of injections that resulted in dye transfer from the injected cell to more than one neighboring cell. The coupling index was calculated as the mean number of cells to which the dye spread.

Data analysis

Data are expressed as means ± SD. Statistical differences were assessed by unpaired Student's *t* test (Mann-Whitney *U*) and by 1-way ANOVA for multiple comparisons, followed by Dunns *post hoc* test, and considered significant at values of $P < 0.05$.

RESULTS

To determine *in vivo* to what extent the release of astrocyte gliotransmitters is necessary for fear memory consolidation at the BLA, we targeted Cx43 hemichannels, which are found in astrocytes and absent in neurons of the adult central nervous system (16–18). To corroborate that Cx43 is present only in astrocytes, BLA slices were immunostained for Cx43 with either astrocytic (GFAP) or neuronal (MAP) markers. Cx43 exclusively colocalized with GFAP, indicating that Cx43 is exclusively present in astrocytes (see **Fig. 1*A*, *B***).

To test whether gliotransmitter release through astrocytic Cx43 hemichannels is necessary for learning and memory, we induced pharmacological blockade of Cx43 hemichannels during learning by making use of TAT-Cx43L2, a synthetic cell-permeable peptide that corresponds to the Cx43L2 region (aa 119–144) of the CL of Cx43; Cx43L2 is known to selectively inhibit Cx43 hemichannels by interfering with loop/tail interactions that are essential for Cx43 hemichannel opening (19, 20). To rule out direct effects of the peptide on neurons and neuronal synaptic activity, we tested the peptide (1 mM) in hippocampal primary neuronal cultures without astrocytes. TAT-Cx43L2 did not affect ATP or glutamate release in neuronal cultures (concentrations of picomoles per 10^6 cells; see Fig. 1*C*). Since astroglial coupling is mainly mediated by Cx43 gap junction channels, we studied the effects of TAT-Cx43L2 on gap junctional astroglial communication. For this,

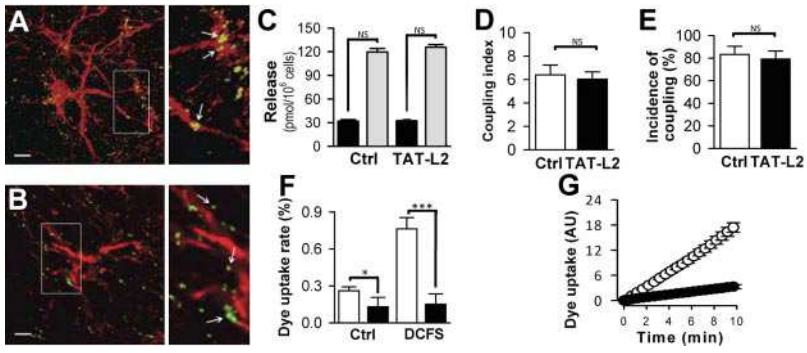

Figure 1. Cx43 in astrocytes but not in neurons in the BLA and TAT-Cx43L2 inhibiting properties. *A*) Cx43 reactivity (green) colocalized with GFAP (red), a marker for astrocytes. Scale bar = 10 μm. Arrows show colocalization. *B*) Cx43 (green) did not colocalize with a neuronal marker (MAP2, red). Arrows show lack of localization. *C*) TAT-Cx43L2 did not affect neuronal synaptic release of ATP (solid bars, right) compared to controls (solid bars, left) or glutamate (shaded bars, right, compared to controls, shaded bars, left) (n=3). *D*) TAT-Cx43L2 had no effects on astrocyte coupling index (solid bar) compared to control (open bar) (n=3). *E*) TAT-Cx43L2 had no effect on astrocyte coupling incidence (solid bar) compared to controls (open bar) (n=3). *F*) TAT-Cx43L2 blocked astrocyte hemichannel activity in control conditions (solid bar, left) compared to controls (open bar, left) but produced complete inhibition of hemichannel activity (dye uptake) when applied to cells bathed with DCFS (solid bar, right) compared to controls (open bar, right) (n=3). *G*) Example of hemichannel dye uptake inhibition in astrocytes exposed to DCFS conditions alone (open circles) and together with TAT-Cx43L2 (solid circles). NS, not significant. *$P < 0.05$; ***$P < 0.001$.

hippocampal astroglial cultures were incubated with TAT-Cx43L2 (1 mM) for 10 min, and then astroglial coupling was assessed by intracellular transfer of LY microinjected into single cells while monitoring its diffusion to neighboring cells. Notably, TAT-Cx43L2 did not affect astroglial coupling (see Fig. 1*D, E*).

