Diminished KCC2 confounds synapse specificity of LTP during senescence

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The synapse specificity of long-term potentiation (LTP) ensures that no interference arises from inputs irrelevant to the memory to be encoded. In hippocampi of aged (21–28 months) mice, LTP was relayed to unstimulated synapses, blemishing its synapse specificity. Diminished levels of the K+Cl− cotransporter KCC2 and a depolarizing GABAA receptor-mediated synaptic component following LTP were the most likely causes for the spreading of potentiation, unveiling mechanisms hindering information storage in the aged brain and identifying KCC2 as a potential target for intervention.

Aging is associated with decreased learning and memory even in the absence of Alzheimer’s disease1. The cellular substrates of this cognitive decline are poorly understood, but may relate to altered long-term potentiation2, regarded as a key mechanism underlying information storage1. A hallmark of LTP is its dendritic clustering4, which provides specific potentiation to synapses activated by a high-frequency stimulus. Currently, LTP is considered a ‘clustered plasticity’ confined to specific dendritic domains where spines or small dendritic areas with diffusional links4 represent computational memory storage units. The spread of plasticity to elements not part of the memory-related cluster interferes with the learning and memory process5. The synaptic specificity of LTP reportedly breaks down with age1,2,6,7. Here we show that the ratio of fEPSP slope values after TBS, was larger than in young (the ratios of fEPSP slope values after TBS to those before TBS administration, old: 2.23 ± 0.16, n = 44 slices, young: 1.67 ± 0.07, n = 37 slices, P = 0.0022, t = 3.321, d.f. = 58.5, two-tailed unpaired t-test, unequal variances) mostly as a result of a larger proportion of rising LTP and some LTP of very large magnitude in the old (Supplementary Fig. 3). Paired-pulse facilitation (PPF) of fEPSPs was similar in young and old, but a small decrease in PPF was detected after potentiation of the untetanized pathway in old (Supplementary Fig. 4). Thus, memory impairments during senescence do not necessarily result from deficits in LTP induction or its decreased magnitude8 but possibly result from the spread of potentiation to unstimulated synapses.

Glutamatergic synapses outnumber GABAergic ones in the apical dendrites of CA1 pyramidal cells10, but when the GABA receptors’ reversal potential (E_GABA) is sufficiently negative, GABAergic synapses can reduce dendritic Ca2+ spiking and signaling11, as well as LTP12. The competitive GABA_A receptor antagonist gabazine (GBZ, 25 µM) marginally enhanced LTP in young, but, unexpectedly, blocked LTP induction and its spread to unstimulated synapses in old (Fig. 1b). A negative allosteric modulator of α5 subunit-containing GABA_A receptors, L-655,708 (200 nM), also partially antagonized LTP in old (Supplementary Fig. 5a). Blocking NMDA receptors, critical for LTP induction6, with D-2-amino-5-phosphonopentanoate (D-AP5, 50 µM) fully blocked LTP in young, but only partially in old (Supplementary Fig. 5b), consistent with the idea of a Ca2+ dysregulation contributing to LTP induction as observed in 12-month-old mice6,7. Applying GBZ before (Supplementary Fig. 6a) and 40 min after (Supplementary Fig. 6b) LTP induction reduced fEPSPs only in old and only in those with the tetanized input. To further probe the participation of GABA receptors in fEPSPs, we applied the antagonist bicuculline methiodide (BMI) by rapid iontophoresis in the stratum radiatum. In young, BMI iontophoresis had no effect on fEPSPs before or after LTP induction. In sharp contrast, BMI reversibly reduced fEPSPs when applied after LTP induction in old, even as early as 12–14 min after TBS (Fig. 1c,d). Our results are consistent with a depolarizing GABA_A receptor-mediated component contributing to the induction (Fig. 1b) and maintenance (Fig. 1c,d) of LTP and potentially even to the spread of LTP to unstimulated synapses in old. A depolarizing dendritic GABA response had to arise during or immediately after the TBS, as BMI iontophoresis (Fig. 1c) or gabazine perfusion (Supplementary Fig. 6a) did not affect dendritic fEPSPs recorded before TBS in old. Accordingly, LTP in untetanized synapses could have ensued through a depolarizing GABA response during TBS that was no longer present 40 min later (Supplementary Fig. 6b).