To demonstrate the inhibitory properties of TAT-Cx43L2 on astroglial hemichannels, cells were incubated for 10 min with TAT-Cx43L2 (1 mM), and then hemichannel activity was evaluated using the Etd uptake assay in control conditions or after the exposure to DCFS, known to increase the open probability of Cx hemichannels (1). As expected, TAT-Cx43L2 inhibited hemichannel activity in astrocytes under control conditions (see Fig. 1*F*, Ctl) and in astrocytes bathed with DCFS, reduced Etd uptake to the same level as in control cells (Fig. 1*F, G*). TAT-Cx43L2 inhibited hemichannel activity to levels comparable to those reported after incubation with La^{3+} (23), a widely used Cx hemichannel blocker (3). In summary, TAT-Cx43L2 inhibited Cx43 hemichannel activity, while maintaining Cx43 gap junction-dependent interastrocyte communication or synaptic release unaffected.

To test whether Cx43 hemichannel activity is required for learning and memory, TAT-Cx43L2 (1 mM) was microinjected bilaterally into the BLA in animals previously implanted with chronic injection cannulas (see Materials and Methods). All animals included in this study had successful bilateral implants into the BLA and showed no histological lesions beyond the diameter of the injection cannula (see **Fig. 2*A*** for a representative Nissl-stained photomicrograph of a representative implant cannula location and Fig. 2*B* for the respective scheme). The BLA was targeted, since this brain region controls emotional memory associations and is crucial for acquisition of fear conditioning memory (24). For fear conditioning memory training, animals are trained to associate a tone (10 s, 5 kHz) with a foot shock. As a consequence, on hearing the tone in a different context, the animals freeze as a predictive response to the shock (for a general outline of experimental procedures used, see Fig. 2*C*). To potentially interfere with fear memory consolidation but avoid any possible infusion-induced discomfort or stress, and to assess the effects of the drug in locomotion, shock reactivity, and short-term memory, TAT-Cx43L2 was injected bilaterally into the BLA 10 min before training. In this study, 3 subsequent trainings were used, each every 30 s. **Figure 3*A*** indicates that TAT-Cx43L2-microinfused rats showed unaffected short-term memory during training, increasing significantly their freezing to the conditioned tone between the 3 conditioning trials ($P<0.01$). Injected animals also showed unaffected locomotion (see Fig. 3*B*) and normal reactivity to the shock (see Fig. 3*C*). Interestingly, when tested 24 h later in a novel chamber with the tone alone, TAT-Cx43L2 microinjected animals showed complete amnesia, as evident by the lack of freezing during tone presentations ($P<0.01$, $F_{(3,20)}=22.07$; Fig. 3*D*). To ensure that the effects of TAT-Cx43L2 are only transient and to rule out a permanent effect of the drug, animals were subsequently retrained in the same setup 48 h after the initial conditioning. Rats previously microinjected with TAT-Cx43L2 recovered their learning capacity (see Fig. 3*E*).

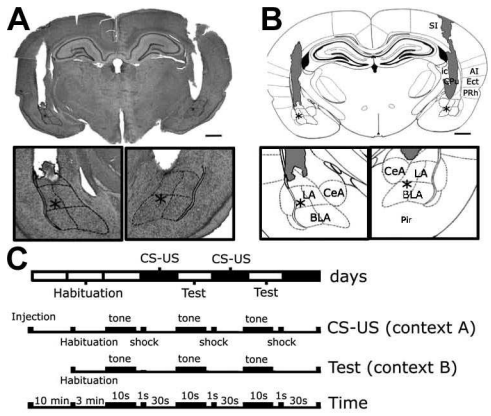

Figure 2. TAT-Cx43L2 microinfusions into the BLA and the fear conditioning paradigm used. *A)* Representative photomicrograph of Nissl-stained brain slice. Zoomed area shows injection cannula tip 1 mm below end of guide cannula (asterisk). BLA, lateral amygdala (LA), and central amygdala (CeA) borders are shown to ease viewing (dashed line). *B*) Scheme of implant location corresponding to *A*, showing relevant areas: somatosensory primary (SI); perirhinal (Prh), ectorhinal (Ect), piriform (Pir), and auditory primary (AI) cortices; caudoputamen (Cpu); internal capsula (ic); and external capsula (ec). Inset: Nissl-based borders of BLA, CeA, and LA relative to location of the injection cannula tip (asterisk). Only successful BLA-LA implants were included in the analysis. *C*) General experimental protocol for fear conditioning used; each rectangle in scale at top corresponds to a day. CS-US signifies training and is shown in more detail in the second scale from the top. Test is shown on the third scale from top. Time lapses are shown in bottom scale. Note change in context between training and test. Scale bars = 1 mm.

It is widely accepted that substances that can induce amnesia after learning have no effects on memory if administered 4 h after the training. Within that 4-h period, known as memory consolidation, stabilization of the short-term memory is believed to occur, as it turns into long-term memory, a process that requires protein synthesis (24). To ascertain that TAT-Cx43L2 is affecting specifically memory consolidation, a different group of rats received the peptide microinjection 6 h after learning, in which case TAT-Cx43L2-microinjected animals showed no memory deficits (see Fig. 3*F*).

To further support the hypothesis that the effects of TAT-Cx43L2 were specific for Cx43-hemichannels, a mutant version of the TAT-Cx43L2 peptide, namely TAT-Cx43$^{L2H126K/I130N}$, was microinfused into the BLA. This mutant peptide differs from TAT-Cx43L2 by 2 amino acids that were found to be essential for binding of L2 to the CT tail of Cx43 (20). Furthermore, this TAT-Cx43L2$^{H126K/I130N}$ peptide has been demonstrated to lack the inhibitory properties of TAT-Cx43L2 on Cx43-hemichannel activity (19).

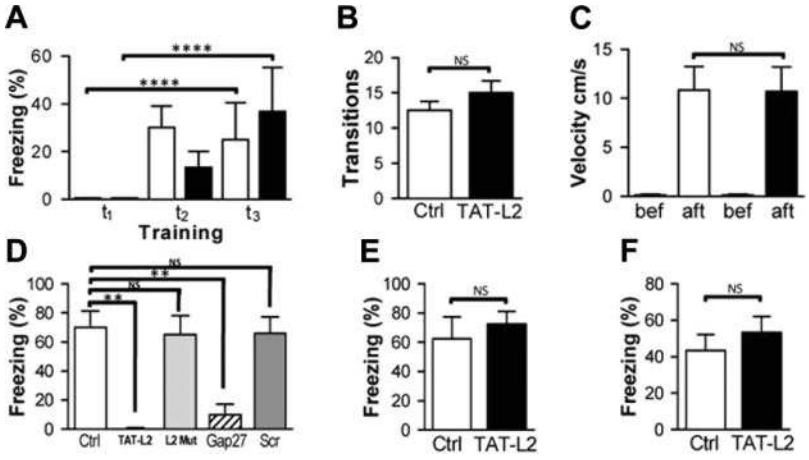

Figure 3. Peptide effect on auditory fear conditioning task *A*) Short-term memory was not affected by TAT-Cx43L2 microinfusions (t_1, t_2, and t_3 correspond to subsequent pairings during training; n=5 or 6). See control group (open bars) and TatCx43L2-microinfused group (Tat-L2, solid bars). *B*) TAT-Cx43L2 did not affect locomotion (n=4 or 5), as measured by the number of crossings (transitions) in the context along 10 cm^2 virtual squares before training. *C*) TAT-Cx43L2 did not affect shock reactivity [TAT-L2 (solid bar) *vs.* controls (open bar); n=6–4], as seen by the escape velocity in response to the footshock [before footshock (bef) and after footshock (aft)]. *D*) Microinfusions of TAT-Cx43L2 (TAT-L2, solid bar) and Gap27 (Gap27, hatched bar) into the BLA strongly impaired fear conditioning memory consolidation when tested 24 h after training (n=5–7) compared to control animals (open bar), while microinfusion of a scrambled Gap27 peptide (Scr; dark shaded bars) or TAT-Cx43L2$^{H126K/I130N}$ peptide (L2 Mut; light shaded bars) had no effects on memory. *E*) TAT-Cx43L2 had no effects on memory when injected 6 h after training (n=4 or 5). *F*) TAT-Cx43L2 microinjected rats recovered their capacity to learn on subsequent training, tested 64 h after the original training (n=4 or 5). NS, not significant. $^{**}P < 0.01$; $^{****}P < 0.0001$.