Phasic and tonic GABAergic events in CA1 pyramidal cells in whole-cell recordings showed no differences between young and old (Supplementary Fig. 7). We next used pharmacological and optogenetic means to alter Cl− in CA1 pyramidal cell dendrites. Preincubating young with the KCC2 antagonist VU0240551 (refs. 13–15) for 1 h (10 µM) enhanced LTP and spread the potentiation to unstimulated synapses (Fig. 2a,b), resembling LTP in untreated old. Conversely, the KCC2 enhancer CLP257 (ref. 13; 1 h preincubation, 100 µM) had little effect on LTP in old but, notably, confined the potentiation solely to the tetanized synapses (Fig. 2a,b). Thus, in

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old, CLP257 restored the properties of LTP to those seen in young, indicating that KCC2 function might be impaired in senescence. CLP257 in young and VU0240551 in old (Fig. 2a,b) were ineffective (Supplementary Table 3). LTP magnitudes in the tetanized pathway were uncorrelated with the potentiation of the non-tetanized pathway; therefore, increased LTP by VU0240551 in young or reduced LTP by CLP257 in old were not responsible for changes seen in untetanized pathways (Fig. 2c).

Quantitative western blots in young and old CA1 mini-slices collected before and at two time points (10 min and 40 min) after LTP induction showed significantly reduced levels of monomeric KCC2 in old at both time points after induction of LTP (Fig. 3a,b). As oligomerization of KCC2 can enhance its function\(^15\), we examined oligomerized KCC2 levels in old and found them to be reduced at 40 min but not at 10 min (Fig. 3c,d) after LTP induction, producing a lower ratio of mono- to oligomeric KCC2 at 10 min (0.07 ± 0.02, n = 7) than at 40 min (0.16 ± 0.04, n = 15) after LTP induction.\(\) We also used another approach to elevate intracellular Cl\(^-\), yielding a depolarizing \(E_{\text{GABA}}\) through prolonged activation of the Cl\(^-\) pump halorhodopsin (eNpHR3.0), which is known to produce excessive Cl\(^-\) loading of neurons\(^16\). In slices from young mice expressing eNpHR3.0 in CA1 pyramidal cells (Supplementary Fig. 8a,b), 568-nm laser illumination (>10 min before and during TBS) resulted in very large LTP (Supplementary Fig. 8c) resembling the LTP found in the outliers among old mice (Supplementary Fig. 3c). As in old controls, robust potentiation of the unstimulated pathway ensued in Cl\(^-\)-loaded young pyramidal cells.

We present evidence for diminished KCC2 levels after LTP in old mice and obtained several signs of altered \(E_{\text{GABA}}\); (i) GBZ antagonized LTP induction in old but had little effect in young; (ii) GBZ reduced potentiated responses after LTP in old but not in young; (iii) after LTP, rapid BMI iontophoresis transiently reduced fEPSPs only in old, revealing a depolarizing component; (iv) blocking KCC2 in young led to the loss of LTP’s synapse specificity, yielding an LTP resembling LTP in old; (v) enhancing KCC2 in old restored the synapse specificity of LTP, as seen in young; and (vi) artificial Cl\(^-\) loading in young resulted in LTP resembling LTP in old, including loss of synaptic specificity. Yet none of our approaches directly measured local Cl\(^-\) changes in small dendritic compartments or even in spines. Somatic recordings only account for the activity of GABAergic synapses close to the cell body\(^17\), whereas \(E_{\text{GABA}}\) may quickly and dynamically change in a small dendritic volume\(^18\) and may be confined to the spines\(^19\). Local dendritic alterations in \(E_{\text{GABA}}\) are impossible to detect with somatic perforated patch recordings or even with the continuously improving Cl\(^-\) indicators, which are still not without problems\(^20\). As KCC2 is subject to complex regulatory events, including phosphorylation, internalization and Ca\(^2+\)-dependent degradation\(^14,15\), the precise mechanisms underlying its failure during repetitive synaptic activation in the aged brain remain to be determined. Pending further verification, we propose that the resulting GABAergic depolarization...
spreads the Ca\(^{2+}\) signal to dendritic branches not an integral part of the clustered plasticity (Supplementary Fig. 9). Consequently, plasticity disperses to synapses not involved in transmitting information intended for storage, severely confounding memory induction and retrieval\(^6\). Conversion of GABAergic inhibition into excitation during repetitive activation of old synapses could also explain why reducing excitability improves cognition in aged humans\(^21\) and rodents\(^22\) alike.