Here, we show that TAT-Cx43L2$^{H126K/I130N}$ microinfusion into the BLA had no effects on memory (see Fig. 3*D*). To further demonstrate the specificity of Cx43 inhibition, we also used Gap27, which is a mimetic peptide of the second extracellular loop of Cx43 hemichannels, previously used to block Cx43 hemichannels when added to the extracellular side (25, 26). This peptide was microinjected into the BLA (1 mM) 10 min before learning. Gap27-microinjected rats showed significant memory deficits compared to control animals (P<0.01; see Fig. 3*D*). Such amnesic effects were comparable to those of the TAT-Cx43L2 peptide. A different group of animals was microinfused with a Gap27-scrambled peptide corresponding to the same amino acids as Gap27 but with a random sequence used to further test the specificity of Cx43 hemichannel inhibition; these experiments convincingly showed no effect on memory (Fig. 3*D*). Taken together, the above experiments show that blockade of Cx43 hemichannels from either the cytoplasmic or extracellular side induces amnesia.

The amnesic effect of Cx43-hemichannel blockage using TAT-Cx43L2 was concentration dependent (**Fig. 4*A***). To determine whether Cx43 hemichannels are involved in memory by allowing gliotransmitter release into the synapse, a lower concentration of TAT-Cx43L2, still capable of inducing amnesic effects (10 nM, P<0.001, $F_{(6,29)}$=13.9) was coinjected with a mixture of potential gliotransmitters including glutamate (100 mM), glutamine (100 mM), lactate (10 mM), D-serine (200 nM), glycine, and ATP (100 μM). The gliotransmitter cocktail was able to reverse TAT-Cx43L2 effects, as microinjected rats showed recovery of their capacity to learn (P<0.05, $F_{(2,17)}$=6.34; see Fig. 4*B*), suggesting that gliotransmitter release through Cx43 hemichannels could be necessary for fear memory consolidation.

DISCUSSION

Our results indicate that astrocytic Cx43 hemichannel activity is required for fear memory consolidation but not for short-term memory in the BLA. This finding is congruent with *in vitro* studies showing astrocyte release of gliotransmitters being necessary for synaptic plasticity (14). The amnesic effect of the lowest effective concentration of TAT-Cx43L2 was prevented by coinjection with a mixture of putative gliotransmitters into the BLA, which suggests that Cx43 hemichannels mediate the release of some of those substances. This notion is supported by an increasing number of *in vitro* studies suggesting that gliotransmitter release from astrocytes is necessary for neuronal plasticity, including ATP, glutamate, and D-serine (6, 9–13). Further research will be needed to identify the exact gliotransmit-

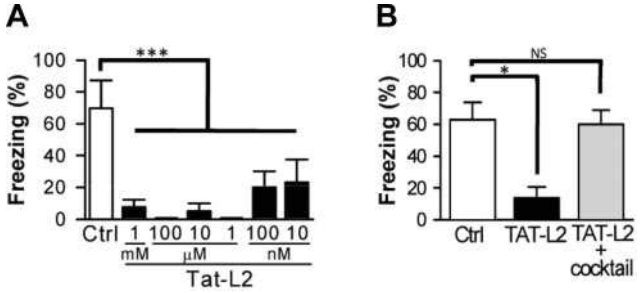

Figure 4. TAT-Cx43L2 dose-response and recovery of learning by comicroinfusion with a cocktail of putative gliotransmitters. *A*) Amnesic effects of TAT-Cx43L2 microinfusions (TAT-L2, solid bars) into the BLA during fear conditioning consolidation (n=5 or 6) compared to controls (open bars). *B*) Recovery of learning was obtained after comicroinfusion of the lowest dose of TAT-Cx43L2 (10 nM) with a mixture of possible gliotransmitters (cocktail, shaded bars) that included D-serine, glutamate, glutamine, glycine, ATP, and lactate (shaded bars) (n=5 or 6). NS, not significant. $^{*}P < 0.05$; $^{***}P < 0.001$.

ters released from the astrocyte during memory consolidation.

To ensure that blockade is specific for Cx43 hemichannels, 2 peptides were used; TAT-Cx43L2 and Gap27, designed for blocking Cx43 hemichannels from the cytoplasmic and extracellular sides, respectively. To further support specificity for hemichannel blockade, the effect of TAT-Cx43L2 peptide on primary neuronal and astroglial cultures was assessed. TAT-Cx43L2 showed no effects on neurons or neuronal synapses, as incubation with the peptide did not affect ATP and glutamate release. This is congruent with our findings and those of others (16–18) in which Cx43 was found only in astrocytes but not in neurons. On the other hand, previous studies have demonstrated that Gap27 blocks Cx43 hemichannels, which are present in astrocytes and absent in neurons (6) and thus, the most conceivable mechanism by which both peptides inhibited memory consolidation is by blockade of Cx43 hemichannels and diminished gliotransmitter release, and not by inhibiting the (vesicular) release of neurotransmitters.

TAT-Cx43L2 peptide also did not affect interastrocyte communication, a process known to be mediated by Cx43 gap junctions. This result is in line with previous *in vitro* studies showing TAT-Cx43L2 selectivity for Cx43 hemichannels without affecting interastrocyte gap junction transmission (19, 20). Thus, our *in vitro* assays showed that TAT-Cx43L2 affected only hemichannel activity, leaving interneuronal and interastrocyte transmission intact.

In our experimental design, peptide infusion was set to only 3 min long and was performed as close as possible to training (10 min prior), in an effort to minimize time of exposure to the peptides and avoid any long-term effects on gap junctions. However, it is possible that Gap27 may have additional inhibitory effects on gap junctions. The prevailing view is that peptides like Gap27 first inhibit free hemichannels and as a consequence of this, prevent the formation of gap junctions. Depending on the turnover rate of gap junctions, the time necessary to observe gap junction inhibition may vary. Thus, we cannot exclude effects of Gap27 on gap junctions, but TAT-Cx43L2, on the basis of data presented, is not expected to have such effects. TAT-Cx43$^{H126K/I130N}$, previously shown not to inhibit Cx43-hemichannel function *in vitro* (19), and a scrambled version of Gap27 were also microinjected into the BLA, and neither had effects on memory, giving further support for the specificity of both mimetic peptides.

Our results suggest a crucial role for astrocytic Cx43 hemichannels in fear memory consolidation. Whether the role of astrocytic Cx43 hemichannels involves direct release of gliotransmitters or contribution to the activation of other release pathways (*e.g.*, exocytic release) remains to be investigated. Furthermore, partial contribution from other glial cells cannot presently be ruled out.

Our results show that Cx43 hemichannel activity is necessary for memory consolidation. This supports the idea that neuronal synapses require the release of gliotransmitters from astrocytes to be fully functional. Originally, the idea that synapses are "tripartite" (2 neurons and an astrocyte) was first presented by Araque *et al.* (27), who suggested that astrocytes release gliotransmitters to modulate neuron to neuron synapses. The release of neuroactive substances from the astrocyte into extracellular space is well documented. These gliotransmitters include glutamate (7), D-serine, ATP, adenosine, γ-amino butyric acid, tumor necrosis factor α (TNF-α), prostaglandins, atrial natriuretic peptide, and brain-derived neurotrophic factor (8, 28). Some of these gliotransmitters have been shown to modulate neuronal activity and synaptic plasticity (6, 8, 10–13). The mechanism by which gliotransmitters are released from astrocytes remains controversial. Candidate pathways that have been proposed include Cx hemichannels (6, 29), pannexin hemichannels (30–32), $P2X_7$ channels (15), Bestrophin 1 anion channel (33), volume-activated anion channels (VAACs; ref. 34), and vesicle exocytosis (12, 35). However, it is accepted that Cx43 hemichannels are permeable to ATP and glutamate (36, 7) and are likely to be permeable to several other gliotransmitters. Here, we show that inhibition of Cx43 hemichannel opening induces amnesia in rats without affecting locomotion, shock reactivity, or short-term memory. This strongly suggests that Cx43 hemichannels mediate or contribute to the release of gliotransmitter to the neuronal synapses. The amnesic effect of the peptide was only observed when the microinfusion was performed during memory consolidation but not 6 h after training.