Reversal of the adverse spread of synaptic plasticity in the aged brain by a KCC2 enhancer may add the treatment of cognitive disorders during senescence to the already wide-ranging and promising prospects of clinical applications for such drugs\(^13\).

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Figure 3 Quantitative analyses of western blots of monomeric and oligomeric KCC2 levels. (a) Representative western blots of monomeric KCC2 and loading controls (GAPDH) from two mini-slices each from young and old, collected after recordings before (C), or 10 min or 40 min after the LTP-inducing TBS (L). (b) Summary data of the optical densities (O.D.) showing reduced monomeric KCC2 levels in old slices at 10 min and 40 min after TBS. Values were normalized to the average of the young unstimulated controls (Ctrl). After one-way ANOVA (10 min after TBS: \(F_{3,30} = 12.34, P < 0.0001\); 40 min after TBS: \(F_{3,50} = 31.75, P < 0.0001\)) multiple comparisons with Tukey's correction indicated that at each time point the old LTP groups are significantly different from every other group (at 10 min: old LTP vs. young control, \(P = 0.0001\); vs. young LTP, \(P = 0.0001\); vs. old control, \(P = 0.0092\); at 40 min: old LTP vs. every other group, \(P < 0.0001\)). (c) Full-length blots of protein samples as in a, but exposure time was shortened to visualize the higher-molecular-weight oligomeric (oligo) KCC2 in comparison to monomeric (mono) KCC2. (d) Summary data as in b for oligomerized KCC2. One-way ANOVA shows a significant difference between the groups 40 min, but not 10 min, after TBS (10 min: \(F_{3,30} = 1.253, P = 0.3080\); 40 min: \(F_{3,50} = 12.39, P < 0.0001\)). At 40 min after TBS, old LTP was significantly lower than every other group (vs. young control, \(P = 0.0008\); vs. young LTP, \(P < 0.0001\); vs. old control, \(P = 0.0002\); Tukey's correction for multiple comparisons). Numbers at the bases of histogram bars show \(n\) values, error bars are s.e.m., and *** indicates \(P < 0.01\).
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
I.F. performed experiments and collected data. I.F., G.C.F. and I.M. conceived the study, designed the experiments, analyzed and interpreted the data, prepared the figures and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Slice preparation and electrophysiology. Hippocampal slices were prepared as previously reported. Briefly, mice were anesthetized with isoflurane and decapitated following UCLA Chancellor’s Animal Research Committee protocol. Coronal slices 350 μm thick were cut on a Leica VT1000S vibratome in ice-cold N-methyl-d-glucamine (NMDG)-based HEPES-buffered solution, containing, in mM: 135 NMDG, 10 glucose, 4 MgCl₂, 0.5 CaCl₂, 1 KCl, 1.2 KH₂PO₄, 20 HEPES, 27 sucrose, 3 kynurenic acid (bubbled with 100% O₂, pH 7.4, 290–300 mOsm/L). Slices were incubated at 32 °C in an interface chamber in a reduced sodium artificial CSF (aCSF), containing, in mM: NaCl 85, d-glucose 25, sucrose 55, KCl 2.5, NaH₂PO₄ 1.25, MgCl₂ 4, NaHCO₃ 26; pH 7.3–7.4 when bubbled with 95% O₂, 5% CO₂. Recordings were done in an interface chamber at 35 °C perfused with aCSF at room temperature, containing, in mM: NaCl 126, glucose 10, MgCl₂ 2, CaCl₂ 2.5, NaHCO₃ 1, NaH₂PO₄ 1.25, sodium pyruvate 1.5, t-glutamine 1, NaHCO₃ 26; pH 7.3–7.4 when bubbled with 95% O₂, 5% CO₂. Recordings were done in an interface chamber at 35 °C perfused with naCSF.

The Schaffer collateral pathway was stimulated every 30 s with two pulses, 50 ms apart. Average stimulation durations (in μs, young: 55.16 ± 0.96, n = 116; old: 56.02 ± 1.38, n = 96, P = 0.6039, t = 0.512, d.f. = 175.3, two-tailed unpaired t-test), stimulus intensities (in μA, young: 58.49 ± 1.01, n = 116; old: 60.77 ± 1.47, n = 96, P = 0.1925, t = 1.308, d.f. = 173.9, two-tailed unpaired t-test) and stimulus charges (in nC, young: 3.27 ± 0.10, n = 116; old: 3.52 ± 0.16, n = 96, P = 0.1747, t = 1.363, d.f. = 163.15, two-tailed unpaired t-test) used in our experiments were all comparable between the two preparations. Similarly, the slopes of the fEPSPs elicited during the control period in the two preparations by the S1 or S2 stimuli were comparable (in V/s, S1 young: 0.543 ± 0.033, n = 37; S1 old: 0.594 ± 0.037, n = 44, P = 0.32, t = 1.001, t = 78.9, two-tailed unpaired t-test; S2 young: 0.381 ± 0.060, n = 8, S2 old: 0.503 ± 0.059, n = 15, P = 0.20, t = 1.33, d.f. = 18.46, two-tailed unpaired t-test). LTP was induced by stimulating the Schaffer collaterals with twice the duration of baseline stimuli with a TBS repeated twice, 30 s apart (4 pulses, 100 s⁻¹ repeated 20 times every 350 ms).

Synaptic specificity of the LTP induction was categorized as follows: (i) no LTP, when the change in the slope of the fEPSP was measured during a 0.5–1.0 ms window of their steepest rise, (ii) rising LTP, if the average fEPSP slope measured between 35 and 40 min after TBS was significantly different from 0, P < 0.05.

Quantitative western blots. The hippocampal CA3 area straddling the recording electrode was microdissected from coronal slices immediately after recording and frozen in liquid nitrogen. Control non-LTP slices were kept under the same conditions of perfusion and temperature as slices that received the tetanus. Protein extraction and western blots were done as previously reported. Briefly, samples were homogenized for 2 min in ice-cold homogenization phosphate-buffered saline containing, in mM, 10 Na₂HPO₄, 100 NaCl, 10 Na₄P₂O₇, 25 NaF, 5 EDTA, 10 EGTA, 1 Na₃VO₄, 2% Triton X-100 and 0.5% deoxycholate, pH 7.4, in the presence of protease inhibitors (for 10 ml, 1 Complete Mini tablet, Roche, and 100 µg phenylmethylsulfonyl fluoride, Sigma). Supernatant was collected after 30 min incubation on ice, and following 20 min centrifugation at 12,000 r.p.m. (13,523g) at room temperature (22–24 °C). Protein concentrations were determined with DC Protein Assay (Bio-Rad). Three micrograms of total protein were diluted in phosphate-buffered saline and Laemmli sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol (Sigma), and later separated by gel electrophoresis (10% precast polyacrylamide gel, Bio-Rad). Proteins were then transferred to a 0.2 µm PVDF membrane (Bio-Rad). Membranes were blocked in 5% nonfat milk and 2% BSA and blotted in rabbit polyclonal anti-KCC2, 1:1,000 (Millipore, 07–432). GAPDH was used as loading control (rabbit monoclonal anti-GAPDH, 1:2,000, Abcam, ab180262). Membranes were then incubated in anti-rabbit horseradish peroxidase-labeled antibody, 1:2,000 (GE Healthcare Lifesciences, NA934), and developed with Clarity Western ECL Substrate (Bio-Rad). Chemiluminescence was detected with CL-Xposure films (Thermo-Scientific). The optical densities of bands were measured with ImageJ software (NIH). Potentiations measured in slices from old mice collected for western blots at 10 min after TBS (1.66 ± 0.70, n = 7) were similar (P = 0.785, two-tailed unpaired t-test, t = 0.285, d.f. = 6) to those seen in other old slices at 10 min after TBS (1.86 ± 0.06, n = 44).