Several mechanisms have been proposed by which astrocytes can modulate synaptic plasticity. Gliotransmitters released from astrocytes can activate receptors in both presynaptic and postsynaptic neurons (27). Glutamate released from astrocytes, possibly through Cx hemichannels (7), activates mGluRs in presynaptic neurons, inducing evoked excitatory postsynaptic currents (37), while ATP released by astrocytes increases interneuronal excitability and potentiates synaptic transmission (38). Another example is the release of D-serine from astrocytes, which has been shown to control NMDA receptor-dependent plasticity (9). Moreover, changes in the release of ATP and adenosine from astrocytes in the hippocampus have been associated with memory consolidation deficits resulting from sleep deprivation (39).

Our results suggest that astrocyte release of gliotransmitters is necessary for higher brain function. To our knowledge, this is the first study showing a physiological role *in vivo* for astroglial Cx43 hemichannels in the brain.

Given that a single astrocyte can extend processes covering thousands of synapses (40), they have crucial roles in maintenance of synaptic transmission through metabolic networks (41), in changing cerebral microcirculation in response to activity-dependent oxygenation needs (for a review, see ref. 42) and can modulate synaptic plasticity through the release of gliotransmit-

ters (28), it seems quite clear that their role in brain function is protagonistic rather than secondary to neurons.

Given the crucial role for astrocytic Cx43 hemichannels in memory proposed here, Cx43-knockout (KO) mice would be expected to show memory impairments. However, Frisch *et al.* (42) reported that Cx43-KO mice tested in the Morris water maze do not show learning impairments. This unexpected result may be explained by an up-regulation of other putative gliotransmitter release mechanisms reported by several *in vitro* studies, which include Cx hemichannels (6, 29), pannexin hemichannels (30–32), $P2X_7$ channels (15), Bestrophin 1 anion channel (33), VAACs (34), and vesicle exocytosis (12, 35). All these mechanisms may normally contribute to functional synapses, and may be up-regulated in Cx43-KO mice. In contrast, the acute and short-term hemichannel blockade using TAT-Cx43L2 peptide is less likely to induce significant compensatory effects on other release mechanisms. Cx43-KO mice may also show up-regulation of other Cxs as well. In that respect, Giaume and colleagues (43), using cultured Cx43-deficient astrocytes, showed that coculture of Cx43-deficient astrocytes with neurons induces the expression of Cx30 and restores gap junctional communication.

Cx30 gap junctions colocalize with most astrocytes expressing Cx43 gap junctions (44–46) and account for ~20% of hippocampal astrocytic coupling (46). This means that the gap junctional activity believed to be dependent on Cx43 may be restored in Cx43-deficient astrocytes by the induction of Cx30 gap junctions. Along the same line, it is probable that Cx43-dependent hemichannel activity may be restored by compensatory Cx30 induction or relocation onto synapses.

Moreover, Cx43-KO mice lack both gap junctional channels and hemichannels, thereby complicating the analysis and interpretation of the phenotype. In this respect, conditional Cx43 mouse mutants with functional gap junctional coupling and impaired hemichannel activity may be helpful in establishing the precise *in vivo* role of Cx43 hemichannels, but have not been reported to date.

All the above may not only explain the lack of memory impairments in Cx43-KO mice, but may also explain the very limited behavioral effects found in Cx43-KO mice by Frisch *et al.* (42), including only increased exploration and temporal motor impairments.

The lack of memory impairments and other behavioral effects in Cx43-KO mice, whether due to compensation by other Cxs or by up-regulation of other putative release mechanisms suggests a redundancy that may reflect on the greater importance of astrocytic release of gliotransmitters into functional synapses required for higher brain function. Further research is needed to decipher the mechanisms by which Cx43-KO mice can still learn in spite of lacking Cx43.

Our report is the first to demonstrate that gliotransmitter release from astrocytes through Cx43 hemichannels is crucial for fear memory consolidation and brings forward Cx43 hemichannels in astrocytes as a novel pharmacological target for the treatment of psychiatric disorders, particularly for memory-related disorders like post-traumatic stress disorder. FJ

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