35 and 40 min after TBS was significantly (P < 0.05, Mann-Whitney U-test) larger than control, but the fEPSP slope did not significantly grow over 20–40 min after TBS (fEPSP slope versus time, linear regression coefficient not significantly different from 0, P > 0.05).

All CA1 pyramidal cell somatic whole-cell recordings were made in naCSF in visually identified neurons (custom-made–made DIC video-microscopy; Olympus 40× water immersion objective) with an Axopatch 200B amplifier. Recording electrodes were pulled from borosilicate glass capillaries with an inner filament (KG-33, 1.12 mm inner diameter, 1.5 mm outer diameter; Garner Glass) to tip diameters of ~1.0 µm in two stages using a horizontal puller (DMZ Universal Puller, Zeitz Instruments GmbH, Munich, Germany). Intracellular solutions contained the following (in mM): 130 cesium methysulfate, 5 CsCl, 10 HEPS, 10 EGTA, 5 Mg-ATP, adjusted to pH 7.25 with CsOH (280–290 mOsm). Voltage-clamp recordings were made at Vₑ = 0 ± 5 mV. The DC resistances of the electrodes were 4–6 MΩ. Series resistance was compensated for by 70–90% using lag values of 7–10 µs. Before compensation, series resistance was <15 MΩ.

All drugs were purchased from Tocris. L655,708 (200 mM), d-AP5 (25 µM) and SR-95531 hydrobromide (gabazine, abbreviated as GBZ, 20 µM) were perfused either 10 min before or 40 min after TBS. In all experiments where GBZ was perfused, the CA1 was mechanically disconnected from CA1 immediately after slices were prepared, in order to prevent development of spontaneous epileptiform activity in the slices. For VU0240551 (10 µM, previously dissolved in DMSO, final vehicle concentration 0.01%) and CLP257 (100 µM, previously dissolved in DMSO, final vehicle concentration 0.1%) slices were incubated in the drug for 1 h, recordings were done in the continued presence of VU0240551 or in aCSF for slices preincubated in CLP257.
Immunohistochemistry and microscopy. Brains were perfused and tissues processed as previously described\(^{23,25}\). Briefly, deeply anesthetized mice were transcardially perfused with 50 ml of 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. Brains were cryoprotected in 30% sucrose solution in Millonig’s modified PBS, frozen and sectioned (coronal, 35 µm). Slices were mounted and coverslipped. Images were collected using a confocal microscope (Leica TCS SP2) using LAS AF software (Leica Microsystems). Digital projection images of 5-µm z-stacks were assembled using NIH ImageJ software. Epifluorescence images were collected using an Olympus BX51 microscope equipped with a Q Imaging Retiga 2000R camera and software. Tiles were captured under the same light intensity and exposure limits and later composed into single images using the Stitching plugin\(^{26}\) for ImageJ.

Statistics. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to or larger than those generally employed in the field. On a daily basis, animals were randomly picked from their home cages by an independent animal technician, and were assigned to the various experimental procedures by the experimenter. Collection of data was not randomized and was not done blindly, but experiments were repeated over a long study period (>2 years). Data analyses were done by a blinded investigator, and the conditions of the experiments were revealed only after all data were analyzed. No animals were excluded from the study. As indicated in Supplementary Figure 3, 7 young and 6 old slices representing 16% and 12% respectively of the total number of slices in each group, all originating from different animals, showed no LTP and hence were excluded from further LTP analyses. Statistical tests (one-way and two-way ANOVAs, two-tailed paired or unpaired t-tests with unequal variances and sample sizes, two-tailed χ² tests, and nonparametric tests) and exact P-values are clearly indicated in the text, figure legends, tables and checklist. Significance was set at \(P < 0.05\). The distribution of individual data points is shown in most figures. In general, data distribution was assumed to be normal but this was not formally tested.

A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study, including original films of the western blots, are available from the corresponding author upon request.

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